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Spectral and thermodynamic properties of the two hemes of the D1D2cytochrome *b-559* complex of spinach

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Abstract

In agreement with previous work [Shuvalov, Heber and Schreiber (1988) FEBS Lett. 258, 27–31] two hemes (low potential (LP) and extra low potential (XLP)) per two pheophytins were found in isolated D1D2Cyt *b-559* complexes. Reductive and oxidative redox titrations demonstrate that the E_m of the LP form is at about +150 mV. It is independent of pH between pH 7.2 and 9.4. The XLP heme is autoxidizable at pH 7.2 and displays, at this pH, an E_m of −45 mV. Both the LP and XLP hemes show absorption peaks at 559 nm. They are proposed to have bis-histidine ligation of the heme iron. At pH 9.4, the XLP heme splits into two forms. One of them has an E_m of +40 mV, and absorption peaks at 559 nm showing the bis-histidine ligation. The other displays an E_m of −220 mV and the peak is shifted to 562 nm. This last form is proposed to be due to the incorporation of OH^- which occupies the 6th coordination position of the heme Fe(III) at high pH. The p*K* value for the conversion of the XLP heme is close to 7.7. In a structure simulation of the α-helices of α- and β-polypeptides, the β-polypeptide, but not the α-polypeptide, reveals a distance between the histidine N and the heme Fe which permits stable N-Fe coordination. In the α-polypeptide, OH^- can be incorporated between N and Fe. The functional role of the two hemes of cyt *b-559* is briefly discussed with respect to water oxidation and cyclic electron transfer.

Key words: Cytochrome *b-559*; D1D2 Cyt *b-559* complex; PS-II

1. Introduction

Although PS-II has been shown many years ago [1] to be able to oxidize Cyt *b-559* with high quantum yield, the function of this Cyt, which is a component of the reaction center of PS-II, is still a matter of discussion [2–9]. Cyt *b-559* consists of two polypeptides with molecular masses of approximately 9 (α-polypeptide) and 4 (β-polypeptide) kDa. They are encoded by the psbE and psbF genes, respectively (see [10,11] for refs.). The two polypeptides are present in 1:1 stoichiometry [12]. Both have a single membrane-spanning region [3] with the following amino acid sequence:

NH_2...RY(2)WVIH(6)SITI(10)PSLFIAGWLFVSTGL AYD(28) α

NH_2...RW(2)LAIH(6)GLAV(10)PTVSFLGSISAMQ FIQR(27) β

It was found that the α-polypeptide has its carboxy terminus on the lumenal side of thylakoid membrane [13]. The Cyt has been suggested to be an $(\alpha\beta)_2$ heterodimer [12] or to consist of $(\alpha)_2$ and $(\beta)_2$ homodimers [14], since each polypeptide contains only a single histidine residue capable of occupying a coordination position of the Fe in the two hemes [3].

The redox potential of Cyt *b-559* is heterogeneous. In intact chloroplasts it displays two forms with redox potentials of +380 mV (high-potential, HP) and +80 mV (low potential, LP) [1]. In hydroxylamine-treated chloroplasts the HP heme displays an intermediate redox- potential (IP) of +240 mV [20]. The high potential form is affected by pH lowering [15], Mn removal [16], ADRY reagents [17], etc.

In isolated D1D2 Cyt *b-559* complexes, Cyt has been reported to have two hemes: LP with an E_m not far from +80 mV and an 'extra-low potential' (XLP) with E_m as low as −500 mV at pH 7.0 [18]. The nature of both has so far not been understood.

Very recently, another redox potential titration of Cyt *b-559* in isolated RC-2 was published by Ahmad et al. [21] who found only one heme (1 Cyt:5.7 Chl) with three redox forms (430 mV, 180 mV and 25 mV). However, in this study no attempts were made to reduce Cyt at redox potentials below −50 mV.

We have performed a detailed further analysis of the two hemes of Cyt *b-559* in isolated RC-2. The LP and XLP hemes displayed E_m values of +150 mV and −45 mV, respectively, at pH 7.2. We have performed a detailed further analysis of the two hemes of Cyt *b-559* in isolated RC-2. The LP and XLP hemes displayed E_m

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Abbreviations: Cyt, cytochrome; E_m, midpoint redox potential; HP, IP, LP and XLP, high, intermediate, low and extra low potential forms of hemes, respectively; P680, primary electron donor; Phe, pheophytin; RCs, reaction centers; PS-II, photosystem-II.

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values of +150 mV and −45 mV, respectively, at pH 7.2. The very low redox potential of the XLP heme reported earlier [18] is an artifact caused by the extreme sensitivity of this heme to oxidation by oxygen. At pH 9.4, the XLP heme splits into two forms with different spectral and thermodynamic characteristics. The relation of these features to Cyt *b-559* function in PS-II is discussed.

2. Materials and methods

RCs of PS-II were isolated as described earlier [18] using Triton X-100 for PS-II particles and RCs solubilization. DEAE-chromatography with Fractogel TSK DEAE-650 (S) (Merck, Darmstadt) was used for purification of RCs. Triton X-100 was replaced by 0.1% of *n*-dodecyl β-D-maltoside (Sigma) on the column. RCs were stored at −80°C in the presence of 10% glycerol, 0.1% maltoside and 50 mM Tris-HCl (pH 7.2). The absorbance measurements at different E_m and pH values were done in 50 mM Tris-HCl buffers containing 0.05% Triton X-100, 30 mM NaCl and RCs diluted to an optical density of 0.01 at 542 nm (Phe band).

Absorbance spectra of the samples were measured at 10°C using an Aminco DW-2a spectrophotometer (Silver Springs, USA).

Redox potential titrations were performed at 10°C using a platinum electrode (Ingold, Germany). Each titration was finished within 2–3 h. The following mediators were used (10 μM): diaminodurene, 1,2-naphthoquinone-4-sulphonate, duroquinone, menadione, 2,3-dimethoxy-5-methyl-1,4-benzoquinone, 2-hydroxy-1,4-naphthoquinone, anthraquinone-2-sulphonate, neutral red, 1 or 10 μM benzyl viologen and 1 or 10 μM methyl viologen. Ascorbate, dithiothreitol and dithionite were used as reductants and ferricyanide as an oxidant. Anaerobic conditions were created by glucose/glucose oxidase in the presence of catalase. A stream of oxygen-free nitrogen gas was directed over the solution.

Beef liver catalase was from Boehringer Mannheim (Germany).

For α-helix structure simulation an IBM-486 computer supplied by a HyperChem program (Autodesk, USA) with molecular mechanics optimization was used.

3. Results

A chlorophyll/pheophytin ratio of 1.7 ± 0.3 was measured using 80% aceton extraction and subsequent acidification by HCl [18]. The ratio of differential extinction coefficients of the Cyt *b-559* band at 556 nm and the Phe band at 545 nm was measured at 77K for the 1 electron transfer reaction Cyt P680 Phe → Cyt$^+$ P680 Phe$^-$ as described earlier [18]. This ratio was found to be 2.4 ± 0.2, in reasonable agreement with previous measurements [18]. It is assumed that this ratio is not changed when temperature increases to 10°C since the ratios of

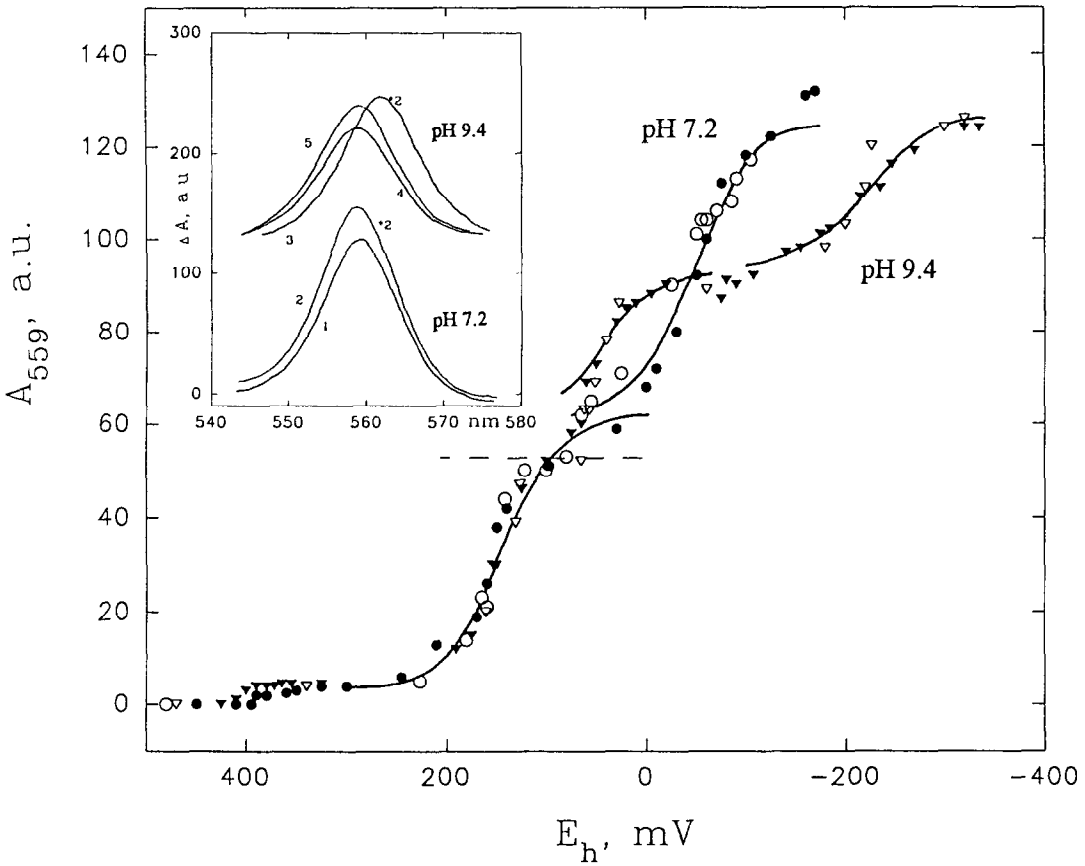

Fig. 1. Reductive (closed symbols) and oxidative (open symbols) redox titrations of Cyt *b-559* in RC complexes at 10°C under strictly anaerobic conditions (for reaction mixture see Section 2). The titration of RC Cyt was carried out at pH 7.2 from +480 to − 180 mV (○,●) and at pH 9.4 from +480 mV to −350 mV (▽,▼). The initial absorbance of Phe band at 542 nm (0.01) is shown by the dashed line. The solid curves represent one electron Nernst behavior for E_m of +150 mV and −45 mV (○,●); +150 mV, +40 mV and −220 mV (▽,▼). The inset shows the difference absorption spectra of α-bands of RC Cyt *b-559* between the following redox potentials of the medium: −106 mV and +480 mV at pH 7.2 (curve 1); +97 and +480 at pH 7.2 (curve 2); −243 mV and −27 mV at pH 9.4 (curve 3); +40 mV and +480 mV at pH 9.4 (curve 4) and −90- mV and +480 mV at pH 9.4 (curve 5). Symbol *2 indicates the multiplication of the spectrum by a factor of 2.

the widths of the Phe and Cyt bands are similar at 77K and 10°C.

Fig. 1 shows reductive and oxidative redox titrations of Cyt *b-559* under anaerobic conditions at different pH values, with the amplitude of the Cyt band at 559 indicating the extent of reduction. At pH 7.2, an E_m of +150 mV is observed which corresponds to that of the LP heme [18]. The maximal amplitude of this wave at 559 nm is 1.2 ± 0.1 times larger than that of the Phe band at 542 nm showing that approximately one LP-heme is present per two Phe molecules. A small fraction of the HP form (~ 6% of the LP-form) is also observed with an E_m of ~ +380 mV. At lower E_h reduction of the XLP-heme is observed with an E_m of −45 mV. The maximal amplitude of this wave at 559 nm is the same as that of the LP-heme showing that one XLP heme is present per two Phe molecules and per one LP-heme. It is important to note that the XLP heme is extremely autoxidizable. Therefore, its reduced form is not observed up to −200 mV in the presence of traces of O_2. Under these conditions the reduction of the heme is only observed in the presence of dithionite [18].

At pH 9.4 the LP heme has the same E_m of +150 mV. However, the XLP heme splits into two forms with different E_m and spectra. One of them has an E_m of +40 mV and the absorption peak is 559 nm (Fig. 1). It can be reduced even in the presence of traces of O_2. The other displays an E_m of −220 mV and the absorption peak is shifted to 562 nm (Fig. 1).

Fig. 2 shows a pH titration of the −45 and +40 mV waves of RC Cyt *b-559* at low redox potentials in the presence of ascorbate and dithiothreitol (from −20 to −180 mV). Traces of O_2 were present owing to the absence of nitrogen flow over the solution. With gradually increasing pH the appearance of the ferroheme *b-559* shows an apparent p*K* of 7.7. This band disappears again when the pH is returned to 7. The solid curve shows the theoretical titration curve assuming cooperative binding of two OH^- per heme with a p*K* of 7.7. The effect is due to the conversion of the autoxidizable −45 mV form (not observed because some oxygen was present) of the XLP heme to the nonoxidizable +40 mV form at high pH.

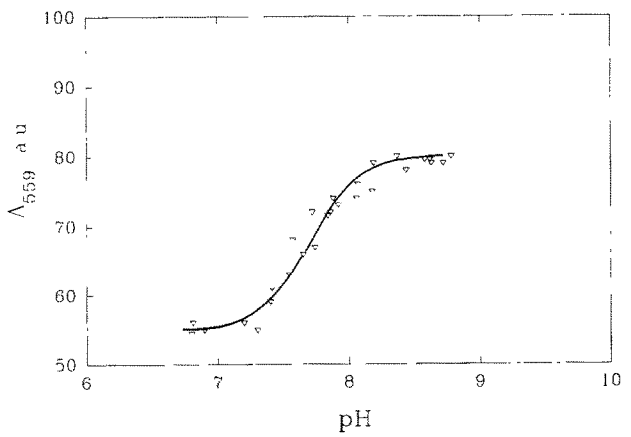

Fig. 2. pH titration of RC Cyt *b-559* from pH 6.8 to 8.8 in the presence of ascorbate and dithiothreitol (from −20 to −180 mV) at 10°C. Some O_2 was present. For reaction mixture composition see Section 2. The solid curve shows calculated cooperative binding to the heme of two hydroxyl ions with p*K* = 7.7 (see text for discussion).

Measurements of absorbance changes near 695 nm where there is an absorption band associated with methionine-heme coordination (extinction coefficient of ~ 800 $M^{-1} \cdot cm^{-1}$ [22]) did not show any band development when the pH was changed.

4. Discussion

Five different forms of two hemes (LP and XLP) of Cyt *b-559* can be distinguished in our D1D2 reaction center preparation by redox and pH titrations. The observed midpoint potentials range from about +380 to −220 mV (Fig. 1) and, hence, are substantially lowered with respect to the in vivo system, in which the HP-form with E_m = +380 mV predominates. The presented data suggest that the variation in redox potentials results from differences in polypeptide and ligand binding of the hemes.

A very small fraction (~ 3% of two hemes) of the HP-form has an E_m ~ +380 mV (Fig. 1). The redox potential wave with an E_m of about +150 mV is close to that of the well-known LP heme [1]. But it may also correspond to the IP form of the HP heme [20]. The waves with the E_m of −45 mV at pH 7.2 and of +40 mV and −220 mV at pH 9.4 are related to the XLP heme [18]. Taking into account the ratio (~ 2.4) of the differential extinction coefficients for the heme at 559 nm and for Phe at 542 nm which is measured during the 1 electron oxidation of Cyt *b-559* at 77K [18], one can see from Fig. 1 that the RCs have ~ 1 LP and ~ 1 XLP hemes per two molecules of Phe. This agrees with earlier findings [18]. The small difference in the E_m values found for the LP heme in [18] and in the present work may be due to the slightly different isolation procedure of RC-2. Ahmad et al. [21] reported the presence of only ~ 1 heme (1 Cyt:5.7 Chl) with three redox forms (430, 180 and 25 mV) at pH 8.0 in isolated RC-2 in the presence of 2–4 mM maltoside. These forms are reasonably consistent with our findings in [18] and with the results of the present work obtained at pH 9.4. However, these authors could not achieve complete reduction of the XLP heme. Their lowest redox potential was −50 mV at pH 8.

The increase of pH from 7.2 to 9.4 leads to the conversion of the XLP heme, which consists of only one form with an E_m of −45 mV at low pH, into two forms, which are almost equal in amplitude and display E_m values of +40 and −220 mV at pH 9.4 (Fig. 1). The p*K* value for this conversion is close to 7.7 (Fig. 2). The absorption peak at 559 nm is characteristic of the +40 mV form as

well as of the LP heme at any pH and of the XLP heme at low pH. In these forms the Fe is ligated in its 5th and 6th coordination positions by histidine nitrogen according to a model system study [22]. The appearance at pH 9.4 of a XLP heme with the E_m of −220 mV and an absorption peak shifted to 562 nm suggests a change in ligation of the heme Fe in this form. We propose that this ligand is OH^- which accepts a proton when Fe^{3+} is reduced. Since the histidine N is a strong-field ligand [23], the EPR spectrum shows low spin Fe(III) for bis-histidine ligation [22]. OH^- is a weak-field ligand [23]. High-spin Fe(III) should be expected for this ligand. Indeed, a replacement of the histidine ligand by OH^- when the pH was increased from 6.8 to 9.4 in D1D2Cyt *b-559* complex was proven by EPR measurements which are reported in a forthcoming paper [24].

The physiological significance of the XLP heme is not clear. There is the possibility that it is an isolation artifact. However, the existence of two different hemes per RC and the possibility of OH^- binding between Fe(III) of the heme and histidine N in RC Cyt *b-559* are consistent with a computer simulation of α-helices of the α- and β-polypeptides of the psbE and psbF gene products (see Introduction and Materials and Methods). Fig. 3 shows the helical structure in the region of the first 13 amino acids of α-helices of α- and β-polypeptides after energy optimization. One can see that the α-polypeptide has three amino acids Tyr-2, His-6, and Ileu-10 with atoms O (Tyr), τ-N (His) and C (Ileu) being placed in an almost straight line forming an angle of ~48° with the α-helical axis and separated by distances of 5.52 Å (C-N), 6.74 Å (N-O) and 12.26 Å (C-O). In the β-polypeptide the His-6 τ-N extends ~1.5 Å beyond the line connecting atoms N (Trp-2) and C (Val-10) in the direction of the heme. The line connecting N-2 and C-10 forms an angle of ~35° with the α-helical axis (Fig. 3).

Thus we can suggest that in a homodimer $(\beta)_2$ the His of the β-polypeptide can form a ligand to Fe of RC Cyt, as in this case the distances between protoheme and amino acids are appropriate (Fig. 3). Such arrangement would correspond to the LP heme. In the case of the α-polypeptide, however, a close contact between τ-N and Fe is sterically hindered by the formation of a hydrogen bond between the OH group of Tyr-2 and the carboxyl oxygen of protoheme on one hand and by hydrophobic interaction between the methyl group of Ileu-10 and methyl and vinyl groups of protoheme on the other hand. Hence, in the α-polypeptide the His ligand of Fe(III) is not as spatially stable as in the β-polypeptide. At high pH one His N in a homodimer $(\alpha)_2$ may be replaced by OH^- in part of the XLP heme (Fig. 3).

There are indications that in isolated RC-2 the reduced LP-heme is photo-oxidized by $P680^+$ at 77K [18] whereas another heme is photoreduced in the presence of added quinone both at positive temperatures [25] and at −40°C [26]. The latter heme cannot be oxidized by $P680^+$ at 77K [26]. Then one can suggest that the LP heme is located close to P680 whereas the XLP heme more close to Phe or Q. In chloroplasts (where the hemes are probably transformed into the chloroplast HP and LP forms, respectively) the LP heme may be located on the lumenal side and the XLP heme on the stromal side of the thylakoid membrane providing a pathway for cyclic electron transfer in PS-II (see e.g. [8]). This suggestion is consistent with findings that the α-polypeptide carboxy terminus is oriented on the lumenal side of the thylakoid membrane providing the orientation of the histidine on the stromal side [13].

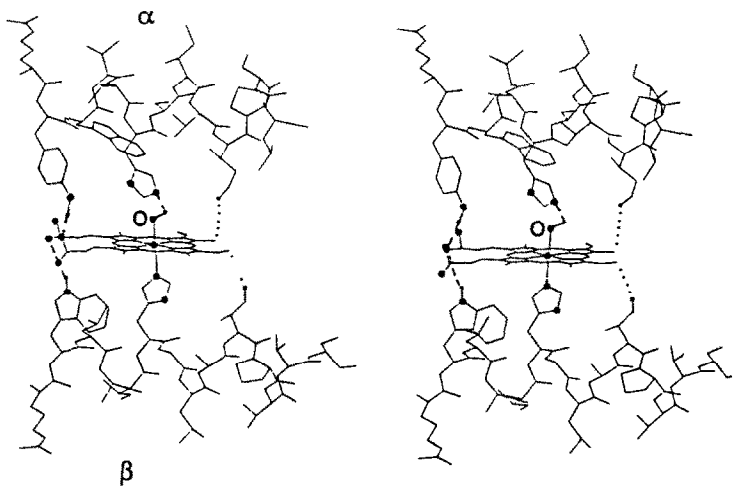

Fig. 3. Stereo image illustrating the position of the heme of RC Cyt *b-559* between the α-helices of α- and β-polypeptides. Computer simulation was done on the basis of known amino acid sequences using a HyperChem program with energy optimization of less than 0.1 kcal (Å mol). For both polypeptides the amino-terminus is on the left side. The protoheme was placed between Tyr-2, His-6 and Ileu-10 of the α-polypeptide α-helix and Trp-2, His-6 and Val-10 of the β-polypeptide α-helix according to the distances between atoms revealed by computer simulation (see text for further details). Oxygen, nitrogen and iron atoms are shown by balls. Hydrogen bonds are indicated by dashed lines and hydrophobic interactions by dotted lines. The letter O indicates the position of oxygen of OH^- as the 6th ligand of Fe.

Our proposal can also be envisaged in connection with the water-splitting process. The OH^- ligation of Fe(III) in the XLP-heme might be accompanied by the appearance of a specific cation (Mn?) near the heme and close to the stromal surface of the thylakoid membrane which is exposed to high pH during the illumination. If the oxidation of two OH^- were involved in a cooperative oxygen evolving process the E_m value for water oxidation could be in the range of < +400 mV. This is covered by the Cyt *b-559* E_m in native systems. The effects of Mn-cluster removal [20] and DCMU [15] on the redox-potential shift of the Cyt *b-559* are consistent with this assumption. The connection between these proposals and the well established role of the Mn-cluster in the oxygen-evolving process [10] requires further investigation.

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