Electron-Transfer Pathways in Stellacyanin: A Possible Homology with Plastocyanin†

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Received February 27, 1987; Revised Manuscript Received June 29, 1987

ABSTRACT: Reduction of the blue Cu(II) protein stellacyanin (*Rhus vernicifera*) with aquochromium(II) ions yields stoichiometrically a substitution-inert chromium(III)–stellacyanin Cu(I) complex. To identify the amino acid residues to which the Cr(III) ion is coordinated, we digested the ^{51}Cr-labeled protein with thermolysin and separated the peptides by gel filtration and high-performance liquid chromatography. A single peptide labeled with chromium was obtained. This peptide consists of Asp, Val, and Lys in a 1:1:1 ratio. Hence it is identified as the stretch Val-48, Asp-49, and Lys-50 in the stellacyanin sequence. The Cr(III) is most probably coordinated to the carboxylate and the amino side chains of Asp-49 and Lys-50, respectively. Asp-49 is homologous to the invariant Asp-42 in all known plastocyanin sequences. Affinity labeling of plastocyanin by aquochromium(II) ions has shown that Asp-42 is one of the four possible ligands for the Cr(III) label. From sequence homology relationships among stellacyanin and the plastocyanins combined with examination of the known three-dimensional structure of poplar plastocyanin, several structural features of stellacyanin are suggested: Tyr-85 may, by analogy to Tyr-83 in plastocyanin, be close to the binding site of Cr(III) as well as being exposed to solvent. On the basis of this, we propose that in stellacyanin the electron transfer from Cr(II) to Cu(II) proceeds through a weakly coupled π^* system provided by the aromatic ring of Tyr-85 and the sulfur of Cys-87 to the probably solvent inaccessible Cu(II) redox center.

Copper ions bound at the type 1 coordination site of the blue copper proteins serve as electron-mediating centers and have attracted considerable interest primarily because of their unusual spectroscopic properties (Fee, 1975; Lontie, 1984). Our current understanding of these properties has been decisively advanced by the high-resolution three-dimensional structure determination of members from two classes of this family of proteins: poplar tree plastocyanin and bacterial azurins (Colman et al., 1978; Guss & Freeman, 1983; Adman et al., 1978; Adam & Jensen, 1981; Norris et al., 1983). Stellacyanin is a glycoprotein member of a third class of blue single copper proteins. It is noteworthy from several points of view. First, besides being a glycoprotein with more than 40% of its mass carbohydrate, stellacyanin has been shown by sequence determination to lack methionine (Bergman et al., 1977). This amino acid has been shown to provide one of the four copper ligands in plastocyanins and azurins (Colman et al., 1978; Guss & Freeman, 1983; Adman et al., 1978; Adman & Jensen; 1981; Norris et al., 1983; Frank et al., 1985). Hence, the question of which residue (if any) serves as the fourth ligand in stellacyanin is an open one. Further, the redox potential of the Cu(II)/(I) center is significantly lower than that in the other group members (184 mV vs. 350–500 mV) (Fee, 1975; Lontie, 1984). Finally, stellacyanin seems to be one of the more redox reactive copper proteins as judged by its electron-transfer rates with a range of proteins or transition metal complexes (Holwerda et al., 1976; Tollin et al., 1986).

All these features make stellacyanin an attractive candidate for direct structure determination by X-ray crystallography. However, so far, attempts to grow suitable crystals have been unsuccessful. Other research methods are therefore being employed to gain insights into structural features of stellacyanin (Engeseth et al., 1984; Hill & Lee, 1979). A central issue in understanding the mode of action of redox proteins is the role played by the protein itself; e.g., which amino acid residues (or the polypeptide backbone) participate in the electron-transfer reaction and how and where is specificity expressed? These questions are then related to another one, namely, where do electrons get in and out of the protein (Farver & Pecht, 1984)?

In a large number of proteins the answer to the latter question may be inherent in their structure. Namely, their redox centers are exposed and accessible to solvent molecules and most probably also to their respective physiological reaction partners (e.g., *c*-type cytochromes). In plastocyanin and azurin, the copper ligands were shown to be relatively inaccessible, with the exception of one imidazole ring which, to a limited extent, is solvent exposed (Colman et al., 1978; Guss & Freeman, 1983; Adman et al., 1978; Adman & Jensen, 1981; Norris et al., 1983). This imidazole residue was indeed proposed to be an electron-transfer locus in these proteins. Evidence for a second electron-transfer site on the surface of these proteins emerged primarily from two types of experimental approaches. NMR measurements, using paramagnetic redox-inert coordination compounds, showed cation binding at a negatively charged cluster to carboxylate residues on plastocyanin (Cookson et al., 1980a,b). The second approach involved an affinity-labeling procedure and provided independent evidence corroborating the role of the above-mentioned negative patch as an electron-transfer locus on plastocyanin (Farver & Pecht, 1981a). Further application of this procedure to *Pseudomonas aeruginosa* azurin revealed an electron-transfer site distinct from the partly exposed imidazole ligand (Farver & Pecht, 1981b). This affinity-labeling approach takes advantage of the facts that chromous ions are strong reductants and rather substitution labile, while chromic ions are highly substitution inert. Hence, ligand(s) bound to Cr(II) during an inner-sphere electron transfer remains (remain) bound to the product Cr(III) ions. The extensive studies by Taube and his associates have elegantly established this pattern (Taube, 1984).

† O.F. acknowledges the support of the Danish Natural Science Research Council.

0006-2960/87/0426-7317$01.50/0

In view of the described features of stellacyanin, we have examined the possibility of affinity labeling this protein using Cr(II) ions (Morpurgo & Pecht, 1982). Cu(II)–St[1] was shown to undergo stoichiometric reduction, and 1 mol of Cr(III) was found coordinated to the protein. Reoxidation of the copper center and its subsequent rereduction by Cr(II) caused neither binding of further Cr(III) ions nor chromium exchange. This implied the presence of a single, specific binding site for the Cr(III) ion on St and, therefore, of a specific electron-transfer site (Morpurgo & Pecht, 1982; Pecht et al., 1983, 1985). In the present study we have carried out proteolysis of the Cr(III)-labeled St in order to identify the site of its coordination.

Experimental Procedures

Materials

Stellacyanin was isolated from the acetone extract of Japanese *Rhus vernicifera* lacquer tree sap and purified according to the procedure of Reinhammer (1970). The final $A(280)/A(604)$ ratio was always less than 6.0. The concentration of oxidized stellacyanin [St(II)] was determined from the absorbance at 604 nm by using an extinction coefficient of 4030 M^{-1} cm^{-1} (Malmström et al., 1970). ^{51}Cr was purchased as aqueous sodium chromate (specific activity 3.7×10^9 Bq/mg) from the Radiochemical Centre, Amersham, England. Thermolysin (3× recrystallized) was obtained from Calbiochem. Trypsin (TPCK) and chymotrypsin were purchased from Worthington Biochemical Corp. All other materials were of analytical grade and used without further purification. Triply distilled water was used throughout.

Methods

Protein Labeling. Cr(II) stock solutions (0.05 M in 0.1 M $HClO_4$) were made by anaerobic reduction of the analogous $Cr(ClO_4)_3$ solution with mossy Zn/Hg amalgam and labeled with ^{51}Cr (specific activity 3.7×10^5 Bq/mg). The concentration of Cr(II) was determined by titration with MnO_4^- solutions in 0.5 M H_2SO_4; the decrease in absorbance was monitored at 545 nm (ϵ = 2340 M^{-1} cm^{-1}).

Stirred stellacyanin solutions (0.1–0.2 mM in 0.1 M HEPES, pH 6.0) were deoxygenated by repeated evacuation and flushing with purified argon. A stoichiometric amount of Cr(II) was then slowly added to the vigorously stirred protein solution by using a gas-tight syringe. After the addition of 1 equiv of reductant, the Cu(II) in stellacyanin was completely reduced as monitored by the disappearance of the 604-nm absorption. The solution was then dialyzed against 0.1 M HEPES, pH 6.0, for at least 24 h, during which the buffer solution was changed several times. During the dialysis, stellacyanin was reoxidized by O_2 as evidenced by the reappearance of the blue color. The Cr(III) binding stoichiometry was established by measuring the radioactivity of ^{51}Cr in the protein solution.

Enzymatic Proteolysis and Peptide Separation. Native stellacyanin like plastocyanin and azurin is relatively resistant toward digestion by proteases. Therefore, following the above-described labeling procedure, the protein was denatured in a 10% trichloroacetic acid solution cooled in ice (Farver & Pecht, 1981a,b). Typically, 25 mg of labeled protein was used and the time allowed for denaturation, as monitored by the loss of the blue color was $\sim^1/_2$ h. The copper and trichloroacetic acid were then separated from the protein by passing the solution through a 20 × 1.0 cm Sephadex G-25 column, eluting with 0.1 M HEPES, pH 6.0.

For thermolytic digestion, the pH was adjusted to 7.0 in 0.1 M HEPES buffer containing 10 mM $Ca(NO_3)_2$. The proteolysis was performed at room temperature for a total time of 16 h with 1% (w/w) enzyme. The solution was then passed through a 90 × 2.0 cm Sephadex G-25 column, with 0.1 M acetic acid as eluent, and the eluate was monitored by its absorption at 230 and 280 nm as well as for its γ activity.

Trypsin and chymotrypsin were also employed for proteolysis. However, the yields of digested protein were invariably very low. Thus, approximately 90% of the intact protein was recovered in the void volume. Thermolytic digestion gave much higher yield (25–50% digest). It was therefore decided to only pursue the thermolytic proteolysis in the following work.

Following separation of the thermolytic peptides, the radioactive peak (cf. Figure 1) was pooled, lyophilized to dryness, and dissolved in 0.1 M acetic acid. For further purification the radioactive pool was then separated by reverse-phase HPLC using a C-8, 20 × 0.45 cm column. The elution was performed with 0.02 M NaOAc, pH 4.0, in 10% methanol and 90% water. The flow rate was 0.2 mL/min, and absorbances at both 230 and 280 nm along with γ activity were monitored. Since a second purification step seemed necessary (cf. Results and Discussion), the radioactive peak was once more passed through the above HPLC column, this time with a flow rate of 0.05 mL/min; each drop was collected in a separate test tube, and the same parameters as above were followed for detection.

The ^{51}Cr-labeled samples were then hydrolyzed with 6 M HCl for 24 h at 120 °C and analyzed for amino acid content.

Results and Discussion

We have earlier employed the reductive labeling procedure to show that oxidized stellacyanin can be reduced stoichiometrically by 1 equiv of Cr(II) ions yielding a substitution-inert Cr(III)-labeled stellacyanin (Morpurgo & Pecht, 1982). It was further demonstrated that, upon repeated reduction of the reoxidized protein with another equivalent of Cr(II) ions, neither exchange nor further labeling was attained. Thus, only the first chromium ion was found to be attached by this method, which suggested that one specific redox-accessible site is present on stellacyanin. In the current work, oxygen-free aqueous solutions of stellacyanin (at pH 6.0, 0.1 M HEPES buffer) were reduced with 1 equiv of $^{51}Cr(II)$. In agreement with the earlier results we have repeatedly (n = 9) observed the formation of a 1:1 Cr–St adduct, while any excess of chromium could easily be removed by dialysis against the HEPES buffer.

One way of identifying the residue(s) to which the Cr(III) ion is coordinated on the labeled Cr–St is by its enzymatic cleavage into peptide fragments. This is followed by separation of these peptides, tracing those that carry the ^{51}Cr label, and then performing amino acid analysis of the ^{51}Cr-containing products. Comparison of the amino acid content of the labeled peptide with known sequences of proteolytic fragments of stellacyanin then allowed identification of Cr(III)-coordinating residues. Thus, after isolation of the radioactively labeled peptide it was subjected to amino acid analysis. However, as it turned out from the large number of analyses done, and as is apparent from Figure 1B, this peak constituted a mixture of at least two different peptides with some variation in total amino acid content. Therefore, this peak was further purified by an additional HPLC fractionation, utilizing a much reduced

[1] Abbreviations: Az, azurin; Pc, plastocyanin; St, stellacyanin; TPCK, tosylphenylalanine chloromethyl ketone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography.

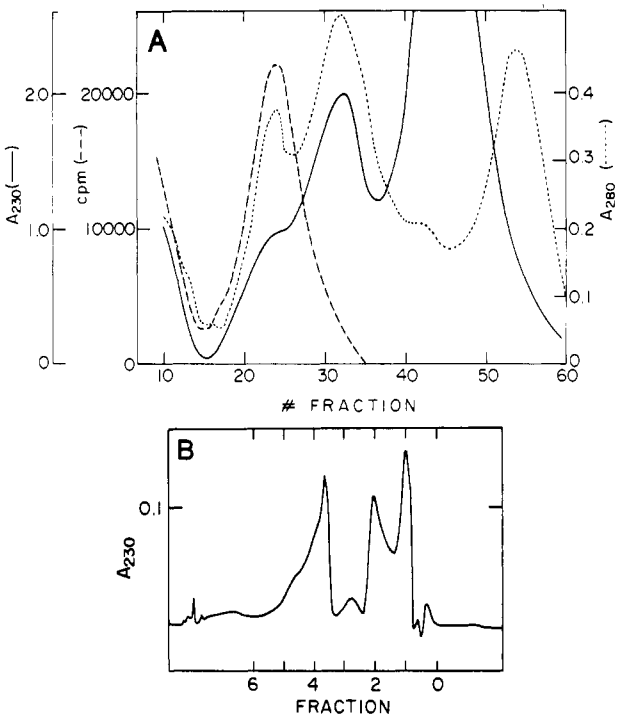

FIGURE 1: Panel A: Elution pattern of Cr(III)–St thermolysin proteolysis products on a Sephadex G-25 (2.0 × 90 cm) column. Eluent 0.1 M HOAc, 5 mL per fraction. Panel B: HPLC fractionation of the pooled radioactive peak. All ^{51}Cr was found in fraction 1, and the activity was 9035 cpm. For further experimental details, cf. Experimental Procedures.

pressure. This procedure successfully separated the initially obtained peak into three fractions. The major part of the radioactivity (>80%) was contained in the front of the leading peak, while the remaining 20% was eluted with the next fraction. Amino acid analysis showed that the first fraction was invariantly made up of one specific Cr-labeled peptide while the second was a mixture of a smaller amount of the Cr peptide together with one or more unlabeled peptides.

Four independent experiments unambiguously and consistently yielded the same amino acid composition of the radioactively labeled peptide: Val, Asp, and Lys (at a 1:1:1 ratio) which is only found in one stretch of stellacyanin sequence. In their sequence determination, Bergman et al. (1977) also observed that thermolytic digestion produces this Val-Asp-Lys peptide. That this peptide is indeed the single labeled site can also be seen from the following more quantitative consideration: From Figure 1A one can calculate the ratio between the cpm value and absorption at 230 nm to be ca. 22 000 at the maximum of the radioactive peak. (A more precise value would require a Gaussian resolution of the bands.) After the HPLC fractionation, the cpm/absorption ratio of this fraction increased by nearly a factor of 3 to ~60 200 (cf. Figure 1B). The HPLC fractionation shows that the initial pool containing the ^{51}Cr-labeled peptide in fact consisted of three peptides with an approximately equal contribution to the 230-nm absorption. The factor 3 is therefore to be expected and corroborates that the chromium is bound to a single peptide. It can therefore be concluded that the chromium label is coordinated to a unique site in stellacyanin, namely, the Val-48–Asp-49–Lys-50 peptide. The actual coordination probably involves a chelate between the γ-carboxylate of Asp-49 and the ϵ-amine of Lys-50, similar to the chelate probably formed upon chromium labeling of *P. aeruginosa* azurin (Farver & Pecht, 1981b).

Structural Aspects. Since crystallographic structure determination of stellacyanin has not been achieved so far, we use an assumed polypeptide fold (Wherland, Farver, and Pecht, unpublished results) as the basis of the following discussion. The available amino acid sequence of St (Bergman et al., 1977) is compatible with that of plastocyanin.

^1H NMR studies have provided significant insights into the structure of St (Hill & Lee, 1979). Thus, resonances in the aromatic region of the holoprotein show the presence of only two freely titrating imidazoles out of the four histidine present in St. It could therefore safely be concluded that the two other imidazole residues are ligands for the copper ion, as in Az and Pc. This also was further supported by extended X-ray absorption fine structure (EXAFS) spectroscopy which suggested that there are two nitrogen atoms in the first coordination sphere of the oxidized protein with normal Cu–N distances of 1.95–2.05 Å (Peisach et al., 1982). The EXAFS spectra also suggested that one or two sulfur atoms coordinate to the copper. The characteristic thiolate to copper(II) charge-transfer absorption which is responsible for the deep blue color of Az and Pc is also exhibited by oxidized St, and this unambiguously implicates a cysteine thiolate as a ligand (McMillin et al., 1974a,b).

The cysteine that coordinates to the copper ion in the three crystallographically characterized blue copper proteins is conserved in all the known sequences of plastocyanins and azurins. In *Rhus* stellacyanin, which contains a total of three cysteines, the homologous residue is Cys-87 (Ryden & Lundgren, 1979). However, in the sequence determination of St (Bergman et al., 1977) it was found that the single free –SH group belongs to Cys-59, which has very little homology in its vicinity as compared to sequences of the other blue copper proteins. Instead, the conserved Cys-87 was found to form a disulfide bridge with Cys-93. This apparent problem found its solution when McMillin and co-workers (Engeseth et al., 1984) showed that, in the native protein at pH 7, it is Cys-59 and Cys-93 which are linked by a disulfide bridge, while at pH >8, where the original sequence work had been performed, a disulfide switch occurs. This finding resolved the apparent discrepancy and makes the thiolate of Cys-87 available for copper coordination.

The fourth Cu ligand in Pc and Az is a methionine sulfur. St however lacks this amino acid. On the basis of spectral analysis, McMillin and co-workers (Tennent & McMillin, 1979; McMillin & Morris, 1981) have therefore suggested that the fourth ligand in stellacyanin may be a disulfide sulfur. A similar suggestion has been made by Ferris et al. (1978), who analyzed the resonance Raman spectra of stellacyanin.

It is noteworthy that the polypeptide loop connecting strands 7 and 8 (cf. Figure 2) contains three of the four copper ligands in plastocyanin and azurin, and a similar folding may also provide the copper binding site in St. As seen from Figure 2, the constellation Cys to Pro to His to Met of the above loop, which is conserved in all azurins and plastocyanins, is also present in stallacyanin with the exception that methionine is replaced by Cys-93 in St. Taken together, all the above observations therefore lead us to the conclusion that the fourth Cu ligand in stellacyanin is indeed the Cys-59/93 cystine disulfide.

Figure 2 also illustrates the many similarities existing between Pc and St in strand 4, which contains His-37, one of the two ligating imidazoles of Pc (Colman et al., 1978; Guss & Freeman, 1983). Particularly noteworthy is our conclusion that the reductive labeling by chromium(II) of St–Cu(II) is at Asp-49 since this carboxylate residue is homologous to the chromium coordinating Asp-42 in plastocyanin affinity labeling (Farver & Pecht, 1981a).

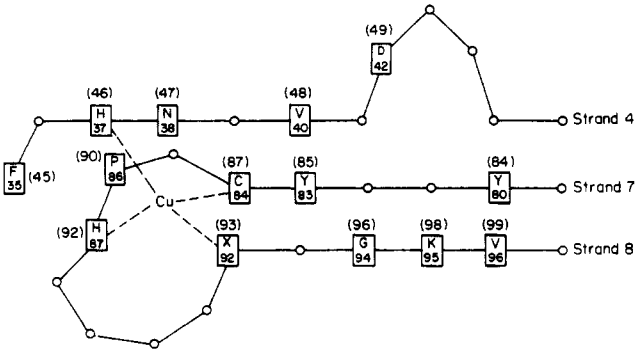

FIGURE 2: Comparison of stellacyanin and plastocyanin structures. Topological structure of three peptide strands (4, 7, and 8) of poplar plastocyanin after Colman et al. (1978). The residues that are invariant in all higher plant plastocyanins and further conserved in *Rhus* stellacyanin are labeled with the one-letter notation, and the numbering is according to the plastocyanin sequence. Numbers in parentheses correspond to the stellacyanin sequence (Bergman et al., 1978). The chromium(III) label is proposed to be coordinated at the aspartate carboxylate in both Pc and St [D-42(49)]. X means Met-92 in plastocyanin and Cys-93/59 in stellacyanin.

In conclusion, all the above structural considerations make the proposed structural comparison of stellacyanin and plastocyanin as illustrated in Figure 2 very plausible.

Electron-Transfer Site on Stellacyanin. As seen in Figure 2, there is only one potential ligand for Cr(III) in strands 4, 7, and 8 of stellacyanin that is close enough to ensure rapid and specific electron transfer to the Cu(II) center, namely, Asp-49. The figure also suggests that Tyr-83, proposed to be functionally important in plastocyanin, is conserved in stellacyanin (Tyr-85). From computer model building studies on stellacyanin (Wherland, Farver, and Pecht, unpublished results) we can estimate a distance from the phenol ring oxygen of Tyr-85 to the copper ion of ~11 Å. This distance is not excessively long for effective electron transfer. Thus, long-distance intramolecular electron transfer of ~12 Å has been reported in Ru-labeled cytochrome *c* and azurin with rate constants of 25 s^{-1} and 1.9 s^{-1}, respectively (Kostič et al., 1983). In plastocyanin, the phenol group of this tyrosine protrudes into solution (Colman et al., 1978; Guss & Freeman, 1983), and the proximity of this residue to the Cr(III) binding site makes it likely to participate in an electron-transfer pathway by electronic delocalization through a weakly coupled π system (Colman et al., 1978; Farver & Pecht, 1981a). Considering the close resemblance between the plastocyanin and stellacyanin sequences in this region, we suggest that electron transfer from the solvent–protein interface to the copper center proceeds via homologous sites and reaction pathways in both proteins.

The carbohydrate content (40% of the molecular weight) of *Rhus* stellacyanin is much higher than in any other known blue copper protein. The carbohydrate chains are attached to three asparagine residues (Bergman et al., 1977), none of which are in the vicinity of the chromium binding site. However, since it is not known how the carbohydrate moieties fold and interact with amino acid residues, it is premature to comment on the possible effects of the carbohydrates on the reactivity of stellacyanin.

In French bean plastocyanin, the immediate vicinity of Tyr-83 contains four exposed carboxylate side chains (Asp-42, Glu-43, Asp-44, and Glu-45), making this region negatively charged at physiological pH. These charged residues probably serve as a recognition site for its biological partner, the photosystem I reaction center, P-70 (Farver et al., 1982). In *Rhus* stellacyanin, which is an extracellular protein, however, only one carboxylate residue, Asp-49, is conserved out of the above four. Moreover, the local negative charge it provides is fully compensated by the neighboring protonated ϵ-amino group of lysine-50. Therefore, it does not seem to be due to any advantageous electrostatic effect that stellacyanin is specifically labeled by chromium(III) at this site. Rather, the specificity of this reaction is most probably caused by a facile electron-transfer pathway between Cr^{2+} and Cu^{2+} via the π-electron system of the Tyr-85 phenol ring and the Cys-87 sulfur atom in stellacyanin. If so, then a π^* orbital electron-transfer pathway may exist in all three types of single blue copper redox proteins.

ACKNOWLEDGMENTS

We are grateful to Dr. S. Blumberg for very helpful advice and discussions concerning the enzymatic proteolysis of stellacyanin. We thank Drs. S. Wherland and P. Frank for their interest in and rather thoughtful and thorough criticism of this work.

Registry No. Cu, 7440-50-8; Cr, 7440-47-3; Tyr, 60-18-4; Cys, 52-90-4; Lys, 56-87-1; Asp, 56-84-8.

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Kinetics of O_2 Evolution from H_2O_2 Catalyzed by the Oxygen-Evolving Complex: Investigation of the S_1-Dependent Reaction†

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Received February 23, 1987; Revised Manuscript Received June 24, 1987

ABSTRACT: The evolution of O_2 from H_2O_2 catalyzed by the oxygen-evolving complex (OEC) in darkness was examined with photosystem II reaction center complex preparations from spinach. Flash illumination of dark-adapted reaction centers was used to make S_0-enriched or S_1-enriched complexes. The membranes catalyzed O_2 evolution from H_2O_2 when preset to either the S_0 or S_1 state. However, only the S_0-state reaction was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone and dependent on chloride. These results indicate that (1) the S_0-dependent and S_1-dependent catalytic cycles can be separated by flash illumination, (2) the S_0-dependent reaction involves the formation of the S_2 state, and (3) the S_1-dependent reaction does not involve the formation of the S_2 or S_3 states. A kinetic study of the S_1-dependent reaction revealed a rapid equilibrium ordered mechanism in which (1) the binding of Ca(II) must precede the binding of H_2O_2 to the OEC and (2) the reaction of Ca(II) with the free enzyme is at thermodynamic equilibrium such that Ca(II) does not necessarily dissociate after each catalytic cycle.

The oxygen-evolving complex (OEC)[1] catalyzes the oxidation of water to molecular oxygen in order to provide the supply of electrons for photosynthetic electron transport. The OEC causes single-electron reductions of the reaction center following each charge separation. As a result, molecular oxygen is evolved only once per four photoevents (Kok et al., 1970). To accomplish these single-electron donations, the OEC cycles through five S states (S_0–S_4) and yields oxygen only upon formation of the S_4 state (Forbush et al., 1971).

The S_1 state is stable in darkness, while the S_2 and S_3 states will deactivate in the dark to S_1. The S_2 and S_3 states are in redox equilibrium with D, the precursor to signal IIs. Although the $t_{1/2}$ of deactivation of the S_2 and S_3 states is on the order of seconds, this rate can be accelerated greatly by the addition of reagents like carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) that convert D^+ to D (Renger, 1972; Yerkes & Crofts, 1983). It has been shown recently that D^+ can oxidize the S_0 state to S_1 in the dark such that 50% of the S_0 state initially formed in PSII preparations is converted to S_1 in about 1 h (Styring & Rutherford, 1987).

The S_2 state has been the most highly characterized of all the S states. Formation of the S_2 state results in the appearance of two low-temperature EPR signals known as the $g = 2$ multiline signal (Dismukes & Siderer, 1980a,b; Brudvig et al., 1983) and the $g = 4.1$ signal (Zimmermann & Rutherford, 1984; Casey & Sauer, 1984). Depletion of chloride by sulfate causes the formation of an abnormal S_2 state that lacks the multiline signal (Ono et al., 1986) and is incapable of undergoing further reactions until Cl^- has been restored (Sandusky & Yocum, 1984; Itoh et al., 1984; Theg et al., 1984). High concentrations of Tris (Frasch & Cheniae, 1980) or hydroxide (Briantais et al., 1977) will inactivate the OEC by a specific interaction with the S_2 state.

The OEC contains four manganese that are bound to intrinsic membrane proteins in the thylakoid (Cheniae & Martin, 1970), and the presence of Ca(II) is required for the enzyme to be catalytically competent (Ghanotakis & Yocum, 1986). The proteins that compose the OEC have not been positively identified to date. Mutants of *Scenedesmus*, which are in-

† This work was supported by grants to W.D.F. from the National Science Foundation (DMB-8604118), the Rackham Foundation, and the Office of Energy Research and a National Institutes of Health Biomedical Research Support Grant to the Vice President for Research.

[1] Abbreviations: PSII, photosystem II; OEC, oxygen-evolving complex; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MES, 4-morpholineethanesulfonic acid; EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton; Chl, chlorophyll.

0006-2960/87/0426-7321$01.50/0