Uptake and Release of Protons during the Reaction between Cytochrome *c* Oxidase and Molecular Oxygen: A Flow-Flash Investigation†

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ABSTRACT: Changes in pH during the reactions of the fully reduced and mixed-valence cytochrome oxidase with molecular oxygen have been followed in flow-flash experiments, using the pH indicator phenol red. Solubilized enzyme as well as enzyme reconstituted into phospholipid vesicles has been studied. With the solubilized enzyme, a biphasic uptake of one proton from the medium was observed, whereas the reconstituted enzyme gave release of 1.3 protons to the extravesicular medium. It is concluded from these results that a total of two to three protons are taken up during oxidation of the fully reduced enzyme. Kinetic analysis suggests that the proton uptake is initiated by the transfer of the third electron to the oxygen binding site. A reaction scheme that integrates proton transfers and oxygen chemistry is presented.

Cytochrome *c* oxidase is the terminal enzyme of the cellular respiration chain in eukaryotic cells and in some bacteria. The enzyme is a redox-linked proton pump which catalyzes the electron transfer from cytochrome *c* to molecular oxygen [for a recent review, see Chan and Li (1990)]. The catalytic cycle involves both translocated protons and protons used in water formation. The stoichiometry for proton pumping is, on average, one proton translocated per electron transferred from cytochrome *c* to oxygen (Casey, 1984). In order to isolate the reactions involving protons in the overall redox cycle, the pH dependence of kinetics and equilibria has been studied. In this way, Wikström (1988) was able to show release of protons from the oxygen binding site during partial reversal of oxygen reduction. The pH dependence of the reaction between the reduced/mixed-valence enzyme and oxygen was studied by Oliveberg et al. (1989) using the flow-flash method. These more or less indirect methods have led to some insight into the electron transfers and their coupling to the proton chemistry, but the mechanism of proton pumping is still largely unknown.

In the present work, we have used the flow-flash method, in combination with a pH indicator, to study directly the uptake and release of protons during oxidation of cytochrome oxidase. This provides a comparison between the rates of the transient proton reactions and the rates of the internal electron-transfer reactions. Both solubilized enzyme and enzyme reconstituted into phospholipid vesicles have been investigated.

With the solubilized enzyme, proton uptake was biphasic and was preceded by a lag. The total amplitude of the absorbance change corresponded to the uptake of one proton per enzyme molecule. With the reconstituted enzyme, a monophasic release of 1.3 protons to the extravesicular medium was obtained. We conclude from these results that the total proton uptake during reoxidation is two to three H^+ per functional unit.

A comparison of the kinetics of proton uptake with earlier work on electron-transfer kinetics in cytochrome oxidase and with the chemistry of oxygen reduction suggests that the proton uptake is triggered by the formation of a ferrous–cupric peroxide intermediate at the oxygen binding site.

Materials and Methods

Materials. All reagents used were of analytical grade or further purified. Carbon monoxide (99.94%) was from Alfax, and asolectin (type IV-S) was obtained from Sigma and further purified as described by Kagawa and Racker (1971). Phenol red was used as pH indicator (Casey, 1986) and was purchased from Merck. Beef heart cytochrome *c* oxidase was isolated according to the method of van Buuren (1972). Enzyme concentrations were calculated from ΔA_{red-ox} at 605–630 nm using an extinction coefficient of 27 mM^{-1} cm^{-1} (Hill & Greenwood, 1984a).

Dialysis of the Solubilized Enzyme. To minimize the buffer capacity, the stock solution of the enzyme (approximately 250 μM) was dialyzed against 1000 volumes of 50 mM choline chloride, 50 mM KCl, and 0.5% Tween 80 for about 15 h at pH 7.4 and 4 °C.

Reconstitution of Cytochrome Oxidase into Phospholipid Vesicles. Prior to reconstitution, the enzyme was further purified by sucrose gradient ultracentrifugation as described by Maison-Peteri and Malmström (1989). The purified enzyme was reconstituted by cholate dialysis using the protocol of Maison-Peteri and Malmström (1989). The last dialysis buffer was 0.1 mM 4-(2-hydroxyethyl)-1-piperazinesulfonic acid (Hepes), 22 mM K_2SO_4, and 75 mM sucrose, adjusted to pH 7.5 just before use. Respiratory control ratios were measured as specified by Nilsson et al. (1988), and values in the range 4–6 were obtained. Anaerobic outside reduction measurements (Nilsson et al., 1988) indicated a right-side-out orientation of around 70%.

Flow-Flash Kinetics. Anaerobic reduction and formation of the fully reduced CO complex of the solubilized and reconstituted enzyme was done according to Nilsson et al. (1990). The mixed-valence CO complex of solubilized cytochrome oxidase was prepared by incubation of the oxidized enzyme in a Thunberg cuvette under O_2-free CO atmosphere at 4 °C for 24 h. Formation of the mixed-valence complex was verified by optical spectroscopy. Mixing was as in Oliveberg et al. (1989), except that a 1:1 mixing ratio was used.

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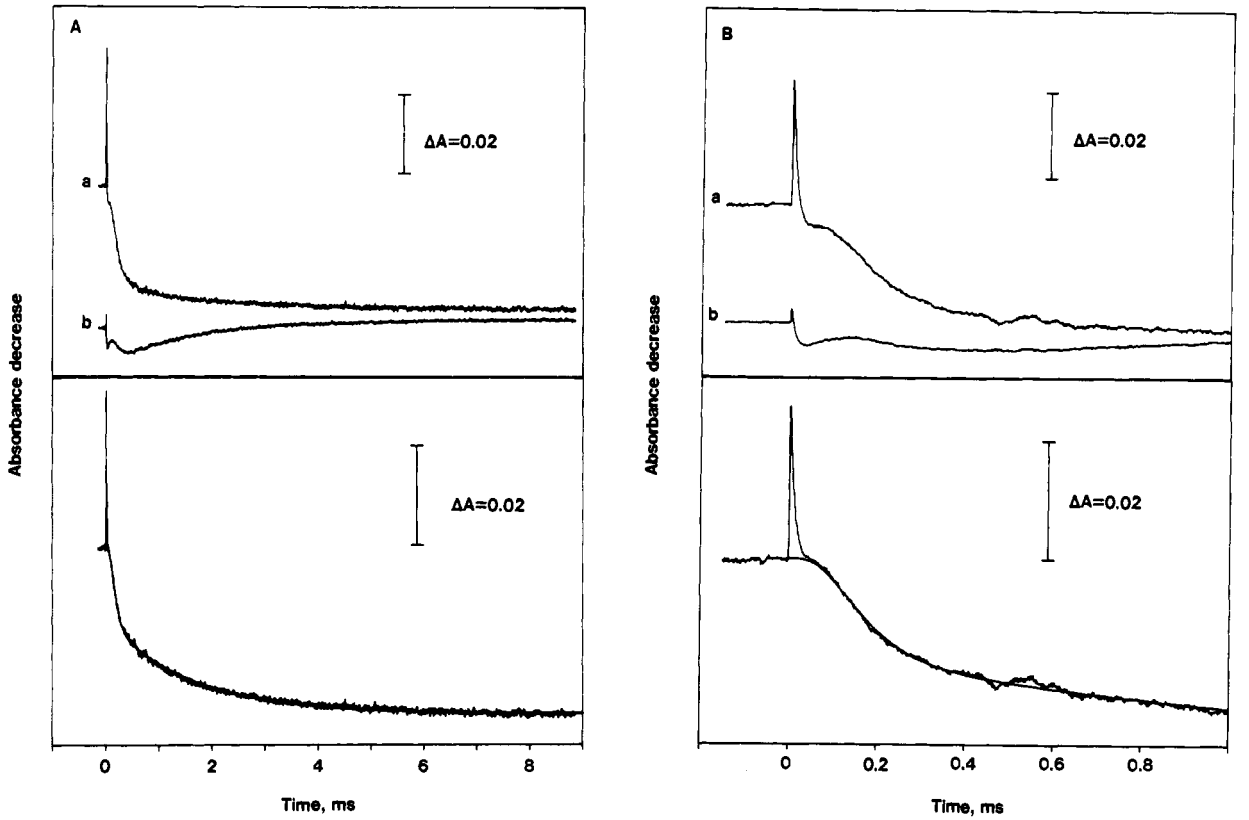

FIGURE 1: Transient absorption changes at 563 nm following flash photolysis of the fully reduced CO complex of solubilized cytochrome oxidase in the presence of oxygen. In (A), the reaction was followed to 9 ms whereas in (B) only the initial part to 1 ms was followed. The upper panels show the transient obtained in the presence (trace a) or absence (trace b) of phenol red, and the lower panels give the differences between traces a and b compared with a simulated curve obtained from a reaction model (smooth line) presented under Discussion. The initial lag can be explained as a number of preceding consecutive electron-transfer reactions not coupled to proton uptake or release. The spike at $t = 0$ is due to scattered light from the laser flash, and the data at $t < 0$ are the preflash absorbance level. The concentrations of the enzyme and oxygen after mixing were about 4.8 μM and 0.6 mM, respectively.

Syringe A contained 4–5 μM cytochrome oxidase, 40 μM phenol red, 50 mM choline chloride, and 50 mM KCl. Syringe B contained 1.2 mM oxygen, 40 μM phenol red, 50 mM choline chloride, and 50 mM KCl. In experiments with the solubilized enzyme, both syringes contained 0.5% Tween 80, and in experiments with the vesicular enzyme, syringe A was complemented with 40 μM valinomycin. All experiments were performed at pH 7.5 and 22 °C. The pH of the exhaust from the flow cell was measured with a pH electrode connected directly to the stop syringe and found to be 7.5. The exhaust was kept in a glass syringe to prevent CO_2 uptake prior to subsequent calibration. For photolysis, a Nd-YAG laser from Quantel was used. Its output wavelength was 532 nm, the duration of the pulse was 10 ns, and the total energy was about 0.3 J. To ensure a saturating laser pulse, an anaerobic solution of the CO complex was photolyzed in the flow cell at varying laser output power. Absorbance changes were followed at 563 nm where the basic form of the dye absorbs and contributions from chromophores in the enzyme are small. No absorbance changes were observed on exposure of a solution containing dye and oxidized enzyme to a laser flash, which excludes contributions from dye photochemistry. Data collection was started 150 μs before the laser pulse. The time constant of the detection system was about 10 μs. All traces shown are averages of 10–15 transients.

Calibration Procedure. To correlate the absorbance changes of the dye to the amount of proton release or uptake per functional unit of the enzyme, a relevant amount of acid was added. The flow cell was replaced with a stirred cuvette, a known quantity of HCl was added to the solution collected from the flow cell exhaust, and the resulting absorbance changes were recorded. Before calibration, the pH was adjusted to 7.5 if necessary.

Data Processing. Curve fitting and simulations were done by using algorithms provided in the Matlab software package from The MathWorks, Inc. (South Natick, MA).

RESULTS

Fully Reduced, Solubilized Enzyme. The net proton exchange between the enzyme and the medium was examined during the flash-induced reaction between the fully reduced enzyme, and oxygen was followed by using the pH indicator phenol red. The total reaction can be divided into two parts: the rapid oxidation of the fully reduced enzyme followed by the much slower steady-state reaction between the small excess of ascorbate present and oxygen. In the steady-state reaction, net consumption of protons led to a absorption increase on the longer time scale (several seconds; not shown). The upper panels of Figure 1 show the absorption changes obtained in the presence and absence of the indicator. The difference, displayed in the lower panels, shows the absorption increase specifically due to the dye. Repeating the experiment with phenol red in the presence of buffer led to a signal identical with the one obtained without the dye (not shown). Figure 1B (lower panel) clearly demonstrates a lag, which is followed by a biphasic increase in absorption. Kinetic analysis of the trace, taking the preceding lag reactions into account, gave apparent first-order rate constants of about 20×10^3 and 600 s^{-1} for the two phases. The total amplitude of the absorption increase due to the indicator (Figure 1A, lower panel) corresponds to the uptake of close to one proton per functional unit of the enzyme. The amplitude was obtained as the dif-

Table I: Summary of the Number of Protons Taken Up or Released during the Reaction of the Fully Reduced Enzyme with Oxygen

	rapid (2×10^4 s^{-1})	slow (600 s^{-1})	total
protons released to "C-side"	0[a]	1–2[a]	1–2[a]
protons taken up from "M-side"	0.5[a]	1.5–2.5[b]	2–3[b]
net proton uptake	0.5[c]	0.5[a]	1[a]

[a] Experimental values. [b] Calculated from comparison of the amplitudes obtained with the solubilized and reconstituted enzyme. [c] Calculated from the difference between the total and the slow amplitude.

ference in absorbance between the preflash level and the absorbance at 9 ms. Curve fitting gave an amplitude of 0.5 proton for the slow phase, suggesting that the rapid and the slow phases of the uptake reaction correspond each to 0.5 proton.

Fully Reduced Enzyme Reconstituted in Phospholipid Vesicles. Previous work from this group (Nilsson et al., 1990) demonstrates a release of protons from the fully reduced, reconstituted enzyme on a time scale similar to the slow phase of proton uptake by the solubilized enzyme. The latter is thus the net result of both uptake and release. To obtain an estimate of the total uptake, we have repeated the vesicle experiment using the present experimental conditions which gives a better signal to noise ratio and provides for a more direct calibration procedure.

Figure 2 shows the absorption decrease of the dye following the flash-induced reoxidation of the vesicular enzyme. The amplitude corresponds to the release of 1.3 protons per right-side-out oriented enzyme. The first-order rate constant for the release is similar to that of the second phase in the uptake reaction and was about 1000 s^{-1}. The total uptake of protons, taking into account the 1.3 protons released, will then be 2.3, with 0.5 proton in the first phase and 1.8 protons in the slow phase. A summary of proton uptake and release is shown in Table I.

Solubilized and Reconstituted Mixed-Valence Enzymes. Reoxidation of the solubilized and reconstituted mixed-valence enzymes gave no detectable pH change in the medium (not shown). It has earlier been reported that the rate constants of the electron transfers in the mixed-valence reaction are independent of pH (Oliveberg et al., 1989) which is in agreement with our results. This suggests that the formation of a stable peroxide complex of the enzyme takes place without uptake of protons.

Discussion

The result obtained here shows a biphasic net uptake of one proton per functional unit during the reaction between the solubilized, fully reduced protein and oxygen. With the reconstituted enzyme, on the other hand, a monophasic release of 1.3 protons to the vesicular outside is observed. This value is in good agreement with that found earlier (Nilsson et al., 1990), but the present determination is significantly more accurate due to experimental improvements. The determination of the stoichiometry of proton release is, nevertheless, liable to systematic underestimation due to, for example, the presence of a small amount of unreconstituted enzyme or an insufficient rate of charge compensation by the K^+–valinomycin system. In the following, we shall therefore consider a range of 1–2 for the number of protons released to the outside of the vesicles.

With the solubilized enzyme, about half of the net uptake occurs in the rapid phase and the remainder at about 600 s^{-1}. With reconstituted enzyme, the release of protons to the vesicular outside takes place at a rate close to that of the slow

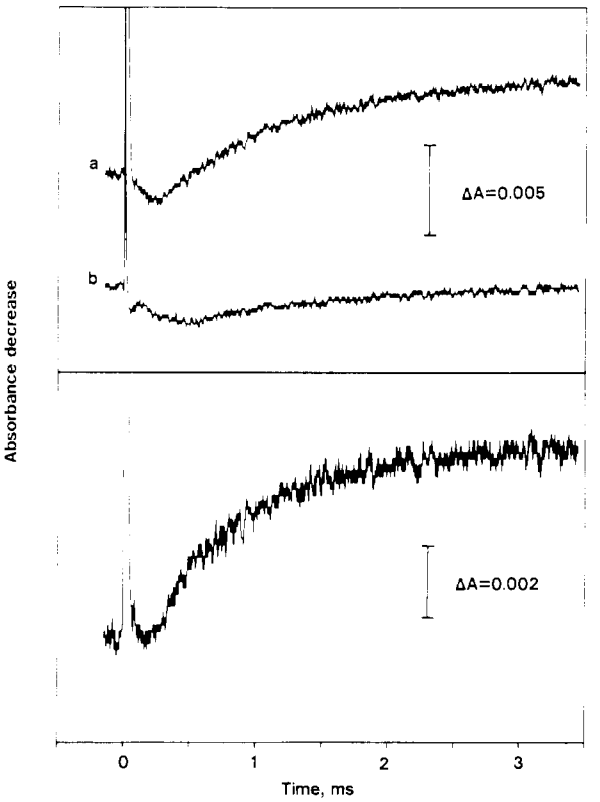

FIGURE 2: Transient absorption changes at 563 nm following flash photolysis of the fully reduced CO complex of reconstituted cytochrome oxidase in the presence of oxygen. The upper panel shows the absorbance changes obtained in the presence (a) or absence (b) of phenol red in the extravesicular medium. The lower panel shows the difference between the traces a and b. The concentrations of fully reduced enzyme and oxygen after mixing were 1.1 μM and 0.6 mM, respectively.

part of proton uptake in solubilized enzyme, i.e., about 1000 s^{-1}. Since the electron-transfer kinetics for the reconstituted enzyme, followed at 605 nm, are similar to those obtained with the solubilized enzyme (Nilsson et al., 1990), it is unlikely that they would differ in reaction mechanism. Therefore, the slow phase of proton uptake in the solubilized enzyme is probably the net result of an uptake of 1.5–2.5 protons concomitant with the release of 1–2 protons. Table I shows a summary of the uptake and release of protons in the rapid and slow phases.

The lag in the beginning of the reaction (best seen in the lower panel of Figure 1B) indicates that the rapid phase of proton uptake from the medium is preceded by other steps. Together with the finding that no detectable proton uptake occurs during the reaction between the mixed-valence CO complex and oxygen, this suggests that it is the arrival of the third electron at the oxygen binding site that initiates proton uptake. As suggested by Blair et al. (1985), the initial result of this electron transfer is most likely a ferrous–cupric peroxide complex. This species is then converted into the so-called ferryl intermediate by cleavage of the oxygen–oxygen bond. Since the rate of the latter reaction at room temperature is not known, the observed proton uptake could be the result of the formation either of the ferrous–cupric peroxide or of the ferryl species. However, cleavage of the oxygen–oxygen bond most probably requires protonation of one of the oxygen atoms in the peroxo bridge (Chan et al., 1988), and therefore we find it more likely that proton uptake precedes formation of the ferryl species.

The amplitude of the rapid phase of proton uptake corresponds to about 0.5 proton/enzyme. This is consistent with the appearance of a base with a pK_a close to the pH of the

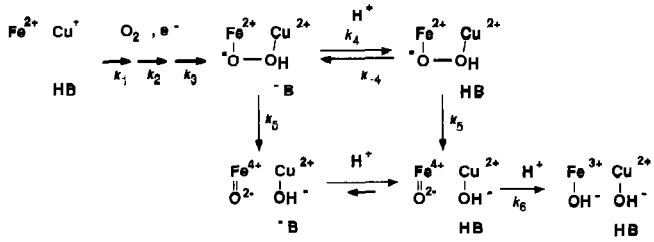

FIGURE 3: Model for proton uptake during the reaction between the fully reduced enzyme and oxygen. For clarity, the release of protons observed with the reconstituted enzyme is not included in this scheme.

medium as the result of the formation of the ferrous–cupric peroxide intermediate. Although the peroxo bridge would be a natural candidate for such a base, this idea is not consistent with the pH dependence of the electron-transfer kinetics. If, as suggested by Chan et al. (1988), only the protonated form of the oxygen–oxygen bridge is reactive, one expects in this case a marked pH dependence for the subsequent steps with a pK_a around 7.5. Since this is not observed (Oliveberg et al., 1989), it is more likely that the required proton is donated from the protein and that the observed uptake reflects partial reprotonation of the proton donor from the medium. After additional steps, the group then becomes fully protonated. A possible scenario for the interplay between oxygen and proton chemistry is shown in Figure 3. This scheme was examined for its ability to reproduce the present results as well as previously observed electron-transfer kinetics and their pH dependencies.

The effect of various choices of parameters in the scheme shown in Figure 3 was investigated by calculating the predicted proton uptake kinetics using numerical integration techniques. To account for the initial lag, at least one reaction has to precede the first step where protons are taken up. From simulations, we found it necessary to include three steps prior to proton uptake. With fewer preceding steps, it was not possible to reproduce the length of the lag together with the rate of the following rapid phase of proton uptake. The solid line in Figure 1 (lower panels) shows the result of a numerical simulation with $k_1 = 4 \times 10^4\ s^{-1}$, $k_2 = 2.8 \times 10^4\ s^{-1}$, $k_3 = 1.4 \times 10^4\ s^{-1}$, $k_4 = k_{-4} = 10^4\ s^{-1}$ (i.e., assuming a pK_a value of 7.5), $k_5 = 640\ s^{-1}$, and $k_6 = 10^3\ s^{-1}$. The rate constants for the first three steps were chosen to conform with rate constants obtained in earlier flow-flash work: k_1 is close to that obtained from the oxygen on rate (Hill & Greenwood, 1984b) and the actual oxygen concentration; k_2 and k_3 are similar to those obtained by Oliveberg et al. (1989). Fairly large values of k_4 and k_{-4} were found necessary, and those used correspond to diffusion-controlled uptake and release of protons in the absence of buffer (Gutman & Nachliel, 1990). The rate constant for cleavage of the oxygen–oxygen bond has been taken to be independent of the protonation state of base B in the ferrous peroxide intermediate. Different rate constants would predict a pK_a of 7.5 in the electron-transfer kinetics, which has not been observed (Oliveberg et al., 1989). However, a small difference would not have been detected in their work. In the final step, the fourth electron is transferred with a rate constant k_6. As a result, an additional proton is taken up from the medium, and at least one proton is released. Since the slowest electron-transfer step during reoxidation takes place at about $600\ s^{-1}$ (Oliveberg et al., 1989), the minimal value for k_6 is $600\ s^{-1}$. However, a comparatively high activation energy was also found for the transfer of the fourth electron, which suggests the possibility that this electron transfer is rate-limited by the cleavage of the oxygen–oxygen bond. In that case, k_6 could be appreciably larger. An interesting consequence is that the maximal occupancy of the ferryl state during reoxidation may be rather low.

The protonation step subsequent to oxygen–oxygen bond cleavage was taken to be in rapid equilibrium, and a pK_a value of 8.8 for the buffering group B in the ferryl intermediate was chosen to take into account the pH dependence of the transfer of the fourth electron (Oliveberg et al., 1989). A similar or higher value in the fully reduced state is necessary to ensure full protonation initially. We note that if the protonation state of base B affects the transfer of the third electron, this assumption can also explain the pH dependence of the intermediate phase (Oliveberg et al., 1989).

We emphasize that the particular set of rate constants given above should not be regarded as a unique solution. Rather, the model should be taken as an attempt to bring together the present result with earlier work on kinetics (Oliveberg et al., 1989; Hill et al., 1986) and oxygen chemistry (Blair et al., 1985; Chan et al., 1988). It is, however, clear that experiments of this kind can contribute to the elucidation of the course of oxygen reduction as well as to the clarification of the catalytic cycle for proton pumping.

The total number of protons taken up per functional unit is at least two but probably not greater than three. Although the protonation state of the end product, the oxidized, pulsed enzyme, is not known, an uptake of two protons per enzyme would seem to be the minimum required for oxygen chemistry. This would give the final state suggested by Chan et al. (1988) and Oliveberg et al. (1989), namely, two hydroxide ions coordinated to heme and copper, respectively. This assumes pK_a values well below 7.5 for water molecules coordinated to Cu_B and cytochrome a_3. Given a pK_a value around 9 for metal-coordinated water in ferric heme (Antonini & Brunori, 1971) and typical values of 8–9 for copper-coordinated water (Hanzlik, 1976), it seems likely that the oxygen binding site contains more than two protons in the oxidized enzyme. It is thus possible that all of the presently observed proton uptake can be accounted for by scalar protons. For simplicity, we have shown only the minimum of two protons in Figure 3.

The involvement of protons in the interconversion between different redox states of the oxygen binding site has been studied earlier by Wikström (1988). In his work, the intermediates were generated by reversed electron transfer in energized mitochondria. From the pH dependencies of the midpoint redox potentials, it was concluded that the conversion of the ferric–cupric peroxide to the ferryl species is linked to the uptake of two protons. Figure 3, on the other hand, accounts only for the uptake of one proton on the formation of the ferryl species from the fully reduced enzyme and oxygen. As discussed above, this number is a minimum since the number of protons taken up in the slow phase could well be higher. Such a case can easily be included in the model, for example, by replacing the copper-coordinated hydroxide ion in the ferryl species with a water molecule. A comparison of the proton stoichiometry associated with the transfer of the fourth electron is more difficult, since this was found to be highly pH dependent in the work of Wikström. Although his model predicts an uptake of two protons at the present pH, this relies on a rather indirect estimate of a pK_a value. Clarification of this issue will have to await a precise determination of the protonation state of the oxygen binding site in the fully oxidized enzyme.

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Deletion Mutagenesis in *Synechocystis* sp. PCC6803 Indicates That the Mn-Stabilizing Protein of Photosystem II Is Not Essential for O_2 Evolution†

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ABSTRACT: The photosystem II (PSII) reaction center complex coordinates a cluster of Mn atoms that are involved in the accumulation of oxidizing equivalents generated by light-induced charge separations within the intrinsic portion of the PSII complex. A 33-kDa extrinsic protein, termed the Mn-stabilizing protein (MSP), has been implicated in the stabilization of two of the four Mn atoms of the cluster, yet the precise role of this protein in O_2 evolution remains to be elucidated. Here we describe the construction of a mutant of the cyanobacterium *Synechocystis* sp. PCC6803 in which the entire gene encoding MSP has been deleted. Northern and immunoblot analyses indicate that other PSII proteins are expressed and accumulated, despite the absence of MSP. Fluorescence emission spectra at 77 K indicate PSII assembles in the mutant, but that the binding of MSP is required for the normal fluorescence characteristics of the PSII complex, and suggest a specific interaction between MSP and CP47. Fluorescence induction measurements indicate a reduced rate of forward electron transport to the primary electron donor, P680, in the mutant. It is concluded that in contrast to previous reports, MSP is not required for the assembly of active PSII complexes nor is it essential for H_2O-splitting activity in vivo.

The photosystem II (PSII)[1] complex found in higher plants, eukaryotic algae, and cyanobacteria catalyzes the light-driven transport of electrons from H_2O to the mobile lipophilic electron carrier plastoquinone [for a review, see Babcock et al. (1989)]. The catalytic site of H_2O oxidation is known to contain a cluster of Mn atoms involved in the accumulation of oxidizing equivalents utilized for the four-electron oxidation of two molecules of H_2O. Biochemical resolution of the PSII complex has resulted in the identification of five intrinsic membrane proteins (CP47, CP43, D1, D2, and cytochrome b_{559}) which bind the pigments and cofactors involved in light capture and primary photochemistry. While the reaction center components mediating the initial charge separation and stabilization events are now thought to be located within a heterodimer formed by the D1 and D2 proteins, the proteins involved in binding the Mn cluster remain to be identified. The Mn cluster is coupled to the photooxidizable reaction center chlorophyll (P680) via an oxidizable tyrosyl residue (Y_z) of the D1 polypeptide (Debus et al., 1988; Metz et al., 1989). Portions of the D1/D2 heterodimer situated on the lumenal side of the thylakoid membrane are proposed to provide ligands for the Mn cluster (Babcock et al., 1989).

In addition to the membrane-spanning polypeptides, the PSII complex possesses one or more extrinsic polypeptides situated on the lumenal side of the thylakoid membrane in

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[1] Abbreviations: Chl, chlorophyll; CP47, 47-kDa PSII chlorophyll protein encoded by the *psbB* gene; cyt, b_{559}, cytochrome b_{559}, encoded by the *psbE/F* operon; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; D1 and D2, PSII reaction center proteins encoded by the *psbA* and *psbD* genes, respectively; FeCN, $K_3Fe(CN)_6$; F_{max}, maximum fluorescence yield, with all Q_A reduced; F_0, initial fluorescence yield, with all Q_A oxidized; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P680, chlorophyll molecule(s) which act(s) as the primary electron donor of the PSII reaction center; HQ, hydroquinone; MSP, manganese-stabilizing protein, extrinsic 33-kDa PSII protein encoded by the *psbO* gene; PSII, photosystem II.

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