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# Low temperature photochemistry and spectral properties of a photosystem 2 reaction center complex containing the proteins D1 and D2 and two hemes of Cyt b-559

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A D1/D2/Cyt b-559 complex isolated from spinach chloroplasts contained 4 chlorophylls, 2 pheophytins and 2 hemes of Cyt b-559 with different redox potentials measured to be +70 mV(LP) (pH 7.2) and about −500 mV (XLP). Prereduced Cyt(LP) is photooxidized at 77 K due to the interaction with the state $P680^+Ph^-$ which produces the accumulation of $Cyt^+$ P680 $Ph^-$. Another Ph molecule is reduced with a midpoint potential of −450 mV and can interact with Cyt (XLP) forming a second electron transfer chain.

Photosystem 2; Reaction center; Cytochrome b-559; Charge separation

## 1. INTRODUCTION

The process of primary light energy conversion in photosynthesis occurs in reaction center protein (RC). Two types of RC exist in the thylakoid membranes of higher plants. One is located in the photosystem II complex which is involved in the photolysis of water. Its principal structure appears to be similar to that of RCs from photosynthetic bacteria which have been studied by X-ray analysis [1-3]. Bacterial RCs consist of 3 protein subunits (L, M and H). The L and M subunits include 4 bacteriochlorophyll molecules, 2 bacteriopheophytins, 2 quinones ($Q_A$ and $Q_B$) and 1 iron atom. A bacteriochlorophyll dimer forms the primary electron donor P. The L and M subunits in *Rhodopseudomonas viridis* RCs are connected to one cytochrome (Cyt) protein subunit containing 4 hemes.

A close relationship between bacterial RCs and the reaction center of photosystem II (RC-2) is suggested by a considerable homology between the amino acid sequence of the L and M bacterial subunits and the so-called D1 and D2 proteins of RC-2 from green plants [4-7]. A complex isolated from thylakoids and containing D1/D2/Cyt b-559 displays properties expected from RC-2 [8,9]. It includes 4-5 Chl molecules, 2 pheophytin (Ph) molecules, 1 iron atom and one $\beta$-carotene molecule.

In bacterial RCs, bacteriopheophytin (BPh) can be stably reduced on illumination of RCs in which the $Q_A$ and $Q_B$ have been prereduced in the dark [10]. In *Chromatium* (as well as in *R. viridis*) an electron is transferred from Cyt associated with the reaction center to the light-induced state $P^+BPh^-$ to form and trap the $Cyt^+P$ $BPh^-$ state. The formation of this state decreases nanosecond luminescence which results from the nanosecond recombination of $P^+$ and $BPh^-$ [10]. Photoreduction of Ph was also observed in PS-2 particles with a similar decrease of recombination luminescence [11]. It has been demonstrated with the isolated RC-2 complex that following illumination P680 is oxidized [9] and $P680^+Ph^-$ is formed within picoseconds [13] and that $Ph^-$ can be accumulated [8,9]. It has been shown further that $Ph^-$ accumulation requires electron donation from some source to the state $P680^+Ph^-$ [12]. However the nature of this electron donor was not known.

The present work shows that the isolated RC-2 complex contains two hemes of Cyt b-559 with different redox potentials (+70 mV and about -500 mV), four Chl and two Ph molecules. $P680^+Ph^-$ formation is observed at 77 K. $P680^+$ is reduced by a Cyt b-559 heme which has an $E_m$ (pH 7.2) of +70 mV. Fluorescence bands at 680 and 685 nm have different excitation spectra in the 500 nm region. There is an additional carotenoid band at 498 nm for 685 emission. The data are discussed in terms of the existence of two quasi-symmetrical cofactor chains, each containing a Ph, two chlorophylls and a heme, with the hemes of the two chains differing in redox potential.

## 2. MATERIALS AND METHODS

The D1/D2/Cyt b-559 complex was isolated at 4°C by the method

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described in [8,9]. Briefly, spinach chloroplasts were isolated in 50 mM Na/K phosphate buffer (pH 7.6) containing 100 mM sucrose and 200 mM NaCl. They were suspended at a chlorophyll concentration of 3 mg · ml$^{-1}$ in 50 mM Na/K phosphate buffer (pH 7.9) containing 300 mM sucrose and 50 mM NaCl.

PS-2 particles were freed from the thylakoids by Triton X-100 (3.3%) treatment (30 min) in the same buffer and isolated by differential centrifugation. A pellet of PS-2 particles obtained by centrifugation at 35 000 × g was resuspended in 50 mM Tris-HCl buffer (pH 7.2). The chlorophyll concentration was 1 mg Chl · ml$^{-1}$. Treatment of PS-2 particles with Triton X-100 (4.8%) and subsequent chromatography on a column of Fractogel TSK DEAE-650 (S) (Merck, Darmstadt, FRG) yielded the D1/D2/Cyt b-559 complex. The preparation was stored at −80°C in 50 mM Tris-HCl buffer (pH 7.2) in the presence of 0.05% Triton X-100 and 10% glycerol. For measurements at 77 K the glycerol concentration was increased up to 60%. Absorbance spectra of RC-2 preparations were very similar to those described in [8,9].

Absorption spectra ($1-T$) ($T$ is optical transmission), difference absorption spectra and excitation and emission spectra of fluorescence were measured at 293 K and 77 K using a modified Aminco SPF-500 spectrofluorometer (Silver Springs, USA) equipped with multibranched fiberoptics connected to a Dewar cuvette. Data acquisition, averaging, subtraction and division of spectra were done by computer (Hewlett Packard, HP 85). Absorbance spectra of RC-2 and their acetone extracts were measured at 293 K using a Shimadzu spectrophotometer (Spectronic 210 UV).

For redox potential titrations the following mediators (10 $\mu$M) were used: 2,6-dichlorophenolindophenol, methylene blue, 2-methyl-1,4-naphthoquinone, phenazine methosulfate, safranine, benzyl viologen, Neutral red and methyl viologen. Ascorbate, dithiothreitol and dithionite were used as reductants. Redox potential titrations in the range from −100 to −600 mV were performed under anaerobic conditions with increasing pH from 7.2 at −400 mV up to 10.0 at −600 mV.

## 3. RESULTS

Pigments were extracted from RC-2 fractions with 80% acetone and absorbance spectra of the extracts were measured. After adding 4 mM HCl (to convert Chl to Ph) to neutral extracts, the amplitude of a Ph band peaking at 535 nm was increased by a factor of 3.02 ± 0.15. The acid dependent increase in the height of the Ph band shows that the ratio of Chl/Ph is 2.02 ± 0.15 in our RC-2 fractions. This agrees with previous estimations of the Chl/Ph ratio in RC-2 [8,9].

Fig.1A shows absorption spectra (470–600 nm) at 77 K and 293 K of RC-2 as a function of ambient redox potential (see section 2). At a redox potential of −100 mV and 77 K, the absorption band of Ph is observed at 543 nm. At 77 K, Cyt b-559 absorbs at 556 nm and $\beta$-carotene around 492 and 510 nm. No Cyt b-559 bands are observed at a redox potential of +175 mV either at 77 K (not shown) or at 293 K (fig.1A). With decreasing redox potential the distinct band of reduced Cyt appears at 559 nm at 293 K (fig.1A). As shown in fig.2, reductive titration of RC-2 yields two waves of a Cyt b-559 absorption increase. The first wave displays one-electron ($n=1$) behaviour. A midpoint potential of +70 mV (pH 7.2) is similar to that of flow potential Cyt b-559 in chloroplasts [14]. At −100 mV, this Cyt(LP) is completely reduced. After correction of the contribution of Cyt to absorption at 543 nm and that of Ph at

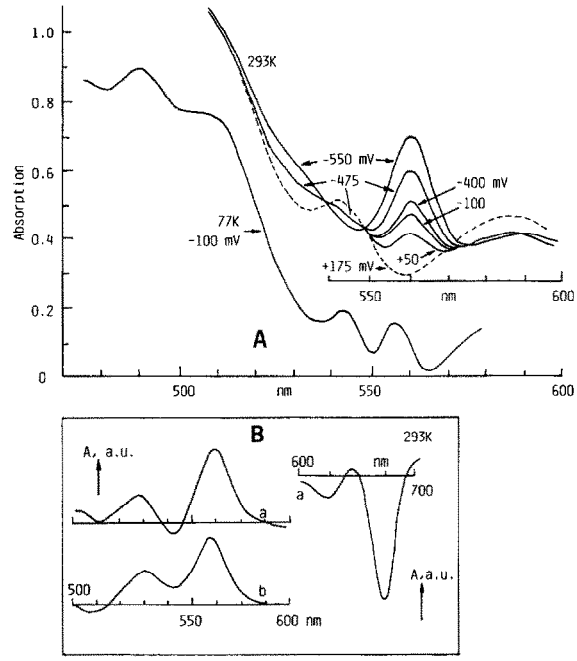


Fig.1. (A) Absorption ($1-T$) spectra of RC-2 at 77 K and a redox potential of −100 mV, and at 293 K and different $E_h$ values (see section 2 for details). (B) Difference between absorption spectra measured at redox potentials of −565 mV and +175 mV (a) and between those at +80 mV and +175 mV (b) at 293 K. The difference absorption spectra in (a) and (b) were normalized at 560 nm. The real difference between maxima at 560 nm corresponds to those plotted in figs 1A and 2. a.u. = arbitrary units.

560 nm, the ratio of amplitudes of absorption bands of Cyt at 560 nm to Ph at 543 nm was 1.16 ± 0.05 (293 K). When the redox potential was decreased below −200 mV, another wave of Cyt absorption appeared. The slope of the redox titration curve was smaller than that of the first wave. The apparent midpoint potential was −500 mV. This type of extra low potential Cyt b-559 we will call Cyt(XLP). It is possible that small fraction of Cyt(XLP) was also observed by Gounaris et al. [20] in the presence of dithionite and oxygen at pH 8.0 ($E_h$ probably was not less than −450 mV) but without measurements of $E_h$. The titration curve of Cyt(XLP) deviates from the behaviour expected from the Nernst equation. This may be caused by heterogeneity of redox potential of this Cyt in RC-2 or by slow redox equilibration with the medium. The amplitudes of the two waves were comparable. This implies that the ratio Cyt(LP)/Cyt(XLP) in RC-2 is 1.

Difference absorption spectra for the reduction of Cyt(LP) and Cyt(XLP) were almost identical in the 500–600 nm region (fig.1B, a and b) except for an additional negative change at 543 nm observed during Cyt(XLP) reduction which is attributed to parallel reduction of Ph. Reduction of Ph under these conditions is indicated by bleaching of the 672 -nm band of Ph with a shoulder at 680 nm (fig.1B). The redox difference spectrum produced during the redox titration differs from that of the photoreduction of Ph (see

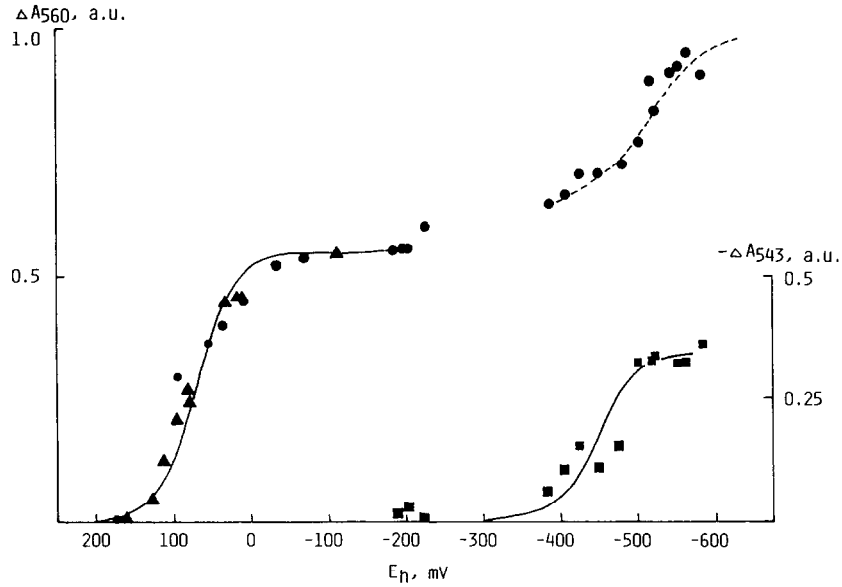


Fig.2. Redox potential titration of the reduction of Cyt b-559 and Ph measured at 293 K via changes in absorption at 560 nm (solid triangles and circles represent two separate experiments) and 543 nm (solid squares). Absorption changes at 543 nm due to Ph reduction were plotted after correction of the contribution of Cyt b-559 absorption. Redox mediators and reductants are indicated in section 2. Solid curves represent one-electron Nernst behaviour for +70 mV (560 nm) and −450 mV (543 nm).

fig.3D). The redox titration curve for the bleaching of the 543 nm Ph band displays a midpoint potential of −450 mV (Ph(HP)), which is very close to that of Cyt(XLP) (fig.2). In contrast, photo-reducible Ph has a redox potential of about −610 mV in PS-2 particles [15]. It might have the same potential in RC-2 since only about 60% of the 543 nm band of Ph is bleached at −580 mV (fig.2).

At 77 K, the maximum of the Cyt(LP) band is shifted to 556 nm (fig.1A). After suitable correction, the ratio of absorptions of Cyt(LP) at 556 nm and Ph at 543 nm is 1.23 ± 0.05, when Cyt(LP) is completely reduced at −100 mV. When RC-2 is illuminated at 77 K with Cyt(LP) prereduced, parallel oxidation of Cyt(LP) and reduction of Ph is induced in some fraction of RC-2 (fig.3C,D). The difference spectrum of $Ph^-$ formation is very similar to that reported for photoreducible Ph(LP) [11]. This spectrum is characterized by bleaching of Ph bands at 545 and 670 nm and by an asymmetric shift of the 680 nm Chl band with maximum bleaching at 684 nm. The appearance of a broad band around 650 nm is due to radical anion formation. In addition, there is a carotenoid band shift at 498 nm (fig.3C). The oxidation of Cyt(LP) is characterized by

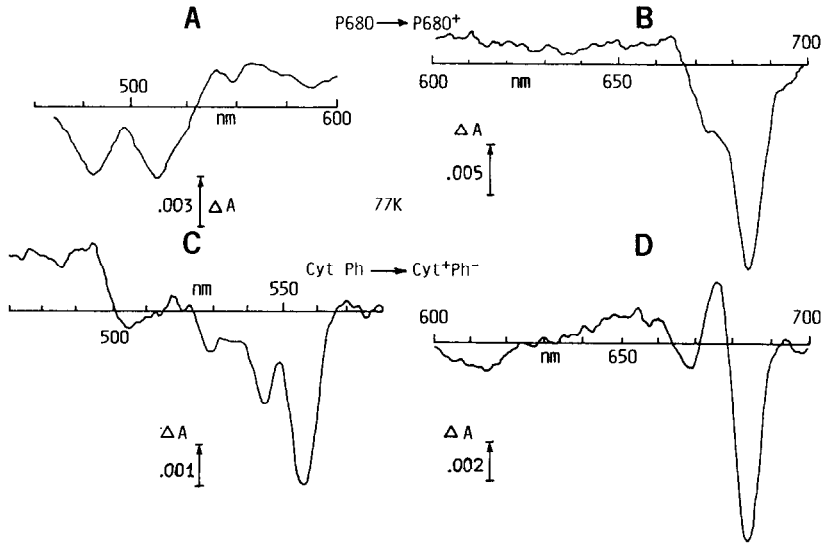


Fig.3. Difference absorption spectra of RC-2 at 77 K for the light-induced accumulation of $P680^+$ after 10 min illumination at 77 K in the presence of silicomolybdate ($10^{-4}$ M) (A and B) and of $Cyt^+Ph^-$ at −100 mV after 30 min illumination at 77 K (C and D). Absorbance at 680 nm was 0.33.

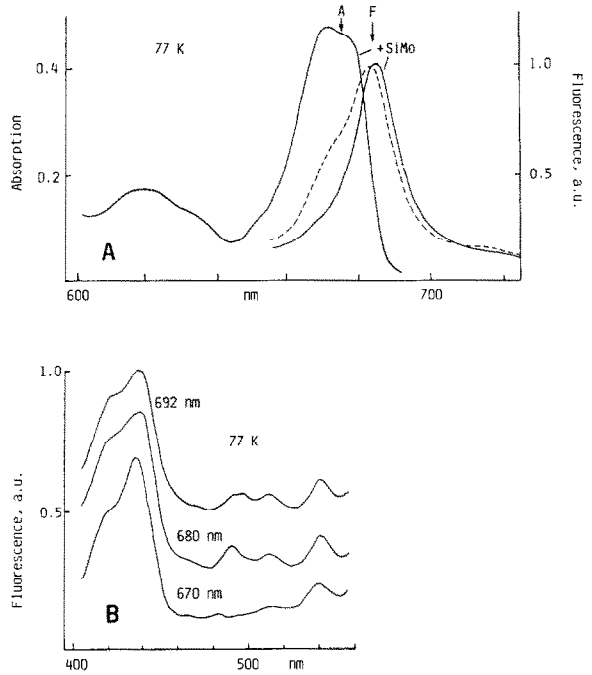


Fig.4. (A) Absorption and fluorescence (F) spectra of RC-2 at 77 K without additions (dashed curve for F) and in the presence of SiMo ($5\times10^{-5}$ M) (solid curves for A and F). Excitation of fluorescence was at 438 nm. Fluorescence spectra were normalized at their maxima. Spectral resolution was 2 nm. (B) Excitation spectra of fluorescence at 77 K for emissions at 692 nm, 680 nm and 670 nm in the presence of silicomolybdate ($5\times10^{-5}$ M). Spectral resolution was 2 nm. Excitation spectra for the 692 and 680 nm emissions were measured with identical sensitivities of the spectrofluorometer, whereas the spectrum for 670 nm was measured with increased sensitivity.

bleaching at 556 nm ($\alpha$-band) and 529 nm ($\beta$-band) (fig.3C). The ratio of absorptions of Cyt(LP) at 556 nm and of Ph at 545 nm is $2.47\pm0.05$ (fig.3C) after suitable corrections. This value is larger than the ratio of absorption of Cyt and Ph ($1.23\pm0.05$) at 77 K by a factor of $2.05\pm0.1$. As one electron can be transferred from one Cyt(LP) to one Ph at 77 K, we conclude that two molecules of Ph are present per one Cyt(LP) or per two hemes of Cyt b-559 (see above). These estimations exclude any errors related to measurements of extinction coefficients of cofactors under different conditions.

Light-dependent oxidation of P680 in some fraction of RC-2 can be observed at 77 K in the presence of silicomolybdate (SiMo). Fig.3A and B show the difference absorption spectrum for this reaction. The bleaching of the band at 684 nm, red shifts of the Ph bands at 670 and 545 nm and bleaching of $\beta$-carotene bands at 485 and 510 nm are observed.

The absorption spectra of RC-2 at 77 K (fig.4A) are practically identical in the presence and the absence of SiMo. The amplitudes of two main bands around 670 and 680 nm are almost equal. Fluorescence spectra of RC-2 at 77 K include at least 3 bands at 671 nm, 680 nm and 685 nm (fig.4A; see also [16]). These bands display differences in their excitation spectra. This is shown in fig.4B. The emission at 680 nm is sensitized by the Chl band at 437 nm, by $\beta$-carotene bands at 491 and 512 nm and by the Ph bands at 543 and 512 nm. The emission at 685 nm (measured at 692 nm with the same fluorometer sensitivity as in the 680 nm measurement) is sensitized by the same bands and in addition by another carotenoid band at 498 nm. The emission at 670 nm which was measured with a higher sensitivity is not sensitized by the 491 and 512 nm carotenoid bands but by the 437 nm Chl band and by the Ph bands at 512 and 540 nm. The relative decrease of the 420 nm band can be related to reduced excitation transfer from carotene to chlorophyll. In the presence of silicomolybdate, the emission at 670 nm is considerably suppressed (fig.4A). This emission is probably related to a small fraction of Chl/Ph not attached to the RC-2 interior.

Illumination of RC-2 for 10 min in the presence of SiMo at 77 K leads to the bleaching of $6\pm0.8\%$ of the 684 nm band. It is accompanied by a decrease of the fluorescence at 685 nm by $19\pm1.0\%$.

## 4. DISCUSSION

The presented data show that photosystem 2 reaction centers (RC-2) contain two hemes of Cyt b-559 with very different redox potentials, i.e. +70 mV (LP) and about −500 mV (XLP). There are two Ph and four Chl molecules per two hemes. The ratio of Chl/Ph is 2/1 as is the BChl/BPh ratio in bacterial RCs [17]. However, whereas histidines liganding the Mg atoms of the special BChl pair in the L and M subunits of bacterial RCs are conserved in the protein sequence of D1 and D2, no similar conservation of the histidines liganding Mg of the other BChl molecules in the L and M subunits is observed in D1 and D2 [4,18]. This means that the two Chl molecules which do not correspond to the special pair in bacterial RCs are in positions which differ from those in the bacterial RCs (see below). For this reason, differences in the interaction between these Chl and Ph must be expected in RC-2 in relation to the situation in bacterial RCs.

Reduced Cyt(LP) is photooxidizable at 77 K. Its electron is transferred to Ph(LP). This means that Ph(LP), P680 and Cyt(LP) form a cofactor chain: Cyt(LP)/P680/Ph(LP). The reaction between P and Ph leads to the light-induced formation of $P680^{+}Ph^{-}$ observed earlier in nanosecond [12] and picosecond [13] time domains. The midpoint redox potential of photoreducible Ph(LP) (−610 mV [15]) differs from that of the other Ph (Ph(HP) with −450 mV, see fig.2). Also, the difference spectrum for the formation of $Ph(LP)^{-}$ (fig.3C,D) is not identical to that of $Ph(HP)^{-}$ (fig.1B) which is reduced in parallel with Cyt(XLP). This suggests that Chl, Ph(HP) and Cyt(XLP) form another cofactor chain. It might be in a quasi-symmetrical arrangement in relation to the chain whose photochemical activity can easily be observed.

However, its low redox potential suggests that in this arrangement Cyt(XLP) may be an electron acceptor. It would be consistent with an electron cycling around PS-2 in chloroplasts which includes Cyt b-559 [19].

The two main bands of fluorescence at 680 and 685 nm show a small but significant difference in their excitation spectra around 498 nm (fig.4) where an additional carotenoid band excites 685 nm emission. This particular band is shifted by the field of $Ph^-$ at 77 K (fig.3C). It indicates that fluorescence at 685 nm is probably emitted by a cofactor chain consisting of Cyt(LP)/P680/Ph(LP)/Car498. If this conclusion is correct, the emission at 680 nm would come from the chain consisting of Cyt(XLP)/Chl(?)/Ph(HP)/Car491. These emissions are probably not completely independent and some excitation energy transfer can be expected.

There are two bands with almost equal intensities in the absorption spectrum of RC-2 around 670 and 680 nm. Probably Ph(LP) mostly contributes to the 680 nm band (fig.3D) and Ph(HP) to the 670 nm band (fig.1B). As the amplitudes of the bands at 670 and 680 nm are almost equal, it is necessary to assume that two Chls contribute to the absorption at 670 nm and the other two to the absorption at 680 nm.

The oxidation of P680 at 77 K decreases the absorption at 680 nm by about 6%, but quenches the fluorescence around 685 nm in the same sample by about 19%. If $P680^+$ quenches the fluorescence in RC-2 completely this would mean that 1/3 of the absorption at 680 nm is related to P680. If the quenching is less than 100% the fraction of P680 bleaching would be less too. We suggest therefore that P680 is a Chl monomer, not a dimer situated in the center of D1/D2. In the latter case, 2/3 of the absorption at 680 nm should be bleached, not 1/3 as calculated above. Considerable interaction of monomer Chl(P680) with Ph(LP) can be expected if Mg of P680 is liganded by histidine-118 in $\alpha$-helices of D1. Then tyrosine-161 can promote electron transfer from Cyt(LP) to $P680^+$. To some extent, a similar arrangement might be possible for the second cofacter chain since in D2 histidine-118 and tyrosine-161 are also conserved [18].

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