ISOLATION AND PROPERTIES OF REACTION CENTERS FROM PHOTOSYNTHETIC BACTERIA

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A. E. COHEN, HOOGLERAAR IN DE FACULTEIT DER LETTEREN, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP WOENSDAG 27 JUNI 1973, TE KLOKKE 14.15 UUR.

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CONTENTS

CHAPTER I

INTRODUCTION: EARLY PHOTOSYNTHETIC REACTIONS AND THE STRUCTURE OF THE PHOTOSYNTHETIC APPARATUS IN BACTERIA

1.1 Introduction

The photosynthetic bacteria fall into three taxonomic categories, viz. the Thiorhodaceae, the Athiorhodaceae and the Chlorobacteriaceae. The Thiorhodaceae, which are purple, and the Athiorhodaceae, which are purple or brown, contain Bchl a. There are two species of Athiorhodaceae known however, which are green and contain Bchl b. The Chlorobacteriaceae, which are green, contain mainly chlorobium chlorophyll, and a little Bchl a (ref. 56). The experiments to be communicated in this thesis were done with Rhodopseudomonas spheroides and Rhodospirillum rubrum, which belong to the Athiorhodaceae. We will confine our discussions to the purple and brown bacteria belonging to the Thiorhodaceae and Athiorhodaceae. In this chapter we will give some data concerning the earliest photosynthetic reactions and concerning the structural organization of the photosynthetic system in these bacteria.

The photochemical reactions in bacteria result in the reduction of NAD^+ (ref. 25) and in the phosphorylation of ADP to ATP (ref. 27). The photosynthetic system is located mainly in an intracytoplasmic membrane system (ICM) which arises probably (see below) as an invagination of the cytoplasmic membrane (CM). When species, which are able to grow both in photosynthetic and in respirative growth conditions, are grown at normal oxygen pressure in the dark, the ICM is absent

(e.g. R. rubrum[74]) or present in only small amounts (e.g. Rps. capsulata[40]). In that case the ICM develops after transfer to photosynthetic growth conditions. The appearance of ICM under the electron microscope depends upon the taxonomic species and upon the growth conditions. For example, under photosynthetic growth conditions it is vesicular in R. rubrum[19,74] and Rps. spheroides[74] and lamellar in Rps. palustris[55]. In Rps. capsulata[40] it is vesicular under photosynthetic growth conditions and tubular under aerobic growth conditions.

Chromatophores will be defined as the isolated membrane fragments which are capable of carrying out photochemical reactions[26,27], and which arise from the ICM during the breakage of cells (see below). They appear as closed vesicles under the electron microscope (e.g. ref. 71).

On the basis of function the different compounds present in chromatophores and required for photosynthesis can be divided into three categories, viz. (1) light-harvesting pigments, Bchl and carotenoids, (2) electron transfer components, e.g. P870 (to be defined in the next section), cytochromes, ubiquinone and non-heme iron protein, and (3) coupling factors (ATP-ase), which couple ATP-synthesis to electron transport through specific sites.

The light absorbed by the light-harvesting pigments is transferred to a pigment called P870; the photooxidation of P870 is the first of a series of reactions which result in photophosphorylation and NAD^+ reduction.

1.2 Early photosynthetic reactions

The primary photochemical reaction results in the bleaching of a

pigment[22] called P890 (ref. 76) or P870 (ref. 15). This bleaching gives a difference spectrum with a minimum at 870-890 nm, the exact wavelength depending upon the taxonomic species. The bleaching at about 870-890 nm is accompanied by a blue shift of a pigment absorbing at about 800 nm (ref. 22), designated as P800. It was suggested that P800 and P870 are Bchl molecules in a special environment[24]. This was confirmed later[13,4].

The light-induced bleaching of P870 signifies its oxidation[24,30]. This photooxidation is a one-electron reaction resulting in the formation of the Bchl radical cation[46,47]. The quantum efficiency for this reaction is high, approaching 1 electron transferred per absorbed light quantum[44,6]. The photobleaching of P870 occurs within 50 nsec after absorption of light[65], and at temperatures as low as 1° K (ref. 2). This indicates a tight coupling between P870 and the substance which functions as the primary electron acceptor during the photooxidation of P870.

Another reaction occurring in whole cells and chromatophores is the light-induced oxidation of cytochrome[11,23]. There are several cytochromes present in photosynthetic bacteria, which can be characterized by their absorption spectra, by their differential absorption spectra (reduced minus oxidized) and by their midpoint potential. (For a review see ref. 28). The oxidation of a cytochrome c is coupled to the reduction of photooxidized P870 (ref. 57). The half time of this reaction at room temperature varies between 0.3 and 40 µsec depending upon the taxonomic species[38]. At 77° K the rate varies from 2 µsec (Rps. species NW) to 2 msec (Chromatium)[38]. This indicates that at least in some spe-

cies there is also a tight coupling between this cytochrome and P870.

In Chromatium at room temperature, the electron transported in the primary photoreaction is transferred from the primary acceptor to a secondary acceptor in a reaction with a half time of 80 µsec (chromatophores)[58] or 300 µsec (cells)[59]. This reaction does not occur at 77° K (ref. 58,20). At this low temperature the electron moves from the primary acceptor to the photooxidized P870 in a direct back reaction with a half time of 20-25 msec[20,21,58]. This occurs only if the (faster) rereduction of oxidized P870 by cytochrome c is inhibited; this can be accomplished by raising the redox potential of the medium in order to oxidize cytochrome c chemically before the onset of light.

1.3 The arrangement of functional chromatophore subunits

As a reaction center we will now define a pigment-protein complex combining in vivo the functions of "energy-trapping" and "energy-conversion" center. More specifically, a reaction center is a pigment-protein complex containing a small number of specific pigment molecules, which function in vivo as a trap for light-energy absorbed by other pigment molecules not belonging to the reaction center. This "trapping" function is correlated with the "energy-conversion" function of the reaction center and arises from the capability of a specific (pair of) pigment molecules of the reaction center to photoreduce another substance in a one-electron reaction, which is commonly called the "primary reaction of photosynthesis". The pigment molecules mentioned here may be chlorophyll or bacteriochlorophyll, depending upon the taxonomic category. The words between brackets refer to a problem which

will be discussed in Ch. IV, namely, whether the electron donor (in our case P870) is a monomer or a monovalent dimer.

Reaction centers have been isolated from chromatophores of certain Athiorhodaceae and separated from light-harvesting components and coupling factors. This was done first by Reed and Clayton[61] and Reed[60]. Reaction centers from purple or brown Athiorhodaceae contain minimally P870 as electron donor, P800, Bph and a substance functioning as an electron acceptor during the "primary reaction". Other electron transfer components (e.g., cytochromes) may also be present.

P800 and Bph do transfer excitation energy to P870 as will be shown in Ch. III. The reason for classifying P800 and Bph nevertheless as reaction center pigments, as against light-harvesting pigments, is not only because P800 and Bph always accompany P870 when this is isolated, but also because in chromatophores P800 and Bph transfer energy to P870 rather than to light-harvesting Bchl, as will be discussed in Ch. III. Apparently Bph, P800 and P870 are associated more tightly with one another than with the remaining, light-harvesting pigment molecules.

Incubation of chromatophores from Rps. spheroides R-26 (a carotenoidless mutant) with a low concentration of Triton X-100 resulted in the release of particles of 9 nm diameter with ATP-ase function (coupling factor)[62]. At higher Triton concentrations reaction center particles were released, appearing as flakes of 12 nm diameter[62]. The remaining fragments containing light-harvesting Bchl could be split into particles of 5 nm diameter by treatment with SDS[62]. This would correspond to a molecular weight of 50 kilodaltons. Reed and Raveed[62] determined also the ATP-ase activity and the Bchl, P870 and protein con-

tent of chromatophores from the R-26 mutant of Rps. spheroides cultured at different light intensities. The result indicated that chromatophores contain per reaction center, which had a molecular weight of 440 kilodaltons[60], one ATP-ase particle with a molecular weight of about 280 kilodaltons, and (depending upon the culture conditions) a variable number of light-harvesting Bchl-protein complexes containing protein to a weight of 10 kilodaltons per molecule of Bchl. These three types of particles accounted for all of the chromatophore protein.

The reaction centers isolated[61,60] from Rps. spheroides R-26 contained P870 and other electron transfer components present in chromatophores but no light-harvesting Bchl. However, it is also possible to convert chromatophores from R. rubrum[42] and Rps. spheroides[43] into small particles, in which all the light-harvesting Bchl is still attached to P870, but which presumably have lost a number of electron transfer components. This was suggested by the low molecular weight of these particles and by the release of protein, observed during the preparation of these particles[42,43].

Th R-26 mutant of Rps. spheroides contains only one type of light-harvesting Bchl, in contrast to the wild type, which contains three types of Bchl, called B800, B850 and B870 after their absorption maxima in the near-infrared. Chromatophores from wild type Rps. spheroides cells grown under different light intensities contained per reaction center a fixed amount of B870 and a variable amount of B800 and B850. The ratio of B800 : B850 was constant, however[1]. After fractionation of Rps. spheroides chromatophores treated with Triton, we found in accordance with earlier results[10] that P870 was recovered

in a fraction containing predominantly B870. Another fraction contained B800 and B850 only. Light absorbed by B800 in this fraction was still transferred to B850 with an efficiency close to 1 (Slooten, unpublished).

An analogous situation may exist in Chromatium, which also contains several types of light-harvesting Bchl, designated[75] as B800, B*800, B820, B850 and B890. After detergent treatment and fractionation of chromatophores the reaction center was found to be associated with B890 only[73].

1.4 The photosynthetic unit: lake model or separate unit model

The chromatophore pigments can be thought to be arranged in photosynthetic units consisting each of a reaction center and the average number of Bchl (and carotenoid) molecules found per reaction center. These units may or may not occur as separate entities.

The excitation energy absorbed by Bchl can either be used for photochemistry (the oxidation of P870) or it can be lost in non-photochemical deexcitation including fluorescence. If P870 is oxidized it does not function as an energy trap and consequently the fluorescence yield (ϕ_f) of light-harvesting Bchl increases. Working with whole cells of R. rubrum, Vredenberg and Duysens[76] showed that $1/\phi_f$ decreased linearly with an increasing ratio of oxidized P870/total P870. This was consistent with a so-called "lake-model" in which a light-harvesting Bchl molecule is able to transfer its energy to any one of a large number of nearby reaction centers. Clayton[14] found that this situation occurred only in aerobic cells and in fresh chromatophore preparations

of R. rubrum. In other conditions deviations from the Vredenberg-Duysens relationship occurred, some of which were consistent with a "separate unit" model, in which reaction centers and light-harvesting pigments are arranged into units in such a way that each pigment molecule is able to transfer excitation energy only to the reaction center which belongs to the same unit as this pigment molecule.

1.5 Membrane conformation in chromatophores

ATP-ase (coupling factor) is located at the outside of chromatophores, where it can sometimes be seen under the electron microscope as protruding knobs[62,45].

After thin-sectioning and negative staining the chromatophore membrane, like other membranes, shows up as a double layer. Freeze-fracturing of membraneous material usually yields fractures in the plane of a membrane. This results in exposure of the two inner faces of a double membrane[7,77,49]. The two outer faces can be exposed in addition by subsequent etching (see Fig. 1.2). Upon freeze-fracturing of chromatophores from Rps. spheroides R-26 (ref. 62) and Thiocapsa roseopersicana[71], the concave inner membrane face appeared to be covered with many particles with a diameter of about 13 nm in Rps. spheroides[62] and about 6-8 nm in Thiocapsa[71]. The convex inner side contained very few particles. It is perhaps too early to relate these particles to reaction centers.

Chromatophores, like most other biomembranes, contain predominantly proteins and lipids. These compounds account for 85 - 90% of the total dry weight of chromatophores (For detailed analyses see ref. 55).

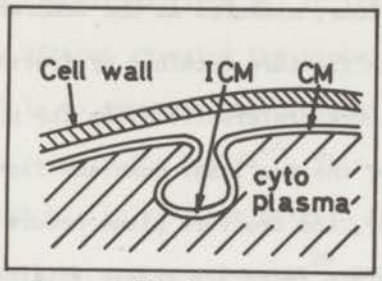

Fig 1

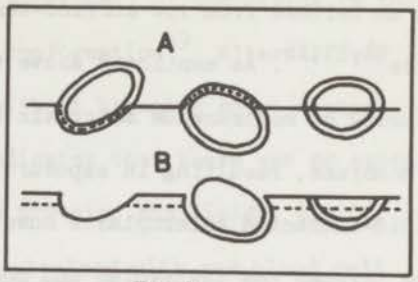

Fig 2

Fig. 1.1 Schematic representation of part of a cell of R. rubrum or Rps. spheroides. The "inside" of the ICM is continuous with the "outside" of the CM.

Fig. 1.2 (A) Schematic representation of the fracture planes occurring in 3 chromatophores as a result of freeze-fracturing, according to Branton[7]. A concave (left) and a convex (middle) inner membrane are exposed.

(B) Subsequent etching (sublimation of water to the level of the dashed line) reveals a convex (left and middle) and a concave (right) outer membrane face.

The weight of proteins is about twice the weight of lipids in chromatophores. The ratio of polar to apolar aminoacids (as classified by Hatch[36]) in chromatophore protein from Th. roseopersicana[72], Rps. spheroides[32] and R. rubrum[32] is 1.18. Such a low ratio is often found in globular proteins with a compact internal volume and a high helix content[36]. The lipids in chromatophores are mainly phospholipids[55]. About 80% (w/w) of the lipids from Rps. spheroides chromatophores consists of C_{16} and C_{18} fatty acids (data from ref. 31).

Apart from these specific data, some general remarks can be made concerning the arrangement of these membrane constituents.

Membranes are generally hydrophilic at the membrane-water inter-

face, as follows from low surface-tensions, measured at the membrane surface[34,35,16]. As mentioned above the fracture obtained by freeze-fracturing of membraneous materials occurs preferentially in the plane of a membrane, resulting in exposure of the two inner membrane faces. In lipid-extracted chloroplasts however, the fracture plane occurs at random through the thylakoids and membrane faces are rarely obtained[9]. This indicates that lipid-soluble substances are responsible for the weak hydrophobic bonds which are apparently broken during freeze-fracturing of normal membranes, and suggests (see above and Fig. 1.2A) that the membrane interior consists largely of lipid-soluble substances. This finding is in line with earlier hypotheses[17,70] of the hydrophobic nature of the interior of cytoplasmic membranes. These hypotheses were based on measurements of the permeability of compounds of different hydrophobicity.

The particles seen after freeze-fracturing of membranes (including chromatophores) are probably not lipid micelles because these do not exist at the low temperatures (-100° C) at which the replicas are made. Lipid micelles undergo a transition to lamellar gels below a critical temperature[63,39,12]. Rather, we favor the suggestion[8,37] that the Danielli-Davson model[18] is still applicable to membranes, including chromatophores, in that the membrane consists basically of a double layer of lipids, of which the polar heads are turned outwards. The particles seen at the concave inner membrane face (see above) are thought to be proteins embedded in the outer membrane half in a lipid matrix. Such a mosaic distribution of proteins and lipids was also suggested to occur in cytoplasmic membranes from Mycoplasma. Here it was possible to induce a

phase transition (from gel to liquid crystal) of the lipids in the membrane without changing the protein conformation[69]. Alternatively it was possible to denature the proteins without bringing about a phase transition in the lipids[69]. This indicates that there was no extensive hydrophobic interaction between the proteins and lipids. Similar results (refs 41,29) and conclusions[29] were obtained with red blood cell membranes.

The particulate nature of mitochondrial[33] and chloroplast[3,78] membranes, as revealed by negative and positive staining techniques in electron microscopy have been interpreted alternatively in terms of a model in which membranes consist of a monolayer of lipoprotein subunits. These models imply extensive hydrophobic interactions between proteins and the fatty acid chains of the lipids. As indicated above, recent results are not in favor of such interactions on an extensive scale. Another argument against the lipoprotein subunit model is the occurrence of a preferential fracture plane, in the freeze-etching technique, in the plane of a membrane.

1.6 The relation between cytoplasmic and intracytoplasmic membrane

Cytoplasmic and intracytoplasmic membrane (CM and ICM) seem to form a continuum in the cell and to develop out of one another. The evidence can be summarized in the following 4 points.

1) The ICM is sometimes directly visible under the electron microscope as an invagination of the CM (see e.g. refs. 71,5).

2) The membrane faces exposed by freeze-fracturing and freeze-etching are recognizable by their being covered with either few or many

particles of a typical size. The situation of these membrane faces on the CM and ICM of Th. roseopersicana were also consistent with the idea that the ICM arises as an invagination of the CM[71]. (Compare Fig. 1.1 and 1.2)

3) There is no qualitative difference between CM and ICM. Photosynthetic and respiratory functions are present in either one of these membrane types, only the ratios are different. The ICM contains oxidative functions such as oxidative phosphorylation[54] or NADH oxidase activity (refs. 54,53). The proteins and functions associated with respiration are present in small or large amounts depending upon the culture conditions[40]. For instance, the amount of proteins[54] associated with respiration, and the rate of certain respiratory reactions[52], were high in the ICM of R. rubrum just after a transition from aerobic to anearobic growth; subsequently they decreased in time. Conversely, after a transfer of the cells from anaerobic to aerobic growth the proteins[53] and functions[52] associated with respiration increased with time in the ICM. On the other hand, the CM contains some Bchl[50] and under special conditions it is the only place where Bchl occurs. (viz. just after a transition from aerobic to anaerobic growth[50]).

4) Intact cells of R. rubrum[64], whether grown under photosynthetic or respirative growth conditions, behave like mitochondria[48] as far as the polarity of H^+-transport is concerned: during photosynthetic or respirative electron transport protons are driven out of the cell. But chromatophores[64] take up H^+ from the medium. This is easy to understand if one assumes that the ICM is an invagination of the CM. After cell breakage the ICM is separated from the CM and closes in such a way that what was outside now becomes inside. (compare Fig. 1.1)

1.7 This work

In this thesis we will describe experiments done with reaction center preparations isolated from chromatophores of Rps.spheroides and R.rubrum. Similar preparations were made by other authors and we shall relate our data to those obtained by these authors. Our results have been partly published elsewhere[66,67,4,68].

The isolation of reaction center particles can be done with several aims in mind: (1) It may answer questions about the organization of the chromatophores into subunits. Examples have been given above[62,72,42,43]. Our work does not give much new information on this point. (2) The isolation of particles allows the study of properties which cannot be studied easily in vivo because they are masked by the properties of other chromatophore constituents. Such properties are described in Ch. II (absorbance spectra and results of chemical analysis), in Ch. III (transfer of light-energy to P870 and fluorescence of P870 and P800) and in Ch. IV (analysis of the absorbance spectrum of reaction centers). (3) Finally, the isolated particles allow the study of partial reactions, which are not seen in chromatophores; this may be because the product formed during the reaction is rapidly consumed in a subsequent reaction in chromatophores, but not in the isolated particles; alternatively, the "partial reaction" may be an artifact, a side reaction which simply does not occur in chromatophores. Ch. V describes the function of endogenous ubiquinone as a (primary or secondary) electron acceptor for the photooxidation of P870 in reaction centers. Ch. VI evaluates literature data pertinent to the question whether this reaction occurs in vivo.

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CHAPTER II

ISOLATION AND SOME SPECTRAL AND CHEMICAL PROPERTIES OF REACTION CENTERS

2.1 INTRODUCTION

During the last few years much attention has been paid to the treatment of bacterial chromatophores with agents that yield so-called reaction center particles, (defined as in Ch. I, section 1.3), which do not contain the bulk or light-harvesting Bchl components normally present in chromatophores. In these relatively simple systems, spectral investigations can be done more precisely, and one may hope to be able to identify the primary electron acceptor of P870 through successive purification steps of the particles.

Kuntz et al.[31] treated chromatophores with iridic chloride, which bleaches most of the bulk Bchl of Chromatium D and Rhodospirillum rubrum, and all of the bulk Bchl in Rhodopseudomonas spheroides. Beugeling[6] in an independent study, bleached the bulk Bchl of R. rubrum chromatophores completely with ferricyanide. In both cases, the reaction center Bchl could be oxidized completely in the light.

Another line of investigation is treatment of chromatophores with detergents. Bril[9] used Triton X-100 to disrupt chromatophores of Rps. spheroides into two types of fragments, one containing B800 and B850 and the other containing B870. Garcia et al.[29,21-23] working with four other species of bacteria added Triton X-100 to chromatophores and obtained a particulate fraction, consisting of small particles, sometimes in a linear or planar arrangement, and a membrane fraction.

The membrane fraction was always photochemically less active than the particulate fraction, and in some cases was not active at all. Thornber[53] used the detergent sodium dodecyl sulphate to fragment chromatophores of Chromatium. After separation on a hydroxylapatite column he obtained particles of different pigment composition, achieving a partial separation of the various Bchl types. One of the particles contained P870.

Reed and Clayton[42] and Reed[41] isolated reaction center particles from a carotenoidless mutant of Rps. spheroides by means of Triton X-100, followed by density gradient centrifugation. At about the same time Gingras and Jolchine[24] reported the isolation of a reaction center particle from a carotenoidless mutant of R. rubrum , using Triton X-100. Thornber et al.[54] made a reaction center preparation from a wild strain of Rps. viridis, using SDS as detergent.

In this Chapter we will describe the isolation and some spectral and chemical properties of reaction center preparations from wild strains of Rps. spheroides and R. rubrum. While this work was in progress, other authors succeeded also in preparing reaction center particles from Rps. spheroides (refs 28,48,45,18,19,13,43) and R. rubrum[37,51,56] and we shall compare our data with the results obtained by these authors.

2.2 MATERIAL AND METHODS

2.2.1 Preparation of the reaction center particles

Rps. spheroides and R. rubrum were cultured anaerobically in a medium after Cohen-Bazire et al.[14] supplied with yeast extract and

peptone. After 3-4 days of growth the cells were harvested by centrifugation, washed in 0.05 M Tris (pH 8.0) and stored at liquid nitrogen temperature until use.

Fragmentation of the cells was carried out by sonication with a Branson type S125 sonifier during 10 min at 8 A. The temperature was not allowed to rise above 8°C during sonication. In some cases the cells were passed through a French press at 5000 kg/cm2 after precooling of the pressure cell to 4° C. The suspension was centrifuged for 30 min at 20000 x g to remove cell debris. CsCl and $MgSO_4$ were added to the supernatant to give final concentrations of 27% and 0.05 M, respectively, and the suspension was centrifuged for 1.5 h at 144000 x g.

This resulted in the precipitation of light brownish material, presumably cell wall fragments, and of whitish, perhaps ribosomal material. Omission of this step resulted in a much higher protein content of the reaction center particles (see section 2.3.5). The chromatophores floated at the top of the solution after this centrifugation. This top layer was diluted, sedimented by centrifugation during 1 h at 200000 x g, resuspended in 0.05 M Tris (pH 8.0) containing 0.01 M $MgCl_2$, and dialyzed overnight against the same buffer.

The absorbance of the chromatophore suspension at 590 nm was adjusted to 12.5/cm and 0.11 vol. of 3% SDS was added. (At lower concentrations of Bchl the SDS-concentration was taken proportionally lower.) After 2 h of incubation at room temperature NaCl was added to give a final concentration of 0.2 M (this was done because it stops the working of SDS[9]), and the suspension was layered on a 0.5 M sucrose solution in 0.05 M Tris (pH 8.0) supplied with 0.2 NaCl. After 4 h of

centrifugation at 200000 x g the chromatophores were sedimented and a greenish band containing the reaction center particles remained in the supernatant layer on top of the sucrose solution. This treatment was also successful with chromatophores of Rps. viridis (cf. ref. 54), and of R. rubrum. The reaction center particles were dialyzed for 2 days against three 25-vol. quantities of 0.05 M Tris (pH 8.0), to which 0.01 M $MgCl_2$ was added in order to improve the reversibility of the light-induced absorbance changes (see below). The reaction center fraction was purified further by centrifugation at 200000 x g on a 0.4-2.0 M linear sucrose density gradient. This procedure resulted in the separation of a small fraction of less purified reaction center. The purified reaction center was dialyzed against 0.05 M Tris (pH 8.0) containing 0.01 M $MgCl_2$ to remove sucrose. The reaction center preparation purified in this way will be called hereafter SDS-RC.

A further purification was obtained by a procedure analogous to the so-called AUT-treatment used by Loach et al.[34] for chromatophores. SDS-RC preparations were adjusted to a concentration corresponding with an absorbance of 1 per cm at 803 nm and layered in 4-ml quantities on top of a linear 0.1-1.0 M sucrose gradient in 0.05 M Tris containing 0.3% Triton X-100 and 1 M urea. The pH had been adjusted to 10.0 after the addition of urea. At lower concentrations of reaction centers, the Triton concentration had to be taken correspondingly lower, because a higher Triton/reaction center ratio resulted in the destruction of Bchl. The gradients (8 ml) had been prepared in tubes for the 50-Ti rotor of the Beckman L2 ultracentrifuge. After 6 h of centrifugation at 200000 x g the original preparation had been separated into an upper green

band and a lower brown band. Both bands were situated about halfway down the sucrose gradient. The lower band consisted of the purified reaction center preparation and contained about 85% of the amount of Bchl recovered after the centrifugation. The recovery of Bchl in the two fractions was about 80% of the original amount. Both fractions were dialyzed against three 25-vol. quantities of 0.05 M Tris (pH 8.0) containing 0.01 M $MgCl_2$. The reaction center particle purified in this way will be denoted AUT-RC. The reaction centers were stored at 4° C in 0.05 M Tris (pH 8.0) containing 0.01 M MgCl. This will be referred to hereafter as Tris buffer. No significant loss of activity was observed after a storage of several months.

Mg ion was present throughout the purification procedure because this had a favourable effect on the reversibility of the light-induced absorbance changes. After some publications had come to our attention which described an Mg-dependent binding of ribosonal material to membraneous fractions[38,3] we did one experiment in order to check whether ribosomes or other proteinaceous components were bound to reaction center particles. In this experiment 1 mM EDTA was present in stead of 10 mM MgCl throughout the preparation procedure, to start with the sonication of the cells. The EDTA was removed from the SDS-RC by dialysis. However, the protein content of the SDS-RC particles obtained in this way was not significantly lower than when Mg was present during the preparation.

It should be noted that the success in isolating reaction centers seems to depend on many factors of which not all are understood. For instance, it appeared to be obligatory to freeze the cells of R. rubrum

and thaw them again prior to the SDS-treatment. With cells of Rps. spheroides this was not necessary during the first year of our experiments but later the yield of reaction centers was much lower if the cells were not given a cold treatment. Apart from these difficulties the yield of SDS-RC and AUT-RC fractions was quite reproducible.

2.2.2 Chemical and physical methods

Protein was measured by the method of Lowry et al.[35]. Cytochrome was measured with the ferrihemochrome method as used by Cusanovitch and Kamen[16]. Ubiquinone was determined by the method of Pumphrey and Redfearn[40], as modified by Takamiya and Takamiya[52]. P870 was estimated from the absorbance at 804 nm and 867 nm in reaction centers, using specific extinction coefficients of 272 $mM^{-1}.cm^{-1}$ and 113 $mM^{-1}.cm^{-1}$ for the absorbance at these wavelengths[12], or it was estimated from the maximum light-induced bleaching at 867 nm, using a differential extinction coefficient of 93 $mM^{-1}.cm^{-1}$ (ref. 8).

Iron was determined with the ferrous o-phenanthroline complex method[47] after a mineralization procedure as used by Konings[30]. The sample was concentrated to near dryness at 120°, then 0.2 ml conc. H_2SO_4 (Merck, p.a.) was added and the samples were heated at 170° for 2 h. They were then heated on a Bunsen burner while $HClO_4$ was added dropwise until a bright yellow colour appeared and disappeared again. This was a rather delicate step because the presence of too much perchlorate in the sample resulted in the precipitation of a reddish ferrous-orthophenanthrolin-perchlorate complex[47]. It appeared however that if this was done carefully and ample time was allowed for

digestion, the final amount of perchlorate was low as indicated by the fact that no precipitate was formed when rubidium chloride was added to an aliquot of the digested sample, and a normal colour development took place with orthophenanthrolin, provided the tubes were shaken immediately after addition of orthophenanthrolin and after adjustment of the pH.

Absorbance spectra were measured on a Cary model 14 R spectrophotometer. Light-induced absorbance changes were measured with a split-beam differential spectrophotometer described earlier[1], or with a similar apparatus built in this laboratory, with which absorbance changes were measured at two wavelengths simultaneously. Actinic light was provided by a 250 W quartz-iodine lamp in front of which suitable filters were placed.

2.3 RESULTS

2.3.1 Absorbance spectra and absorbance difference spectra

Fig. 2.1A (solid line) shows the absorbance spectrum of chromatophores from Rps. spheroides. The light-harvesting Bchl types B800, B850 and B870 (see Ch. I, section 1.3) are apparent by their respective maxima and shoulder in the near-infrared. The maxima at 590 and 375 nm are also due to Bchl; those at 440, 475 and 505 nm are due to carotenoid (mainly spheroidene, see refs 26 and 36).

The dashed line of Fig. 2.1A shows the absorbance spectrum of the same chromatophore preparation after 2 h incubation at room temperature with SDS (final concentration 0.22%). The dilution factors have been

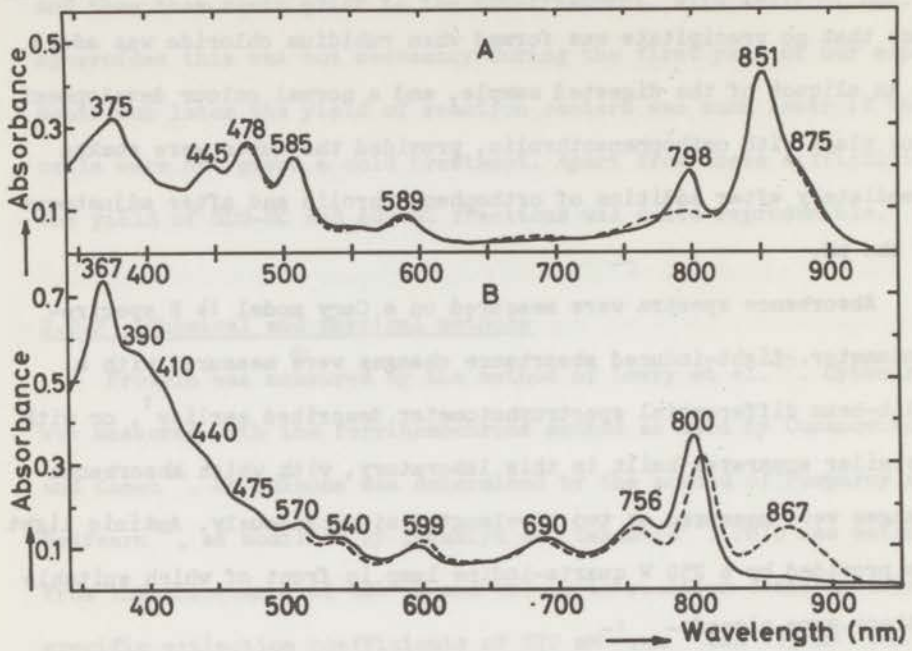

Fig. 2.1A Solid line: Absorbance spectrum in a 1 mm cuvette of a chromatophore preparation from Rps. spheroides in Tris buffer. No additions. Dashed line: same preparation, but the chromatophores had been incubated with 0.11 vol. 2.2 % SDS for 2 h at room temperature. The spectra represent equal concentrations of chromatophore material.

Fig. 2.1B Absorbance spectrum of an SDS-RC preparation from Rps. spheroides in Tris buffer. Solid line: 100 µM ferricyanide present. The ferricyanide contribution was subtracted by putting 100 µM ferricyanide also in the reference cuvette. Dashed line: 100 µM ferricyanide and 2 mM ferrocyanide present.

chosen such that these spectra represent equal concentrations of chromatophore material. The SDS-treatment did not change the amount of Bchl significantly, judging from the 375 and 590 nm peaks, but the 800 nm peak had become much lower and the shoulder at 870 nm had also

decreased somewhat. Small absorbance increases were observed around 770 and 690 nm. This may reflect the formation of solubilized and oxidized Bchl, respectively. The 850 nm peak was not much affected. Usually we used higher concentrations of SDS and Bchl (see section 2.2.1) and this had about the same effect, except that B870 disappeared to a larger extent.

Fig. 2.1B shows the absorbance spectra of an SDS-RC preparation isolated from another batch of chromatophores, and suspended in Tris buffer. The solid line shows the absorbance spectrum in the presence of 100 µM ferricyanide, which caused a maximal bleaching of P870. (The ferricyanide contribution has been subtracted already from this spectrum.) Upon addition of ferrocyanide to a final concentration of 2 mM the absorbance around 870 nm due to reduced P870 was completely restored. Other wellknown features are the blue shift of P800 from 804 to 800 nm (see Ch. I, section 1.2) and the bleaching at about 600 nm concomitant with the bleaching of P870. Prolonged illumination causes the same absorbance changes as chemical oxidation (see below). The absorbance spectrum of Fig. 2.1B indicates the presence of Bph (with maxima at 756 and at about 540 nm) and of carotenoid (with maxima at 440, 475 and 510 nm). The maximum at about 600 nm is due to P800 and P870 (see Ch. IV). The maximum at 367 nm and the shoulder at 390 nm are caused by P800, P870 and Bph (see Ch. IV, Fig. 4.6). The absorption band at 690 nm was probably due to one or more degradation products of Bchl. The shoulder at 410 nm was associated with this absorption band (see below).

Fig. 2.2 shows the absorbance spectrum of an AUT-RC preparation.

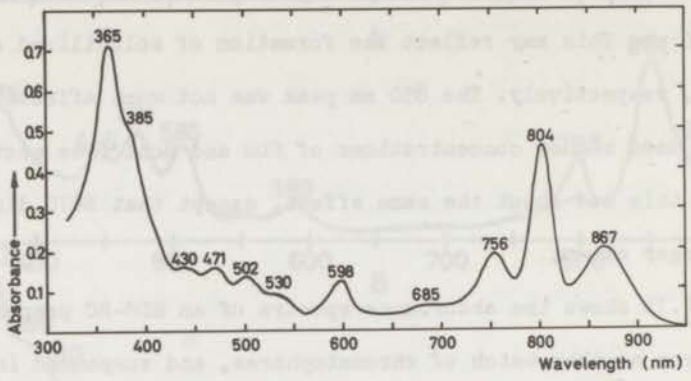

Fig. 2.2 Absorbance spectrum of an AUT-RC preparation in Tris buffer. No additions.

The main difference with an SDS-RC preparation was that the peak at 690 nm and the shoulder at 410 nm were absent from the AUT-RC preparation. At one occasion we measured the difference in absorbance between the upper and lower fraction which arise in the centrifuge tube during the AUT-treatment (see section 2.2.1). Fig. 2.3 shows the

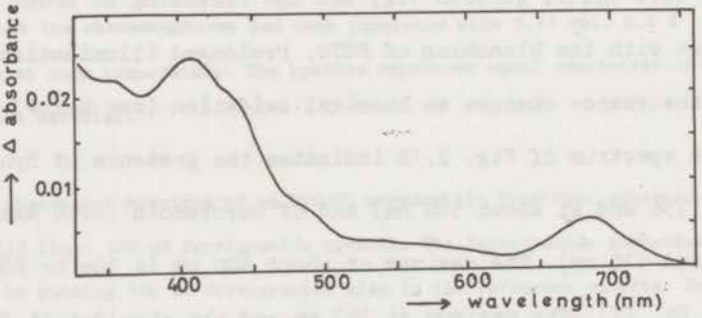

Fig. 2.3 Spectrum of the difference in absorbance between the "upper" and the "lower" band, respectively, which arise during the AUT-treatment in the centrifuge tube. The "upper" band was put in the sample cuvette and the "lower" band in the reference cuvette. The concentration of P870 was equal in both cuvettes. The absorbance at 804 nm was 0.025. This spectrum reflects probably a mixture of products which arise during the oxidative degradation of Bchl.

difference in absorbance of the upper and lower fraction, as observed after dilution to equal concentrations of P870. It is difficult to estimate how much of this absorbance was due to the substance(s) of which the fluorescence excitation spectrum is shown in Fig. 4.1. The absence of carotenoid and Bph from the spectrum of Fig. 2.3 indicates that the AUT-treatment affected neither the carotenoid nor the Bph content of reaction centers significantly. As will be shown in Ch. III, literature data indicate that Bph is an integral part of reaction centers in vivo.

The procedure used for the isolation of reaction centers from Rps. spheroides was also successful when applied to R. rubrum. Fig. 2.4 shows the absorbance spectrum of an SDS-RC preparation from R. rubrum.

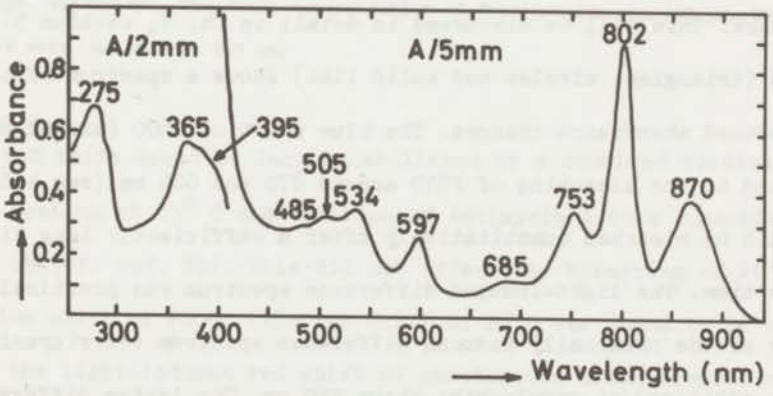

Fig. 2.4 Absorbance spectrum of an SDS-RC spectrum from R. rubrum in Tris buffer. No additions. The spectrum was taken in a 5 mm cuvette from 940 - 450 nm and in a 2 mm cuvette at shorter wavelengths.

Fig. 2.5 shows kinetics of the light-induced absorbance changes at 600 nm and 767 nm observed in reaction centers from Rps. spheroides. The absorbance changes showed biphasic kinetics both in the light and

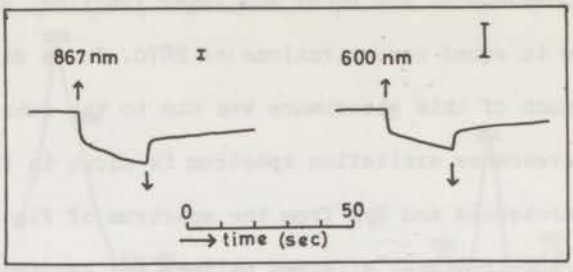

Fig. 2.5 AUT-RC preparation from Rps. spheroides in Tris buffer. No additions. Kinetics of the light-induced absorbance changes observed in a 1 mm cuvette at 867 nm and 600 nm. Actinic illumination, 20 sec with light of about 400 - 540 nm, intensity about 3 nEinstein.cm^{-2}.sec^{-1}. The light was switched on and off at the times indicated by the upward and downward pointing arrows, respectively. The vertical bars represent an absorbance change of 0.001 optical density units. A downward deflection means an absorbance decrease.

in the dark. This will be discussed in detail in Ch. V, section 5.3.1. Fig. 2.6 (triangles, circles and solid line) shows a spectrum of the light-induced absorbance changes. The blue shift of P800 (around 800 nm) is coupled to the bleaching of P870 around 870 and 600 nm (see below). P870 could be bleached quantitatively after a sufficiently long illumination time. The light-induced difference spectrum was practically the same as the chemically induced difference spectrum (ferricyanide minus no addition) at wavelengths above 550 nm. The latter difference spectrum is shown by the dashed line of Fig. 2.6. The absorbance changes below 600 nm will be discussed in Ch. V.

Above 600 nm, the difference spectra obtained with reaction centers of Rps. spheroides were about the same as those observed in chromatophores (see Fig. 2.7) except that in the latter a red shift of B850 was superimposed on the bleaching of P870 (cf. refs.55 and 11). However

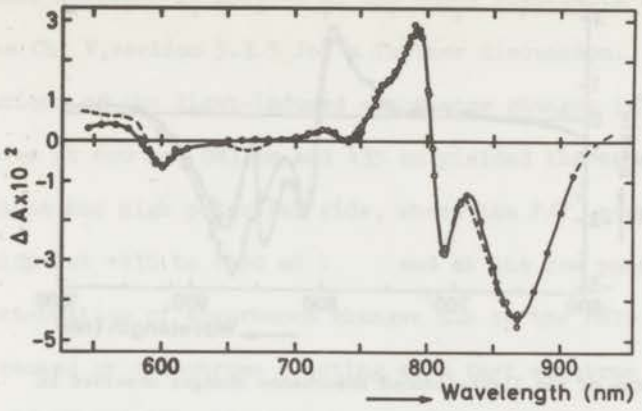

Fig. 2.6 Solid line: spectrum of the absorbance changes observed after 2 sec of illumination. Further legends see Fig. 2.5. The open symbols refer to measurements done with the AUT-RC preparation used for Fig. 2.5. The solid symbols represent measurements done with another preparation. Dashed line: chemically induced difference spectrum (ferricyanide minus no additions) observed in an SDS-RC preparation from Rps. spheroides. The final concentration of ferricyanide was 100 μM. The spectra were matched at 867 nm.

this red shift could be largely abolished by a combined treatment of 5 min heating at 65° C and addition of Antimycin A to a concentration of 10 μM (cf. ref. 20). This did not affect the bleaching of P870 and the blue shift of P800. (The red shift of B850 was found to be correlated to the light-induced red shift of carotenoids under several experimental conditions[55,2,20]. This is probably because both phenomena are correlated to an intermediate in the photophosphorylation process[20,4,27]; this "intermediate" may be a membrane potential formed during the photosynthetic electron transport[27,50].)

Another difference between reaction centers and chromatophores is of course that in reaction centers 85% of the absorbance of 867 nm

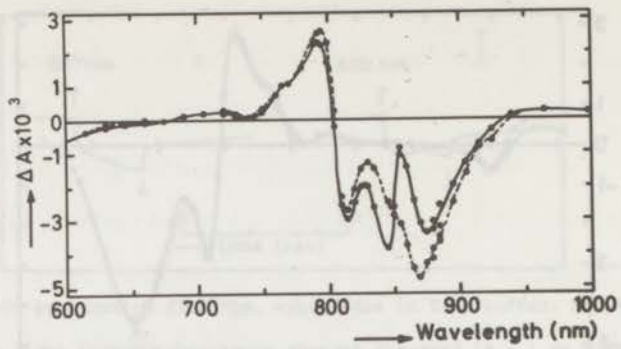

Fig. 2.7 Spectrum of the light-induced absorbance changes observed in chromatophores from Rps. spheroides suspended in Tris buffer. Solid line, normal chromatophores, no additions. Dashed line, 10 μM Antimycin A; the chromatophores had been heated for 5 min at 65° C. Illumination 2 sec with light of 430 nm, intensity 0.6 nEinstein.$cm^{-2}sec^{-1}$.

could be bleached by illumination (compare Fig. 2.1B). The maximum light-induced bleaching at 867 nm in chromatophores was not more than a few percent of the total absorbance.

2.3.2 The origin of the 600 nm absorbance change

The 600 nm absorption band in reaction centers is due to P800 and P870, as will be shown in Ch. IV. There are several indications, outlined below, that the light-induced or chemically induced bleaching around 600 nm is due to the oxidation of P870. Firstly, the light-induced absorbance changes at 600 nm and in the near-infrared region have almost identical kinetics in reaction centers under a variety of conditions, such as long or short illumination times (cf. Fig. 2.5) and illumination in the presence of an exogenous electron donor or electron acceptor (see Ch. V). (A small portion of the near-infrared absorbance changes had different

kinetics. This concerns at most 4% of the total absorbance change at 867 nm. (See Ch. V,section 5.3.5 for a further discussion.) Secondly, redox titrations of the light-induced absorbance changes in Chromatium chromatophores at 600 nm, 883 nm and 435 nm yielded the same titration curves, both at the high potential side, where the P/P^+ couple was titrated (midpoint +470 to +490 mV) and at the low potential side, where the attenuation of absorbance changes due to the P870 photooxidation was caused by cytochrome c acting as a fast electron donor to P^+870 (refs 49,17,15) (see also Ch. I, section 1.2). Furthermore it has been shown that P800 and P870 are Bchl a molecules[12,7]. Finally, the spectrum of the light-induced and chemically induced absorbance changes (see Fig. 2.6 and Ch. V) are, with exception of the blue shift around 800 nm, similar to the difference spectrum (oxidized minus reduced) of Bchl[33].

All these data indicate that the bleaching around 600 nm is due to oxidation of P870. However it does not seem unlikely that there occurs also a small blue shift of the 600 nm absorption band due to P800, concomitant with the blue shift of P800 around 800 nm, which accompanies the oxidation of P870 (see below).

2.3.3 Analysis of the 600 nm absorption band

For experiments to be discussed in Ch. III we needed an analysis of the 600 nm absorption band observed in reaction center preparations.

Fig. 2.8A shows absorbance spectra of an AUT-RC preparation from Rps. spheroides around 600 nm. The solid line shows the total ab-

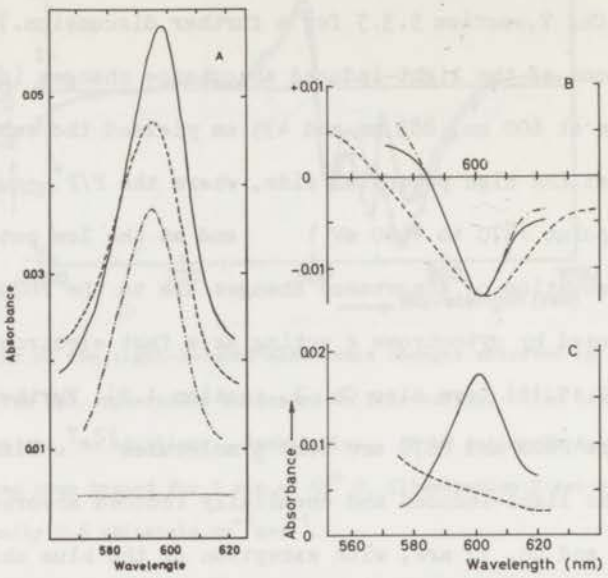

Fig. 2.8 (A) Solid line, absorption spectrum of an AUT-RC preparation, with P870 reduced (no additions); dashed line, same with P870 oxidized (0.1 mM ferricyanide); -.-.-, absorption band due to P800. ...., absorption due to Bph (from Fig. 4.6, Ch. IV). (B) (a) ---, absorption difference spectrum (ferricyanide minus no additions) of Bchl in methanol (data from ref. 25); (b) -.-.-, same as (a) but the band width was narrowed by a factor 2.0 and the whole spectrum was shifted 0.5 nm towards shorter wavelengths; (c) ——, absorption difference spectrum (ferricyanide minus no additions) of the AUT-RC preparation shown in (A). (C) —— and ----: absorption spectrum of reduced and oxidized P870, respectively, of the AUT-RC shown in (A). These spectra were calculated from the spectra of reduced and oxidized Bchl (ref. 25), using the same transformations as in Fig. 2.8B(b). Note that in this analysis it is assumed that no shift of the P800 absorption band occurs concomitant with the bleaching of the P870 absorption band.

sorption, due to Bph (see Ch. IV, Fig. 4.6), P800 and reduced P870 (no additions). This spectrum has a maximum at 598 nm. The dashed line, with a maximum at 595 nm, shows the total absorption due to

Bph, P800 and oxidized P870 (ferricyanide added). The difference between these two spectra (Fig. 2.8B, solid line) has a minimum at 601 nm. These data were insufficient to resolve the spectra into absorption due to P800, oxidized P870 and reduced P870. However, as discussed already, oxidized minus reduced difference spectra (Fig. 2.6) and light minus dark difference spectra of reaction centers from Rps. spheroides (e.g. Fig. 5.5) and R. rubrum (Fig. 5.11) are rather similar to the oxidized minus reduced difference spectrum of Bchl in methanol[25]. In all these difference spectra there is an absorption decrease centering around 600 nm, and an absorption increase at shorter wavelengths. (The maximum at about 540 nm in the difference spectra of reaction centers from Rps. spheroides (e.g. Fig. 5.5) and R. rubrum (Fig. 5.11) is perhaps analogous to the maximum at about 515 nm of the difference spectrum of Bchl in methanol[25].) Because of the similarity of the difference spectra, we supposed that around 600 nm the absorption spectra of reduced and oxidized P870 would be similar to the absorption spectra of reduced and oxidized Bchl in methanol. This hypothesis led us to the analysis outlined below, in which we considered 2 alternative possibilities: (1) There is no blue shift of P800 in this region; (2) There is a blue shift of the 600 nm absorption band of P800, concomitant with a bleaching of the 600 nm absorption band of P870.

Assuming that no blue shift of the P800 absorption band occurs, we compared the oxidized minus reduced absorbance difference spectrum of an AUT-RC preparation (Fig. 2.8B, solid line) with the oxidized minus reduced difference spectrum of Bchl in methanol[25] (Fig.

2.8B, dashed line). The difference spectra obtained with Bchl in methanol and with AUT-RC preparations, could be made to correspond fairly well with each other if the following transformations were carried out with the difference spectrum of Bchl in methanol: (1) The whole difference spectrum was shifted over 0.5 nm to shorter wavelengths; (2) Without further changing the location of the minimum, the band width was narrowed by a factor 2.0 (see Fig. 2.8B). It was assumed that the absorption spectra of P870 (reduced and oxidized) can be obtained from the corresponding absorption spectra of Bchl in methanol[25] by carrying out the same transformations along the wavelength axis (see Fig. 2.8C). It was found that reduced P870 had a peak at 601 nm and a half width of 25 nm. The ratio of the peak heights of reduced P870 and reduced AUT-RC was 0.3:1. The absorption peak of P800 was obtained by subtracting the absorption of P870 and of Bph (see Fig. 4.6, Ch. IV), from the total absorption of the AUT-RC preparation. In this way an absorption band of P800 was found with a maximum at 595 nm and a half width of 31 nm (Fig. 2.8A). From the product of the absorbance at peak value and the half width, we estimated that the area under the absorption band of P800 was about 2.7 times larges than the area under the absorption band of reduced P870.

In the second approach we assumed that the P800 absorption band shifts to shorter wavelengths concomitant with the bleaching of the P870 absorption band. A good fit to all data was obtained by assuming that when P870 is reduced, the P800 absorption band and the P870 absorption band have the same shape (with a half width of 28 nm) and the

same peak location (at 598 nm), but that the P800 absorption band is two times larger than the P870 absorption band (see Fig. 2.9). This enabled us to calculate the P870 absorption band and thus, by comparison with Bchl in vitro as above, the oxidized minus reduced difference spectra of P870. The discrepancy between the calculated difference spectrum (of P870, with a minimum at 599 nm) and the observed difference spectrum (of an AUT-RC preparation, Fig. 2.8B, solid line) could

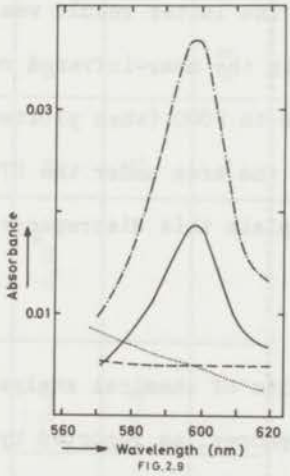

Fig. 2.9 Analysis of the 600 nm absorption band (shown in the solid line of Fig. 2.8A) into the following three components: ——: reduced P870; -.-.-: P800, as found when P870 is reduced; ----: residual absorption due mainly to Bph (from Fig. 4.6, Ch. IV). In this analysis it was assumed that at any wavelength in this region P800 absorps twice as much as P870 when the latter is reduced. This Figure shows also the spectrum of oxidized P870 (....), as calculated in a way similar to the spectra shown in Fig. 2.8C. This analysis leaves open the possibility that a blue shift of the P800 absorption band occurs concomitant with the bleaching of the P870 absorption band.

be explained largely by assuming that the absorption band due to P800 shifts 1-1.5 nm to shorter wavelengths concomitant with the bleaching of the absorption band due to P870.

Both approaches yielded the result that when P870 is oxidized about 15% of the area under the absorption band between 575 and 620 nm is due to oxidized P870, and suggested that the 600 nm absorption band due to P800 had a 2 to 2.7 times larger area than the 600 nm absorption band due to reduced P870. The latter result was somewhat surprising because it appeared that in the near-infrared region the area under the 800 nm absorption band due to P800 (when plotted on a wave number scale) was 1.2 times larger than the area under the 870 nm absorption band due to P870. We cannot explain this discrepancy.

2.3.4 Chemical data

Table 2.1 gives results of chemical analyses of reaction center preparations and chromatophores, as reported by us (first four columns), and by other authors (last four columns).

If we look at our results with Rps. spheroides (first three columns), it appears that SDS-RC particles contained on a P870 basis a smaller amount of protein and of ubiquinone than chromatophores, whereas the amount of cytochrome c, and probably also of iron (cf. Reed[41]) was not affected. Cytochrome b was present in chromatophores: a chemically induced difference spectrum, dithionite minus no additions, indicated the presence of 0.5 moles of cytochrome b per mole of P870, based on a differential extinction coefficient of $30.mM^{-1}.cm^{-1}$ in the maximum at 560 nm (refs 5,39,46). No cytochrome b could be detected in SDS-RC particles.

	Rps. spheroides			R. rubrum	Rps. spheroides R-26			R. rubrum	
	chromato-phores	SDS-RC	AUT-RC	SDS-RC	reaction centers			reaction centers	
protein	0.6	0.15-0.25**	0.12	0.11*	0.44	0.07		0.071	< 0.08
cytochrome c	0.95	0.90	0.25	0.10*	1	0		0	1
ubiquinone	8-12	3.3	1.2		13		≈ 1	0.3	1.7
iron		20*			16	0.8	< 0.23		
Reference cited	this work				44	18	19	37	51

* one determination

** six determinations

Table 2.1 Chemical composition of chromatophores and reaction center preparations from Rps. spheroides and R. Rubrum. The quantities are expressed in moles per mole of P870, except protein, which is expressed in grams per μMole of P870. The values reported in this work are an average of three determinations, unless otherwise indicated.

AUT-treatment of SDS-RC particles from Rps. spheroides resulted in the formation of two colored bands about halfway down the sucrose gradient (see section 2.2.1). Table 2.1 (3^{rd} column) gives data concerning the composition of the lower band. In this fraction the amounts of protein, cytochrome and ubiquinone were even further reduced on a P870 basis. Table 2.2 shows that the recovery yield of protein and of ubiquinone were about 80% of that of P870 in the two colored bands which were formed during the AUT-treatment, suggesting that these components were mainly particle-bound in SDS-RC fractions.

The splitting of SDS-RC fractions into two colored bands, viz. a lower one containing "purified" reaction centers and an upper one containing some P870 and relatively large amounts of protein, ubiquinone

	SDS-RC	AUT-RC (upper band)	AUT-RC (lower band)	Recovery
P 870	0.13	0.014	0.083	75 %
ubiquinone	0.34	0.11	0.10	62 %
cyt c	0.12	0.02	0.02	33 %
protein	21	3.5	10	64 %

Table 2.2 Recovery of some SDS-RC components in the two colored bands, which arose in the centrifuge tube during AUT-treatment of an SDS-RC fraction (see section 2.2.1).
Absolute amounts are expressed in µMoles, except protein which is expressed in milligrams.

and cytochrome, did not occur when Triton was omitted during the AUT-treatment. Two incompletely separated bands were found when Triton was present and urea had been omitted.

The protein content of reaction center preparations was rather variable, especially with SDS-RC preparations from Rps. spheroides in which we found values mostly between 0.15 and 0.25 g protein per µMole of P870. Values between 0.3 and 0.5 g/µMole were found in SDS-RC preparations if the CsCl centrifugation step of the chromatophores had been omitted (see section 2.2.1).

In SDS-RC preparations the absorbance at 276 nm relative to the absorbance at 867 nm was correlated to the protein/P870 ratio as determined by the Lowry method. At a protein/P870 ratio of 0.20 g per µMole of P870 we found an A 276 nm/A 867 nm ratio of 7.8. In one SDS-RC preparation we found 0.096 g protein per µMole of P870 by the Lowry method and here the A 276 nm/A 867 nm ratio was 4.6. Similar results were obtained with an SDS-RC preparation from R. rubrum (compare Fig. 2.4 and the fourth column of Table 2.1). These data are in fair agreement with data published earlier on ultraviolet absorption and protein content of reaction center preparations[41,28]. In AUT-RC particles the absorbance at 276 nm was due for a large part to the presence of Triton X-100, which could not be removed by dialysis.

Finally we may mention some experiments done in collaboration with Dr T.Beugeling and Mrs P.G.M.M. Barelds-van de Beek in order to determine the chemical nature of the pigments present in reaction centers. To this end AUT-RC particles from Rps. spheroides were dialyzed against distilled water, freeze-dried, extracted with methanol and subjected

to thin-layer chromatography. The three largest spots obtained in this way were due to Bchl (blue), Bph (purple) and carotenoid (yellow). Ubiquinone was also present. Comparison of absorbance spectra and Rf values with literature data indicated that we were dealing with Bchl a, Bph a, spheroidene and ubiquinone-10, respectively. For details see ref. 7.

2.4 DISCUSSION

Table 2.1 gives also the results of chemical determinations carried out by other authors on reaction center preparations (last four columns). The method used for the isolation of reaction centers was similar in all cases reported: chromatophores were incubated with a detergent and were then fractionated by centrifugation.

The reaction centers prepared by Reed, Raveed and Israel[44] (see also Reed[41]) are the most "complete" particles that have been obtained so far. In this case it appeared that the reaction center particles, after their detergent-induced release from the chromatophore membrane, were stripped of the light-harvesting Bchl-protein complexes and of ATP-ase, but they contained all of the remaining chromatophore protein[43], including cytochrome b and cytochrome c[41,44] (see Ch. I, section 1.3).

Other preparations, including those prepared by us, had a lower content of proteins and electron transfer components. Cytochrome b, which could not be demonstrated in our reaction center preparations, was also absent from other preparations (refs 18,37,51,19): Cytochrome c was present in some preparations (our SDS-RC particles from Rps.

spheroides, and refs 44 and 51), and absent in other preparations[18,37]. Our SDS-RC fractions from R. rubrum and AUT-RC fractions from Rps. spheroides had only a low content of cytochrome c (see Table 2.1).

Ubiquinone, which has been postulated earlier[10,6] to function as the primary electron acceptor during the photooxidation of P870, was present in most cases investigated (refs 44,19,51 and this work). A low ubiquinone content (less than 1 mole per mole of P870) has been reported in only one case[37].

Iron and magnesium were shown[18] to be the only metals present in more than trace amounts in purified reaction centers from Rps. spheroides. ESR measurements reported recently suggested that iron functions as the primary electron acceptor in Chromatium chromatophores[32]. Photoreduction of iron was also suggested by ESR measurements with reaction centers obtained by Feher[18] which contained about 1 mole of iron per mole of P870. From these particles Feher et al. prepared "iron-free" reaction centers[19], which showed photoreduction of ubiquinone in a one-electron reaction (see Ch. VI, section 6.1). Our SDS-RC particles from Rps. spheroides had a high iron content, as was also the case with the particles obtained by Reed et al.[44] (see Table 2.1). The iron content of AUT-RC particles from Rps. spheroides was not checked, but in later experiments we got indications for photoreduction of ubiquinone in all types of reaction centers, but not for photoreduction of iron (see Ch. V).

It should be noted that so far no case has been reported of reaction center particles containing neither iron nor ubiquinone. This leaves room for speculation that one of these two serves as the primary electron acceptor in vivo. (See Ch. VI).

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CHAPTER III

TRANSFER OF EXCITATION ENERGY TO P870 IN REACTION CENTERS FROM RPS. SPHEROIDES

3.1 INTRODUCTION

In this Chapter we will describe experiments on transfer of electronic excitation energy, carried out with reaction center preparations from Rps. spheroides. The transfer of excitation energy from various pigments to P870 was studied by a comparison of the efficiency of light of different wavelengths in eliciting either the bleaching (indicating oxidation) or the fluorescence of P870. Some characteristics of the fluorescence from P870 will be given, viz. (1) emission spectra under different conditions (2) kinetics of the fluorescence changes in comparison with light-induced absorbance changes and (3) the fluorescence yield and lifetime. Moreover an estimate is given of the yield and lifetime of the fluorescence from P800. The results indicate that transfer of energy from P800 to P870 may proceed by a faster mechanism than that of Försters inductive resonance transfer. The lifetime of the fluorescence of P870 was 3-8 picosec when the reaction center was photochemically active. This is difficult to reconcile with recent measurements of the lifetime of the fluorescence of chromatophores of which all reaction centers were photochemically active (50 picosec, according to refs. 5 and 6).

3.2 MATERIALS AND METHODS

The preparation of the reaction center particles was described in Ch. II, section 2.2.1. Light-induced absorbance changes were measured with a splitbeam apparatus described earlier[1]. The fluorescence measurements described in section 3.3.2 (Fig. 3.4) were also done with this apparatus. In this case a 4-side clear cuvette of 1 cm thickness was put at the place of the sample cuvette. The fluorescence was excited with weak, chopped light of 590 nm (band width 20 nm); the fluorescence yield of the sample was modified by irradiation with strong, continuous blue light. The light emitted by the sample was filtered by 2 far-red cut-off filters and a broad-band interference filter (maximum transmission at 905 nm) put in front of the photomultiplier. The photomultiplier tube was colled with ethanol of 5° C in order to improve the signal-to-noise ratio. The signal from the photomultiplier was fed into an AC amplifier (Brookdeal) which transmitted a band of 10-1000 Hz. The light-induced absorbance changes in the experiment of Fig. 3.4 were measured under similar conditions: The 1-cm cuvette was placed at sample side of the cuvette holder, and the actinic light was provided by the two light beams which served as excitation and actinic beam, respectively, in the fluorescence set-up. Both beams were continuous and the intensity of the first beam was adjusted to the same average value as used in the fluorescence set-up (where this beam was chopped).

The other fluorescence experiments described in this and the next Chapter were done with an apparatus similar to one described

earlier[12]. The excitation light, chopped at 50 Hz, was provided by a 900 W Xenon lamp or a 600 W Tungsten-Iodine lamp and passed through a Bausch and Lomb grating monochromator (1200 lines per mm grating). An image of the exit slit of the excitation monochromator was focussed upon a 1 mm glass or perspex cuvette. This image was focussed upon the entrance slit of a second, analyzing monochromator. The image of the grating of the analyzing monochromator was focussed upon the cathode of a photomultiplier with an S1 type of response which was operated at -80° C. The excitation and analyzing monochromators transmitted pass bands of 7 and 8.5 nm width, respectively. It was usually necessary to place additional filters both in the excitation beam and in the emitted beam, in order to cut off "false" light and stray excitation light, respectively. The intensity of the excitation light was measured with an RCA photocell type 925, which was calibrated against a YSI-Kettering Radiometer (model 65) of which the sensor was put in the place of the cuvette. The radiometer was calibrated against a standard thermopile. The base line for the excitation and emission spectra was provided by the signals obtained with water instead of sample in the cuvette. These signals, due to "false" excitation light, were usually less than 10% of the signals obtained with the sample in the cuvette.

The emission spectra were corrected for the wavelength-dependence of the relative sensitivity of the detecting system by putting a surface covered with powdered MgO instead of the cuvette in the sample holder. This surface was illuminated with light of a calibrated tungsten band lamp with known intensity distribution[25].

A correction for reabsorption of the emitted light was carried out using an equation as given by Duysens[11], with a modification to account for the different geometry of our apparatus. The absorbance of the samples at the maximum at 803 nm did not exceed 0.14 in a 1 mm cuvette.

3.3 RESULTS

3.3.1 The quantum yield for the photooxidation of P870

Transfer of light energy from the different pigments present in reaction center preparations to P870 was studied first by comparing the quantum efficiencies of light of different wavelengths for the P870 photooxidation. This photooxidation was illustrated already in Fig. 2.5 (Ch. II). The difference spectrum for this reaction had a minimum at about 600 nm (Ch. II, Fig. 2.6 and section 2.3.2). Assuming that the differential extinction coefficient of P870 (reduced minus oxidized) at 867 nm is 93 $mM^{-1}.cm^{-1}$ (ref. 4; see also Ch. IV, DISCUSSION), we derived a differential extinction coefficient of 16.3 $mM^{-1}.cm^{-1}$ for the oxidation of P870 at 600 nm. With actinic light of 870 nm, the initial quantum efficiency for the photooxidation of P870 was independent of the light intensity within the measured range (0.35 - 16 $nEinstein.cm^{-2}.sec^{-1}$).

The values found for the initial quantum efficiency for the photooxidation of P870 varied somewhat in different reaction center preparations, but our results were in general agreement with the data of Bolton et al.[4] and of Yau[28], who also worked with reaction center

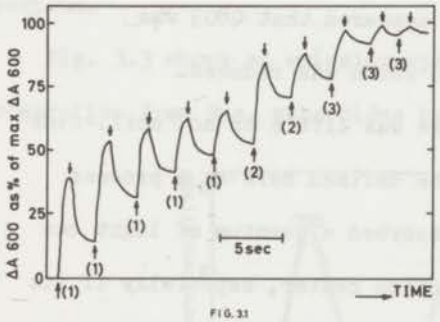
particles from Rps. spheroides. Light of 872 nm had a quantum efficiency ranging from 0.86 - 1.19. Light of 803 nm seemed to be slightly less active than light of 872 nm. In order to eliminate systematical errors involved in these calculations (errors in the estimation of the molar extinction coefficient of P870, in the measurement of the actinic light intensity, and in the calibration of the magnitude of the absorption decrease) the quantum yield of 803 nm light was expressed relative to the quantum yield of 872 nm light for the bleaching at 600 nm. Putting the quantum yield of 872 nm light equal to 1, we derived a quantum yield of 0.89 ± 0.05 for 803 nm light as an average for 7 different preparations. This is taken to be the efficiency of the energy transfer from P800 to P870.

Bacteriopheophytin was present in all reaction center preparations studied so far. The amount was rather variable, but the minimum amount (observed repeatedly) was such that the ratio of the absorbancies at 756 and 867 nm was approximately 0.9 : 1. In preparations containing such a low amount of Bph, light absorbed by Bph was transferred to reaction center Bchl with a high efficiency. Light of 764 (absorbed mainly by Bph), 803 and 872 nm had a relative quantum efficiency of 1.21 ± 0.07, 0.87 ± 0.06, and 1, respectively, for the bleaching at 600 nm (average for three different preparations). The low efficiencies measured by Yau[28] at about 760 nm were probably due to contaminations which absorbed in that region and did not transfer energy to P870 (see DISCUSSION and Ch. IV).

The question arose whether transfer of excitation energy was possible between pigment molecules within one reaction center only,

or wether energy could be transferred from one reaction center to another. To answer this question we studied the initial quantum yield of 803 nm light (Q803) for the photooxidation of P870 as a function of the fraction of P870 which was reduced at the time of the onset of the actinic light.

Fig. 3.1 shows schematically how the experiment was carried out. The dark decay of the light-induced absorbance change was biphasic;



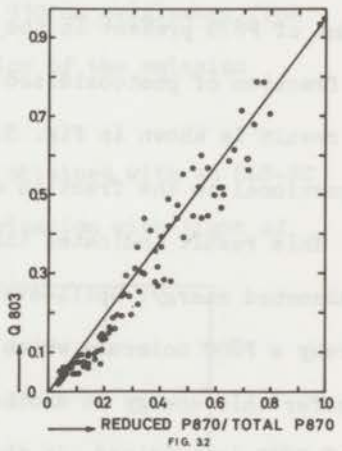


Fig. 3.1 SDS-RC preparation from Rps. spheroides suspended in Tris-buffer. No additions. Schematic representation of the kinetics of the 600 nm absorbance change caused by actinic light of 803 nm (band width about 15 nm). Upward and downward pointing arrows indicate when the light was switched on and off, respectively. Actinic intensity, (1) 1.0 nEinstein, (2) 3.1 nEinstein, and (3) 6.0 nEinstein.cm^{-2}.sec^{-1}.

Fig. 3.2 Initial quantum yield of 803 nm light for the photooxidation of P870 (measured at 600 nm) as a function of the fraction of P870 which was reduced at the time of the onset of the light (see text).

the reason for this will be discussed in Ch. V. By choosing the dark time after a light period long enough, it was possible to obtain a reliable base line for the slope of the subsequent light-induced absorbance change. From this slope we determined Q803. It was confirmed (1) that the amount of actinic light absorbed did not depend on the redox state of P870; (2) that the maximum light-induced bleaching obtained in this way corresponded to the total amount of P870 present in the preparation; this enabled us to obtain the fraction of photooxidized P870 from the abscissa of Fig. 3.1. The result is shown in Fig. 3.2. It appeared that Q803 was proportional to the fraction of P870 which was reduced.

This result indicates that there was little or no "spill-over" of absorbed energy. Spill-over may be defined here as a process whereby a P800 molecule which has absorbed a quantum of light can transfer this energy to another reaction center, especially if its "own" P870 is oxidized. In that case light absorbed by P800 associated with oxidized P870 would also have some effect in photo-oxidizing P870, and the result of Fig. 3.2 would have been a curved line, with all values of Q803, except those at the extremes (at $P_{red}/P_{tot} = 0$ and 1, respectively) lying above the diagonal of Fig. 3.2.

Thus, the fact that Q803 was directly proportional to the fraction of reaction centers in which P870 was reduced at the time of the onset of the actinic light, indicates that there was no measurable energy transfer between different reaction centers, suggesting that each P800 molecule was coupled tightly to its "own"

P870. Measurements of circular dichroism[22] suggested a strong interaction between specific P800 and P870 molecules, and seem therefore in agreement with this notion.

3.3.2 Emission spectra and kinetics of the fluorescence from P870

Before proceeding to give excitation spectra for the fluorescence from P870 (see section 3.3.3) we will show in this section that a fluorescence band centered around 910 nm originates from P870, and we will give some characteristics of the emission spectrum.

Fig. 3.3 shows an emission spectrum obtained with an SDS-RC preparation from Rps. spheroides by illumination with light of

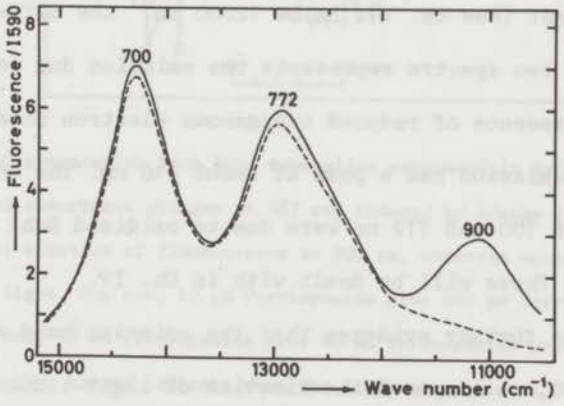


Fig. 3.3 Fluorescence emission spectra of an SDS-RC preparation from Rps. spheroides suspended in Tris buffer. ----- : No additions. ——— : with 15 mM ascorbate. Excitation light, 370 nm, intensity 8.4 nEinstein.cm^{-2}.sec^{-1}. For the ordinate scale see the legends to Fig. 3.9.

370 nm. Without additions (dashed line) P870 was kept photooxidized by the excitation light. The tail which is seen below about 12000 cm^{-1} when P870 is oxidized (dashed line) is probably due to a degradation product with an emission maximum at 860 nm. The excitation spectrum for the fluorescence of this impurity has peaks at about 845 nm, 760, 690 and 590 nm, and also small carotenoid peaks at 510, 475 and 445 nm (Slooten, unpublished). The location and relative magnitude of these peaks indicated that the tail in the emission spectrum below 12000 cm^{-1} was not due to photooxidized P870. It is suggested that it was due to reaction centers with degraded pigments. In the presence of 15 mM ascorbate (solid line) both P870 and the primary acceptor were reduced in the excitation light (see Ch. V). Below 12000 cm^{-1} the difference between these two spectra represents the emission due to reduced P870 in the presence of reduced endogenous electron acceptors (see below). This emission has a peak at about 910 nm. The emission peaks with maxima at 700 and 772 nm were due to oxidized Bchl and to Bph, respectively. These will be dealt with in Ch. IV.

To obtain further evidence that the emission band around 910 nm is due to P870, we compared the kinetics of light-induced absorbance changes (at 867 nm) and fluorescence changes (at 905 nm) in an AUT-RC preparation from Rps. spheroides. These measurements were all done with the apparatus normally used for the measurement of light-induced absorbance changes as described in section 3.2. The results are shown in Fig. 3.4. In the presence of 40 µM ferricyanide and 400 µM ferrocyanide all reaction centers were in a state with

reduced P870 prior to the onset of the orange light (as indicated by the absorbance spectrum). Weak orange light caused a partial bleaching of P870, which reached a steady state after about 20 sec (top left part of Fig. 3.4). The blue light which was switched on subsequently caused a complete bleaching of P870 after 15 sec of

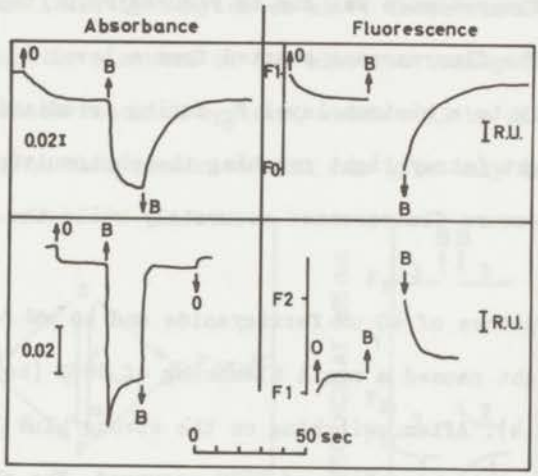


Fig. 3.4 AUT-RC preparation from Rps. spheroides suspended in Tris buffer. Left: kinetics of absorbance changes at 867 nm, induced by orange (O) and blue (B) light. Right: kinetics of fluorescence at 905 nm, observed upon excitation with orange (O) light. Top row, 40 µM ferricyanide plus 400 µM ferrocyanide present. Bottom row, 40 µM ferricyanide plus 40 mM ferrocyanide present. Upward and downward pointing arrows indicate when the light was switched on and off, respectively. The time scale is indicated at the bottom of the Figure. Vertical bars (left column) indicate an absorbance change of 0.02 OD units.The fluorescence measurements are all on the same scale as indicated by the vertical bar representing a R(elative)U(nit). The average intensity of the orange light (580 - 600 nm, selected with a Schott 596 AL filter) was 0.42 nEinstein.cm^{-2}.sec^{-1}. The intensity of the blue light (selected with Corning CS 4-97 and CS 4-76 filters) was about 20 nEinstein.cm^{-2}.sec^{-1}. Further details see section 3.3.2.

illumination. After switching off the blue light the bleaching decayed with a half time of 6 sec to the level occurring prior to the onset of the blue light. The kinetics of the 905 nm fluorescence elicited by orange light were very similar to the kinetics of the light-induced absorbance changes, indicating that the variable part of the 905 nm fluorescence was due to reduced P870 (top right part of Fig. 3.4). The fluorescence started from a level F_1 and decreased probably to a minimum level F_o during irradiation with strong blue light (stray light reaching the photomultiplier did not permit us to measure fluorescence accurately while the actinic blue was on).

In the presence of 40 µM ferricyanide and 40 mM ferrocyanide weak orange light caused a small bleaching of P870 (bottom left part of Fig. 3.4). After switching on the strong blue light a large, but still incomplete bleaching of P870 occurred. The absorbance at 867 nm attained a minimum value after about 1 sec of illumination and increased subsequently to a somewhat higher level while the light was still on. After switching off the blue light the bleaching decayed within a second to the level occurring prior to the onset of the blue light.

On the other hand, the 905 nm fluorescence elicited by orange light, which started from the same level F_1 as above, increased slightly during the irradiation with orange light (bottom right part of Fig. 3.4). The fluorescence increased to an even higher level during illumination with blue actinic light (not shown) and, after switching off the blue light, decayed from a level F_2 to a lower level, with a half time of 3 sec.

In the presence of 10 mM dithionite light-induced absorbance changes did not occur and the 905 nm fluorescence elicited by orange light was high (at a level F_3 in Fig. 3.6) and was not modified by irradiation with strong blue actinic light.

Similar results were obtained earlier by Zankel et al.[29] and Reed et al.[21], and later by Clayton et al.[9]. These results give additional support for a scheme of the electron transport cycles operating in isolated reaction centers, which will be discussed in detail in Ch. V. For this reason we will now give only an ad hoc

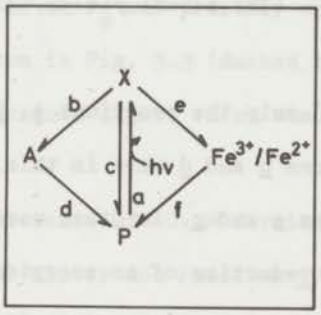


Fig 3.5

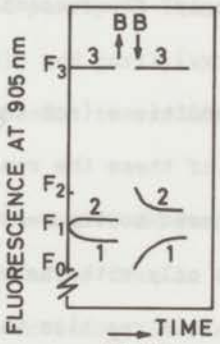


Fig 3.6

Fig. 3.5 Reaction scheme explaining the results shown in Fig. 3.4. Arrows indicate the direction of the electron transport. The light quantum is indicated by hν. R The letters a, b, c and d have been used here as in Ch. V (Fig. 5.3). Further details see text.

Fig. 3.6 Schematic representation of the fluorescence kinetics discussed in connection with Fig. 3.4. The orange excitation light is switched on at zero time. B, blue actinic light. Curve (1), 40 μM ferricyanide and 400 μM ferrocyanide; curve (2), 40 μM ferricyanide and 40 mM ferrocyanide; curve (3), 10 mM dithionite.

explanation (see Fig. 3.5). In this scheme, P is P870 and X and A are endogenous electron acceptors. P can be oxidized to P^+ and X and A can be reduced to X^- and A^-. As far as we know (see Ch. V and VI), the primary reaction is the photoreduction of X by P870 (reaction a). As shown above (see also Fig. 3.3), oxidized P870 does not emit fluorescence at 905 nm. It has been shown[21,9] that reduced P870 in reaction centers in the state PX is "weakly" fluorescent because fluorescence competes with photochemical activity. Reaction centers in the state PX^- are photochemically blocked and therefore P870 is "strongly" fluorescent[21,9]. (The actual yields will be discussed below.)

Without additions (not shown) only the reactions a, b, c and d occurred, and of these the reactions b and d were in this preparation very slow compared to the reactions a and c. In other words we were dealing almost only with the photoreduction of an acceptor X by P870 (reaction a); this reaction was reversible in the dark (reaction c). Reaction c has a half time of about 0.2 sec. For further details see Ch. V.

In the presence of 40 μM ferricyanide and 400 μM ferrocyanide, reactions a, e and f occurred. In this case, reaction e ($P^+X^- + Fe^{3+} \longrightarrow P^+X + Fe^{2+}$) was apparently faster than reaction c ($P^+X^- \longrightarrow PX$), as indicated by the absence of a decay component with a half time of about 0.2 sec, whereas reaction f was still rather slow (half time 6 sec). As a result, reaction centers in the state P^+X^- did not accumulate under our experimental conditions and the net reaction was a photoreduction of ferricyanide by P870 (reaction a

and e) which was reversible in the dark (reaction f). This explanation was supported later by measurements of light-induced absorbance changes at 325 nm under the same conditions (compare Ch. V). The results were similar to those shown in the middle row of Fig. 5.1. The fluorescence, coming only from reaction centers in the state PX in this case, was proportional to the fraction of P870 which was reduced. Our results indicated that F_1 and F_o were fluorescence levels observed when all reaction centers were in the state PX and P^+X, respectively (top right part of Fig. 3.4). The invariable fluorescence level F_o was probably caused partly by the spectral component shown in Fig. 3.3 (dashed line), and partly by false light, or by stray actinic light, which was difficult to exclude in these measurements.

In the presence of 40 μM ferricyanide and 40 mM ferrocyanide reaction e was slower and reaction f was faster than in the former case. After P870 had photoreduced X in reaction a, it was itself rereduced by ferrocyanide (reaction f), so that in the light an equilibrium was established where all reaction centers were in the state PX, PX^- or P^+X^-. The fluorescence level observed in this mixture was higher than the level F_1, observed when all reaction centers were in the state PX (as was the case prior to the onset of the orange light). After switching off the blue light reaction a became much slower and the state PX was largely restored. The fluorescence decrease observed after switching off the blue light was probably due to the reaction PX^- + ferricyanide $\longrightarrow$ PX + ferrocyanide.

After addition of dithionite all reaction centers were in the state PX^- prior to the onset of the orange light. As a result the fluorescence was at an invariant high level F_3.

The conclusions from these experiments were that the "variable" fluorescence centered around 910 nm originates from reduced P870, and that, in accordance with earlier results[21,9], reaction centers in the state PX^- have a higher fluorescence yield than reaction centers in the state PX. (See Fig. 3.6). We found that $F_3 - F_o$ (reflecting the fluorescence yield of PX^-) was 6-8 times higher than $F_1 - F_o$ (reflecting the fluorescence yield of PX). From these numbers one might conclude (see e.g. refs. 9,18) that the quantum yield of light absorbed by P870 for the primary photochemical reaction in reaction centers in the state PX was 0.84-0.88 in this preparation. Since, however, the addition of dithionite caused the preparation to become slightly more turbid, it is possible that the high fluorescence level F_3 was caused partly by an increase of F_o, due to an increased amount of false light or stray actinic light.

The shape of the emission spectra of reaction centers in the state PX and in the state PX^-, respectively, was the same within the limits of accuracy, as is shown in Fig. 3.7. The left hand part shows the kinetics of the decrease in emission of 920 nm light, as observed in an SDS-RC preparation, suspended in Tris-buffer, upon excitation with light of 510 nm. Without additions the fluorescence yield decreased from a level F_1' to F_o. The variable part of the fluorescence in this case arose from reduced P870 in the presence of oxidized electron acceptors, and the fluorescence decrease

corresponded probably with phase b of the photooxidation of P870, as defined in Ch. V, section 5.3.1 and Fig. 5.1. (The time constant of our apparatus was too slow to allow detection of the fluorescence transient corresponding with phase a of the P870 photooxidation.) In the presence of ascorbate the fluorescence increased to a level F_3. As discussed earlier in this section the emission difference

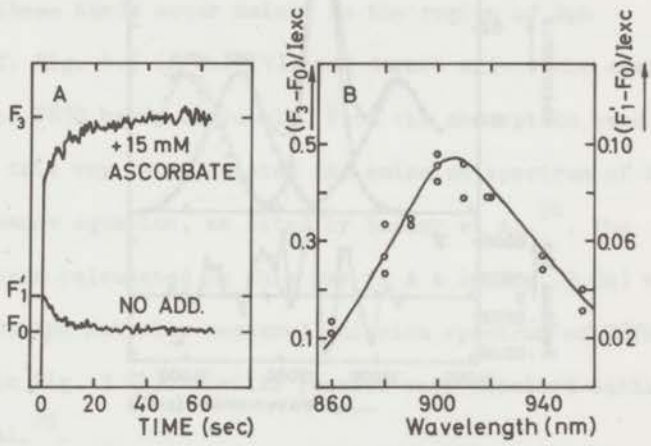


Fig. 3.7A Kinetics of the fluorescence changes measured at 920 nm upon irradiation of an SDS-RC preparation with light of 510 nm (intensity 31 nEinstein.cm^{-2}.sec^{-1}). The time constant of the detecting system was 0.5 sec.

Fig. 3.7B Same preparation. Solid line: Emission difference spectrum (reduced minus oxidized) obtained by taking the difference between the emission spectra as measured in the presence and in the absence of 15 mM ascorbate (left hand scale). Excitation with light of 510 nm, intensity 31 nEinstein.cm^{-2}.sec^{-1}. Open points: Spectrum of the variable part of the fluorescence ($F'_1 - F_o$) as observed without additions (right hand scale). Excitation with light of 510 nm, intensity 29 nEinstein.cm^{-2}.sec^{-1}. See the legend to Fig. 3.9 for the ordinate scales.

$(F_3 - F_o)$ arose from reduced P870 in the presence of reduced electron acceptors. Fig. 3.7B shows that the emission difference spectra corresponding with $(F_1' - F_o)$ and $(F_3 - F_o)$, respectively, had the same shape, within the limits of accuracy.

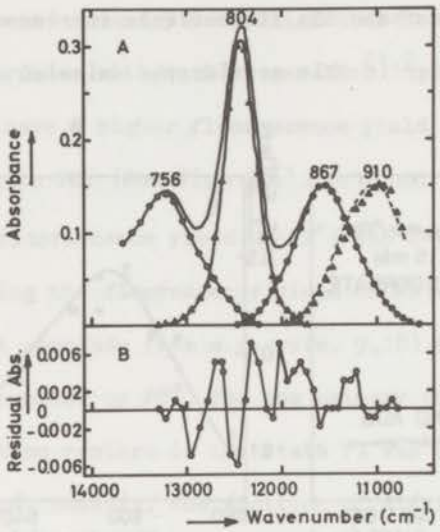


Fig. 3.8A Solid line: Absorbance spectrum of an SDS-RC preparation from Rps. spheroides plotted on a wavenumber scale. Maxima have been indicated in nm. This spectrum was analyzed into three components, viz. O——O : Bph; Δ——Δ : P800; and ●——● : P870. Dashed line: Emission spectrum of P870 (as PX^-) obtained by taking the difference in emission spectra as observed prior to, and after addition of 15 mM ascorbate. Excitation light, 500 nm, intensity 30 nEinstein.cm^{-2}.sec^{-1}. ▲▲▲ : Emission spectrum of P870 calculated from the absorption band of P870 using the Stepanov equation (see text). The emission spectra were adjusted arbitrarily to the same peak values as that of the P870 absorption band.

Fig. 3.8B Residual absorption remaining after the analysis of the absorbance spectrum as explained in Fig. 3.8A.

Fig. 3.8A (solid line) shows the absorbance spectrum of an SDS-RC preparation from Rps. spheroides plotted on a wave number scale. This spectrum was analyzed by hand into three components, P870, P800 and Bph, assuming that the bands were symmetrical. This analysis is incomplete in that there are in the near-infrared region probably also weak satellite bands due to P800 and P870, but we suppose that these bands occur mainly in the region of Bph absorption (cf. Fig. 4.6 in Ch. IV), and do not affect the analysis of the P800 and P870 bands seriously. From the absorption band of P870 found in this way we calculated the emission spectrum of P870, using the Stepanov equation, as cited by Szalay et al.[24]. The emission spectrum calculated in this way (▲ ▲ ▲ in Fig. 3.8A) was very similar to the actually measured emission spectrum of P870 (dashed line in Fig. 3.8A). Similar results were obtained earlier by Zankel et al.[29].

3.3.3 Excitation spectrum for the P870 fluorescence

Transfer of light energy from the different pigments present in reaction center preparations to P870 was studied also by measuring an excitation spectrum for the fluorescence at 920 nm due to reduced P870.

Fig. 3.9 (upper curve) shows the absorbance spectrum of the SDS-RC preparation of which emission spectra were shown in Fig. 3.3. See Ch. II, section 2.3.1 for a discussion of a similar spectrum. The preparation shown here was somewhat turbid and the zero line runs probably between the indicated extremes. This approximation

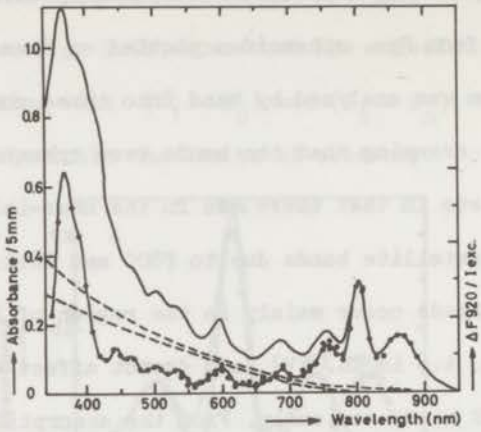


Fig. 3.9 ——— : Absorbance spectrum of an SDS-RC preparation. ----- : Extreme values suggested for the base line of the absorbance spectrum (see text). Open symbols: Differential excitation spectrum for the fluorescence of P870 (in the state PX^-), measured with the same preparation, at 920 nm (see text). Solid symbols: Same, but with another SDS-RC preparation. The excitation spectra match the absorbance spectrum at 800 nm. The vertical scales in Figs. 3.3, 3.7B and Fig. 3.9 (fluorescence intensity divided by the intensity of the excitation light at unit concentration of P870), are all expressed in the same relative units. The same scale applies also to Figs. 4.1 - 4.5 of the next Chapter.

is based on the experience that in clear preparations the absorbance at 950 nm is zero and the absorbance at the 600 nm maximum is 3 times higher than the absorbance at 640 nm and 565 nm. Moreover the absorbance spectrum of a clear preparation is almost horizontal between 620 and 650 nm.

To obtain with this preparation the excitation spectrum for the fluorescence of reduced P870 in reaction centers in the state PX^-, we took the difference in emission at 920 nm as observed upon irradiation with excitation light before and after addition of 15 mM ascorbate, respectively (compare Fig. 3.3). The result is shown

in the open and solid circles of Fig. 3.9. The open symbols refer to measurements with the preparation represented by the absorbance spectrum of Fig. 3.9. The solid symbols are points measured with another SDS-RC preparation.

As shown by a comparison of the absorption and excitation spectra of Fig. 3.9, light absorbed by P800 at 800 nm was transferred to P870 with an efficiency close to 1. Light of around 760 nm was transferred to P870 with an efficiency of 0.8-0.9. This lower efficiency is attributed to the presence of non-transferring contaminations (see Ch. IV), which account for 10-20% of the light absorbed in this region. The remaining light absorption in this region is due mainly to reaction center Bph (see DISCUSSION), which transfers energy to P870 with an efficiency close to 1, as shown in section 3.3.1. The peak at 690 nm suggests the presence of a substance designated as S690, which absorbed light of 690 nm and transferred energy to P870. The excitation spectrum of Fig. 3.9 does not show a peak in the blue region attributable to S690. However, excitation spectra for the fluorescence of 700 and 710 nm had a maximum at 685 nm and another maximum at 420 nm, which was larger than that at 685 nm (see Fig. 4.1, Ch. IV). This was ascribed to the presence of a substance called P690; apparently P690 did not transfer energy to P870, whereas S690 did.

Fig. 3.9 shows that light absorbed by carotenoids was also transferred to P870, with an efficiency which was estimated to be at least 0.7.

The excitation spectrum shown in Fig. 3.9 was, apart from the

peak at 690 nm, practically identical with the absorbance spectrum of an AUT-RC preparation, shown in Fig. 2.2 (Ch. II). Excitation spectra for the fluorescence of P870 were similar in AUT-RC and SDS-RC preparations, except that in the former S690 or P690 were practically absent (cf. Ch. II, section 2.3.1).

3.3.4 The fluorescence yield and fluorescence lifetime of P870 and P800

The fluorescence yield of P870 was measured in an SDS-RC preparation from Rps. spheroides by comparison with chromatophores from Rps. spheroides, in which P870 was kept photooxidized by the excitation light. Both measurements were done with excitation light of 590 nm, intensity 30 nEinstein.$cm^{-2}.sec^{-1}$. The reaction centers were brought in the state PX^- by addition of 10 mM dithionite. Assuming that the fluorescence yield of chromatophores is 5% at high light intensities[27,17] we derived a fluorescence yield of 2.5×10^{-3} for P870 in reaction centers in the state PX^-. At another occasion, using a different batch of SDS-RC particles from Rps. spheroides we found a fluorescence yield of 1.0×10^{-3} for P870 in reaction centers in the state PX^-, indicating that the fluorescence yield of P870 (as PX^-) varied from preparation to preparation. This was also apparent by direct comparison of two batches of reaction centers from Rps. spheroides. A ratio of 0.4:1 was found for the fluorescence yield of P870 (as PX^-) in these preparations. The reason for these variations is not known.

Assuming that the ratio of the fluorescence yields of P870 in

reaction centers with oxidized and reduced electron acceptors, respectively, is 0.2:1 (cf. refs. 29 and 9 and section 3.3.2 and Fig. 3.6 of this Chapter) we derived a value of $2 - 5 \times 10^{-4}$ for the fluorescence yield of P870 in the presence of oxidized electron acceptors.

The application of the Stepanov formula allowed us to estimate the emission spectrum of P800 from the absorbance spectrum of P800 shown in Fig. 3.8A. The result was an emission band with a peak at 814 nm (10 nm further than the absorption maximum) and a halfwidth of 30 nm (470 cm^{-1}). Unexpectedly, such an emission band was not detected, not even when P870 was oxidized. From Fig. 3.3 and other similar emission spectra we estimated that the magnitude of the peak of the P800 emission band was less than 0.04 times the magnitude of the peak of the P870 emission which was observed in the presence of strong reductants. Since the estimated half width of the P800 emission band was about 2 times smaller than the measured halfwidth of the P870 emission band (820 cm^{-1}), this meant that the total emission from P800 was less than 0.02 times the total emission from P870 in the presence of strong reductants.

Now if P870 is reduced, light absorbed by P870, P800 and Bph contributes to the P870 fluorescence. It was assumed that if P870 is oxidized, only light absorbed by P800 and Bph contributes to the P800 fluorescence. The separate absorbance spectra of P870, P800 and Bph in the region of 600 nm have been calculated in Ch. II, section 2.3.3. Using excitation light of 596 nm (band width 20 nm)

we concluded that the fluorescence yield of P800 was less than 4.10^{-5} even when P870 was oxidized.

The fluorescence lifetime can be calculated by multiplying the intrinsic lifetime, τ_o, (occurring if the fluorescence yield is 100%) with the actual fluorescence yield. The intrinsic lifetime τ_o can be computed by a formula derived by Strickler and Berg[23]:

$$\frac{1}{\tau_o} = n^2 . \frac{2.88 \times 10^{-9}}{(k^{-3})_{av}} . \frac{g_l}{g_u} . \int \left(\frac{\varepsilon_k}{k} \right) . dk$$

where n is the refractive index of the medium at the mean wavelength of the emission band, g_l and g_u are the degeneracies in the upper and lower state respectively, ε_k is the extinction coefficient ($M^{-1}cm^{-1}$) at a particular wavenumber k (cm^{-1}), and

$$(k^{-3})_{av} = \frac{\int k^{-3} . I_f . dk}{\int I_f . dk}$$

where I_f is the fluorescence intensity in quanta per unit frequency range.

For these calculations we used the absorption band of P800 as observed when P870 is reduced (with a maximum at 804 nm). Apart from a shift of 4 nm to shorter wavelengths, the results would probably have been about the same if the P800 absorption band (maximum at 800 nm) corresponding with oxidized P870 had been taken. For the computation of $(k^{-3})_{av}$ we took the P800 emission spectrum calculated by means of the Stepanov equation (see above). The maximum extinction coefficient of P800 was taken to be 136000 $M^{-1}.cm^{-1}$ at 804 nm (ref. 4). In the case of P870 we took the

actually measured emission spectrum (see Fig. 3.8A) for the computation of $(k^{-3})_{av}$; the maximum extinction coefficient of P870 was taken to be 113000 $M^{-1}.cm^{-1}$ (ref. 4)(see however Ch. IV for a discussion of these extinction coefficients). With $g_l/g_u = 1$, and assuming that n = 1.33, we found values of 17 and 21 nsec for the intrinsic lifetime of the fluorescence of P870 and P800, respectively. The value for P870 corresponds well with the value of Zankel et al.[29], who found 18 nsec. However, these authors omitted n^2 from their reproduction of the formula of Strickler and Berg. Since Zankel et al.[29] started from practically the same extinction coefficients and emission spectra as used by us, we presume that they also used a value of 1.33 for the refractive index in their calculations.

From the known fluorescence yields of P870 (as PX^-), P870 (as PX) and P800 (see above), and from the intrinsic lifetimes of the fluorescence from P870 and P800, we found actual lifetimes of 17-42 picosec for the fluorescence of P870 in the state PX^-, 3-8 picosec for the fluorescence of P870 in the state PX, and less than 0.8 picosec for the fluorescence of P800. The latter value is probably the time within which energy is transferred to P870 since this transfer occurs with high efficiency (see section 3.3.1 and 3.3.3). It should be noted however, that the very short lifetime of the fluorescence from P800 may be in conflict with one of the assumptions on which the calculation was based, namely, that thermal relaxation of the vibration levels of the excited state of P800 has been established at the time when emission takes place. This

assumption is implicit in the application of the Stepanov equation for the calculation of the emission spectrum of P800.

3.3.5 Distance between P800 and P870

The data obtained in section 3.3.1 and 3.3.4 were used to estimate the distance R between P800 and P870. To this end it was assumed (1) that the energy is transferred from P800 to P870 via dipole-dipole interaction, and (2) that the vibration levels of the excited state of P800 are thermally relaxed at the time when the transfer takes place. With these assumptions the Förster equations[15] for inductive resonance transfer, in which the rate of energy transfer varies as R^{-6}, may be applied. These equations were applied earlier to photosynthesis by Duysens[11]. It should be noted however, that the second assumption is somewhat doubtful, as we saw in section 3.3.4, and it is possible that a faster type of energy transfer than that of inductive resonance occurs from P800 to P870. We did not attempt to calculate the distance between P800 and P870 for such a faster type of energy transfer, but it may be estimated roughly that the distance between P800 and P870 is smaller than 20 Å (ref. 8).

However if it is allowed to apply the Förster equations, a more specific estimate can be made as follows. The distance R_o between two molecules which transfer energy to each other via the Förster mechanism is defined as the distance at which the rate of de-excitation by energy transfer (K_t) equals the rate of de-excitation by fluorescence (K_e). Then

$$\frac{K_t}{K_e} = \left(\frac{R_o}{R}\right)^6$$

in which R is the actual distance between the molecules. In the case of energy transfer from P800 to P870, K_t and K_e are properties of P800; their ratio was determined from (cf. ref. 11):

$$\frac{K_t}{K_e} = \frac{Q}{(1 - Q)\phi}$$

in which Q is the efficiency of the energy transfer from P800 to P870 and ϕ is the fluorescence yield of P800 in the absence of energy transfer. With $\phi < 4.10^{-5}$ (see section 3.3.4) and Q=0.89 (average of section 3.3.1) we found that $R < 0.13\ R_o$. It should be noted that in the above mentioned relation between K_t, K_e, Q and ϕ it is assumed that the internal conversion processes of the excited state of P800 have an average rate constant which is independent of whether P870 is reduced or oxidized, and that P800 does not transfer energy to oxidized P870. We will return to this below.

The distance R_o was calculated from the overlap between the emission band of P800 (calculated with the Stepanov equation, see section 3.3.2) and the absorption band of P870, using the Förster equation (Eqn. 9.1 in ref. 11). This equation contains factor k^2 which depends on the position of the red dipoles of P800 and P870 relative to one another. Fluorescence polarization measurements (ref. 14 and own observations) indicate an angle of approx. 25^o between the red dipoles of P800 and P870. This means that k^2 may vary from 0.81 to 3.24 and so R_o varies from 77 to 94 Å and $R < 10$ Å if the red dipoles of P800 and P870 are situated opposite

to one another; R < 12 Å if the red dipoles of P800 and P870 are in line.

It is difficult to understand why the fluorescence yield of P800 is so low (less than 4.10^{-5}) when P870 is oxidized. Dimer formation can be the cause of lowering of the fluorescence yield, but circular dichroism measurements[22] indicated no interaction between the two molecules of P800 when P870 is oxidized. One might suppose that the fluorescence from P800 is quenched not only by reduced P870, but also by oxidized P870. In that case one has to use another relationship to calculate the ratio of K_t/K_e. This is

$$\frac{K_t}{K_e} = \left(\frac{R_o}{R}\right)^6 = \frac{Q}{\phi'}$$

in which ϕ' is the fluorescence yield of P800 in the presence of energy transfer. Then one arrives at a higher upper limit for R, namely, $R < 0.18\ R_o$ but on the other hand, in that case the oxidized P870 has to be at a very short distance of P800 anyway to quench the fluorescence from P800 within 0.8×10^{-12} sec.

A further correction may be necessary because the simple dipole approximation is not correct at distances that are comparable to the dimensions of the system of conjugated bonds of the individual molecules. In summary, the assumption of "slow" energy transfer (see above) leads to the result that P800 transfers its energy to P870 along a distance which may be less than 10-12 Å. Again, this result may be inconsistent with the assumption of "slow" inductive resonance transfer. Our results are not inconsistent with the conclusion reached by Sauer et al.[22] on the basis of circular dichroism

measurements, that 1 molecule of P870 and 2 molecules of P800 form a trimer when P870 is reduced.

3.4 DISCUSSION

All reaction center preparations made so far from Rps. spheroides and R. rubrum contain Bph. We have seen in section 3.3.1 and 3.3.3 that energy absorbed by reaction center Bph (see below) is transferred to P870 with an efficiency approaching 1. One may ask whether Bph is a degradation product formed as a result of the procedure followed in isolating the reaction center, or whether it occurs as such in the reaction center in vivo. Evidence in favor of the latter idea can be collected from the literature. Firstly, the absorption spectrum of chromatophores of Rps. spheroides strain R-26 exhibits shoulders at about 760 and 800 nm; these shoulders are absent in chromatophores depleted of reaction centers[20]. Secondly, in the same organism, the action spectrum for the photooxidation of P870 exhibits shoulders at about 760 and 800 nm (ref. 10); these shoulders are absent in the action spectrum for the fluorescence of "light-harvesting" Bchl (refs. 10,27,13). These data indicate that Bph and P800 occur as such in the reaction center in vivo and that these pigments transfer energy to P870 rather than to "light-harvesting" Bchl.

We will show however in Ch. IV that distinction should be made between two kinds of Bph: firstly reaction center Bph, which is part of the reaction center in vivo and transfers energy to P870,

and secondly "free" Bph, which arises supposedly as a degradation product from bulk Bchl during the preparation of reaction centers and does not transfer energy to P870. Reaction center Bph occurs in a fixed ratio to P870, which is exemplified by the absorbance spectrum shown in Fig. 2.2 (Ch. II). "Free" Bph is present in variable amounts, but in our hands it does not exceed 1/10 or 1/5 of the amount of reaction center Bph, judging from absorbance spectra. It has however a high fluorescence yield, in contrast to reaction center Bph (see Ch. IV).

The quantum efficiency of light absorbed by P800 for the photooxidation of P870 was proportional to the fraction of P870 which was reduced. This suggests that each P800 molecule transfers energy to its "own" P870 only. This follows also from the conclusion obtained by Sauer et al.[22], that P800 and P870 form a trimer (2 P800 and 1 P870 molecules, according to these authors. However see Ch. IV for a discussion of these numbers).

The lifetime calculated for the fluorescence of P800 was shorter than 0.8 picosec. This is probably the time within which energy is transferred to P870. The calculated distance between P800 and P870 was 12 Å or less. Both values are probably only approximations because the results are in conflict with an assumption on which the calculations are based, namely, that thermal relaxation of the vibration levels of the excited state of P870 occurs prior to energy transfer.

A similar situation may exist in chromatophores. Borisov and Godik[5,6] found that the fluorescence of chromatophores of several

species, including Rps. spheroides, had two components with different lifetimes, viz. a non-variable "background" component with a lifetime of the order of 1 nanosec and a variable component of which the lifetime increased with the fluorescence yield, ranging from 50 picosec when all reaction centers were in a "trapping" state (see below), to 500 picosec when all reaction centers were in a state with oxidized P870[5,6]. Apparently, when all reaction centers in the chromatophores are photochemically active (i.e. with P870 reduced and the primary electron acceptor oxidized), the absorbed energy is transferred within 50 picosec to the reaction center. Consequently, according to the authors, the time within which energy is transferred from one bulk Bchl molecule to another is "comparable with lattice relaxation times"[5,6]. This will be considered below.

An absorbed quantum of light remains during a time t_m in a photosynthetic unit before it is trapped in a reaction center. During this time t_m it makes a number of "jumps" among bulk Bchl molecules before it encounters a reaction center. We assume that if all reaction centers are photochemically active, the quantum efficiency for the primary photochemical reaction approaches 100%. It is also assumed that the excitation energy has a probability approaching 1 of being trapped upon encountering a photochemically active reaction center. Under these conditions the number of jumps, n, which the excitation energy made from one bulk Bchl molecule to another before it encountered a reaction center, is determined by: $n = t_m/t_{jk}$, where t_{jk} is the jump time of excitation energy from the j^{th} to the k^{th} bulk Bchl molecule. Once arrived in the reaction center, the

excitation energy remains there during a time t_t before trapping occurs: trapping is defined here as the excitation energy dependent transition of the reaction center pigment to a state from which no back transfer of excitation energy occurs. The high trapping probability which was assumed above requires that $t_t \ll t_{rj}$, where t_{rj} is the time within which energy is transferred back from the reaction center to a nearby bulk Bchl molecule. This requirement reduces to $t_t \ll t_{jk}$ if it is assumed that t_{rj} is approximately equal to t_{jk}. In Rps. spheroides the latter assumption ($t_{rj} \sim t_{jk}$) seems reasonable in view of the similarity of the absorption and emission spectra of P870 and the bulk Bchl species B870 (see Table 3.1). In isolated reaction centers, t_t is indicated by the lifetime of the fluorescence from P870 in the state PX, and we have seen in section 3.3.4 that this was 3-8 picosec (cf. ref. 29). However, in view of the requirement that $t_t \ll t_{jk}$, a value of 3-8 picosec for t_t in chromatophores is difficult to reconcile with the short jump time t_{jk} (1.4-2 picosec) which follows from t_m = 50 picosec (refs. 5,6) if the equations derived* by Bay and Pearlstein[3] are applied. There are several explanations for this discrepancy, as enumerated below.

(1) The formulas derived by Bay and Pearlstein[3] were based on random

*) These equations, obtained by a "delocalized" treatment of the transfer of excitation energy in chloroplasts, apply to separate units and give t_m/t_{jk} as a function of the size of the photosynthetic unit, N. Thus, in a 2-dimensional array of bulk Bchl molecules[3], $t_m/t_{jk} = 1.3\,N - N^{\frac{1}{2}}$, and in a 3-dimensional array[3], $t_m/t_{jk} = 2.4\,N^{2/3}$. In chromatophores, N is about 30.

	Values in cm^{-1}			
	Absorption band		Emission band	
Pigment	peak	half width	peak	half width
B870	11370	500	11150	520
P870	11550	800	11050	820

TABLE 3.1. Absorption and emission characteristics of P870 and B870. The values for P870 were taken from Fig. 3.8A. The values for the absorption band of B870 were obtained by analysis of an absorption spectrum of a fraction from Rps. spheroides chromatophores containing predominantly B870 and P870, and little B850 or B800. This fraction was prepared by centrifuging chromatophores incubated with Triton as described earlier[7,15]. The values for the emission band of B870 were obtained with normal chromatophores. It should be noted that the absorption and emission band of B870 fitted even better with the Stepanov equation than those of P870 (see section 3.3.2).

walk calculations assuming that the pigment system consisted of identical molecules. A homogeneous pigment system was also assumed by Borisov and Godik[6] when they calculated jump times "comparable with lattice relaxation times" for transfer between bulk Bchl molecules. However, in many species of bacteria the pigment system is not homogeneous. For instance, in Rps. spheroides, energy transfer occurs predominantly from B800 to B850 and from B850 to B870 (cf. ref. 7 and the 1st alinea of page 15 of this Thesis. This may result in a lower number of jumps, n, and a longer jump time, t_{jk}, between like pigment molecules than calculated with the Bay and Pearlstein[3] formulas.

(2) The fluorescence lifetime of P870 in reaction centers in the trapping state (indicating t_t) is perhaps lower in chromatophores than in isolated reaction centers.

(3) The values obtained by Borisov and Godik for the lifetime of the variable fluorescence component (50 picosec at low light intensities) were challenged by Govindjee et al.[17] who found values of about 1-2 nanosec. The reason for this discrepancy is not understood. If the values of Govindjee et al.[17] are correct, the jump time t_{jk} would rise to about 30-40 picosec, according to the equations derived by Bay and Pearlstein[3]. Each of the latter two explanations, in combination with the first one, may be sufficient to meet the requirement $t_{rj} >> t_t$ (where t_{rj} is assumed to be equal to t_{jk}), which was necessary to obtain a high trapping probability of excitation energy encountering a reaction center.

Finally, it is interesting to compare our estimates of the transfer time of energy from P800 to P870, and of the distance between P800 and P870 molecules, with data obtained by Philipson and Sauer[19] with Bchl a-protein particles from Chloropseudomonas ethylicum. The authors found that the particles consisted of subunits containing 5 Bchl molecules. The Bchl molecules were 12 - 15 Å apart. Circular dichroism measurements indicated a strong coupling between the molecules, suggesting that excitation energy was delocalized over the whole array. The authors suggested that in most photosynthetic organelles there is heterogeneous arrangement of photosynthetic pigments, with strong excitation delocalization predominant among chlorophylls within subunits, and with Försters resonance transfer between the widely separated subunits.

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CHAPTER IV

FLUORESCENCE PROPERTIES AND RELATIVE NUMBERS OF PIGMENT MOLECULES IN REACTION CENTERS FROM RPS. SPHEROIDES

4.1 INTRODUCTION

In an attempt to get an impression of the "purity" of our routinely prepared reaction center preparations from Rps. spheroides, we used fluorescence excitation and emission spectra to detect small amounts of contaminating pigments present in our preparations. The low fluorescence yield of P870 (see Ch. III) enabled us to observe small amounts of contaminating substances with fluorescence yields of the order of 5 % or more quite easily. We will give fluorescence excitation spectra reflecting the absorbance spectra of one or more Bchl degradation products called P690, of Bph and of solubilized Bchl, respectively.

The contaminating form of Bph ("free" Bph) can be distinguished from reaction center Bph, which apparently forms an integral part of reaction centers from purple bacteria (see Ch. I, section 1.3, Ch. II, section 2.3.4 and Ch. III, section 3.4). Reaction center Bph has a low fluorescence yield compared to "free" Bph (The ratio is smaller than 1 : 100). This is thought to be due to a high efficiency of energy transfer from reaction center Bph to P870. Excitation spectra for the fluorescence of P870 at 77° K support earlier indications[5] for the presence of 2 molecules of Bph per reaction center.

Finally we analyzed an absorbance spectrum of an AUT-RC preparation from Rps. spheroides into absorbances due to Bph, to carotenoid and to P800 and P870 together. The results indicate that there is at least 1 molecule of Bph per 2 molecules of Bchl present in reaction centers. The number of Bchl and carotenoid molecules per reaction center is also discussed. The average number of caronoid molecules per reaction center is less than 1.

4.2 MATERIALS AND METHODS

Reaction centers were prepared as described in Ch. II. Fluorescence measurements were done as described in Ch. III, section 3.2 (second alinea). For measurements at 77° K the cuvette was immersed in liquid nitrogen in a Dewar vessel with a transparant quartz window. A correction for reabsorption of emitted light (as described in Ch. III) was carried out only at room temperature. At 77° K, the increased scattering by ice crystals may have increased the optical path by an unknown factor.

4.3 RESULTS

4.3.1 Fluorescence of P690

In the sections 4.3.1, 4.3.2 and 4.3.3 we will summarize our results concerning the identification of contaminating pigments present in reaction center preparations. The amounts of the contaminations, and their respective fluorescence yields will be dealt with in section 4.3.4. Section 4.3.5 gives an analysis of the absorbance spectrum of reaction centers.

The emission spectrum shown in Fig. 3.3 (Ch. III) had an emission maximum at 700 nm. Fig. 4.1 shows an excitation spectrum for this emission band (measured at 710 nm). This spectrum has maxima at about 690 nm and 420 nm, but no appreciable absorption band between 500 and 600 nm such as are present in Bph and Bchl. The spectrum has characteristics intermediate between those of the oxidation product of Bchl, 2-desvinyl-2-acetyl-chlorophyll a and its pheophytin[16]. We will call the substance, or mixture of substances, giving rise to this excitation spectrum P690.

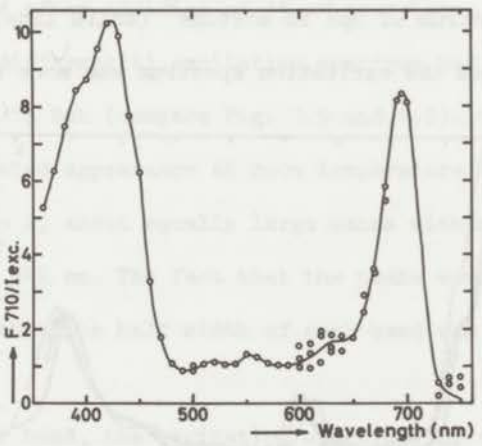

Fig. 4.1 Excitation spectrum for the fluorescence of P690, measured at 710 nm. The excitation spectrum was measured with the preparation of which absorbance and emission spectra were shown in Fig. 3.9 and Fig. 3.3, respectively. No additions. The ordinate scale of Figs. 4.1 - 4.5 is the same as that of Fig. 3.9.

4.3.2 Fluorescence of "free" Bph and reaction center Bph

The maximum at 772 nm in the emission spectrum of Fig. 3.3 was due to Bph (Ch. III, section 3.3.2). This appeared from the shape of an excitation spectrum for the fluorescence emitted at 790 nm. However, because in this preparation solubilized Bchl was also present (cf. section 4.3.3), we give in Fig. 4.2 an excitation spectrum for the emission of 790 nm light measured with another preparation (an AUT-RC preparation). Fig. 4.2 shows that apart from small differences in the position of the peaks, the excitation spectrum for the fluorescence at 790 nm was virtually the same as the absorbance spectrum of Bph in acetone[2] (solid line). In the region of 650-770 nm the excitation spectrum was more similar to

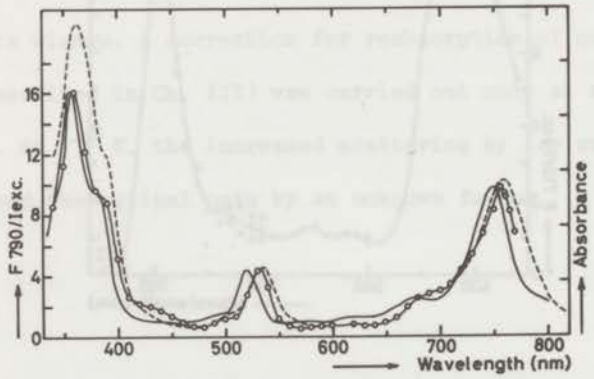

Fig. 4.2 o——o Excitation spectrum for the fluorescence of Bph, measured at 790 nm, with an AUT-RC preparation, (left hand scale). No additions.

——— : Absorbance spectrum of Bph a in acetone (from ref. 2).

----- : Absorption spectrum of a Bph-protein complex (from ref. 10). This spectrum coincides with our excitation spectrum between 580 and 710 nm.

the absorbance spectrum of a Bph-protein complex[14,10] (dashed line).

Since Bph occurs probably as an integral part of reaction centers, both in isolated form and in vivo (see Ch. I, section 1.3, Ch. II, section 2.3.4 and Ch. III, section 3.4), one might conclude that the excitation spectrum shown in Fig. 4.2 corresponds with reaction center Bph. However, this was not the case, as appeared from a comparison of the excitation spectra, in the region of 520-560 nm, for the fluorescence of Bph and of P870, respectively. As discussed in Ch. III, the excitation spectrum for the fluorescence of P870 (with reduced X) was given by the difference in emission observed before and after addition of 15 mM ascorbate. Fig. 4.3A shows that this differential excitation spectrum had a band at about 540 nm due to Bph (compare Fig. 3.9 and 4.2). This band had a somewhat truncated appearance at room temperature; at 77° K it was resolved into 2, about equally large bands with maxima at about 535 nm and 542 nm. The fact that the peaks were about 8 nm apart indicates that the half width of each band was at most 8 or 9 nm.

On the other hand, the excitation spectrum for the fluorescence of Bph at 770 nm (Fig. 4.3B) and at 790 nm (not shown) had an approximately symmetrical band with a single maximum both at room temperature and at 77° K. The half width of this band was 18 nm at 77° K.

These results indicate that the Bph which transfers energy to P870 (the reaction center Bph, see Ch. III, section 3.4) is different from the Bph which emits fluorescence at 770 nm ("free"

Bph). Apparently the fluorescence emitted by reaction center Bph was negligible compared with that emitted by "free" Bph (see below).

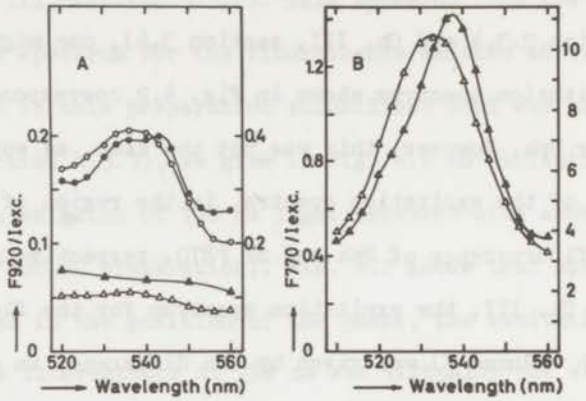

Fig. 4.3A Excitation spectra for the fluorescence of P870, measured at 920 nm, with an AUT-RC preparation. Open symbols: room temperature (left hand scale). O——O : with 15 mM ascorbate; Δ——Δ : no additions. Solid symbols: 77° K (right hand scale). ●——● : with 15 mM ascorbate; ▲——▲ : no additions. In the latter two cases the sample was illuminated first at room temperature for 1 min with light from a 125 W quartz-iodine lamp immediately before the sample was rapidly frozen. This was done in order to ensure either that both P870 and the primary acceptor were fully reduced (●——●), or that P870 was completely photooxidized (▲——▲) before freezing (cf. Ch. V).

Fig. 4.3B Excitation spectrum for the fluorescence of Bph, measured at 770 nm, with the preparation used in Fig. 4.3A. Δ——Δ room temperature (left hand scale); ▲——▲ 77° K (right hand scale).

4.3.3 Fluorescence of P760

In some cases when emission spectra were taken repeatedly with one sample, the results were not quite reproducible in the region of 760-830 nm. This appeared to be due to the photodestruction of

a pigment of which the fluorescence emission spectrum had a maximum at about 790 nm. The photodestruction (probably an oxidation) occurred with reaction center preparations in Tris buffer without additions, but not in the presence of 15 mM ascorbate. This enabled us to identify the pigment concerned as indicated below. The results suggested that it was solubilized Bchl (referred to hereafter as P760).

The kinetics of the fluorescence decrease observed upon illumination with 585 nm light were the same throughout the region of 760 - 830 nm, as is illustrated in Fig. 4.4A. This suggests that this fluorescence change reflected the disappearance of one fluorescing molecular species. The kinetics of the 920 nm fluorescence change, due to the photobleaching of P870 were quite different, as is also shown in Fig. 4.4A.

Fig. 4.4B shows the spectrum of the decrease in emission as observed after 90 sec of excitation with light of 585 nm (intensity 23 nEinstein.cm^{-2}.sec^{-1}). The spectrum consists of a symmetric band with a maximum at 795 nm and a half width of 42 nm.

In order to obtain an excitation spectrum corresponding to the P760 absorption spectrum, we measured two excitation spectra for the fluorescence emitted at 820 nm, viz. 1) in the presence of 15 mM ascorbate, which prevented the photodestruction of P760, and 2) without additions, after 5 min preillumination with light of 585 nm (intensity 23 nEinstein.cm^{-2}.sec^{-1}). As a result of this preillumination, P760 had been destroyed and the yield of 585 nm light for the emission at 820 nm had decreased to 47 % of the original

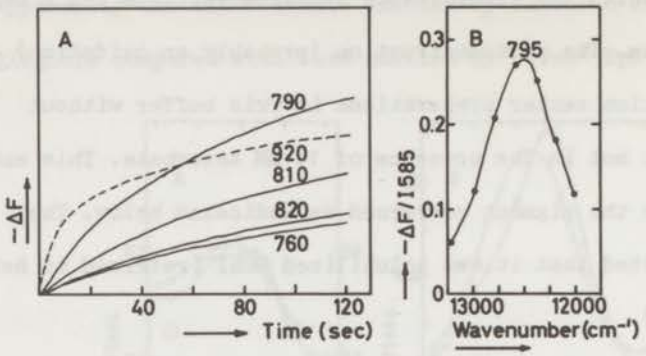

Fig. 4.4A Kinetics of the fluorescence decrease at the indicated wavelengths, induced in an AUT-RC preparation by excitation with light of 585 nm (intensity 23 nEinstein.cm^{-2}.sec^{-1}). The tracings, which were copied directly from the recording paper, have all different scales. A fresh sample was taken for each point.

Fig. 4.4B Same preparation. Spectrum of the fluorescence decrease observed after 90 sec of illumination with 585 light of 22 nEinstein.cm^{-2}.sec^{-1}. Obtained from the tracings of Fig. 4.4A by correcting for the wavelength-dependent sensitivity of the detecting system.

value. It was checked that this yield did not decrease further during the time that the second spectrum was taken. The difference between these two spectra, corresponding to the material which disappeared during the preillumination without ascorbate, is shown in Fig. 4.5. The position of the bands, their rather large width and the splitting of the near-infrared band suggest that this is aggregated rather than monomeric Bchl (see for example the absorption spectra of monomeric and dimeric Bchl in carbon tetrachloride[22], the spectra of dissolved and colloidal Bchl[12] and the spectrum of a dry film of monohydrated Bchl trimer[1].

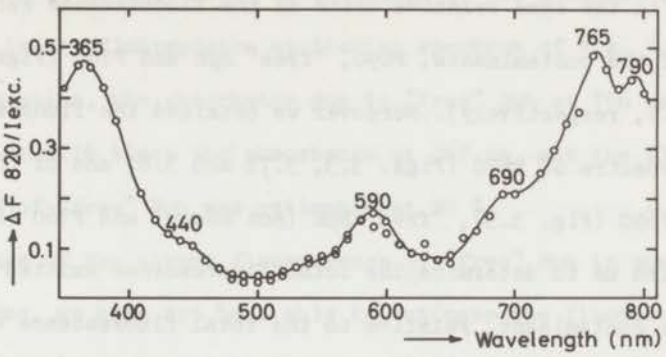

Fig. 4.5 Excitation difference spectrum for the fluorescence change at 820 nm measured with the preparation shown in Fig. 4.4. Further details see text.

However, it is known that chlorophyll a dimers in solution emit no, or very little fluorescence[24,15] and according to Goedheer[12] colloidal Bchl emits no fluorescence either. Moreover, the near-infrared band of the excitation difference spectrum is maximal at 762 nm and half-maximum at 725 nm, suggesting a halfwidth of this band of 60 - 70 nm. The emission difference spectrum however had a halfwidth of 42 nm (see Fig. 4.4B). It is therefore suggested that weakly fluorescent aggregated Bchl transferred excitation energy to strongly fluorescent monomeric Bchl, that the emission spectrum which is given in Fig. 4.4B was due to monomeric Bchl, and that the excitation difference spectrum of Fig. 4.5 reflects mainly the photo-destruction of aggregated Bchl.

4.3.4 Relative amounts and fluorescence yields of P690, Bph and P760

The fluorescence excitation spectrum of P870 (Fig. 3.9) was

expressed in the same relative units as the fluorescence excitation spectra of the contaminants, P690, "free" Bph and P760 (Figs. 4.1, 4.2 and 4.5, respectively). Moreover we obtained the fluorescence emission spectra of P870 (Figs. 3.3, 3.7B and 3.8) and of the contaminants, P690 (Fig. 3.3), "free" Bph (not shown) and P760 (Fig. 4.4B). This enabled us to determine the total fluorescence emitted by a particular contaminant, relative to the total fluorescence emitted by P870, upon excitation with light of a certain wavelength. The fluorescence yield of the contaminant, relative to that of P870, could then be calculated after estimating the absorbance of the contaminant at the excitation wavelength, relative to the absorbance of P870. The absorbance of the contaminant was estimated (with possible errors of about 20 %) by comparing the absorbance spectrum of the contaminated preparation with the absorbance spectrum of an AUT-RC preparation (Fig. 2.2, see also Fig. 4.6) which happened to contain a negligible amount of contaminants. Absolute values for the fluorescence yields of the contaminants were obtained using a fluorescence yield of 2×10^{-3} for P870 as PX^- (cf. Ch. III, section 3.3.4).

Thus, in the preparation used for the measurements of Fig. 3.3, 3.9 and 4.1, the absorbance at 685 nm due to P690 was estimated to be 0.3 times the absorbance due to P870 at 867 nm. Since part of the energy absorbed by P690 was transferred to "free" Bph and/or P760 in this preparation (not shown), the fluorescence yield of P690 would have been more than 8 % in the absence of energy transfer.

The AUT-RC preparation used for the measurements of Fig. 4.2

contained no detectable P760, as indicated by the absence of a peak at 590 nm in the fluorescence excitation spectrum of Fig. 4.2. In this preparation, the absorbance due to "free" Bph at 756 nm was estimated at 0.16 times the absorbance at 867 nm, and the fluorescence yield of "free" Bph was estimated at 20 %.

Because of the strong fluorescence of "free" Bph in most preparations, we have not been able to estimate the fluorescence yield of reaction center Bph. An upper limit could be estimated only, using an SDS-RC preparation of which the absorbance spectrum did not reveal a detectable amount of "free" Bph. In this preparation the integrated P870 fluorescence in the presence of 15 mM ascorbate was 1.6 times stronger than the integrated Bph fluorescence, using 525 nm excitation light. Since the absorption due to Bph was about 0.5 times the total absorption at 525 nm (see Fig. 4.6), this indicates that the fluorescence yield of reaction center Bph was at most 0.6 times the fluorescence yield of P870, or at most $1{,}2.10^{-3}$. This means that the fluorescence yield of reaction center Bph was less than 0.01 times the fluorescence yield of "free" Bph. This might mean that the efficiency of energy transfer from reaction center Bph to P870 is better than 0.99.

In the preparation shown in Fig. 4.5 the absorbances at 760 nm due to "free" Bph and P760 were estimated at 0.05 and 0.09, respectively, if the absorbance at 867 nm was put at 1. (The absorbance due to "free" Bph was estimated from the fluorescence due to Bph, assuming a fluorescence yield of 20 % for "free" Bph.) The fluorescence yield of P760 was estimated to be at least 3 %.

The estimates given in this section are probably fairly representative for the amounts of contaminating pigments absorbing in the near-infrared region, which could be found in our reaction center preparations from Rps. spheroides. Here we should recall that fluorescence measurements suggested that reaction centers with degraded pigments were present in some preparations (see Ch. III, section 3.3.2). The concentration of this contamination was too low to allow detection by measurement of absorbance spectra.

4.3.5 Analysis of the absorbance spectrum of a reaction center preparation

The absorbance spectrum of an AUT-RC preparation (taken from Ch. II, Fig. 2.2) was analyzed as shown in Fig. 4.6. For this analysis first the absorption due to Bph was subtracted from the total absorption, assuming that reaction center Bph has the same absorption spectrum as "free" Bph, and that this absorbance spectrum is identical with the excitation spectrum for the 790 nm fluorescence shown in Fig. 4.2. As follows from section 4.3.2, the first assumption may have introduced an error in the region of the 535 nm absorption band, because of the partially resolved band splitting in the spectrum of reaction center Bph. Next allowance was made for a small amount of P690, of which the absorbance spectrum was taken from Fig. 4.1.

Finally the absorption spectrum of carotenoid was subtracted. This spectrum was obtained from the spectrum of a heptane extract of a lyophilized AUT-RC preparation (unpublished). It was assumed that

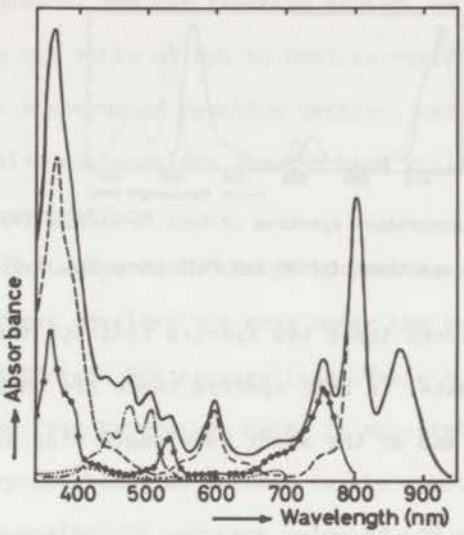

Fig. 4.6 Analysis of the absorbance spectrum (———) of the AUT-RC preparation shown in Fig. 2.2. •———• : Bph; ..... : P690; -.-.- : carotenoid; ----- : residual absorption due to P800 and P870 together. The amount of Bph was taken such that after subtraction there remained a shoulder at about 760 nm, in analogy with the shoulder in the absorption spectrum of Bchl in acetone at about 705 nm (ref. 2). The amount of P690 was taken such that after subtraction of the Bph and P690 contribution the residual absorbance spectrum was approximately horizontal in the region of 675 - 720 nm, in analogy with the region of 620 - 660 nm of the Bchl absorption spectrum in acetone. Further details see text.

the absorption spectrum of this carotenoid was the same in vivo as in vitro, except that in vivo the maxima and minima were shifted along a distance of about 20 nm to longer wavelengths.

The residual absorption (----- in Fig. 4.6), which is due to P800 and P870, was plotted again in Fig. 4.7, in order to compare it with the absorption spectrum of Bchl a in acetone (taken from ref. 2).

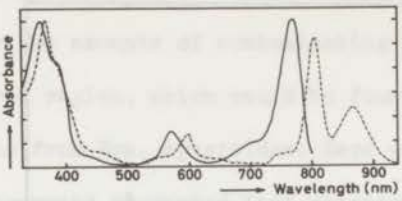

Fig. 4.7 Solid line : Absorbance spectrum of Bchl in acetone (from ref. 2). Dashed line : Absorbance spectrum of P800 and P870 (from Fig. 4.6).

The similarities between these two spectra indicate that the analysis was reasonably adequate. In both spectra there are shoulders at 385 nm, at about 450 nm and at the short wavelength side of the orange band.

For further comparison the spectra shown in Fig. 4.7 were plotted on a wavenumber scale (cm^{-1}). The areas under the Soret bands of Bchl in acetone (from 330 to 420 nm) and of reaction center Bchl in situ (from 340 to 430 nm) had a ratio of 1.10 : 1, indicating that these spectra represented approximately equal amounts of Bchl. In accordance with this, the areas under the near-infrared bands of Bchl in acetone and of P800 and P870 had a ratio of 1.06 : 1.

4.4 DISCUSSION

4.4.1 The ratio of Bph to Bchl in reaction centers

The excitation spectrum for the fluorescence of P870 (with reduced X) exhibited a band splitting in the 535 nm absorption band of Bph at 77° K. This finding was in line with an earlier observation[5] that the absorbance spectrum of reaction center Bph exhibits a band splitting at 77° K. Clayton et al.[5] proposed for this reason that

there are two molecules of Bph per reaction center. We checked this point by calculating the ratio of Bph to Bchl in reaction centers, using the absorbance spectrum of reaction centers, and also by considering alternative explanations for the band splitting of Bph in reaction center preparations.

In order to estimate the ratio of Bph to Bchl in reaction centers, we will first consider the area under the near-infrared absorption band in different Bph preparations. Table 4.1 summarizes the results collected from literature data. It appeared that the area under the absorption band, as defined in the subscript of Table 4.1, has an approximately constant value of $(38 \pm 2) \times 10^3$ $mM^{-1}.cm^{-2}$, despite large variations in ε_{max}.

ref.	system	λ_{max} (nm)	ε_{max} ($mM^{-1}.cm^{-1}$)	halfwidth (cm^{-1})	area ($mM^{-1}.cm^{-2}$)
9	Bph in chloroform	750	58	700	40×10^3
23	Bph in ether	749	63	620	39×10^3
10	Bph-protein	756-760	36	1000	36×10^3
this Thesis	"free"Bph	756	≤ 42	≥ 910	

TABLE 4.1

Spectral characteristics of the near-infrared absorption band of different bacteriopheophytin preparations. The half width of the absorption band was calculated from figures published in the references mentioned. The area under the absorption band (last column) is defined for our purpose as the product of the extinction coefficient in the absorption maximum (ε_{max}) and the half width.

For this reason we assumed that reaction center Bph and "free" Bph have a near-infrared absorption band with an area (as defined above) of 38 x 10^3 $mM^{-1}.cm^{-2}$. This enabled us to calculate an extinction coefficient of at most 42 $mM^{-1}.cm^{-1}$ for "free" Bph, given a half width of at least 910 cm^{-1} for the near-infrared absorption band (as calculated from Fig. 4.2). Moreover we assumed (as was done in the analysis of Fig. 4.6) that the shape of the near-infrared absorption band is the same in reaction center Bph and "free" Bph.

Clayton and coworkers[5] determined the amount of Bchl which can be extracted from an aliquot of a watery suspension of reaction centers with known absorbance at 803 and 867 nm. Knowing that the Bchl corresponded to P800 and P870, they determined the absorbance coefficients of reaction centers in the absorption maxima of P800 (at 803 nm) and of P870 (at 867 nm), assuming that there are 3 or 4 or 5 molecules of Bchl per reaction center[5].

We extended these calculations to the assumption of 2 molecules of Bchl per reaction center[19]. The results are shown in the first 3 columns of Table 4.2. According to the analysis of Fig. 4.6, the absorbance at 756 nm due to reaction center Bph is 0.71 times the absorbance at 867 nm. This enabled us to calculate the absorbance at 756 nm of reaction centers ($mM^{-1}.cm^{-1}$) which was due to reaction center Bph. This is shown in the 4th column of Table 4.2. With the assumption that the specific extinction coefficient of reaction center Bph at 756 nm is 42 $mM^{-1}.cm^{-1}$ (see above) we were able to estimate the best fitting number of Bph molecules per reaction center (last column of Table 4.2).

Assumed molar ratio Bchl/RC	Absorbance of RC ($mM^{-1}.cm^{-1}$)		Absorbance of RC ($mM^{-1}.cm^{-1}$) due to Bph	Molar ratio Bph/RC
	803 nm	872 nm	756 nm	
2	176	76	54	1
3	264	113	80	2
4	352	152	107	2 or 3

TABLE 4.2

Calculation of the molar ratio of Bph per reaction center (RC), starting from a given number of Bchl molecules per reaction center. The middle and bottom row of the first three columns were taken from ref. 5. The top row of the first three columns was obtained in the same way as in ref. 5. Further details see text.

It appears from Table 4.2 that, whatever the number of Bchl molecules per reaction center, the molar ratio of Bph : Bchl is at least 1 : 2. A 1 : 2 ratio was also reported recently by Reed and Peters[20] on the basis of extraction experiments. We favor the assumption of 2 molecules of Bph per reaction center because of the band splitting of the 525 nm absorption band of Bph at low temperature (see below), and because the assumption of 1 molecule of Bph is, according to Table 4.2, only compatible with 2 molecules of Bchl per reaction center, and this leads to rather low specific extinction coefficients of the reaction center particles in the near-infrared. This is not likely because it would mean that the quantum yields measured for the photooxidation of P870 (number of

electrons transported/number of quanta absorbed) are in reality higher than reported in Ch. III of this Thesis (section 3.3.1) and in refs. 5 and 3 (i.e. higher than 1) and would approach 1.5. The reported values were based on an extinction coefficient of 113 $mM^{-1}.cm^{-1}$ for the P870 absorption maximum. (A lower extinction coefficient means that a certain absorbance change represents a larger number of oxidized P870 molecules.)

4.4.2 The band splitting of Bph at 535 nm

Absorption spectra[5] and fluorescence excitation spectra (this Thesis) of reaction center preparations measured at 77° K revealed a splitting in the 535 nm absorption band of Bph. This could be taken as indicating the presence of 2 molecules of Bph per reaction center (one might assume that there are 2 kinds of reaction centers, with slight differences in the position of the 535 nm absorption band of Bph, but then one would have to explain why there are two kinds, and why these two kinds always occur in a 1 : 1 ratio).

One might assume that the band splitting arises because some of the Bph molecules are subject to pigment-solvent interactions resulting in a shift of the 535 nm absorption band. Such interactions are known to occur with Bchl in polar solvents[25], where a shift of 1 nm of the far-red absorption band (from 772 to 771 nm) is accompanied by a shift of 34 nm of the "yellow" absorption band (from 575 to 609 nm). However, Goedheer[11] showed that the position of the 535 nm absorption band of Bph in polar solvents does not vary more than the position of the near-infrared absorption band as a result

of pigment-solvent interactions, and the near-infrared absorption band of Bph in reaction center preparations does not show a splitting at 77° K (ref. 7).

A third possibility is that one electronic transition is responsible for two peaks in the 535 nm absorption band of monomeric Bph in reaction centers at low temperatures. However, such a band splitting is not apparent at room temperature in monomeric Bph (refs. 9,23), in "free" Bph (Fig. 4.3B) and in a Bph-protein complex (refs. 14,10), whereas even at room temperature the 535 nm absorption band of reaction center Bph has a truncated appearance, suggesting an unresolved band splitting, as shown in absorption spectra (e.g. ref. 7) and fluorescence excitation spectra (Fig. 4.3A) of reaction centers.

Therefore we favor the hypothesis[6] that the band splitting in the 535 nm absorption band of reaction center preparations is due to dimer interaction between two Bph molecules. Although circular dichroism measurements[21] with reaction center preparations in the near-infrared region did not indicate a strong interaction between two Bph molecules, one might assume that such an interaction shows up mainly in the electronic transition responsible for the 535 nm absorption band of Bph.

4.4.3 The number of Bchl and carotenoid molecules per reaction center

The literature data concerning the number of Bchl molecules per reaction center are conflicting. Mauzerall (as cited in ref. 5), Straley (as cited in ref. 5) and Reed and Peters[20] did extraction

experiments showing that the stoichiometry of Bph : Bchl in reaction center is 1 : 2, according to Mauzerall the best estimate was that there are four molecules of Bchl per reaction center. Recent analyses of light-induced ESR signals in photosynthetic bacteria[17,8] and higher plants[18] suggested that the electron donor molecule in photosynthetic reaction centers is not a monomer, but a (bacterio) chlorophyll dimer. Furthermore, an analysis of the absorption spectra shown in Fig. 4.7 and Fig. 3.8 indicates that the area under the P800 absorption band was of about the same magnitude as the area under the P870 absorption band, when plotted on a wavenumber scale (the actual ratio found in two preparations was 1.2 : 1). Clayton and coworkers[5] stated that the areas under the P800 and P870 absorption bands have an approximately 2 : 1 ratio, but from data reported earlier by Clayton (Fig. 2 and Table 2 of ref. 4) we find a ratio of 1.46 : 1 for these areas. All these data suggest that 4 molecules of Bchl (viz. 2 P870 and 2 P800 molecules) are present per reaction center. On the other hand, extraction of Bchl from reaction centers with bleached and unbleached P870 respectively, gave molar ratios of 2 : 3 of the amount of Bchl found, irrespective of whether the bleaching occurred through addition of ferricyanide[4] or by illumination (Straley, as cited in ref. 5). This suggests the presence of 3 rather than 4 molecules of Bchl per reaction center (viz. 2 molecules of P800 and 1 molecule of P870). A 2 : 1 ratio of P800 and P870 was also suggested by the results of an analysis of the 600 nm absorption band of reaction centers (this Thesis, Ch. II, section 2.3.3).

The uncertainty about the number of Bchl molecules does not allow us to estimate the number of carotenoid molecules per reaction center. Assuming a maximum value of 150 $mM^{-1}.cm^{-1}$ for the extinction coefficient of carotenoid[13] we arrive from Fig. 4.6 at a value of 0.43 or 0.58 molecules of carotenoid per reaction center, assuming that there are 3 or 4 molecules of Bchl per reaction center respectively. Since both values are significantly less than 1, some reaction centers apparently did not contain carotenoid at all.

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CHAPTER V

ELECTRON ACCEPTORS IN REACTION CENTER PREPARATIONS FROM PHOTOSYNTHETIC BACTERIA

5.1 INTRODUCTION

The primary reaction in the photosynthesis of purple bacteria is the photooxidation of P870 (see Chapter I, section 1.2). The role of primary electron acceptor during this reaction has often been attributed to ubiquinone. However, a practical difficulty in testing this assumption was that the reduction of ubiquinone to ubiquinol can be monitored only by absorption changes in the ultraviolet region of the spectrum. Light-induced absorption changes in this region do occur in photosynthetic bacteria and in chromatophore preparations, but so far it was not clear which part of these changes was due to reduction of ubiquinone or other substances and to P870 photooxidation, respectively.

Several authors (e.g. ref. 5,3,2) assumed that all of the light-induced absorption change at 275 nm was due to ubiquinone reduction. This led to the conclusion[3] that one mole of ubiquinone was reduced per mole of photooxidized P870. However, this hypothesis was difficult to reconcile with the fact that ubiquinone requires two electrons for reduction to ubiquinol, and P870 is a one-electron donor[14]. Loach, Bambara and Ryan[17], on the other hand, assumed that the light-induced 275 nm absorption change was completely due to P870 photooxidation. Ke and Chaney[12], working with subchromatophore

particles from Chromatium observed different decay kinetics at 881 and at 280 nm, respectively. This indicated that the change at 280 nm was due in part to a substance other than P870. Since, however, these measurements were not extended to other wavelengths, they provided no information about the nature of this substance.

Better interpretable results were obtained with reaction center preparations. Clayton and Straley[8] and Clayton[6] measured light-induced absorbance changes in reaction center particles from a carotenoidless strain of Rhodopseudomonas spheroides in the presence of exogenous oxidants or reductants. They found absorbance changes that could be ascribed to reduction or reoxidation of an electron acceptor. McElroy, Feher and Mauzerall[18] and Feher[11] working with the same particles, observed light-induced ESR signals at 1.4° K, which they tentatively ascribed to reduction of iron.

This Chapter gives the results of experiments with reaction center preparations from Rps. spheroides (wild strain) and Rhodospirillum rubrum (wild strain). The results can be summarized by the following scheme:

$$PXA \overset{h\nu}{\rightleftharpoons} P^{+}X^{-}A \rightleftharpoons P^{+}XA^{-}$$

in which P is P870, and X and A are endogenous electron acceptors. The light quantum is indicated by hν. Absorbance difference spectra (reduced minus oxidized) were obtained for P870, A and X or X + A. It is suggested that X is ubiquinone and X^{-} is the anion of ubisemiquinone. P and X are suggested to occur in a complex into which protons cannot penetrate. The function of X in vivo is discussed.

5.2 MATERIALS AND METHODS

Reaction center fractions were prepared as described in Chapter II. Light-induced absorbance changes were measured aerobically in 1-mm cuvettes with an apparatus described earlier[1]. The concentration of P870 was about 3 μM. If necessary the signals obtained at a certain wavelength were fed into a signal averager (Nuclear Chicago, model 7100 Data Retrieval Computer) to improve the signal-to-noise ratio. Actinic light provided by a 150 W quartz-iodine lamp was filtered by a Balzers B-40 803 nm interference filter in combination with a Schott RG10-2 and a Kodak 89B far-red cut-off filter.

5.3 RESULTS

5.3.1 Light-induced absorbance changes observed without additions

Fig. 5.1 shows the light-induced absorbance changes at a few representative wavelengths, observed in AUT-RC particles from Rps. spheroides, suspended in Tris buffer without additions (A), with 30 μM ferricyanide (B) and with 10 mM ascorbate (C). Fig. 5.1B and 5.1C will be discussed in section 5.3.2. Fig. 5.1A shows that without additions the kinetics at 600 and 325 nm both in the light and in the dark were biphasic. (The 345 nm change will be dealt with later in this section). The fast and the slow phase in the light will be referred to hereafter as phase a and b, respectively. The fast and the slow phase of the decay kinetics will be called phase c and d, respectively. Phase a was complete within 1 sec after the onset of the actinic light. Phase b reached a steady state level

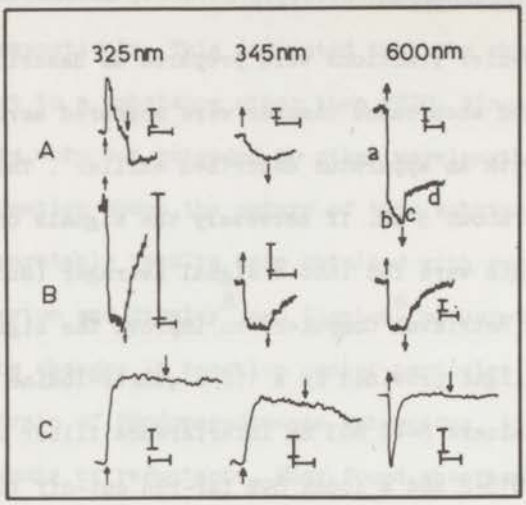

Fig. 5.1A

Light-induced absorbance changes at 325, 345, and 600 nm in AUT-RC particles from Rps. spheroides, suspended in Tris buffer. No additions. Actinic light of 803 nm (intensity 4.5 nEinstein.cm^{-2}.sec^{-1}) was switched on and off at the times indicated by the upward and downward pointing arrows. The horizontal bars indicate a time interval of 10 sec. The vertical bars indicate an absorbance change (ΔA) of 0.001. Each sample was illuminated only once and then allowed to stand in the dark for at least two hours, before a second period of illumination was given.

1B Same as Fig. 1A, except that 30 µM ferricyanide was present. In order to avoid the exhaustion of ferricyanide, a fresh sample was taken after three illumination cycles consisting of 10 sec light and 60 sec dark. Each point is the average of three, six or nine illumination cycles.

1C Same as Fig. 1A, except that 10 mM ascorbate was present. Each sample was illuminated only once.

after 2 - 5 min of actinic illumination. The decay kinetics of phase c and d were first order when plotted semilogarithmically, with half times of 0.12 - 0.15 sec and 30 - 70 sec, respectively, depend-

ing upon the preparation. In sections 5.3.1 and 5.3.2 we will show that the reactions corresponding to the phases a, b, c and d can be summarized in the scheme shown in Fig. 5.2, in which P is P870 and X and A are electron acceptors. It is implied that the rate of reaction a is high compared to that of reaction b, which in turn is high compared to that of reaction d. According to this scheme, the

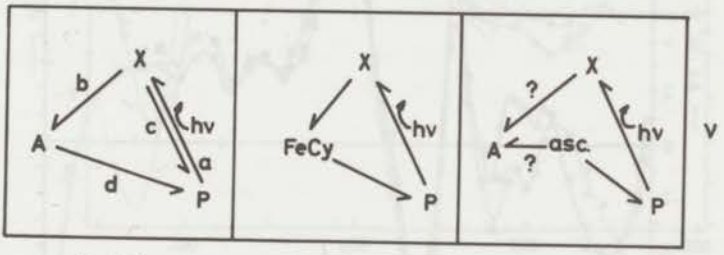

fig. 5.2 fig. 5.3 fig 5.4

Fig. 5.2

Scheme of the electron transport chains operating in reaction centers from Rps. spheroides and R. rubrum suspended in Tris buffer. P is P870, X and A are electron acceptors. Arrows indicate the direction of the electron transport. The light requirement is indicated by hν. The letters a, b, c and d refer to the phases of the light-induced absorbance changes which correspond with the indicated reactions (see Fig. 1A and text).

Fig. 5.3

Same as Fig. 5.2, but in the presence of 30 μM ferricyanide.

Fig. 5.4

Same as Fig. 5.2 but in the presence of 10 - 15 mM ascorbate. Question marks refer to the uncertainty whether the reduction of A by ascorbate proceeds in a direct reaction or via X and P (see text).

spectrum of the absorbance changes obtained in phase a represents the difference spectrum (P^+X^- - PX), and the spectrum of the absorbance changes observed in phase d represents the difference spectrum (P^+A^- - PA). These spectra are shown in Fig. 5.5. The spectra were matched at 600 nm.

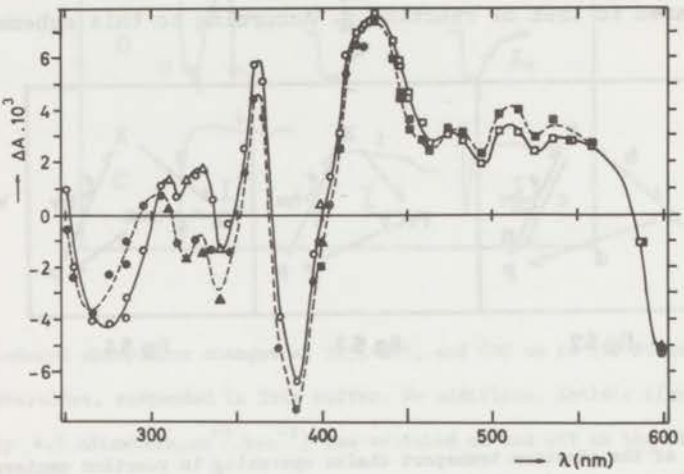

Fig. 5.5

Light minus dark absorbance difference spectra of AUT-RC particles from Rps. spheroides, suspended in Tris buffer. The dark absorbance is that at the time just before the actinic light is switched on. No additions. Actinic light, 803 nm. Intensity, 4.5 nEinstein.cm^{-2}.sec^{-1}. Illumination time 10 sec. Open symbols, solid line: difference spectrum of phase a, as observed at 0.7 sec after switching on the light. Solid symbols, dashed line: difference spectrum of phase d, as observed at 2 sec after darkening. The illumination time was chosen such that at 600 nm phase d was approximately as large as phase a. The circles, triangles and squares refer to experiments with three different batches of particles. The spectra were matched at 600 nm; the ordinate scale regers to the spectrum with the open circles.

The kinetics of the light-induced absorbance changes were (apart from a certain small deviation, discussed in section 5.3.5. identical in the regions of 580 - 720 nm and 780 - 950 nm, so it

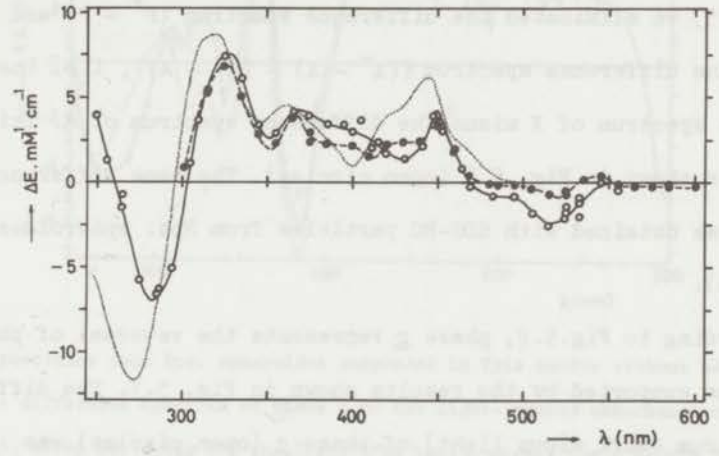

Fig. 5.6

O —— O: AUT-RC particles from Rps. spheroides. Spectrum of $\{(X^- - X) - (A^- - A)\}$, as determined by the difference between the two difference spectra shown in Fig. 5.5. The ordinate is given in molar extinction units. To this end, the spectra of Fig. 5.5 were expressed per millimole of photooxidized P870, and thus, according to our model, per millimole of X and A, before subtraction. The differential extinction coefficient of P870 at 600 nm (reduced minus oxidized) was taken to be 16 $mM^{-1}.cm^{-1}$.

● - - - ●: AUT-RC particles from Rps. spheroides. Spectrum of $(X^- - X)$ or $\{(X^- - X) + (A^- - A)\}$, as determined by the light-induced absorbance changes observed in the presence of 10 mM ascorbate. Actinic illumination 30 sec with 803 nm light. Intensity 4.5 $nEinstein.cm^{-2}.sec^{-1}$. In order to ensure that P870 was completely reduced, the absorbance changes were measured at 1.3 sec after switching off the actinic light. The data were expressed as the maximum absorbance change per millimole of P870 present. The concentration of P870 was determined from the maximum light-induced absorbance change at 600 nm, as observed without additions.

Dotted line: reduced minus oxidized difference spectrum of ubisemiquinone anion minus ubiquinone. Data from ref. 16.

could be safely assumed that the bleaching at 600 nm was due to P870 oxidation only (see Chapter II, section 2.3.3). Consequently, by taking the difference between the two difference spectra shown in Fig. 5.5, we eliminated the difference spectrum ($P^+ - P$) and obtained the difference spectrum $\{(X^- - X) - (A^- - A)\}$, i.e. the difference spectrum of X minus the difference spectrum of A. This spectrum is shown in Fig. 5.6 (open circles). The same difference spectrum was obtained with SDS-RC particles from Rps. spheroides (not shown).

According to Fig.5.2, phase c represents the reversal of phase a. This was supported by the results shown in Fig. 5.7. The difference spectrum (dark minus light) of phase c (open circles) was proportional to the difference spectrum of phase a (solid line). (The (The dashed line representing phase d was drawn just for comparison).

The kinetics of the P870 photooxidation, as observed at 600 nm upon illumination with 803 nm light (intensity 1.5 nEinstein $cm^{-2}sec^{-1}$), are shown in Fig. 5.8 as a function of the illumination time. After a short illumination time most of the photooxidized P870 decayed as phase c, with a half time of about 0.12 sec. The remaining part decayed as phase d. Upon prolonging the illumination time, the light-induced signal exhibited biphasic kinetics (phase a and b), and the fraction of photooxidized P870 decaying in the dark as phase d increased with increasing illumination time. Our interpretation is (see also Fig. 5.2) that an electron transferred from P870 to X in the primary photoreaction, returns from X^- to P^+870 either in a direct rapid back reaction (phase c) or via a secondary acceptor A^-.

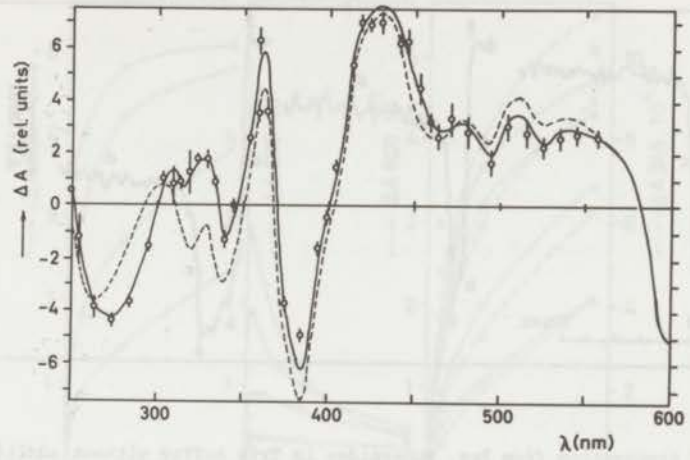

Fig. 5.7

AUT-RC particles from Rps. spheroides suspended in Tris buffer without additions. Open circles: difference spectrum of phase c of the light-induced absorbance change (see Fig. 1A), after switching off the light. The bars indicate the standard deviation of the mean. Solid and dashed line: difference spectra of phase a and phase d, respectively, as copied from Fig. 5.5. The data concerning phase c were obtained with the same samples as those of Fig. 5.5. The spectra were matched at 600 nm. At this wavelength, the magnitude of phase c was about one third of the magnitude of phase a and d.

Since A^- returns electrons to P^+870 very slowly (phase d), the acceptor A functions as an electron "trap" during the continued cycling of electrons which takes place during prolonged illumination. Thus after a brief illumination period the oxidized reaction centers are mainly in the form P^+X^-A, but after prolonged illumination they are mainly in the form P^+XA^-.

The experiments shown in Fig. 5.8 were extended to include actinic illumination times up to 30 sec and were done at four different light intensities. The results are summarized in Fig. 5.9. The total amount

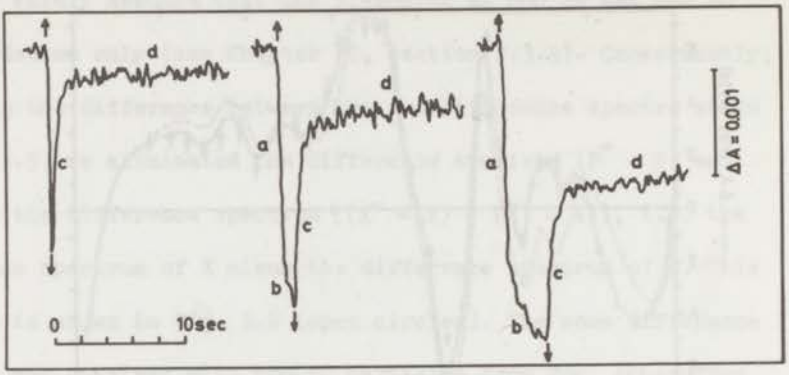

Fig. 5.8

AUT-RC preparation from Rps. spheroides in Tris buffer without additions. Kinetics of the light-induced absorbance change at 600 nm. Actinic light (803 nm, intensity 1.5 nEinstein.cm^{-2}.sec^{-1}) was switched on and off at the times indicated by the upward and downward pointing arrows. A fresh sample was taken for each curve. A downward deflection means an absorbance decrease.

of photooxidized P870 after a certain period of illumination (left) was analyzed into the fractions decaying as phase c (middle) and as phase d (right, solid line), respectively, after switching off the actinic light. Fig.5.9 shows that after a short illumination time most of the photooxidized P870 decayed as phase c, and after 30 sec of illumination most of the photooxidized P870 decayed as phase d. The data fitted with a model of the form

$$P\ X\ A \underset{k_3}{\overset{k_1.I.\sigma}{\rightleftharpoons}} P^+X^-A \underset{k_4}{\overset{k_2}{\rightleftharpoons}} P^+X\ A^- \qquad \text{(Eq. 5.1)}$$

in which the k's are rate constants, I is the incident light intensity and σ is the absorption cross section of a reaction center particle. In this model the phases a, b, c and d of the absorbance changes re-

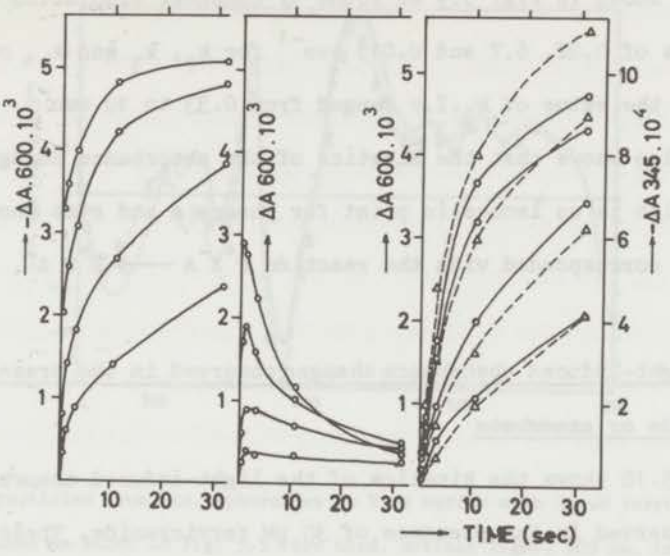

Fig. 5.9

Same preparation as in Fig. 5.8. No additions. Kinetics of the light-induced absorbance changes at 600 nm (o —— o) and at 345 nm (Δ - - - Δ). Actinic light, 803 nm. Intensities from upper to lower curves, 4.5 - 1.5 - 0.45 - 0.15 nEinstein. $cm^{-2}.sec^{-1}$. Left: Total absorbance change at 600 nm, obtained in the light, as a function of the time of illumination. Middle: Magnitude of phase c of the decay of the absorbance change at 600 nm, as a function of the time of illumination. Right: Magnitude of phase d of the decay of the absorbance change at 600 nm (o —— o), and magnitude of the absorbance change at 345 nm (Δ - - - Δ) as a function of the illumination time. The values shown in the open circles were obtained by plotting the observed decay curves on a semilog scale and extrapolating te zero time after switching off the light. Note the inverted signs of the left and right hand scale.

present reactions indicated by $k_1.I.\sigma$, k_2, k_3 and k_4, respectively. In fact we do not know whether A^- donated its electron to $P870^+$ (in phase d) directly or via X, but this was of no consequence for our model because of the widely different values of k_3 and k_4. For the

experiment shown in Fig. 5.9 we found by computer simulation approximate values of 0.22, 6.7 and 0.015 sec^{-1} for k_2, k_3 and k_4, respectivily, and the value of $k_1.I.\sigma$ ranged from 0.33 to 10 sec^{-1}. $Einstein^{-1}$. Fig. 5.9 also shows that the kinetics of the absorbance change at 345 nm (which is an isobestic point for phases a and c as shown in Fig. 5.1A) corresponded with the reaction $P^+X^-A \longrightarrow P^+X\,A^-$.

5.3.2 Light-induced absorbance changes observed in the presence of ferricyanide or ascorbate

Fig. 5.1B shows the kinetics of the light-induced absorbance changes observed in the presence of 30 µM ferricyanide. These absorbance changes were reversible within 50 sec after switching off the light. The observed kinetics could be explained with the scheme shown in Fig. 5.3. The reoxidation of X^- in the light proceeded in the presence of ferricyanide at a much higher rate than without additions. For instance, in the presence of 20 - 25 µM ferricyanide, an initial absorbance increase at 325 nm, due to the photoreduction of X was still just visible but the rate of the subsequent absorbance decrease was strongly enhanced. In the presence of 30 µM ferricyanide, however, the rate of the reoxidation of X^- was so high that accumulation of X^- was not observable and only a negative absorbance change caused by photooxidation of P870 was observed.

Fig. 5.10 shows the light-induced difference spectrum observed in the presence of 30 µM ferricyanide (open circles). As expected, this spectrum was different from the difference spectrum of (P^+X^- - PX) (solid line, copied from Fig. 5.5). It was however also different

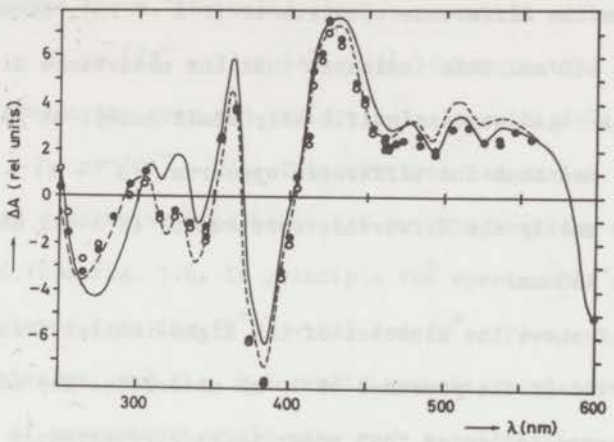

Fig. 5.10

AUT-RC particles from Rps. spheroides in Tris buffer with 30 μM ferricyanide. The same batches as shown in Fig. 5.5 were used. Actinic light, 803 nm, intensity 4.5 nEinstein.$cm^{-2}sec^{-1}$.

0 0 0: Absorbance changes as observed after 10 sec of illumination. • • •: Absorbance changes after subtraction of the difference spectrum of ferrocyanide minus ferricyanide, assuming that one mole of ferricyanide was reduced per mole of photooxidized P870. —— and - - -: Absorbance changes of phase a and d, respectively, as copied from Fig. 5.5. The spectra were matched at 600 nm. The light-induced absorbance changes observed with ferricyanide were about half the maximum absorbance changes observed with additions. Further legends: see Fig. 5.1B.

from the difference spectrum of $(P^+A^-$ - PA)(dashed line), notably at wavelengths below 280 nm and above 460 nm. This indicated that in this case ferricyanide had reoxidized X^- in the light, the net result being a light-driven oxidation of P870 by ferricyanide. After subtraction of the absorbance changes due to ferricyanide reduction there remained a reduced minus oxidized difference spectrum which was attributable to P870 alone (see DISCUSSION). This spectrum was not significantly

different from the difference spectrum of (P^+A^- - PA), except at wavelengths above 460 nm. This indicates that the absorbance difference spectrum of (A^- - A) was insignificantly small except at wavelengths above 460 nm, and that the difference spectrum {(X^- - X) - (A^- - A)} (Fig. 5.6) is mainly the difference spectrum of (X^- - X) at wavelengths below 460 nm.

Fig. 5.1C shows the kinetics of the light-induced absorbance changes observed in the presence of 10 mM ascorbate. The 600 nm absorbance change indicates that under these circumstances the photooxidation of P870 by X was followed by reduction of $P870^+$ by ascorbate (see Fig. 5.4). Consequently, the absorbance differences observed in the steady state at other wavelengths reflect the reduction of X, or possibly of X and A.

Fig. 5.6 (solid circles, dashed line) shows the spectrum of the light-induced absorbance changes observed in the presence of 10 mM ascorbate, at 1.3 sec after switching off the actinic light. The spectrum was similar to the one of {(X^- - X) - (A^- - A)} (Fig. 5.6, open circles), and the fact that these two difference spectra are equal in magnitude indicates that, in the presence of ascorbate, only 1 mole of X was reduced per mole of P870 present. Apparently the photoreactive pool of X is identical in size to that of P870. This was also found by Clayton, Fleming and Szuts[7].

The difference spectrum given by the solid circles was similar in the blue region to those obtained by Clayton and Straley[8]. In later experiments these authors[9] came independently to about the same analysis of the ultraviolet absorbance changes as outlined above.

As discussed above, the solid circles represent either the spectrum ($X^- - X$) or $\{(X^- - X) + (A^- - A)\}$. Consequently, the difference between the open and solid circles should give either the spectrum ($A^- - A$) or ($2A^- - 2A$). This spectrum had maxima at 515 nm and 430 nm and a negative band below 415 nm. The accuracy was low as can be judged from Fig. 5.6. In principle the spectrum ($A^- - A$) could also be calculated from the ($P^+A^- - PA$) and ($P^+ - P$) spectra (Fig. 5.10, solid circles, and Fig. 5.5, solid symbols, respectively), but the accuracy of measurement would make such a calculation practically meaningless.

5.3.3 Reaction center particles from R. rubrum

Measurement of light-induced absorbance changes in SDS-RC particles from R. rubrum gave essentially the same results as in AUT-RC particles from Rps. spheroides, except that the conversion of fast decaying (half time about 0.2 sec) P^+X^-A into slowly decaying (half time about 30 sec) P^+XA^- proceeded at a 5 - 10 times higher rate. The difference spectrum $\{(X^- - X) - (A^- - A)\}$ was very similar to the corresponding spectrum of AUT-RC particles from Rps. spheroides (see Fig.5.11), indicating that for these two organisms X is identical. Fig. 5.11 also shows the difference spectrum ($P^+A^- - PA$), which approximates the difference spectrum ($P^+ - P$) fairly well, as discussed above.

5.3.4 The chemical nature of X

Fig. 5.6 (dotted line) shows the difference spectrum (reduced

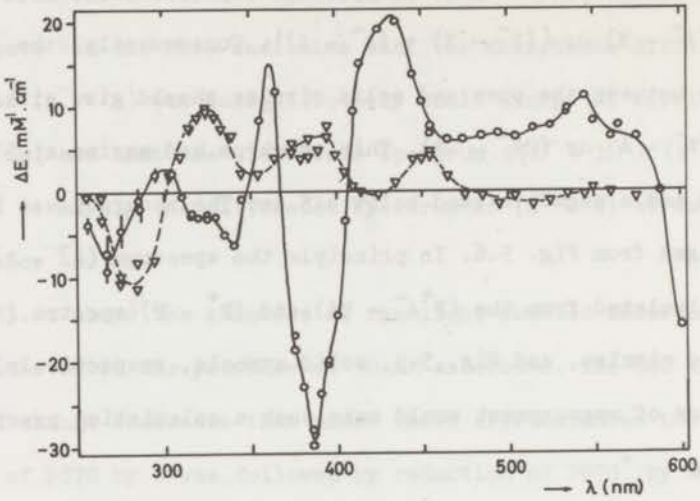

Fig. 5.11

SDS-RC particles from R. rubrum suspended in Tris buffer without additions. Actinic light, 803 nm, intensity 4.5 nEinstein.cm^{-2}.sec^{-1}.0 —— 0: Difference spectrum (P^+A^- - PA) of the slow part of the dark decay after 3 sec of actinic illumination. ∇ - - - ∇: Spectrum of {(X^- - X) - (A^- - A)}, obtained in a similar way as the spectrum of Fig. 5.6 (open circles), viz. by subtracting, after normalization at 600 nm, the (P^+A^- - PA) spectrum from the (P^+X^- - PX) spectrum that was obtained by measuring the fast part of the dark decay after 0.20 sec of actinic illumination.

minus oxidized) of the ubisemiquinone anion minus ubiquinone, as measured in methanol. The difference spectrum was calculated from data given by Land, Simic and Swallow[16]. This spectrum is remarkably similar to the difference spectra of {(X^- - X) - (A^- - A)} and of (X^- - X) or {(X^- - X) + (A^- - A)}, obtained with AUT-RC particles from Rps. spheroides (Fig. 5.6, open and solid symbols). One difference is, however, that the latter spectra seem to be shifted to longer wavelengths by about 10 nm, as compared to the ubiquinone difference

spectrum. In addition, the absorbance changes around 285 nm and in the region of 420 - 450 nm were smaller in the in vivo difference spectra than in the ubiquinone difference spectrum. Apart from these discrepancies the shape and the magnitude of the absorbance difference spectra shown in Fig. 5.6 and 5.11 are so similar that we conclude that presumably X is ubiquinone and X^- is the anion of ubisemiquinone in reaction center particles from Rps. spheroides and R. rubrum. The discrepancies observed in the regions of 280 nm and 420 - 450 nm might be due to a tight coupling between ubiquinone and the reaction center Bchl. Such a tight coupling may also be suggested by the observation that the $(X^- - X)$ or $\{(X^- - X) + (A^- - A)\}$ difference spectrum, as obtained by illumination in the presence of ascorbate, showed small absorbance changes in the region of 730 - 900 nm (maximum ΔA,5 $mM^{-1}.cm^{-1}$ as expressed on a P870 basis) which were interpretable as small shifts in the absorption bands of bacteriopheophytin, P800 and P870, perhaps due to electric field interactions between these molecules on the one hand and X^- on the other hand (see next section).

5.3.5 Near-infrared absorbance changes

This Chapter deals mainly with absorbance changes at wavelengths below 600 nm. Near-infrared difference spectra corresponding with P870 oxidation (light-induced and chemically induced) have been presented in Ch. II. The kinetics of the light-induced 600 nm absorbance change were under various conditions (illumination without additions, or in the presence of ascorbate or ferricyanide) very similar to those of

the near-infrared absorbance changes (compare Ch. II, Fig. 2.5). Small differences occurred however, depending upon the wavelength. Notably after illumination in the presence of 10 mM ascorbate during a period sufficiently long to ensure that all reaction centers were in a state with reduced P870 and reduced X, there remained near-infrared absorbance changes corresponding with the spectrum of (PX^- - PX) (●----● in Fig. 5.6). These near-infrared absorbance changes were small (maximum ΔA, 5 $mM^{-1}.cm^{-1}$ at 770 nm) compared to the near-infrared absorbance changes due to P870-oxidation (maximum ΔA, 93 $mM^{-1}.cm^{-1}$ at 867 nm[4]). The spectrum of (PX^- - PX) was very similar to the spectrum given by Clayton and Straley (Fig. 6 in ref. 9). These authors attributed these changes to shifts in the absorbance spectrum of Bph and Bchl associated with the reduction of the primary acceptor. Importantly, these authors were also able to separate these near-infrared Bchl and Bph shifts kinetically from an absorbance change at 455 nm reflecting presumably the reduction of ubiquinone. This indicated that the small near-infrared absorbance changes and the 455 nm absorbance change are of a different origin. The results suggested that under certain conditions the small near-infrared absorbance changes were associated with a secondary reaction, in contrast to the 455 nm which was associated with the primary reaction.

We observed similar shifts in Bph and Bchl absorbance by comparing the light-induced difference spectra of phase a (P^+X^- - PX) and phase d (P^+A^- - PA), as measured without additions (see section 5.3.1), indicating that upon reduction of X the absorbance spectra of Bph and P800 are influenced in a similar way when P870 is reduced (by il-

lumination in the presence of ascorbate) and when it is oxidized (by illumination without additions).

As discussed above, these near-infrared absorbance changes are small and are of a different origin than the absorbance changes shown in Fig. 5.6, and we do not believe that the former contribute much to the latter, because in general changes in the environment of Bchl and Bph are reflected most strongly in the near-infrared part of the absorbance spectra of these molecules.

The fact that near-infrared absorbance changes corresponding with (PX^- - PX) are small compared with the total absorbance of P870 (maximum at 867 nm, 113 $mM^{-1}.cm^{-1}$ according to ref. 4), is in line with our observation (Ch. III, Fig. 3.7) that the P870 fluorescence emission spectra have within the limits of accuracy the same shape whether the fluorescence originates from PX or from PX^-.

5.3.6 pH-dependence of the light-induced absorbance changes

We wanted to check whether the reaction $PXA \xleftarrow{h\nu} P^+X^-A$ was pH-independent, and whether the reaction $P^+X^-A \longrightarrow P^+XA^-$, or the reaction $P^+XA^- \longrightarrow PXA$ (corresponding with phase b and phase d, respectively), involved one or more protons. To this end we measured the pH-dependence of the light-induced absorbance changes at 600 nm and at 325 nm of an AUT-RC preparation, over a pH-range of 4.5 to 9.7. The results indicated that the reaction $PXA \xleftarrow{h\nu} P^+X^-A$ was practically independent of the pH over this range, that the reaction $P^+X^-A \longrightarrow P^+XA^-$ was accelerated 15-fold or less upon lowering the pH from 6.5 to 4.5 and that the reaction $P^+XA^- \longrightarrow PXA$ was accelerat-

ed by a factor 2.2 - 2.7 upon raising the pH from 4.5 to 9.7.

A preparation dialyzed against distilled water was divided into eight 1-ml portions to which 0.1 ml of the required buffer was added (see subscript of Fig. 5.12A). The particles were incubated with these buffers for two hours in the dark at room temperature prior to the experiments. In each experiment the particles were illuminated in a 2-mm quartz cuvette for 0.38 sec with 803 nm light (intensity 5.5 nEinstein.cm^{-2}.sec^{-1}); after a dark recovery of the light-induced absorbance change for more than 97% (in 2 - 5 min) the sample was illuminated for 8 sec with the same light. Simultaneous measurements were done at 600 and 325 nm. The dark decay of the 600 nm absorbance change was analyzed as before (see section 5.3.1) into first order reactions corresponding with phase c and phase d, respectively. The results are summarized in Fig. 5.12. Each point represents one experiment.

The magnitude of the light-induced 600 nm absorbance change was slightly pH-dependent as indicated by the open circles and solid lines of Fig. 5.12A (0.38 sec actinic light) and Fig. 5.12B (8 sec light). The magnitude of the absorbance change at 600 nm was found (within the error of measurement) to be proportional to the rate of the primary reaction, when the pH was varied (the two top curves of Fig. 5.12A). Computer simulations confirmed that these slight pH-dependences were in the pH range of 5.5 to 9.7 accounted for mainly by variations in the rate constant of the reaction corresponding with phase b (see below).

The half time of phase d varied from 47 sec at pH 9.7 to 102 sec

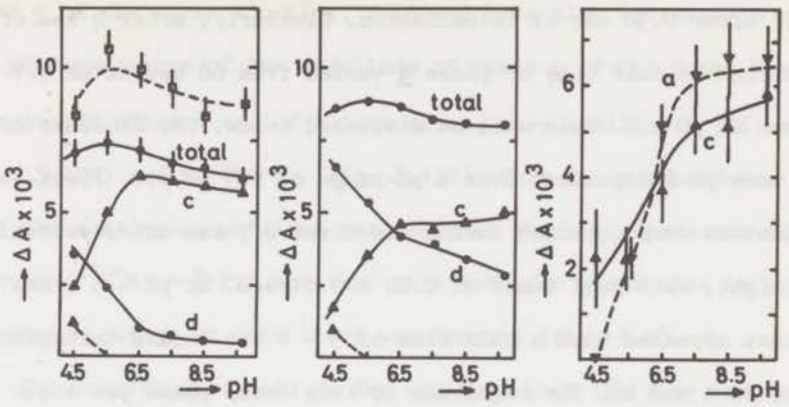

fig. 5.12.A fig. 5.12.B fig. 5.12.C

AUT-RC particles of Rps. spheroides. Kinetics of the light-induced absorbance changes as a function of pH. Actinic light intensity: 5.5 nEinstein.$cm^{-2}.sec^{-1}$.

Fig. 5.12A: Measuring light 600 nm, actinic illumination time 0.38 sec. O—total—O: absorbance change at the end of the illumination time (sign inverted). Δ—c—Δ and ●—d—●: Magnitude of the fractions of the absorbance change decaying as phase c and as phase d, respectively, after switching off the actinic light. ▲: Magnitude of the fraction of the absorbance change decaying with a half time of 3.3 sec. □ - - - □: Initial rate (per 0.2 sec) of the absorbance change in phase a of the light-on kinetics (sign inverted). The vertical bars in this Fig. represent standard deviations calculated as percentage of the mean from 8 experiments at pH 8.0 under otherwise the same conditions. The standard deviations given do not include possible errors made during the dilution procedures.These are estimated at 3 - 4% of the mean values. The buffers used to attain the indicated pH values were (final concentrations in brackets): pH 4.5 - 6.5, citric acid-phosphate (2.9 - 5.5 mM citric acid); pH 7.5, Tris-HCl (10 mM Tris); pH 8.5, tetraborate-HCl (3.5 mM tetraborate); pH 9.7, tetraborate-NaOH (3.5 mM tetraborate).

Fig. 5.12B: Measuring light 600 nm, actinic illumination time 8 sec. O—total—O, Δ—c—Δ and ●—d—●: As in Fig. A. ▲: Magnitude of the fraction of the absorbance change decaying with a half time of 4 sec.

Fig. 5.12C: Measuring light 325 nm, actinic illumination time 8 sec ▼—a—▼: Magnitude of phase a of the rise kinetics. Δ—c—Δ: Magnitude of phase c of the decay kinetics (sign inverted). Vertical bars represent errors of the mean as estimated from the noise level in these experiments.

at pH 4.5 after 0.38 sec of illumination. Similarly, after 8 sec of illumination the half time of phase d varied from 68 sec at pH 9.7 to 180 sec at pH 4.5. This will be discussed below. The kinetics of phase c were pH-independent over a pH-range of 5.5 to 9.7. First-order kinetics were obtained during the first 0.7 sec after switching off the light, with half times of 0.22 ± 0.01 sec. At pH 4.5 a new decay phase appeared with a half time of 3 - 4 sec (solid triangles in Fig. 5.12 A and B). The magnitude of this decay phase was about the same as that of phase c. After subtraction of this new decay phase it appeared that at pH 4.5 phase c had a half time not significantly different from those obtained at higher pH values: 0.20 sec.

The magnitude of phase c and d of the decay kinetics (extrapolated to 0 sec after switching off the light) exhibited a marked pH-dependence especially at pH values below 6.5, where the magnitude of phase c decreased and the magnitude of phase d increased with decreasing pH (Fig. 5.12A and B). At pH values above 6.5 the magnitude of phase c was almost independent of the pH, but the magnitude of phase d decreased with increasing pH, although not as sharply as below pH 6.5. This was seen clearly after 8 sec of illumination (Fig. 5.12B).

At 325 nm the magnitude of phase a of the rise kinetics was approximately constant at pH values above 6.5 and decreased sharply with decreasing pH at pH values below 6.5 (Fig. 5.12C, solid triangles, dashed line). At a pH of 4.5 the fast light-induced increase in absorbance was absent, only a slow light-induced decrease was observed. In all cases an absorbance decrease was observed in

phase c of the decay kinetics, followed by a slow increase in phase d. The pH-dependences of the magnitude of phase c of the decay kinetics, as measured after 8 sec of illumination, were similar at 325 nm (Fig. 5.12C) and at 600 nm (Fig. 5.12B). This is in accordance with the conclusion obtained earlier that phase c represents the reaction $P^+X^-A \longrightarrow PXA$ and that this reaction gives rise to an absorbance change at both these wavelengths (Fig. 5.7).

The results discussed so far indicate that the rate constants of the reactions $PXA \overset{h\nu}{\rightleftharpoons} P^+X^-A$ (phase a and c respectively) were practically independent of the pH over a range of 5.5 to 9.7. This is consistent with the interpretation of section 5.3.4 that these are one-electron reactions. It also suggests that P870 and X occur in a complex into which protons cannot penetrate easily, since protons would probably react rapidly with anionic ubisemiquinone to give the neutral semiquinone. The rate constant for this reaction in methanol* is probably $3 \pm 1 \times 10^{10}\ M^{-1}.sec^{-1}$ and the equilibrium constant is $2.5 - 5 \times 10^{-7}$ M, according to Land and Swallow[15]. The absorbance difference spectra discussed in section 5.3.1 did not point to the formation of neutral ubisemiquinone in phase a of the light-on kinetics. As shown below it is also unlikely that this occurred in phase b of the light-on kinetics.

The ratio of phase c: phase d of the dark decay after a certain illumination time was strongly pH-dependent. For this reason one might suspect that the reaction corresponding with phase b of the

*) According to Dr E.J. Land, these numbers would probably be of the same order of magnitude in water (pers. comm.).

light-on kinetics, which was written in section 5.3.1 as $P^+X^-A \longrightarrow P^+XA^-$, is at low pH a protonation of anionic ubisemiquinone: $P^+X^- + H^+ \longrightarrow P^+XH$. The kinetics of the 325 nm and 600 absorbance changes were at the first sight not inconsistent with this interpretation, since the absorbance difference spectrum of $(X^- - X)$, in which X is ubiquinone, is similar to the absorbance difference spectrum of $(X^- - XH)$ above 300 nm. In the region of 260 - 300 nm, the $(X^- - X)$ difference spectrum is more negative than the $(X^- - XH)$ difference spectrum[16]. We tried to make use of this in a control experiment with another batch of reaction centers (details below) but the accuracy was too low to decide between reoxidation or protonation of X^- in phase b of the light-on kinetics. However, if phase b represents the reaction $P^+X^- + H^+ \longrightarrow P^+XH$, the rate of this reaction would be proportional to the H^+ - concentration. This was not the case as can be derived from Fig. 5.12A. After 0.38 sec of illumination, the ratio of phase c: phase d of the dark decay of the 600 nm absorbance change was 7 : 1 at pH 6.5, and at least 1 : 2 at pH 4.5. (The latter value is a lower limit obtained if the decay phase with a half time of 3.3 sec is included in phase d). Since the rate constant of the reaction corresponding with phase c ($P^+X^- \longrightarrow PX$) had remained constant, this indicates that the rate of the reaction corresponding with phase b of the light-on kinetics increased only at most 14-fold upon lowering the pH from 6.5 to 4.5 (The rate constant of the reaction corresponding with phase d was at both pH values too low to be of any influence in this analysis). For this reason we do not believe that a protonation of X^- occurred in phase b of the light-on kinetics.

These experiments were repeated with another preparation, buffered at pH 4.6 with an acetic acid-acetate buffer (final concentration 20 mM of each) and at pH 6.6 with a Na_2HPO_4 - KH_2PO_4 buffer (final concentration 20 mM of each). The results and conclusions were qualitively the same as above, except that no "new" dark decay phase appeared as a result of lowering the pH to 4.5, and the reaction corresponding with phase b of the light-on kinetics was accelerated only at most 3-fold by lowering the pH from 6.6 to 4.6, again indicating that a protonation of X^- did not occur in phase b of the light-on kinetics. By including absorbance changes at 275 nm in these experiments it was checked that anionic ubisemiquinone was not reduced and protonated to neutral ubiquinol in phase b of the light-on kinetics. This would have given rise to an additional absorbance decrease instead of the observed absorbance increase, in phase b. (Unfortunately the rather high turbidity of the sample at pH 4.5 and the resulting low accuracy did not permit us to exclude directly the possibility that at pH 4.5 neutral ubisemiquinone is formed in phase b of the light-on kinetics.)

The rate constant of the reaction corresponding with phase d ($P^+XA^- \longrightarrow PXA$) increased gradually with increasing pH over a pH-range of 4.5 to 9.7 in the experiments shown in Fig. 5.12. Moreover this rate constant decreased with increasing illumination time (see below). An explanation may be that during prolonged illumination the electrons cycling through P870, X and "A" are gradually accumulated in secondary acceptors with longer half times for the return of electrons to P870. (This should give rise to inhomogeneous decay kinetics

of phase d. One would have to check this point by measuring the decay kinetics over periods of 5 to 10 minutes, but it was difficult to eliminate slow instrument drifts over these periods.) The secondary acceptor(s) may be buried in the reaction center protein. If the number of positive charges in the protein is increased by protonation as a result of lowering of the pH, this could enhance the rate of the reaction $P^+X^-A \longrightarrow P^+XA^-$ by providing an electrostatic attractive force working on the electron on X^-. This electrostatic attractive force would also retard the return of electrons from A^- to P^+. Alternatively, it is possible that the lower pH brings about a structural change in the protein so that A is removed from P and comes in closer contact with X.

5.4 DISCUSSION

The interpretation of the experiments described in sections 5.3.1 and 5.3.2 was supported by a comparison of the kinetics of the light-induced changes in absorbance and fluorescence of P870, as described in Ch. III, section 3.3.2. In those experiments the reoxidation of X^- was indicated by a slow decrease in fluorescence yield without accompanying absorbance changes (Fig. 3.4, bottom row).

The visible and ultraviolet difference spectra ($P^+ - P$) of Rps. spheroides and ($P^+A^- - PA$) of R. rubrum (Fig. 5.10, solid circles, and Fig. 5.11, open circles, respectively) correspond fairly well with the reduced minus oxidized difference spectrum of bacteriochlorophyll in vitro, as obtained by Loach, Bambara and Ryan[17]. The main discre-

pancy is a large blue shift at about 370 nm which is present in vivo, but not in vitro. This shift is probably due to P800, which also shows a blue shift at about 800 nm upon oxidation of P870. In addition, the (P^+ - P) and the (P^+A^- - PA) difference spectra of Rps. spheroides exhibit a few humps at 475 nm and at 510 nm which are lacking in the (P^+A^- - PA) difference spectrum of Rsp. rubrum, and which are thought to be due to carotenoid shifts. Our data indicate that in the ultraviolet region the difference spectrum of (P^+ - P) in vivo has a minimum at 265 nm, a maximum at 305 nm and another, more or less clearly separated maximum at 325 nm, with differential extinction coefficients of -8 to -11, +2 and -3 $mM^{-1}.cm^{-1}$, respectively.

The idea of a one-electron reduction of ubiquinone in the light is in accordance with results of titration experiments[19] which indicated that the primary oxidant is a one-electron acceptor with a pH-independent midpoint potential. It is not necessarily at variance with the observation by Bolton, Clayton and Reed[4] that there is only one unpaired electron per mole of photooxidized P870 in reaction center particles, as indicated by ESR-measurements at room temperature, because the latter measurements seem to have been done under conditions where X^- had been reoxidized by A or another acceptor already, which perhaps cannot be observed by ESR-spectroscopy. This is indicated by the rate of the dark decay of photooxidized P870 in those experiments. The direct return of electrons from X^- to P^+ has a half time of 0.06 sec in reaction center preparations at room temperature, according to Clayton and Yau[10], and 0.12 - 0.15 sec in our preparations. The dark decay of $P870^+$ in the experiments of Bolton et al.,

however, had a half time of 4 sec as shown in Fig. 4 of ref. 4.

Our interpretation that X (ubiquinone) is reduced in a one-electron reaction yielding the anionic semiquinone implies (as discussed in the previous section) that X and P870 occur in a complex which is separated from the surrounding medium by a barrier through which no proton diffusion occurs. The existence of such a proton diffusion barrier may explain different effects observed after extraction and readdition of ubiquinone: (1) The spectra given by Clayton and Yau (Fig. 7 in ref. 10) may be interpreted to indicate that their original reaction center particles were capable of light-induced formation of anionic ubisemiquinone, but that their ubiquinone-depleted and subsequently reconstituted particles showed a light-induced ubiquinol formation. This may indicate that the proton diffusion barrier mentioned above was destroyed during the extraction of ubiquinone. (2) The difference spectrum obtained by Ke et al. (Fig. 2 of ref. 13) with ubiquinone-depleted and subsequently reconstituted Chromatium subchromatophore particles may be interpreted as reflecting the light-induced formation of anionic ubisemiquinone. If this is so, it would indicate that the proton diffusion barrier mentioned above was not destroyed during the extraction of ubiquinone.

It may be speculated that the H^+-inaccessible P870-ubiquinone complex is capable of holding just one ubiquinone molecule and that in chromatophores or in whole cells this specific ubiquinone molecule serves as a link between the primary photoreactant(s) on the one hand and the large ubiquinone pool on the other hand, of which the photoreduction can be observed in Chromatium[20,21] and R. rubrum[2]. This may

be suggested by the positive absorbance change around 320 nm in chromatophores from Chromatium[5] and Rps. spheroides (L. Slooten and C. Noome, unpublished). In both organisms (ref. 13 and this thesis) the difference spectrum of P870 (oxidized minus reduced) is negative in this region. At least in Rps. spheroides the absorbance increase around 320 nm cannot be explained sufficiently by taking cytochrome oxidation and carotenoid shifts into account (L. Slooten and C. Noome, unpublished).

The question whether X is the primary or a secondary electron acceptor in our reaction center particles cannot be answered yet. The half time of 0.12 - 0.15 of the back reaction of X^- with P^+ at room temperature (see section 5.3.1) could be taken as an indication that X is a secondary, rather than the primary electron acceptor, since the back reaction of the reduced primary acceptor with $P870^+$ has a half time of 20 - 30 msec at 77° K and lower temperatures (see Ch. VI). The reaction center particles prepared by Clayton et al. posed a similar problem: the half time for the reaction $P^+X^- \longrightarrow PX$ (in which X appeared to be identical with our X in later experiments[9]) was 60 msec at room temperature and 20 - 30 msec below 193° K (ref. 10). Clayton and coworkers[7] tried to establish the number of electron acceptors in these reaction centers by illumination in the presence of reduced cytochrome c. This kept P870 in the reduced state, the net result being a light-induced reduction of electron acceptors by cytochrome c. Only one electron was transferred in this way per mole of P870 present. As argued by the authors, this could mean either that only one electron acceptor was present, or that there were two elec-

tron acceptors present per mole of P870, and that only the secondary acceptor was photoreduced in this way. The latter possibility would imply that the primary electron acceptor could not be photoreduced if the secondary acceptor was reduced.

In the next Chapter we will collect more literature on the question of the primary electron acceptor. We will discuss that in Chromatium an iron-sulfur protein is the primary acceptor, but that in R. rubrum and Rps. spheroides ubiquinone cannot yet be excluded as a candidate for this function.

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CHAPTER VI

DISCUSSION: "THE" PRIMARY ELECTRON ACCEPTOR

6.1 Literature data concerning the identity of the primary electron acceptor

In the previous Chapter data were presented indicating that ubiquinone functions as an electron acceptor during the photooxidation of P870 in reaction center particles from Rps. spheroides and R. rubrum. Our data are consistent with those obtained by Clayton and Straley[2,3], who worked with reaction centers from Rps. spheroides. In neither of these cases it had been attempted to remove either ubiquinone or iron completely. (See Ch. II, Table 2.1). Although a one-electron reduction of ubiquinone was also demonstrated by other authors in preparations from Rps. spheroides and R. rubrum (see below), the function of ubiquinone as primary electron acceptor has not yet been proven decisively, as will be discussed in this Chapter.

Feher[10] prepared reaction center particles from Rps. spheroides, containing on the average 1 atom of iron per mole of P870. These particles exhibited two reversible light-induced ESR signals at 1.4° K, indicating P870 photooxidation coupled to the reduction of a substance which was tentatively identified as non-heme iron. This interpretation was consistent with the fact that at room temperature only an ESR signal due to P870 was observed, since iron would not give a detectable ESR signal at room temperature.

After removal of iron from these reaction centers as a result of

addition of SDS, Feher et al.[11] obtained reversible light-induced ESR-signals at 1.4° K which corresponded to the photooxidation of P870 and the photoreduction of ubiquinone, respectively, in a one-electron reaction. However, the authors stated that the photochemical activity (probably defined as the fraction of P870 which is photooxidizable) of the "iron depleted" reaction centers at 1.4° K was only 0.2 - 0.3 of the activity measured in the original, iron containing, reaction centers at room temperature. Since not all the iron had been removed from the reaction centers by treatment with SDS, it is possible that at 1.4° K only those reaction centers were photochemically active which contained iron. We feel that if this could be demonstrated, it might suggest that iron is the primary electron acceptor in the iron-containing particles; if the iron is removed, ubiquinone may adopt the role of primary electron acceptor, at least at room temperature, but not at 1.4° K.

Similar results were obtained by Loach et al., who prepared subchromatophore particles from R. rubrum containing 2 atoms of iron per mole of P870. These particles, as well as the chromatophores, exhibited at room temperature only one light-induced ESR signal[19], corresponding with the photooxidation of P870. The iron content of the subchromatophore particles could be reduced to 0.15 - 0.3 atom per mole of P870 by electrophoresis. The particles treated in this way exhibited light-induced ESR signals at room temperature indicating P870 photooxidation coupled to the reduction of a quinone-like substance[20]. This was confirmed by Feher who identified the new ESR signal as due to anionic ubisemiquinone (see footnote in ref. 3).

Clayton and Straley[3], who tried to reproduce the experiments of Feher et al.[11] with reaction centers (see above), measured absorbance changes at room temperature. Upon addition of SDS to a suspension of reaction centers from Rps. spheroides, the absorbance changes attributable to the light-induced formation of anionic ubisemiquinone were abolished, and in contrast to the results obtained by Feher et al.[11], and by Loach and Hall[20] (see above), ESR-signals indicating light-induced ubiquinone reduction did not appear, although P870 was still capable of reversible photooxidation. A possible explanation is that SDS blocked electron transport from the primary electron acceptor (iron?) to the secondary acceptor (ubiquinone). The iron content of these particles before and after SDS-treatment was not checked. However, see Ch. V, section 5.4 for a discussion of the number of trapping sites per mole of P870.

Reaction center preparations obtained by Noel et al.[23] from R.rubrum showed a reversible P870 photooxidation and contained no ubiquinone. The iron content of these particles was not checked.

Neither of these observations is incompatible with the hypothesis (refs.11,20) that in Rps. spheroides and R. rubrum in vivo either ubiquinone or iron functions as the primary electron acceptor during the photooxidation of P870 (see section 6.4). It should be noted however, that although electron acceptors have been identified with some certainty in the cases cited bove (refs.10,11,20, Ch. V of this Thesis and ref. 3), it is not yet proven decisively that they should be qualified as primary acceptors. The time resolution was in most cases too low to afford such a conclusion. Perhaps the strongest indication in this

respect was the observation[1] that the "trapping" capacity of internal electron acceptors in reaction preparations was about 1 electron per mole of P870; this concerned reaction centers in which the light-induced formation of anionic ubisemiquinone is suggested by absorbance changes[3] similar to those described in Chapter V of this Thesis.

More clear-cut evidence concerning the qualification of an electron acceptor as "primary" acceptor has been obtained by Leigh and Dutton, who worked with Chromatium chromatophores. They attributed as ESR signal, with the characteristics required for a primary acceptor, to an iron-sulfur protein[16]. This was not inconsistent with results obtained by Ke et al.[13]. Subchromatophore particles prepared from Chromatium by these authors showed a light-induced absorbance difference spectrum which was rather similar to our (P^+XA^- - PXA) difference spectra (see Ch. V). Ubiquinone reduction was not observed. These difference spectra were obtained with ubiquinone-depleted particles at room temperature and at 77° K, and with untreated particles at 77° K. Ke et al. concluded that ubiquinone is not the primary electron acceptor in Chromatium[13].

Next we will consider whether other types of experimental results suggest that the primary electron acceptor is the same in Chromatium on the one hand and in Rps. spheroides and R. rubrum on the other hand. It will appear that this is not the case.

6.2 The midpoint potential of the primary electron acceptor

A parameter which may yield information about the identity of the primary acceptor is its midpoint redox potential. In this section we

will see that the values known for the midpoint potentials of the primary acceptor are different in Chromatium, R. rubrum and Rps. spheroides and therefore do not suggest that the primary electron acceptor is identical in these three species.

The measurements reviewed in this section were done at pH values of 7.4 - 7.5 unless otherwise indicated.

Values reported for the midpoint potential of the primary electron acceptor in reaction centers form Rps. spheroides are -50 mV (ref. 28) and -30 mV (ref. 22). In the first case, the midpoint potential was pH-independent. Here, the initial fluorescence yield of P870 was measured as a function of the potential of the medium. This method assumes that the initial fluorescence yield of reduced P870 is higher when the primary electron acceptor is reduced, than when it is oxidized[1]. However, Clayton and Straley[3] obtained data indicating that under certain circumstances the quenching of the fluorescence yield upon darkening was associated with the dark reoxidation of a secondary, rather than the primary electron acceptor. The estimate of -30 mV (ref. 22) was made by addition of various redox reagents, with known midpoint potentials, in the oxidized state. The rationale was that these substances would couple between X^- and P^+ and thus accelerate the back reaction from X^- to P^+, provided that the midpoint potential of the added reagent was higher than that of the couple X/X^-. No acceleration of the back reaction would occur if the added reagent had a lower midpoint potential than X. Here one might argue that the midpoint potential of another acceptor than X is measured. In spite of these objections, the measured values fall in the range of most other values obtain-

ed with Rps. spheroides and R. rubrum (see below).

In Chromatium chromatophores at room temperature, the P890 photooxidation by the primary electron acceptor is followed within 2 - 3 μsec by rereduction of $P^{+}890$ by a c-type cytochrome[26], the net result being the reduction of the primary acceptor by a cytochrome c. The midpoint potential of the primary acceptor follows from the attenuation of the cytochrome c photooxidation upon lowering of the redox potential. Values ranging from -127 to -135 mV have been reported[6,7,29].

Loach[17], working with Rps. spheroides and R. rubrum, measured absorbance changes associated with P870 photooxidation as a function of the redox potential. The attenuation of the light-induced signals versus decreasing redox potential was ascribed to reduction of the primary acceptor. Loach obtained the same titration curves whether the measurements were done at 761 nm or 433 nm, indicating that oxidation of a low potential cytochrome (C-428, ref. 12) did not occur. The midpoint potentials found were -25 mV (R. rubrum cells), -18 mV (Rps. spheroides cells) and -22 mV (R. rubrum chromatophores)[17]. Other authors, who measured only near-infrared absorbance changes, found about the same values in chromatophores from R.rubrum:-44 mV(ref.15), -30 mV (ref. 24) and "a value intermediate between +50 and -10 mV" (ref. 18).

In all these cases the values obtained with Rps.spheroides[28,22,17] and R. rubrum[15,24,18,17] were higher than those obtained with Chromatium[6,7,29]. These results are not quite consistent with, and perhaps not quite comparable to results obtained by Cramer[5] who found midpoint potentials of -160 mV, -145 mV and -65 or -95 mV, respectively

(at pH 8.0),for the primary acceptor in cells of Chromatium, R. rubrum and Rps. spheroides. In these measurements[5] the increase in fluorescence yield of light-harvesting Bchl at low light intensities upon lowering the potential was taken as an indication of the reduction of the primary acceptor.

In summary, the results mentioned in this section do not indicate that the primary electron acceptor in Chromatium, Rps. spheroides and R. rubrum is identical.

6.3 The rate of the back reaction of the reduced primary electron acceptor with oxidized P870

Another parameter which one might expect to give information concerning the primary electron acceptor is the back reaction of the reduced primary acceptor with oxidized P870. In this section we will see that different reduced compounds had about the same rate of reaction with photooxidized P870. This means conversely that a certain rate of reduction of $P^{+}870$ might at most suggest that it concerns the reversal of the primary reaction, but it yields no additional information concerning the identity of the primary electron acceptor.

The back reaction of the reduced primary electron acceptor can be studied in Chromatium chromatophores at low temperatures, and only at redox potentials at which cytochrome c is oxidized prior to the illumination (see Ch. 1.2). Chromatophores from R. rubrum pose no problem in this respect, because cytochrome oxidation does not occur at low temperatures[14]. (We confine ourselves to low temperatures because it has been shown in Chromatium[26,27] that at room temperature the elec-

tron is transferred rapidly from the primary to a secondary acceptor).

The available data concerning the rate of the back reaction of reduced electron acceptors with P^+870 show a striking similarity. In Chromatium chromatophores the half time at 77° K was 20-25 msec[8,9,27]. Evidence has been presented indicating that this reaction did proceed from a primary acceptor, identified as an iron-sulfur protein, and not from a secondary acceptor[16]. In R. rubrum the half time for the dark reduction of photooxidized P870 was 17 msec at 77° K (ref. 25). In this case the half time decreased slightly with decreasing temperature. At 38° K the half time was 15.5 msec[25]. In subchromatophore particles from Chromatium the half time at 77° K was 20 msec, independent of whether or not the particles had been extracted with non-polar solvents to such an extent that ubiquinone had been completely removed[13].

In accordance with these values are recent data obtained with reaction center fractions prepared from Rps. spheroides strain R-26 (refs.21,4,11). Between 1.7° K and 77° K, reaction centers suspended in 50% glycerol had a temperature-independent half time of 30 msec for the dark recovery of photooxidized P870 (ref. 21). Absorbance changes attributable to the chemically induced formation of anionic ubisemiquinone had been found at room temperature in these preparations[2]. Other reaction center preparations, of which the absorbance changes suggest that the P870 photooxidation was coupled at room temperature to the formation of anionic ubisemiquinone (ref. 3 and Fig. 7 in ref. 4), had a half time for the dark recovery of P^+870 which was temperature-independent below 193° K, where it was 20-30 msec, even when the particles had been extracted with iso-octane in order te remove ubi-

quinone[4]. In both cases the identity of the substance reacting with P870 at low temperatures is unknown. Iron-depleted reaction centers (containing less than 0.2-0.3 atoms of iron[11,10] per mole of P870) showed at 1.4° K a biphasic decay of the photooxidized P870, with half times of 18 and 124 msec, respectively, for the fast and the slow recovery phase[11]. In this case the back reaction proceeded from anionic ubisemiquinone to P^+870, but the forward reaction may have proceeded from P870 via iron to ubiquinone (see section 1).

6.4 Concluding remarks

The results discussed in section 2 and 3 of this chapter are not inconsistent with those discussed in section 1, but do not provide much additional evidence concerning the identity of the primary electron acceptor. At low temperature, the half time for the P^+870-recovery was in iron-depleted reaction centers[11], in which electrons returned from anionic ubisemiquinone to P^+870, practically the same as in normal chromatophores (refs. 8,27,9,25) and in normal and in ubiquinone-depleted subchromatophore particles[13] and reaction centers[21,4]. This may be taken as an indication that in some preparations[11] ubiquinone is located very close to the site of the primary acceptor, but this was indicated already by the low temperature (1.4° K) at which ubiquinone can be photooxidized (ref. 11).

If ubiquinone acts as a secondary electron acceptor in iron-depleted reaction centers, its reactivity at 1.4° K would be in contrast to the observation that in Chromatium chromatophores secondary acceptors are not photoreduced at low temperatures[8,27]. This decrepancy may be explain-

ed in several ways[11,20]: (1) In Rps. spheroides and R. rubrum (cf. Ch. V and ref. 20) ubiquinone is the primary electron acceptor, in contrast to the situation in Chromatium where an iron-sulfur protein is the primary acceptor; the results obtained by Noel et al.[23] and by Clayton and Straley[3] might be explained by assuming that iron takes over the role of primary acceptor when ubiquinone has been removed. (2) Alternatively one might assume that in Rps. spheroides and R. rubrum ubiquinone takes over the place and the role of primary acceptor (iron) when the latter has been removed. Neither of these hypothesis can be ruled out presently, because fortuitously no particles have been prepared which are known to contain neither iron nor ubiquinone (see Chapter II), and because insufficient data are available in the microsecond time range of light-induced ESR or absorbance changes.

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SUMMARY

(Numbers between brackets refer to Chapter sections)

A definition of a photosynthetic reaction center applicable to photosynthetic bacteria is (1.3): A Bchl-protein complex which functions in vivo as a trapping center for light energy absorbed by Bchl molecules not belonging to the reaction center, and in which light energy is utilized for the photoreduction of a low-potential electron acceptor by a (pair of) Bchl molecule(s) in a one-electron reaction. The Bchl types present in reaction centers of purple bacteria are called P800 and P870; of these, P870 is the photoreductant.

The literature review given in Ch. I deals shortly with reactions occurring in reaction centers and gives information about the structural organization of the bacterial photosynthetic system.

Reaction center particles were isolated from Rhodopseudomonas spheroides and Rhodospirillum rubrum by centrifugation of chromatophores preincubated with SDS (2.2.1). The particles contained P800, P870, carotenoid and also Bph, which is probably an integral component of reaction centers in vivo (3.4). An excitation spectrum for the fluorescence of P870 (4.3.2) and an analysis of the absorbance spectrum of reaction centers (4.3.5) indicated that there are probably 2 molecules of Bph per reaction center (4.4.1, 4.4.2). The molar ratio of Bph, P800 and P870 in reaction centers is 2 : 2 : 2 or 2 : 2 : 1 (see 4.4.3). The results of an analysis of the 600 nm absorption band of reaction centers (2.3.3) favored the latter ratio; the former ratio was suggested by the approximately equal areas under the near-infrared absorption bands due to P800 and P870 (see 4.4).

Chemical analysis (2.3.4) indicated the presence of Bchl *a*, Bph *a*, spheroidene and also ubiquinone-10. The molar ratio of ubiquinone : P870 was minimally 1.2 : 1 (assuming 1 molecule of P870 per reaction center).

The quantum yield for the photooxidation of P870 is about 1 electron per absorbed quantum of light (3.3.1). Light energy absorbed by P800, Bph and carotenoid was transferred to P870 with high efficiency (3.1.1 and 3.3.3). The fluorescence yield of P870 was 0.2 - 0.5 $^{o}/_{oo}$ in reaction centers with oxidized electron

acceptors (3.3.4). The fluorescence yield of P800 was less than 0.04 °/oo (3.3.4), that of reaction center Bph was less than 1.2 °/oo (4.3.4). The fluorescence lifetime, as calculated from the fluorescence yield and the intrinsic lifetime, was 3-8 picosec for P870 (with oxidized electron acceptors) and less than 0.8 picosec for P800. The results suggest that the mechanism by which energy is transferred from P800 to P870 may be faster than that of inductive resonance (3.3.5 and 3.4). The lifetime of 3-8 picosec of the fluorescence of P870 in isolated reaction centers is difficult to reconcile with the recently measured lifetime of 50 picosec for the fluorescence of chromatophores (3.4).

The light-induced absorbance changes observed under different conditions in reaction centers from Rps. spheroides and R. rubrum indicated that the photo-oxidation of P870 was coupled to the photoreduction of an electron acceptor X. X could either react back with oxidized P870, or it could reduce a secondary electron acceptor A, which was then able to reduce oxidized P870 (5.3.1-5.3.4). This scheme was supported by a comparison of the kinetics of the light-induced changes in absorption and fluorescence of P870 (3.3.2). X is presumably ubiquinone, which is photoreduced to anionic ubisemiquinone (5.3.4). The pH-dependence of the rates of the different reactions (5.3.6) suggested that P870 and X are separated from the surrounding medium by a proton diffusion barrier (5.4).

The possibility that X (ubiquinone) is the primary electron acceptor, either in our reaction center particles or in vivo, is discussed in Ch. VI. In Chromatium an iron-sulfur compound has been identified recently as the primary electron acceptor (ref. 16 in Ch. VI). However, there appears to be no reason to maintain that the primary electron acceptor found in Chromatium is the same as the one present in Rps. spheroides and R. rubrum.

SAMENVATTING

(Getallen tussen haakjes verwijzen naar secties van Hoofdstukken)

Een definitie van een fotosynthetisch reactiecentrum die van toepassing is op fotosynthetische bacteriën is (1.3): een Bchl-proteine complex dat in vivo de lichtenergie wegvangt die geabsorbeerd is door Bchl moleculen die niet tot het reactiecentrum behoren, en waarin de opgenomen lichtenergie wordt gebruikt voor de fotoreductie van een electron acceptor met een lage redoxpotentiaal door een (paar) Bchl molecule(n) in een één-electron reactie. De Bchl typen die aanwezig zijn in reactiecentra van purperbacteriën worden P800 en P870 genoemd. P870 is de fotoreductant.

Het literatuuroverzicht in hoofdstuk I behandelt in het kort een aantal reacties die plaats vinden in reactiecentra, en geeft informatie over de structurele organisatie van het bacteriële fotosynthesesysteem.

Reactiecentrum partikels werden geïsoleerd uit Rhodopseudonomas spheroides en Rhodospirillum rubrum door het centrifugeren van chromatoforen die tevoren met SDS waren geïncubeerd (2.2.1). De partikels bevatten P800, P870, carotenoid en ook Bph, dat waarschijnlijk een integraal bestanddeel van reactiecentra in vivo is (3.4). Een excitatiespectrum voor de fluorescentie van P870 (4.3.2) en een analyse van het absorptiespectrum van reactiecentra (4.3.5) wezen erop dat er waarschijnlijk 2 moleculen Bph per reactiecentrum zijn (4.4.1, 4.4.2). De molaire verhouding van Bph, P800 en P870 in reactiecentra is 2 : 2 : 2 of 2 : 2 : 1 (zie 4.4.3). De resultaten van een analyse van de absorptieband bij 600 nm van reactiecentra (2.3.3) pleitten voor de laatste verhouding; de ongeveer gelijke oppervlakken onder de nabij-infrarode absorptiebanden veroorzaakt door P800 en P870, wezen daarentegen op een 2 : 2 : 2 verhouding.

Chemische analyses (2.3.4) wezen uit, dat Bchl *a*, Bph *a*, spheroidene en ook ubichinon-10 aanwezig waren. De molaire verhouding ubichinon : P870 was minimaal 1.2 : 1 (aannemende dat er 1 molecule P870 per reactiecentrum is). De quantumopbrengst voor de fotooxidatie van P870 is ongeveer 1 electron per geabsorbeerd lichtquantum (3.3.1). Lichtenergie geabsorbeerd door P800, Bph en carotenoid werd

met hoge efficiëntie naar P870 overgedragen (3.3.1 en 3.3.3). Het fluorescentie-rendement van P870 was 0.2 - 0.5 °/oo in reactiecentra met geoxideerde electron acceptoren (3.3.4). Het fluorescentie-rendement van P800 was minder dan 0.04 °/oo (3.3.4); dat van reactiecentrum Bph was minder dan 1.2 °/oo (4.3.4). De levensduur van de fluorescentie, zoals berekend uit het fluorescentierendement en de intrinsieke levensduur,was 3-8 picosec voor P870 (met geoxideerde electronacceptoren) en minder dan 0.8 picosec voor P800. De resultaten wijzen erop dat het mechanisme waardoor energie van P800 naar P870 wordt overgedragen, misschien sneller is dan het mechanisme van inductieve resonantie (3.3.5 en 3.4). De levensduur van 3-8 picosec voor de fluorescentie van P870 in geïsoleerde reactiecentra is moeilijk in overeenstemming te brengen met de onlangs gemeten levensduur van 50 picosec voor de fluorescentie van chromatoforen (3.4).

De lichtgeïnduceerde absorptieveranderingen die onder verschillende proefomstandigheden werden waargenomen in reactiecentra van Rps. spheroides en R. rubrum, wezen erop dat de fotooxidatie van P870 was gekoppeld aan de fotoreductie van een electronacceptor X. X kon ofwel terugreageren met het geoxideerde P870, ofwel een secundaire electronacceptor A reduceren, welke dan op zijn beurt in staat was het geoxideerde P870 te reduceren (5.3.1-5.3.4). Dit schema werd gesteund door een vergelijking van de kinetiek van de lichtgeïnduceerde veranderingen in absorptie en fluorescentie van P870 (3.3.2). X is vermoelijk ubichinon, dat in het licht wordt gereduceerd tot het ubisemichinon anion (5.3.4). De pH-afhankelijkheid van de snelheden van de verschillende reacties (5.3.6) wezen erop dat P870 en X van het omringende medium worden gescheiden door een proton diffusie barrière (5.4).

De mogelijkheid dat X (ubichinon) de primaire electron acceptor is, in onze reactiecentrum partikels of in vivo, wordt besproken in Hoofdstuk VI. In Chromatium is onlangs een ijzer-zwavelverbinding geïdentificeerd als de primaire electronacceptor (ref. 16 in Hst. VI). Er blijkt echter geen reden te zijn om aan te nemen, dat de primaire electron acceptor die in Chromatium is gevonden, dezelfde is als die welke in Rps. spheroides en R. rubrum aanwezig is.

ABBREVIATIONS

AUT	An aqueous solution of urea (1M) and Triton X-100 (0.3%) at pH 10.0
AUT-RC	Reaction center particles obtained from SDS-RC particles (see below) by treatment with AUT
Bchl	Bacteriochlorophyll
B800,B850,B...	Bacteriochlorophyll with an absorption maximum at 800,850,... nm
Bph	Bacteriopheophytin
CM	Cytoplasmic Membrane
ICM	Intracytoplasmic Membrane
P690	A pigment with an absorption maximum at about 690 nm, identified as a degradation product of Bchl
P760	A pigment with an absorption maximum at about 760 nm; presumably solubilized Bchl
P800	Reaction center Bchl with an absorption maximum at about 800 nm
P870 (or P)	Reaction center Bchl with an absorption maximum at about 870 nm; the primary photoreductant
SDS	Sodium dodecyl sulphate
SDS-RC	Reaction center fraction obtained by centrifugation of chromatophores preincubated with SDS
Tris	Tris (hydroxymethyl) aminomethane

CURRICULUM VITAE

Overeenkomstig het gebruik in de Faculteit der Wiskunde en Natuurwetenschappen volgt hier een kort overzicht van mijn academische studie.

Na het behalen van het diploma gymnasium β aan het Westfriesche Lyceum te Hoorn in 1962, begon ik datzelfde jaar mijn studie in de biologie aan de Gemeentelijke Universiteit van Amsterdam. Na het afleggen van het kandidaatsexamen (letter k) te Amsterdam, vervolgde ik de opleiding aan de Rijksuniversiteit te Leiden, met als hoofdvak plantkunde en als bijvakken biofysica en hydrobiologie. De doctoraalstudie stond onder leiding van de hoogleraren Dr. A. Quispel, Dr. L.N.M. Duysens en Dr. D.J. Kuenen en werd in december 1968 beëindigd. In december 1968 trad ik in dienst van de Stichting Scheikundig Onderzoek in Nederland in de functie van wetenschappelijk medewerker. Vanaf toen tot 1 maart 1973 werd onder leiding van Dr. J. Amesz het werk verricht waarvan dit proefschrift het resultaat is. Per 1 maart 1973 werd ik benoemd tot assistent in tijdelijke dienst aan de afdeling biofysica van de Vrije Universiteit Brussel.

NAWOORD

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