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Biochimica et Biophysica Acta 1767 (2007) 550–558
Biochimica et Biophysica Acta
BBA
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Two reaction pathways for transformation of high potential cytochrome b559 of PS II into the intermediate potential form

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Received 13 October 2006; received in revised form 4 December 2006; accepted 3 February 2007
Available online 13 February 2007

Abstract

This study describes an analysis of different treatments that influence the relative content and the midpoint potential of HP Cyt b559 in PS II membrane fragments from higher plants. Two basically different types of irreversible modification effects are distinguished: the HP form of Cyt b559 is either predominantly affected when the heme group is oxidized ("O-type" effects) or when it is reduced ("R-type" effects). Transformation of HP Cyt b559 to lower potential redox forms (IP and LP forms) by the "O-type" mechanism is induced by high pH and detergent treatments. In this case the effects consist of a gradual decrease in the relative content of HP Cyt b559 while its midpoint potential remains unaffected. Transformation of HP Cyt b559 via an "R-type" mechanism is caused by a number of exogenous compounds denoted L: herbicides, ADRY reagents and tetraphenylboron. These compounds are postulated to bind to the PS II complex at a quinone binding site designated as Q_C which interacts with Cyt b559 and is clearly not the Q_B site. Binding of compounds L to the Q_C site when HP Cyt b559 is oxidized gives rise to a gradual decrease in the E_m of HP Cyt b559 with increasing concentration of L (up to 10 $K_{ox}(L)$ values) while the relative content of HP Cyt b559 is unaffected. Higher concentrations of compounds L required for their binding to Q_C site when HP Cyt b559 is reduced (described by $K_{red}(L)$) induce a conversion of HP Cyt b559 to lower potential redox forms ("R-type" transformation). Two reaction pathways for transitions of Cyt b559 between the different protein conformations that are responsible for the HP and IP/LP redox forms are proposed and new insights into the functional regulation of Cyt b559 via the Q_C site are discussed.

Keywords: Photosystem II; Cyt b559; Redox potential; Herbicide

Cyt b559 is an integral heme protein in the PSII complex of all oxygenic photosynthetic organisms (reviewed in [1–3]). Each of the two transmembrane protein subunits of Cyt b559, psbE and psbF, provides a His ligand for the non-covalently bound heme. Cyt b559 is considered to participate in protection of PS II from photoinhibition; the details of the protection mechanism however are not yet clarified. Latest reports suggest that Cyt b559 may be involved in oxidation of the pool plastoquinol by molecular oxygen [4–6].

The redox properties of Cyt b559 are unique among b-type cytochromes. In a predominant fraction (~75%) of untreated PS II membrane fragments from spinach and sugar beet at pH 6.5 Cyt b559 is present in the high potential (HP) form with a midpoint potential (E_m) close to +400 mV while the remaining fraction typically represents a mixture of the intermediate potential (IP) and low potential (LP) forms characterized by E_m values of +180–250 mV and 0–100 mV, respectively [7–12]. The origin of the redox heterogeneity of Cyt b559 in PS II preparations and factors which determine the differences between the redox forms are not known although several possibilities have been suggested such as mutual orientation of the planes of the His ligands [13], the difference in the

Abbreviations: PS II, photosystem II; Cyt, cytochrome; His, histidine; E_m, midpoint potential; HP, high potential; IP, intermediate potential; LP, low potential; IM, intermediate; ADRY, acceleration of the deactivation reactions of system Y; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; CCCP, carbonylcyanide-m-chlorophenylhydrazone; Ant2p, 2-(3-chloro-4-trifluoromethyl)aniline-3,5-dinitrothiophene; TPB, tetraphenylboron; PQ, plastoquinone; PQH_2, plastoquinol; MES, 2-[N-Morpholino]ethanesulfonic acid; CHES, 2-[N-Cyclohexylamino]ethanesulfonic acid; β-DM, n-dodecyl β-D-maltoside; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; dinoseb, 2,4-dinitro-6-sec-butylphenol; ox, oxidized; red, reduced

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doi:10.1016/j.bbabio.2007.02.005

protonation and H-bonding pattern [7,12,14–16], polarity of dielectric environment of the heme [17] and a replacement of one of the His ligands [11].

Another so far unexplained property of Cyt b559 is the significant instability of its HP form towards a variety of treatments [1,7–12,18–24]. Procedures such as mild heating, incubation at high or low pH, at the elevated ionic strength, at high concentrations of detergents, ageing and sonication—induce a conversion of the vulnerable HP form to the IP form or a mixture of the IP and LP forms. The effects of transformation of HP Cyt b559 into the IP and LP forms are considered as irreversible dark modifications under normal conditions. Low-yield restoration of the HP Cyt b559 from the IP form can be achieved by illumination of PS II membrane fragments [25].

A conversion of HP Cyt b559 into the LP form is also caused by elevated concentrations of the so called ADRY compounds FCCP, CCCP, ANT2p [7,26], the lipophilic anion TPB [27] and the phenolic herbicide dinoseb at mM concentration [28].

Recently we identified a novel quinone-binding site (Q_C) in the PSII complex interacting with HP Cyt b559 [29,30]. It is suggested to mediate a redox equilibration between Cyt b559 and the PQ pool and to control the level of the pool reduction by opening a temporary pathway of oxidation of PQH_2 by molecular oxygen via an autoxidizable form of Cyt b559. The present study further addresses the peculiar redox properties of Cyt b559 and analyzes the factors governed modification of HP Cyt b559 to the forms with lower midpoint potential.

1. Materials and methods

PS II membrane fragments from sugar beet were isolated according procedures described in [31,32]. All assays were performed in a medium containing 100 mM MES (pH 6.5), 0.4 M sucrose, 15 mM NaCl, 10 mM $CaCl_2$ and 10% glycerol. For high pH treatment suspensions of PS II membrane fragments were incubated for 20 min at 15 °C in the assay medium containing 25 mM CHES (pH 9.1) instead of MES and either 0.5 mM $K_3[Fe(CN)_6]$ or 2 mM $Na_2S_2O_4$. The samples were then diluted by concentrated MES buffer to adjust the final pH to 6.5. DCMU and dinoseb were dissolved in ethanol. Maximal amount of ethanol in samples containing high concentrations of herbicides was 1.6%.

Difference absorbance spectra of Cyt b559 were recorded in spectrophotometer Cary 4000 in the range 520–590 nm as previously described [24]. Anaerobic redox titration was performed as outlined in previous studies [11,24]. The difference spectra of Cyt b559 were recorded at various redox potentials of the medium and related to the absolute spectra taken at redox potentials of either +450 to +480 mV or near 0 mV in reductive and oxidative titrations, respectively. $K_3[Fe(CN)_6]$ and $Na_2S_2O_4$ were used as oxidant and reductant, respectively. In case of the transition of HP Cyt b559, the Nernst fitting parameters, the E_m values and the relative amplitudes, obtained in the oxidative and reductive titrations differ by no more than 10 mV and 5–10%, respectively; if not indicated otherwise, the numbers obtained in the opposite titration waves were averaged.

2. Results

In accordance with earlier reports [7–12], the typical redox composition of Cyt b559 in our preparation of PS II membrane fragments from sugar beet is characterized with predominance (70–80%) of the HP redox form with the E_m of near +390 mV at pH 6.5 (curve a in Fig. 1). Curves b and c in Fig. 1 demonstrate the effect of 0.1% β-DM on the redox pattern of Cyt b559 at pH 6.5. In these experiments concentrated PS II membrane fragment suspensions were diluted by the assay medium containing 0.1% β-DM and 2 mM $Na_2S_2O_4$ and the oxidative redox titration was performed (curve b) followed by the reductive redox titration (curve c). It is seen that the presence of 0.1% β-DM in the assay medium results in a significant diminution of the relative amplitude of HP Cyt b559 in the titration curve (to 54% and 15% in experiments (b) and (c), respectively).

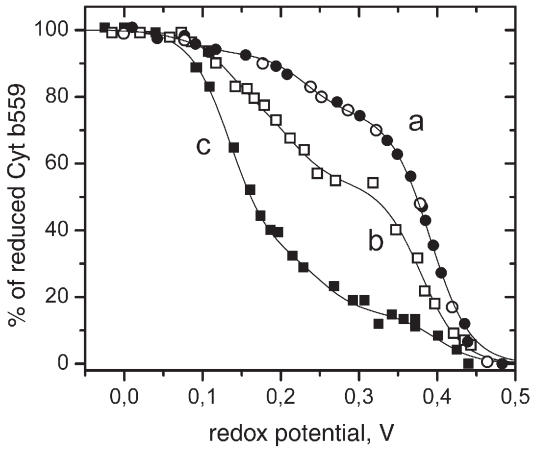


Fig. 1. Relative amplitude of reduced Cyt b559 as a function of the ambient redox potential of suspensions of PS II membrane fragments in the absence (a) and presence of 0.1% β-DM (b, c). Closed and open circles represent the data obtained in reductive and oxidative redox titrations, respectively, of untreated control. Open and filled squares are the data for the first oxidative and the second reductive redox titrations, correspondingly, of samples suspended in the presence of β-DM. The full lines are the Nernst functions for a sum of three one-electron components with the following E_m values and the relative amplitudes (in brackets): +391 mV (75%) , +232 mV (18%) and +82 mV (7%) for curve a; +379 mV (54%), +216 mV (23%) and +132 mV (23%) for curve b; +399 mV (15%), +252 mV (18%) and +135 mV (67%) for curve c. See text for further details.

The data presented in Fig. 1 are qualitatively similar to previous reports on the effects of detergents β-DM [9] and Triton X-100 [7,22] on HP Cyt b559. However marked quantitative difference exists in the earlier study [9] where already 0.05% β-DM has been reported to result in the total disappearance of HP Cyt b559 in spinach PS II membrane fragments in the oxidative wave of titration, while an appreciable fraction of HP Cyt b559 still persists in presence of 0.1% β-DM in our experiments. Importantly, the midpoint potential of the remaining fraction of HP Cyt b559 is not affected at all by the detergent (in average E_m=+389 mV).

A comparison of curves b and c in Fig. 1 suggests that the reduced form of HP Cyt b559 is more resistant to the action of β-DM. Incubation of PS II membrane fragments in presence of 0.5 mM $K_3[Fe(CN)_6]$ and 0.1% β-DM for 15 min resulted in a total disappearance of the HP form of Cyt b559 and the redox titration revealed a fully reversible behavior in oxidative and reductive waves characterized by 47% of the IP form (E_m= +240 mV) and 53% of the LP form (E_m=+113 mV) [data not shown].

Incubation at elevated pH is another procedure to affect the percentage of HP Cyt b559 [11,12]. At pH 9 the relative amplitude of HP Cyt b559 is significantly diminished (concomitant with a corresponding increase of the lower potential redox forms) and a down shift of its E_m value to about +350 mV is observed [11].

Fig. 2 compares the redox pattern of Cyt b559 at pH 6.5 in samples preincubated at pH 9.1 in presence of either the reductant (curve a) or the oxidant (curve b) for HP Cyt b559. In both cases the preincubation at high pH results in the conversion of a fraction of the vulnerable HP Cyt b559 into a mixture of the IP and LP forms. However, the extent of the effect clearly differs between the experiments where the high pH affects the reduced or oxidized HP Cyt b559. If the reduced form of HP Cyt b559 is exposed to high pH, the relative content of HP Cyt b559 decreases by only 11% (a) as compared to untreated samples while it decreases by 40% (b) when HP Cyt b559 stays oxidized during this treatment (see also [11]).

The data of Fig. 2 indicate that the conversion HP → IP/LP caused by high pH is irreversible since the decrease in the relative amplitude of HP Cyt b559 at pH 9 persists after resuspension of the treated samples at pH 6.5. In contrast, the midpoint potential of the remaining HP Cyt b559 exhibits a reversible behavior upon the pH change. Indeed, an average value of +392 mV is gathered for the E_m of HP Cyt b559 at pH 6.5 in samples preincubated at high pH (see Fig. 2), thus indicating that the negative shift in the E_m to a value of about +350 mV at pH 9.1 [11] is fully reversible. This small and reversible pH effect on E_m is assigned to deprotonation/protonation equilibrium of heme-coupled aminoacid residue(s). The experiments described above show that pretreatment of PS II membrane fragments at high pH results exclusively in an irreversible decrease of the percentage of HP Cyt b559 without concomitant influence on its E_m value.

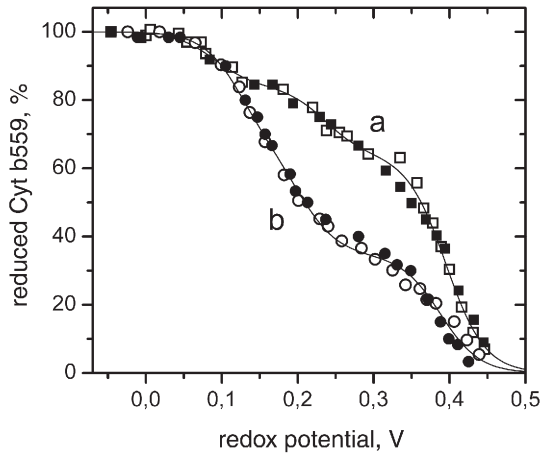


Fig. 2. Relative amplitude of reduced Cyt b559 as a function of the ambient redox potential of suspensions of PS II membrane fragments at pH 6.5 preincubated at pH 9.1 either in presence of 2 mM of $Na_2S_2O_4$ (a) or 0.5 mM $K_3[Fe(CN)_6]$ (b). Closed and open symbols are the data obtained in reductive and oxidative redox titrations, respectively. The best fit of the data points in curves a and b is a sum of the three Nernst components with the E_m values +395 mV (64%), +236 mV (20%), +90 mV (16%) and +388 mV (35%), +205 mV (31%), +124 mV (34%), respectively. For other details, see Materials and methods.

It is interesting to note that even untreated samples of PS II membrane fragments exhibit an effect on the redox behavior of Cyt b559, as reflected by the hysteresis between the first oxidative and a subsequent reductive titration. The contribution of the HP form in the first oxidative titration wave has been reported to be significantly higher than in the second reductive wave in untreated PS II membrane fragments at pH 6 [9]. We also normally observe such kind of behavior if the redox titration experiments in the same sample are of the order of first oxidative, followed by the reductive titration, but typically the difference in the percentage of HP Cyt b559 between the two titration experiments does not exceed 10%. A subsequent oxidative (the third) redox titration in the same sample fully matches the second (reductive) titration curve. To avoid of the small difference between titration curves originating from the effect of "the first oxidative titration" we normally started the redox titrations with the reductive wave. The hysteresis effect is somewhat more pronounced at high pH [11].

The results of Figs. 1 and 2 demonstrate the effects of treatments that cause a decrease of the ratio HP/(IP+LP) of Cyt b559. Apart from the effect on Cyt b559, both treatment types could also lead to a partial depletion of the water oxidizing complex (WOC) of Mn. However, a strict relation between the functional state of the WOC and the redox properties of Cyt b559 does not exist. This is illustrated by a mild treatment with hydroxylamine of PS II membrane fragments that leads to Mn depletion but retains an appreciable amount of Cyt b559 in the HP form [10,33]. Likewise, in PS II core complexes from spinach with high oxygen evolution activity all Cyt b559 is present in the LP form [11].

The results of Figs. 1 and 2 clearly demonstrate that during high pH- or detergent treatment the HP Cyt b559 in the oxidized form is much more vulnerable than in the reduced form. This finding corresponds with an earlier investigation [22] where effects of elevated pH, high concentration of Triton X-100, of heat treatment and of ageing on the reduced and oxidized HP Cyt b559 were compared. In this study, however, direct redox potential measurements were not performed and the percentage of the HP Cyt b559 was estimated only by the three-point checking method ($K_3[Fe(CN)_6]$-hydroquinone-$Na_2S_2O_4$). In fact, this approach does not permit an unambiguous separation of effects that change the relative amplitude of HP Cyt b559 from those that give rise to a downshift of the E_m value. In the present paper we will show that generally two distinctly different modification effects on the HP Cyt b559 can be distinguished. To allow a detailed analysis and straightforward conclusions on the underlying mechanisms of these modification effects, the exact parameters gathered from complete redox titration must be determined. The results of Figs. 1 and 2 have shown that the high pH- and detergent treatments do only affect the relative content but not the midpoint potential of the remaining fraction of the HP Cyt b559.

An entirely different type of modification effects was recently discovered [29,30]. It has been found that gradual concentration-dependent negative shift in the E_m of the HP Cyt b559 is induced by some type of substances (DCMU, dinoseb,

TPB, ADRY reagents) that interact with PSII without exerting an appreciable effect on the relative content of the HP form in a wide range of concentrations of the active compounds. This effect of the compounds symbolized by L has been interpreted as to originate from their binding to a specific site denoted Q_C that is postulated to be a novel quinone-binding site in the PS II complex [29,30].

This second type of the effects on HP Cyt b559 is illustrated in Figs. 3–5 for DCMU, dinoseb and TPB. These figures show redox titration curves of Cyt b559 in the presence of different concentrations of compounds L. The results clearly indicate that the midpoint potential of HP Cyt b559 is a function of the concentration of DCMU, dinoseb or TPB. This effect is specific for HP Cyt b559 because the E_m values of the IP and LP forms are independent of the presence of the examined compounds (see panel A of Fig. 3 and Fig. 5). It is important to note that no hysteresis is observed in the course of the oxidative and reductive titrations in this type of modification of the HP Cyt b559 (see, for instance, the data for TPB in Fig. 5c).

In the experiments described above rather high concentrations of DCMU and dinoseb were used, and therefore the possible effect of the solvent – ethanol – on the redox pattern of Cyt b559 was carefully checked. The E_m values and relative amplitudes of the HP, IP and LP redox forms were found to be virtually unaffected by ethanol at maximal concentration (1.6%) used in this study as compared to the control sample (not shown). Moreover, as illustrated by Fig. 5 the water soluble compound TPB induces a quite similar effect on the E_m of HP Cyt b559 as DCMU and dinoseb. The negative shift in the E_m of HP Cyt b559 caused by DCMU, dinoseb, TPB and ADRY reagents is explained by their preferential binding to the quinone-binding site Q_C when the heme group of HP Cyt b559 is oxidized [29,30].

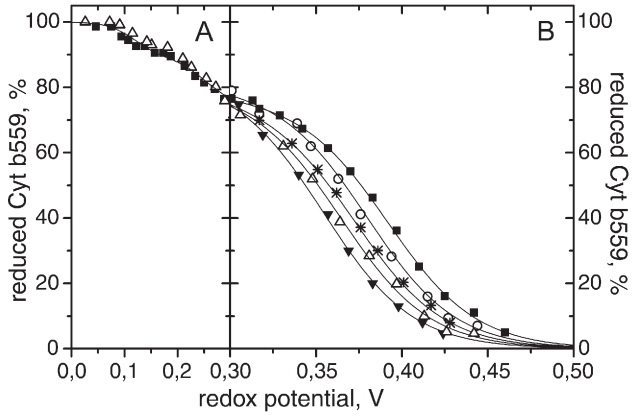


Fig. 3. Effect of DCMU on the redox pattern of Cyt b559 in PS II membrane fragments. The redox titration of Cyt b559 was performed in untreated PS II membranes (filled squares) and in presence of 100 μM (open circles), 160 μM (stars), 320 μM (open triangles) and 640 μM (filled inverted triangles) DCMU. Both oxidative and reductive titrations were performed in each case and no hysteresis was observed; the data obtained in the correspondent titration waves were averaged. Panel A demonstrates the absence of DCMU effects of on the IP and LP forms of Cyt b559. Panel B depicts the data for the high potential region in enlarged abscissa scale. The E_m values and the relative amplitudes for the HP Cyt b559 in the titration curves are the following: +392 mV and 77% (filled squares), +380 mV and 80% (open circles), +373 mV and 77% (stars), +365 mV and 79% (open triangles), +355 mV and 82% (filled triangles).

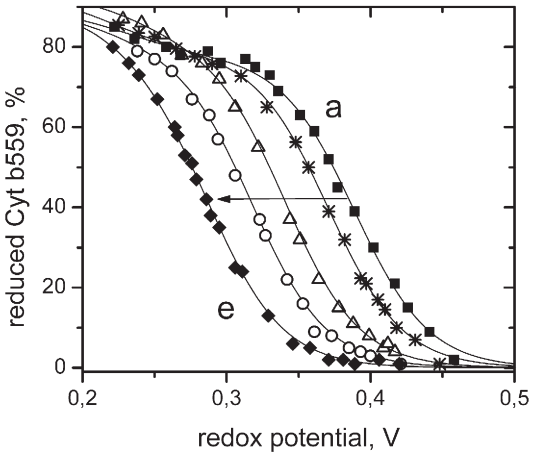


Fig. 4. Relative amplitude of reduced Cyt b559 as a function of the ambient redox potential of suspensions of PS II membrane fragments in the presence of different dinoseb concentrations. The titration curves of Cyt b559 were obtained in presence of 0.1 μM (a), 6.5 μM (b), 53 μM (c), 110 μM (d) and 210 μM (e) dinoseb (the arrow indicates the labeling of the different curves from a to e). The data points represent averaged values gathered from the oxidative and reductive redox titrations as no hysteresis in the titrations has been observed. The E_m values and the relative amplitudes for the HP Cyt b559 in the titration curves are: +389 mV and 78% (a), +371 mV and 79% (b), +341 mV and 82% (c), +318 mV and 81% (d), +289 mV and 77% (e). The IP and LP forms are not affected (data not shown).

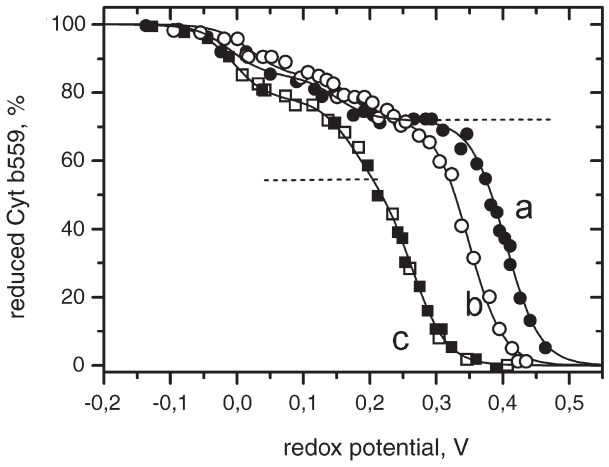


Fig. 5. Relative amplitude of reduced Cyt b559 as a function of the ambient redox potential of suspensions of PS II membrane fragments in the absence (a) and presence of different TPB concentrations (b, c). Curves b and c were obtained in presence of 1 μM and 30 μM TPB, respectively. In curves a and b the data for oxidative and reductive titrations were averaged. In curve c the data for oxidative and reductive titrations are shown by the open and closed symbols, respectively, to indicate of the absence of a hysteresis effect. Dashed lines mark the levels corresponding to contributions of the HP form of Cyt b559 in the titration curves. The best fits of the data points are sums of the three one-electron Nernst components with the correspondent E_m values and the relative amplitudes (in brackets): +404 mV (72%), +150 mV (13%), +10 mV (15%) for curve a; +349 mV (72%), +180 mV (13%), +20 mV (15%) for curve b; +265 mV (54%), +171 mV (24%), −7 mV (22%) for curve c. Note that for the experiments with TPB a preparation of PS II membrane fragments was chosen that is characterized by somewhat lower E_m values of the IP and LP forms.

The effect starts to arise at concentrations of substances L that are close to the constant $K_{ox}(L)$ for binding of L to the HP Cyt $b559_{ox}$-Q_C (the binding constants for the inhibitors in the Q_C site are: K_{ox}(DCMU)=200 μM, K_{ox}(dinoseb)=6.6 μM, K_{ox}(TPB)=72 nM, see [29,30]). In a range of up to 10 $K_{ox}(L)$ the effect of L is restricted to the negative shift of the midpoint potential of HP Cyt b559 without any influence on the ratio HP/(IP+LP) [see Figs. 3 and 4 and curve b in Fig. 5]. In case of DCMU this behavior was observed in the whole range of concentrations tested in this study. However, in cases of a substantial increase in dinoseb and TPB concentrations an additional effect emerges, i.e. the relative amplitude of HP Cyt b559 decreases accompanied by a corresponding increase in the content of the lower potential forms. These effects are illustrated in Figs. 5 and 6.

The data presented in Fig. 5 indicate that at 1 μM TPB the E_m of HP Cyt b559 is shifted to about +350 mV(curve b) while its relative amplitude (72%) is totally unaffected compared to control (curve a). An increase of TPB concentration to 30 μM, however, causes a diminution of the relative content of HP Cyt b559 to 54% accompanied by larger shift in the E_m of this form down to +265 mV (curve c).

Fig. 6 summarizes the data on the effects of DCMU, dinoseb and TPB on the percentage of HP Cyt b559 gathered from the redox titrations. The HP Cyt b559 is characterized by the E_m values, which are functions of the concentration of compounds L (see Figs. 3–5). The decrease in the relative content of the HP Cyt b559 with increasing concentrations of dinoseb and TPB was accompanied by the mirror increment in the percentage of IP and LP Cyt b559. At the maximal concentration of dinoseb of 320 μM used in the present study Cyt b559 was characterized by the following composition: 56% of the HP, 34% of the IP and 10% of the LP forms. At the maximal concentration of TPB of 1 mM the total Cyt b559 consisted of 45% of the HP, 31% of the IP and 24% of the LP forms (not shown).

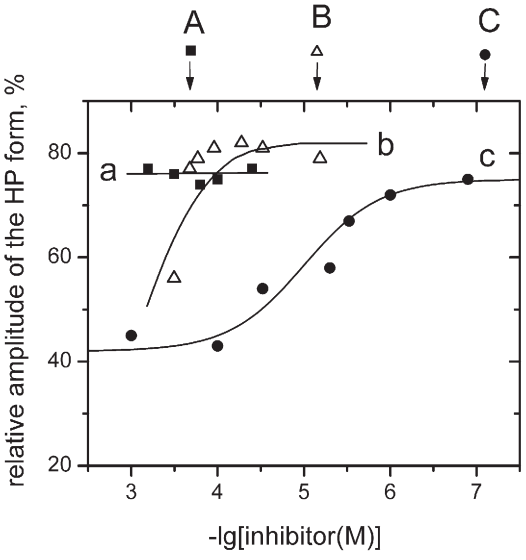


Fig. 6. Relative amplitude of HP Cyt b559 as a function of concentrations of DCMU (a), dinoseb (b) and TPB (c) in suspensions of PS II membrane fragments. The relative amplitudes of the HP form of Cyt b559 were gathered from titration experiments performed in samples containing different amounts of DCMU, dinoseb or TPB. The arrows marked by the capital letters at the top of the figure indicate the positions on the abscissa scale corresponding to the numbers of pK_{ox} (DCMU)=3.70 (A), pK_{ox} (dinoseb)=5.18 (B) and pK_{ox} (TPB)=7.14 (C). For further details, see text.

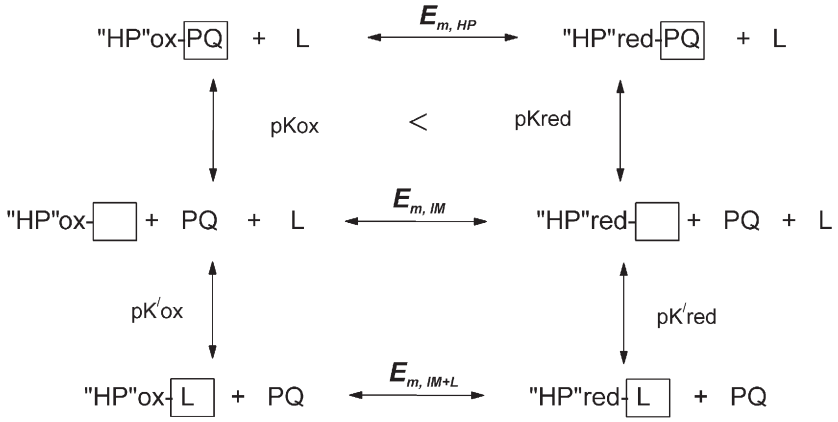
At first glance the curves shown in Fig. 6 might suggest a difference between DCMU and the two other tested compounds (dinoseb, TPB) in their effects on the relative content of HP Cyt b559. However, one should take into account the scaling of the abscissa relative to the correspondent pK_{ox} values of these compounds (indicated by the arrows at the top of the figure). It is seen that an appreciable decrease in the amplitude of the HP Cyt b559 appears when the concentration of L exceeds the value of the correspondent K_{ox} by at least a factor of 10. Therefore, based on this consideration, the data of Fig. 6 appear to be qualitatively similar for all three tested compounds. The data for TPB in Fig. 6 show that the decrease in the relative content of HP Cyt b559 observed with increasing TPB concentrations is characterized with a pK of ~4.9 and thus coincides with the saturation of the binding of the inhibitor with the reduced form of HP Cyt b559 (pK_{red} (TPB)=4.4, see [29,30]).

Therefore, a very interesting conclusion emerges from the analysis of the data of Fig. 6. It follows that when the conversion HP→IP/LP of Cyt b559 is caused by binding of compounds L at site Q_C, the oxidized form of HP Cyt b559 is now more resistant to the transformation as compared to the reduced form of HP Cyt b559. In other words, the primary target for the transformation of HP Cyt b559 caused by the binding of the PS II inhibitors to Q_C is the reduced form of HP Cyt b559. This feature is just the opposite of the sensitivity of the oxidized and reduced form of HP Cyt b559 to the action of high pH and detergents as illustrated in Figs. 1 and 2.

3. Discussion

In this study factors were analyzed that influence the relative content and the midpoint potential of HP Cyt b559 in PSII membrane fragments from higher plants. Two basically different types of modification effects on HP Cyt b559 have to be distinguished.

The typical treatments affecting the ratio HP/(IP+LP) in preparations of PS II membranes include incubations of samples at high pH or at high detergent concentrations (Figs. 1 and 2), mild heating, incubation with chaotropic agents or at high ionic strength, ageing and sonication [1,7–9,11,12,18–24]. Based on the available experimental data this type of HP Cyt b559 modification is characterized by the following features: (1) decrease of the relative content of HP Cyt b559 and a mirror increase in the sum percentage of the lower potential forms, IP and LP, (2) the midpoint potential of the remaining fraction of the HP form is not affected, (3) the modification of the redox pattern of Cyt b559 is an irreversible effect, and (4) among the two redox states of HP Cyt b559 the oxidized form is significantly more vulnerable to the treatment than the reduced form. We propose to denote this kind of the effects on oxidized Cyt b559 influencing exclusively the ratio HP/(IP+LP) as the "O-type" effects (O stands for "oxidized").



Scheme 1. Schematic representation of the interaction of the presumed native cofactor PQ and exogenous substances L at site Q_C for Cyt b 559 characterized by the "HP" protein conformation. The symbols $E_{m,\ HP}$, $E_{m,\ IM}$ and $E_{m,\ IM+L}$ describe the correspondent midpoint potentials of the redox transitions of Cyt b559 in states where the Q_C site (symbolized by a rectangular) is either occupied by PQ, is empty or associated with L, respectively. "HP"ox and "HP"red are symbols for Cyt b559 in the "HP" protein conformation in oxidized and reduced states of the heme group, respectively. For details, see text.

Figs. 3–5 describe effects on the redox pattern of Cyt b559 that are markedly different. The modifications induced by the compounds L (herbicides, ADRY reagents, TPB) are characterized by the following properties: (1) the midpoint potential of the HP Cyt b559 is gradually shifted to lower values with increasing concentration of compound L, (2) in a wide concentration range of L, up to at least ten-fold of the corresponding K_{ox} values, the progressive negative shift of E_m with the increasing concentrations of L is not accompanied by any decrease in the relative amplitude of the HP form, and (3) at higher concentrations ([L] > ~10 K_{ox}) a transformation of HP Cyt b559 into a mixture of forms IP and LP takes place.

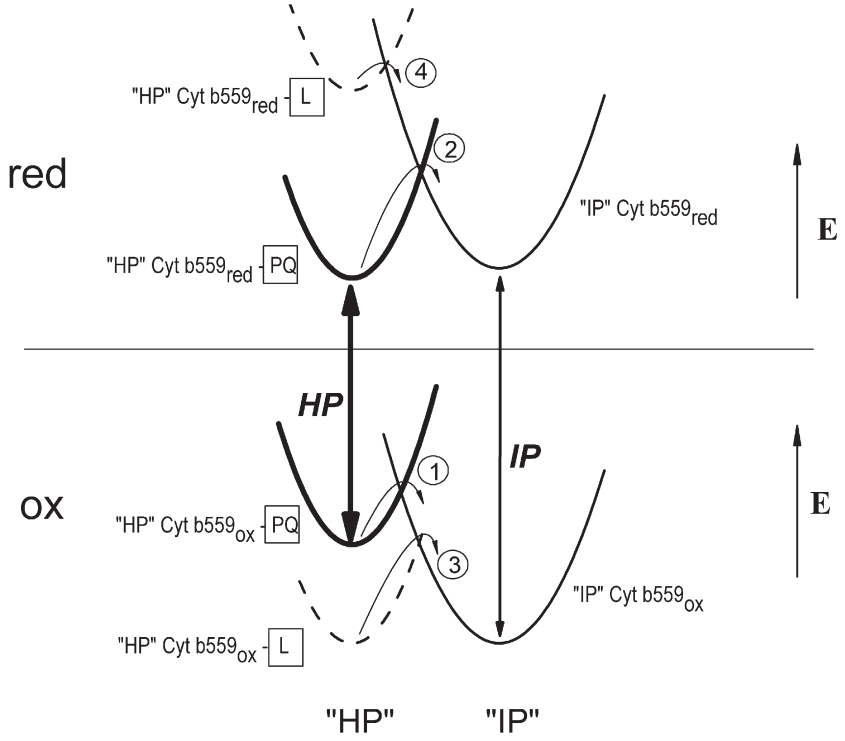
The gradual shift in the E_m of HP Cyt b559 due to increasing concentrations of the PS II inhibitors is indicative of a dynamic equilibrium process implying full reversibility in the effect on the E_m of HP Cyt b559 caused by the binding of L to the Q_C site when the heme group of HP Cyt b559 is oxidized. On the other hand, if HP Cyt b559 is reduced the binding of L to the Q_C site induces an irreversible transformation of the HP form to the forms IP and LP. We suggest to denote this kind of the effects on the ratio HP/(IP + LP) in Cyt b559 as the "R-type" effects (R stands for "reduced").

A comparative analysis of the two types of modification effects on HP Cyt b559 provides deeper insight into the various interrelations between the different redox forms of Cyt b559. We observed that all treatments under mild conditions converted HP Cyt b559 into a mixture of the IP and LP forms while under harsh treatment conditions a complete transformation into the LP form takes place (see Figs. 1 and 2 and Refs. [11,24]). The latter finding implies the order of events HP → IP → LP for the modification of HP Cyt b559 to the lower potential forms. In the following discussion we will concentrate on the first transformation event of the above sequence, i.e. the HP → IP processes.

In our recent study [29] we have assigned the HP form of Cyt b559 to a state of the PS II complex where the proposed binding site Q_C is occupied by PQ (see Scheme 1). We have further suggested that the unique property of Cyt b559 in the "HP" conformation is unequal affinity of the Q_C site to the bound PQ in case of reduced and oxidized heme group, i.e. PQ binds weakly to Q_C when the heme group of HP Cyt b559 is oxidized and tightly when the heme is reduced, i.e. pK_{red} (PQ) > pK_{ox} (PQ). This mechanism provides a stabilization of the reduced form of Cyt b559, thus shifting its E_m to a higher value as compared to a correspondent PQ-free heme protein (the IM form). The latter form, IM, is assumed to maintain the "HP" protein conformation and thus is capable of equilibrium PQ binding (Scheme 1). In contrast, the IP form is considered to possess a modified Q_C site which is either unable to bind PQ in the dark equilibrium reaction or is characterized with similar affinity to PQ in the oxidized and reduced states of the heme group. A redox-linked conformational rearrangement which takes place in HP Cyt b559 influences not only the environment near the Qc pocket but also the optical properties of the oxidized heme chromophore [11].

The data of Figs. 1 and 2 reveal that the two individual redox forms of Cyt b559, HP and IP, are not freely interconvertable under normal conditions. The irreversibility of the transition HP → IP/LP in the dark at pH 6–7.5 was demonstrated also in other studies [7–9,11,21,24]. This feature implies that two different conformational substates (corresponding to the "HP" and "IP" conformations[1]) exist in both oxidized and reduced Cyt b559 and that these states are separated by a significant energy barrier (transitions 1 and 2 in Scheme 2). Obviously, the presumed transition "HP" ↔ "IP" can be considered to occur as molecular switch. The rearrangement of the protein matrix during the "HP" → "IP" transformation may comprise the displacement of an aminoacid residue which acts as a ligand to the PQ bound at Q_C. This displacement could destabilize the bound PQ cofactor resulting in its possible release in the IP Cyt b559. This mechanism may resemble a feature recently revealed by X-ray crystallographic studies on the Q_o site of the

[1] The symbols HP, IP are used to indicate the correspondent redox transitions in Cyt b559 while the symbols "HP", "IP" refer to protein conformation.



Scheme 2. Schematic representation of the free energy profiles corresponding to different protein conformations of Cyt b559 and their modification by different occupants of the Q_C site (PQ, L). The following abbreviations are used: "HP,"IP"—two distinct protein conformations of Cyt b559; red—reduced, ox—oxidized; E—free energy. The numbers 1–4 mark the activated reaction pathways for the conformational change "HP"→"IP" in different states of oxidized and reduced Cyt b559. For details, see text.

mitochondrial bc_1 complex, where a switch has been found between the two different orientations of Glu-272 ligating the bound ubiquinol [34–36].

The gradual concentration-dependent shift in the E_m of HP Cyt b559 induced by compounds L (Figs. 3–5) corresponds to increasing saturation levels in binding of L to the HP Cyt $b559_{ox}$-Q_C. This binding comprises the character of a dynamic equilibrium (Scheme 1) thus giving rise to full reversibility. In this case a deleterious effect on the protein conformation of HP Cyt b559 is absent and no conversion HP→IP occurs. This fact indicates that the binding of L to the Q_C site in the HP Cyt $b559_{ox}$ does not diminish the activation barrier for the transition "HP"→"IP" (transition 3 in Scheme 2). Therefore, the state "HP" Cyt $b559_{ox}$-L may be either nearly isoenergetic to the initial state "HP" Cyt $b559_{ox}$-PQ or be even more stabilized. The latter case is illustrated in Scheme 2. Interestingly, if "HP" Cyt $b559_{ox}$-L is really stabilized compared to "HP" Cyt $b559_{ox}$-PQ, binding of L is expected to exert even a protective effect against the transformation of HP Cyt b559 to the IP form caused by the "O-type" treatments (compare the energy barriers for the transitions 3 and 1 in Scheme 2).

The mechanism by which the "O-type" treatments decrease the ratio HP/(IP+LP) in Cyt b559 likely consists in the lowering of the activation barrier for the molecular switch "HP"→"IP" in the oxidized Cyt b559. This effect arises obviously due to an overall destabilization of the protein conformation in presence of either high concentrations of salts, protons, hydroxyl ions, chaotropic agents, detergents or at elevated temperature or under sonication. The markedly higher stability of the reduced form of HP Cyt b559 to such treatments is explained by a significant stabilization of "HP" protein conformation by the binding of PQ in the Q_C site in HP Cyt $b559_{red}$-Q_C (compare transition 2 with transition 1 in Scheme 2).

On the other hand, substances leading to "R-type" effects (herbicides, TPB, ADRY compounds) do not increase the probability of the transition "HP"→"IP" in oxidized HP Cyt b559 but affect instead the reduced form of HP Cyt b559. These substances are assumed to decrease the activation barrier for the transition 4 (Scheme 2) by an increase of the free energy of the state "HP" Cyt $b559_{red}$-L as compared to the native form "HP" Cyt $b559_{red}$-PQ. A similar destabilization effect on reduced HP Cyt b559 is expected when the bound PQ is released from the Q_C site.

It is known that ADRY-type agents lead to transformation of HP Cyt b559 into an LP form [7,26] and cause autoxidation of Cyt b559 [7,26,37–39]. The transformation effect was attributed to a deprotonation of the Cyt b559 protein [7,14,37]. In good correspondence with the previous reports [27,28], our present study describes a quite similar effect on HP Cyt b559 (HP→IP/LP) induced by dinoseb and TPB. Furthermore, we have shown that autoxidation of HP Cyt b559 is also caused by DCMU in high concentration, TPB and dinoseb and thus is not specific for the ADRY-type reagents (see Ref. 29 and discussion therein). We consider that all these substances (ADRY reagents, PS II herbicides, TPB) induce a conversion of HP Cyt b559 into the lower potential forms (R-type effects)

by one and the same mechanism. The primary event consists in replacement of the native PQ at the Q_C site by the exogenous molecule L. The release of PQ from the Q_C site triggers conformational rearrangements in HP Cyt b559 into states that are responsible for the forms IP and LP. Irreversible transformation HP→IP/LP may be accompanied by changes in protonation/deprotonation equilibria of relevant aminoacids. Therefore our model is not in contradiction with the previous suggestions [7,14,37].

A most intriguing aspect of protein chemistry of Cyt b559 is the possibility to accomplish under experimental conditions the reverse transition IP (LP)→HP. This type of conversion may be a stage in the catalytic mechanism of Cyt b559 assumed to operate via a shuttle between different conformational states [7,14,40–42]. The lower potential forms of Cyt b559 may be involved in the mechanism of plastoquinol oxidation by molecular oxygen [4,5].

An appreciable extent of HP Cyt b559 reconstruction from lower potential redox forms, not reducible by hydroquinone, was achieved in heptane-washed chloroplasts by supplementation of the PQ-extracted samples with exogenous PQ [43]. This experiment most likely suggests that in a substantial fraction of PQ-extracted chloroplasts the protein of Cyt b559 remained in the "HP" conformation (in the presumed IM form) which is capable to bind PQ at Q_C in a dark equilibrium reaction. However, after attaining the "IP" conformation Cyt b559 looses the ability to restore the HP form in the dark although the membrane samples containing Cyt b559 in the IP form retain its intrinsic PQ pool.

Reconstruction of HP Cyt b559 from the IP form was reported to occur at a low extent under illumination [25]. It is found that a functionally competent Q_B site is indispensable for this process. A very interesting conclusion emerges from an analysis of these results within the framework of the considerations of the present study. The strict requirement of the Q_B site function for reconstruction of the HP Cyt b559 could imply that PQH_2 is involved in the restoration process. The PQH_2 formed at the Q_B site is assumed to act not as a reductant for IP Cyt b559 since the chemical reduction of the heme does not lead to the restoration. Instead, the binding of PQH_2 may be important to switch of Cyt b559 to the "HP" conformation by providing a significantly stabilized transient state "HP" Cyt $b559_{ox}$-PQH_2. This situation can be rationalized in Scheme 2 by a substantial down shift of the potential energy curve corresponding to the state "HP" Cyt $b559_{ox}$-L (where L is now PQH_2) until it intersects the minimum of the potential energy curve of the "IP" Cyt b559. An impairment of the Q_B site typically accompanying the conversion HP Cyt b559→IP Cyt b559 may explain the low extent of the light-dependent reconstruction of the HP form [25]. On the other hand, the requirement for the simultaneous presence of oxidized HP Cyt b559 and PQH_2 may account for the inability to accomplish the reconstruction in the dark by chemical reductants.

According to common view Cyt b559 operates in a cyclic flow around PS II accepting electrons from PQH_2 of the pool [37,44–46]. The latter reaction implies a binding interaction between PQH_2 and HP Cyt $b559_{ox}$. In our previous study [29] we have hypothesized that one and the same site Q_C may bind and exchange the quinone and quinol cofactors but with the opposite order of binding affinities to the oxidized versus reduced HP Cyt b559 for quinone and quinole molecules. Aromatic compounds that interact as high-affinity surrogates with the Q_C site of HP Cyt $b559_{ox}$ are the phenolic herbicides and the ADRY reagents [29]. The common structural feature of these compounds is the presence of either an acidic –OH or –NH group connected with an aromatic ring [47]. It is therefore reasonable to suggest that the phenolic herbicides and ADRY reagents may mimic the native PQH_2 in the Q_C site of HP Cyt $b559_{ox}$.

Acknowledgments

The financial supports by grants from Russian Fund of Basic Research (05-04-49488) and DFG (436 Rus 113/635/0-1) are gratefully acknowledged.

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