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A Calcium-Specific Site Influences the Structure and Activity of the Manganese Cluster Responsible for Photosynthetic Water Oxidation†

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ABSTRACT: EPR studies have revealed that removal of calcium using citric acid from the site in spinach photosystem II which is coupled to the photosynthetic O_2-evolving process produces a structural change in the manganese cluster responsible for water oxidation. If done in the dark, this yields a modified S_1' oxidation state which can be photooxidized above 250 K to form a structurally altered S_2' state, as seen by formation of a "modified" multiline EPR signal. Compared to the "normal" S_2 state, this new S_2'-state EPR signal has more lines (at least 25) and 25% narrower ^{55}Mn hyperfine splittings, indicative of disruption of the ligands to manganese. The calcium-depleted S_2' oxidation state is greatly stabilized compared to the native S_2 oxidation state, as seen by a large increase in the lifetime of the S_2' EPR signal. Calcium reconstitution results in the reduction of the oxidized tyrosine residue $^{161}Y_D^+$ (E_m ~0.7-0.8 V, NHE) within the reaction center D_1 protein in both the S_1' and S_2' states, as monitored by its EPR signal intensity. We attribute this to reduction by Mn. Thus a possible structural role which calcium plays is to bring Y_D^+ into redox equilibrium with the Mn cluster. Photooxidation of S_2' above 250 K produces a higher S state (S_3 or S_4) having a new EPR signal at $g = 2.004 \pm 0.003$ and a symmetric line width of 163 ± 3 G, suggestive of oxidation of an organic donor, possibly an amino acid, in magnetic contact with the Mn cluster. This EPR signal forms in a stoichiometry of 1-2 relative to Y_D^+. This state is photoaccumulated, does not evolve O_2, and decays in the dark to the stable S_2' state. The enhanced stability and apparent lowered redox potential of the S states can be explained if calcium depletion exposes the Mn cluster to an increased solvent activity, resulting in the binding and hydrolysis of additional water ligands (hydroxo and oxo). The possibility that this causes disproportionation of Mn^{III} to Mn^{II} + Mn^{IV} is considered on the basis of analogy to the hydrolysis-induced disproportionation observed for synthetic dimanganese complexes. A "gatekeeper" role for calcium in limiting access of substrate water to the catalytic Mn cluster is indicated.

Calcium is required for normal functioning of the photosynthetic water-oxidizing complex. Its precise role has been difficult to elucidate owing to it poor spectroscopic detectability, unlike the active site of this complex which is comprised of four closely arranged Mn ions. This complex can exist in five oxidation states, so-called S states, produced by photooxidation of photosystem II (PSII). The highest oxidation state, S_4, oxidizes water to O_2. The effects of calcium depletion by salt washing with or without chelators have been controversial. Calcium depletion has been observed to slow the kinetics of reduction of the photooxidized tyrosine radical Y_Z^+ by the Mn cluster in PSII (Ghanotakis et al., 1984; Dekker et al., 1984; Cole & Sauer, 1987), to eliminate the characteristic multiline EPR signal associated with the S_2 state (de Paula et al., 1986), and to block photooxidation of manganese beyond the S_3 without the ability to form the S_2 multiline EPR signal (Boussac et al., 1985; Boussac & Rutherford, 1988). On the other hand, recent studies employing low-pH incubation with citric acid to quantitatively remove one of two calciums per PSII have shown from thermoluminescence that a block in photooxidation beyond the S_2 state is produced (Ono & Inoue, 1989a).

At the 8th International Congress on Photosynthesis held in Stockholm, Aug 6-11, three groups presented results demonstrating that a "modified" multiline EPR signal can now be observed in PSII membranes treated to deplete calcium (Ono & Inoue, 1989b; Boussac & Rutherford, 1989; Baumgarten et al., 1989). Results from all three groups, including ours, suggested that the new EPR signal was stable over hours in the dark and could be attributed to a modified S_2' oxidation state, formed by dark adaptation following room temperature illumination. It was also noted to form partially in the dark, possibly by oxidation from an unknown species (Boussac & Rutherford). Ono and Inoue observed that its formation by illumination required a higher temperature than the normal S_2 state and that calcium-depleted PSII was unable to undergo further stable charge separation, owing to a block in the $S_2' \rightarrow S_3'$ reaction. They also found the modified S_2' state to be thermodynamically more stable than the normal S_2 state, as seen by an increased temperature for thermoluminescence

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induced by recombination with the acceptor Q_A^-. In contrast, Baumgarten et al. and Boussac and Rutherford found that illumination of this S_2' state reversibly bleached the modified multiline signal and produced in its place still another new EPR signal having $g = 2.00$ and a symmetric line width of 150–165 G.

In addition, Lockett et al. (1989) and Baumgarten et al. (1989) observed that calcium reconstitution in the dark reduces by 50–80% the intensity of EPR signal II_S for the oxidized tyrosine residue Y_D^+, suggestive of the storage of a reducing equivalent in the Mn cluster. This was interpreted by Lockett et al. in terms of the reduction of the S_1 state to an S_0' state upon calcium depletion which could then serve to reduce Y_D^+ upon calcium reconstitution. Thus, this interpretation and the previous observation of a dark stable S_2' state appear to be inconsistent.

Here we show that these seemingly inconsistent interpretations can be reconciled if it is considered that calcium depletion exposes the Mn cluster to an increased water environment resulting in hydrolysis of manganese and an attendant stabilization of the S_2, S_3, and S_4 states. Synthetic dimanganese(III) complexes which are stabilized by hydrolysis and spontaneously disproportionate to form the $Mn^{II}Mn^{III}$ and $Mn^{III}Mn^{IV}$ oxidation states are presented as simple examples which may account for this stabilization.

Materials and Methods

Spinach PSII membranes were prepared by Triton extraction (Berthold et al., 1981). Depletion of one of two calcium ions was performed with the 10 mM citrate extraction method at pH 3.0 described by Ono and Inou (1988). Treated membranes were incubated for 30 min at 0–4 °C in rigorous darkness prior to adjustment of the pH to 6.5 with separation by centrifugation. Samples were suspended in a buffer consisting of 0.4 M sucrose, 50 mM MES, and 25 mM NaCl, pH 6.5. LDS-PAGE revealed no significant release of the three extrinsic proteins associated with water oxidation (not shown). O_2 evolution rate measurements using a Clark electrode, the Hill reaction ($H_2O \rightarrow$ DCIP), and the intensity of the S_2 multline EPR signal were reduced by 80–90%, 80–85%, and 80–90% in depleted samples, respectively. Reconstitution with 50 mM $CaCl_2$ restored minimally 70–80% of the original activity in each assay. Ono and Inoue (1988) have shown that calcium rebinding and reactivation require higher than physiological concentrations of $CaCl_2$ (5–50 mM). However, after calcium binds the $CaCl_2$ concentration is lowered, the site becomes inaccessible to extraction by EDTA. We observed no restoration of O_2 activity with $MgCl_2$ or $CdCl_2$ up to 50 mM or with 200 mM NaCl; 50 mM $SrCl_2$ restored O_2 activity by 30–40%, in agreement with a prior report (Boussac & Rutherford, 1988). The high $CaCl_2$ concentration required for reactivation is at least partly attributable to enhancing the accessibility of the site, not unlike that observed in the salt requirement for calcium extraction by EDTA. We confirmed the result of Ono and Inoue (1988) showing that the calcium site is coupled to water oxidation as seen by retention of 70% of the electron transport rate through the reaction center, DPC $\rightarrow$ DCIP.

Results and Discussion

Figure 1 compares the light minus dark EPR difference spectra of PSII membranes illuminated at different temperatures: (A) untreated control illuminated at 195 K compared to calcium-depleted membranes, (B) illuminated at 195 K, (C) illuminated at 273 K while freezing to 195 K, and (D) illuminated as in (C) except with 250 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] added prior to illumination to restrict electron transfer to a single turnover at 273 K. Spectra A, and B show that photooxidation to produce the normal S_2-state multiline EPR signal observed in the control membranes at 195 K (A) does not occur in the calcium-depleted sample (B). Reconstitution with $CaCl_2$ restores the normal S_2 multiline EPR intensity to 70–80% (not shown). Light-induced charge separation does occur in the calcium-depleted samples at 195 K. This can be seen by the yield of the EPR signal for $g = 1.9$ for Q_A^-Fe following illumination as shown by the arrows in the insets to Figure 1A,B. The depleted samples, if carefully handled so as to avoid illumination, do not exhibit new features in the spectrum. This changes dramatically if the sample is illuminated at 273 K where multiple turnovers can occur, and then frozen immediately. This produces a new EPR signal centered at $g = 2.004$ with a symmetric line width of 163 ± 3 G. The inset to Figure 1C gives the difference spectrum between calcium-depleted and control PSII membranes also illuminated at 273 K (this helps to remove the sharp central signal arising from Y_D^+). This signal decays reversibly at 273 K in the dark in parallel with the formation of a "modified" multiline EPR signal with the kinetics shown in Figure 2. Neither the kinetics nor the

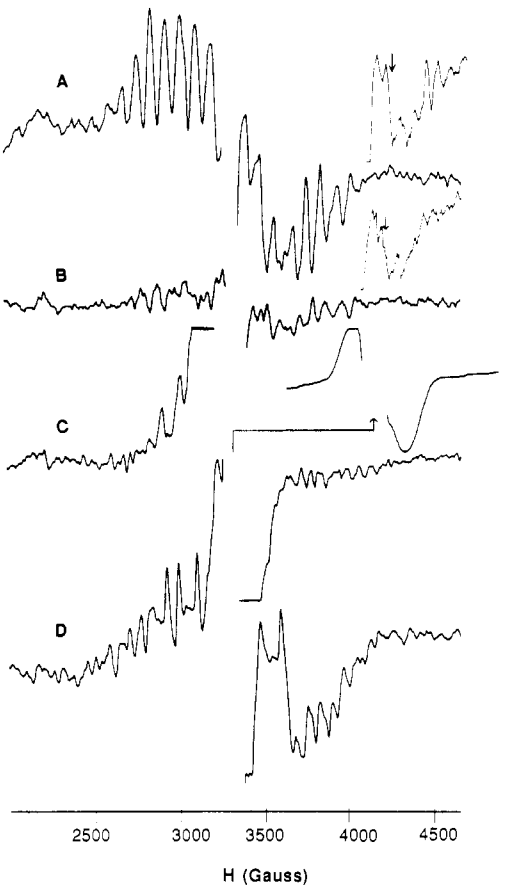


FIGURE 1: Light minus dark EPR difference spectra of untreated (A) and Ca^{2+} depleted (B–D) PSII membranes following illumination at 195 K (A, B) or illumination at 277 K for 20 s and frozen immediately (C, D). Samples A–C contained the exogenous electron acceptor DCBQ (1 mM) prior to illumination, whereas sample D contained the inhibitor DCMU (250 μM) prior to illumination. The insets to curves A and B show the $g = 1.9$ FeQ_A^- EPR signal formed in these samples. The inset to curve C shows the full extent of the 163 G wide $g = 2.0$ EPR signal; the background spectrum used for this difference spectra is the untreated PSII sample illuminated at 273 K for 20 s and frozen immediately. Chlorophyll concentration: 5 mg/mL. EPR conditions: temperature, 8 K; microwave power, 32 mW; 20-G modulation at 100 kHz. The insets for curves A and B were recorded at 4.2 K and a microwave power of 51 mW.

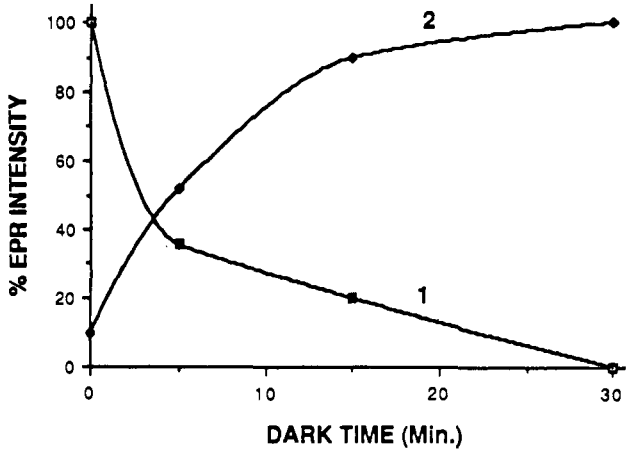


FIGURE 2: Time dependence for decay in the dark of the 163 G wide $g = 2.0$ signal (1) and formation of the modified multiline signal (2) following illumination at 273 K of Ca^{2+}-depleted PSII membranes. The samples contain DCBQ (1 mM). Conditions are the same as for Figure 1.

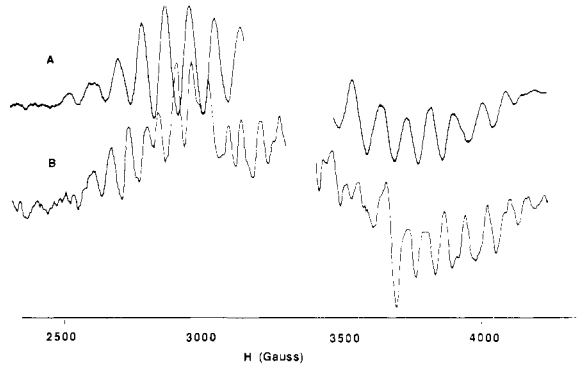


FIGURE 3: Light minus dark EPR difference spectra of (A) untreated PSII membranes following illumination at 195 K and warmed in the dark to 255 K for 2 min and of (B) Ca^{2+}-depleted membranes following illumination at 277 K for 20 s and subsequent dark adaptation for 15 min. Both samples contained DCBQ (1 mM). Conditions are the same as for Figure 1.

EPR spectrum reveals evidence for a long-lived intermediate. The spectrum of this modified multiline signal is compared directly to that of the normal S_2 multiline signal in Figure 3. It exhibits an asymmetrically structured hyperfine field centered at about the same g value, with a greater number of lines (26) and having reduced hyperfine splittings from ^{55}Mn which average about 60–65 G. This suggests an identification with the S_2 oxidation state. Qualitatively, there is similarity between the "modified" S_2 multiline signal observed in Ca^{2+}-depleted samples and that observed upon reconstitution of Ca^{2+}-depleted PSII samples with $SrCl_2$ (Boussac & Rutherford, 1988).

As shown in Figure 1D, if DCMU is present to limit turnover to one electron prior to 273 K illumination, the $g = 2.004$ signal forms in only a minority of centers (<15%), and instead, the modified multiline signal forms directly without the need for subsequent dark adaptation. This state ($S_2'Q_A^-$) is also unusually long lived in the dark like the $S_2'Q_A$ state. This shows that the dark state and the state producing the modified multiline differ by one electron. From this we assign these as S_1' and S_2', respectively. The state producing the symmetric signal at $g = 2.004$ must therefore be a higher state than S_2'; hence, it is either S_3' or S_4'. Both the S_1' and S_2' calcium-depleted states differ from the normal S_1 and S_2 states in that following calcium addition they both lose the oxidized tyrosine Y_D^+ EPR signal. This is shown in Figure 4A for the S_1 state (curve 1) [compare also spectrum A (control) vs

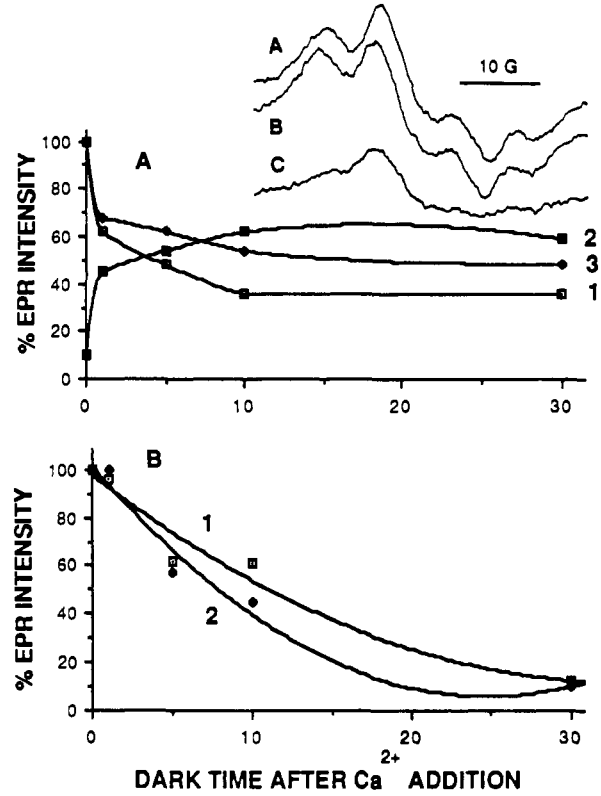
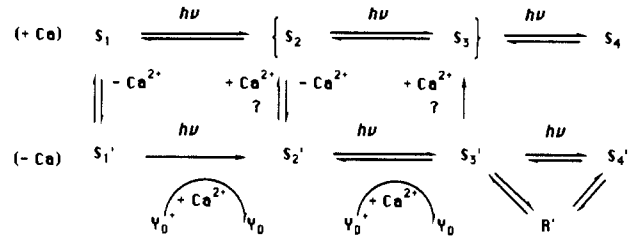


FIGURE 4: Effect of Ca^{2+} reconstitution to Ca^{2+}-depleted PSII membranes in the S_1' state which were not exposed to any light (panel A) and to Ca^{2+}-depleted PSII membranes that were illuminated at 277 K and then dark adapted for 15 min in order to generate the modified S_2' multiline signal (panel B). In all samples Ca^{2+} was mixed within 30 s and then allowed to incubate in the dark for various periods of time. (A1) Yield of Y_D^+; (A2) yield of the normal $S_1 \rightarrow S_2$ reaction at 195 K; (A3) yield of Y_D^+ following 195 K illumination. (B1) Yield of Y_D^+; (B2) yield of the modified S_2' multiline EPR signal. All the samples contained DCBQ. Conditions are the same as for Figure 1, except for signal II_S: microwave power, 20 μW; modulation amplitude, 0.5 G. The lines are quadratic fits to the data points. The inset shows EPR spectra of the oxidized tyrosine Y_D^+ in dark-adapted untreated PSII membranes (A), Ca^{2+}-depleted PSII membranes that were not exposed to any light (B), and Ca^{2+}-reconstituted PSII membranes that were not exposed to any light (C). Conditions are the same as for Figure 1, except for a microwave power of 20 μW and a 0.5-G modulation amplitude.

spectrum B (−Ca) and spectrum C (+Ca)]. These data show that 50% of the EPR signal intensity for Y_D^+ is lost following calcium reconstitution in the dark within 5 min when in S_1 vs 12 min when in S_2'. The initial yield of the Y_D^+ EPR signal is the same in calcium-depleted vs untreated PSII membranes, corresponding to 1 Y_D^+/PSII (Figure 4A, spectra A and B). The loss of Y_D^+ EPR intensity following calcium addition varied from 50 to 80% in the case of the S_1' state in different samples (Figure 4A, spectrum C). Calcium reconstitution in the dark S_1' state resulted in the formation of a normal S_1 state as seen by the recovery of the ability to photogenerate the normal S_2 multiline signal by the usual method of 195 K illumination (Figure 4A, curve 2). The normal S_2 state was not recovered in the dark. Illumination at 195 K also produced a 15–20% increase in the EPR signal for Y_D^+ (Figure 4A, curve 3) analogous to the control sample. Stably oxidized Y_D^+ is recovered in full if the sample is illuminated at 273 K.

In the case of the S_2' state (Figure 4B), the decay of Y_D^+ (Figure 4B, curve 1) upon calcium reconstitution proceeded directly in parallel with the loss of the modified S_2' multiline signal (Figure 4B, curve 2). There appeared to be no formation of the normal S_2-state signal in the dark, although a small extent of recovery would have been difficult to see in

Scheme I



the presence of the modified multiline signal. The fate of the S states is not yet clear in this case. The origin of the electron which reduces Y_D^+ in both the S_1' and S_2' states is undetermined. However, it appears to come directly from manganese. It does not come from either Q_A^- or Q_B^-, which are oxidized by the exogenous quinone DCBQ (2,5-dichlorobenzoquinone), nor from cytochrome *b*-559, as was confirmed by the absence of the well-known EPR signals for these cofactors.

The number of spins giving rise to the $g = 2.004$ signal was estimated by integration of the spectrum using two methods. First, digital integration of the difference spectrum relative to an illuminated control sample gave an area ratio compared to the control Y_D^+ EPR signal following room temperature illumination equal to 1 ± 0.5 spin/Y_D^+. Second, we calculated the area from the line width and amplitude by assuming a Gaussian line shape with area = (constant)(derivative amplitude)(line width)^2 as given in Poole (1983) and compared this to the area of the Y_D^+ signal. This method eliminates errors arising from overlap with the Y_D^+ signal. This gave a ratio of 2 ± 0.5 spins/Y_D^+. Its microwave power dependence (not shown) revealed no saturation up to at least 125 mW at 8 K, indicating strong spin relaxation and hence origination from a transition metal, or an organic radical in magnetic contact with a paramagnetic metal ion. The latter intepretation is favored considering the isotropic *g* value.

These results can be explained by the model given in Scheme I. We propose that extraction of calcium in the dark S_1 state forms an EPR-silent, modified S_1' state which has a lower reduction potential for manganese in the $S_2' \rightarrow S_1'$ reduction. Upon addition of calcium it is proposed that Y_D^+ oxidizes manganese, which we label as the $S_1' \rightarrow S_2'$ path in Scheme I (lower pathway). The S_2' state so formed is converted to a normal S_2 state in a slower nonredox step by binding calcium. S_2 eventually is reduced to S_1 by the normal S_2-state reactions, as seen by the recovery of the ability to regenerate the S_2 multiline signal upon 195 K illumination. After the sample is warmed to 273 K and illuminated, oxidized Y_D^+ is regenerated, and the donor side is fully restored to a normal state. In the model of Scheme I calcium has two functions. Initially, it overcomes a kinetic barrier by bringing Y_D^+ into redox equilibrium with the modified Mn cluster, resulting in Y_D^+ reduction and Mn oxidation. This contrasts with the native S_1 state which does not reduce Y_D^+ (Styring & Rutherford, 1987; Inui et al., 1989). Second, calcium rebinding restores the native structure of the Mn cluster which in turn raises the reduction potential for the S_2–S_1 reduction above that for Y_D^+/Y_D.

An analogous model is given in Scheme I to account for the behavior of the light-adapted, calcium-depleted S_2' state. Here, addition of calcium enables oxidation of S_2' by Y_D^+ forming S_3'. In a slower nonredox step, S_3' is proposed to form S_3 upon rebinding calcium. We have placed a question mark around the S_2 and S_3 states to emphasize that we have not found direct evidence proving their formation upon calcium addition. This may be because of a small steady-state population passing through the EPR-detectable S_2 state at any given time. The kinetic correlations observed in the reduction of Y_D^+ and loss of S_1' and S_2' may also be explained if these species decay by independent but coincidentally identical kinetics. We view this to be less plausible than Scheme I.

Prior studies have assigned the Mn oxidation states of the normal S_2 state to $Mn^{IV} + 3\ Mn^{III}$ ions (Dismukes et al., 1982). The same net oxidation state should be applicable for the S_2' state. The decrease in the average hyperfine splitting observed in S_2' might be due to an increase in ligand covalency or a change in the ground spin state of the Mn cluster (Dismukes et al., 1982).

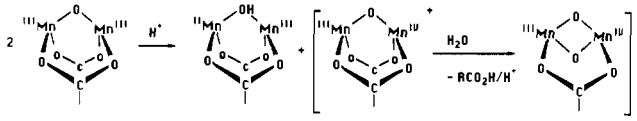
Illumination in the absence of DCMU permits at least an additional turnover, yielding a higher S state responsible for the 163 ± 3 G wide $g = 2.004$ signal. Since only the block imposed by calcium depletion restricts turnover in this case, we are unable to say if the state giving rise to this signal is an S_3' or S_4' state. It decays reversibly in the dark to form the modified S_2' multiline signal, with no evidence for kinetically resolved intermediates (Figure 2). An S_3' oxidation state assignment would agree with that reported by Boussac and Rutherford (1989), who found evidence to support this from the decay of the $g = 2.004$ signal to form a normal S_2 multiline signal in the dark upon addition of calcium. The identity of the paramagnetic species responsible for the symmetric $g = 2.004$ signal is not yet apparent. The most likely candidates include a radical derived from a nonphysiological donor or an oxidized amino acid within magnetic contact with the manganese cluster. This will have to await further studies.

Our results using the citric acid extraction method of Ono and Inoue for calcium depletion (1989) and those of Boussac and Rutherford (1989) using the NaCl/EDTA extraction method differ in one important respect. They do not see reduction of Y_D^+ upon calcium reconstitution with the latter method, using samples in which the dissociated extrinsic proteins of masses 17 and 23 kDa have been rebound. Y_D^+ reduction upon calcium reconstitution has also been observed by Lockett et al. (1989) using the NaCl/EDTA method, although they do not observe formation of the modified multiline signal or the $g = 2.004$ signal. This latter result can be understood because the dissociated extrinsic proteins have not been rebound in their samples. These proteins need to be rebound for observation of the modified multiline signal (Boussac & Rutherford, 1989). The discrepancy with respect to Y_D^+ decay cannot be explained by the absence of the extrinsic proteins in citric acid treated samples. We see little release of these proteins.

Unlike Ono and Inoue (1989a,b) we do not observe a block beyond the S_2' state. At present this is unexplained. However, we do note that in their EPR experiments DCMU is present which would preclude stable photooxidation beyond S_2' even if no block existed.

The attractiveness of Scheme I derives its principal support from its ability to account for the simultaneous reduction of Y_D^+ and decay of the S_1' and S_2' states upon calcium reconstitution. In order for this to occur, the standard reduction potentials of the calcium-depleted S_2' and S_3' states must be decreased below that for Y_D^+, which has been measured in the range +0.7 to 0.8 V vs NHE under denaturing conditions in the presence of calcium (Boussac & Etienne, 1984; Tso et al., 1987). A chemically reasonable explanation for how this could occur can be proposed on the basis of the known chemistry of manganese in solution. We propose that calcium extraction increases the accessibility of water to the Mn cluster in PSII, resulting in hydrolysis—the binding of additional

Scheme II



water ligands and their deprotonation to form hydroxo or oxo ligands. It is known that hydrolysis of free Mn^{III} and Mn^{IV} ions occurs spontaneously in aqueous solution to form more stable hydroxo and oxo species, as seen in the reduction potentials of the following reactions: $Mn(H_2O)_6^{2+} \rightarrow Mn(H_2O)_6^{3+} + e^-$, $E_0 = 1.5$ V, vs $Mn(H_2O)_6^{2+} \rightarrow {}^1/_2Mn_2O_3 + e^- + 3H^+$, $E_0 = 0.2$ V, and $Mn(H_2O)_6^{2+} \rightarrow Mn(H_2O)_6^{4+} + 2e^-$, $E_0 > 1.5$ V, vs $Mn(H_2O)_6^{2+} \rightarrow MnO_2 + 2e^- + 4H^+$, $E_0 = 0.6$ V (*CRC Handbook of Chemistry*, 1982–83).

Stabilization of Mn^{III} and Mn^{IV} by water is also the driving force responsible for the hydrolysis-induced disproportionation of dimanganese(III) complexes which have proven useful as partial models of the PSII Mn cluster. These reactions are summarized in Scheme II, L = hydrotris(pyrazolyl)borate (Sheats et al., 1986, 1987; Dismukes, 1989) or triazacyclononane (Wieghardt et al., 1988). The mechanism of disproportionation has been examined and involves protonation of the μ-oxo bridge to form the Mn^{III}–OH–Mn^{III} species. This is a stronger oxidant than the μ-oxo dimer and initiates a one-electron transfer reaction. The resulting $[Mn^{III}–O–Mn^{IV}]^+$ cation reacts with H_2O to displace the carboxylic acid and deprotonates to form a neutral bis(μ-oxo) (III,IV) species. This spontaneous reaction is driven by the greater stability of the pair of mixed-valence dimers, as seen by a decrease in the reduction potential for $Mn^{IV}Mn^{IV} \rightarrow Mn^{III}Mn^{IV}$ from 1.5 V for the mono(μ-oxo) species to 0.9 V for the bis(μ-oxo) species. Either the thermodynamically driven hydrolysis or disproportionation of $S_1(4Mn^{III})$ to form $S_1'(Mn^{II,III,III,IV})$ upon calcium extraction in PSII could possibly explain the abnormally stable S_2' state reported by Ono and Inoue (1989a,b), on the basis of an increase in the activation temperature for induction of thermoluminescence, and that observed here on the basis of the formation of a modified S_2' EPR signal with extended lifetime.

Conclusions

We have presented results suggesting that the role of calcium is to maintain a structure of the Mn cluster which is capable of activating bound water for O_2 formation. A consequence of this is the ability of calcium to limit the accessibility of water to the Mn cluster—a gatekeeper function. When too much water is exposed to the Mn cluster, a thermodynamically more stable S_2' oxidation state forms which cannot oxidize water. This role contrasts with a hypothesis by Coleman and Govindjee (1987) in which calcium serves to enhance the accessibility of water to a hydrophobic water oxidation site.

Calcium exhibits a second function. Its reconstitution appears to cause the modified S_1' and S_2' states to reduce tyrosine Y_D^+, a reaction which does not occur with the normal S_1 or S_2 states. The structural change produced by calcium depletion appears to break the redox communication between the Mn cluster and Y_D^+. This is analogous to the slowing observed in the rapid phase of reduction of the other oxidized tyrosine, Y_Z^+ (Ghanotakis et al., 1984; Cole & Sauer, 1987; Ono & Inoue, 1989). We would not have seen these rapid changes using the steady-state methods we employed here.

These results and those of others (Boussac & Rutherford, 1989; Ono & Inoue, 1988, 1989a,b; Lockett et al., 1989) need to be assessed in the light of other data showing that, of the two tightly bound calcium ions in PSII membranes from rice, the citrate- or EDTA-extractable one is absent in a rice mutant lacking the Chl-*b*/LHC subunits, yet no loss of O_2 evolution is observed (Shen et al., 1988; Shen & Katoh, 1989). It will be interesting to see if the one tightly bound calcium in the mutant correlates with the extractable calcium in the WT cells when comparisons other than dissociation constants are made. Studies similar to those reported here ought to be attempted on this interesting mutant.

Acknowledgments

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Oxidation of Glycated Proteins: Age-Dependent Accumulation of N^{ϵ}-(Carboxymethyl)lysine in Lens Proteins†

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ABSTRACT: N^{ϵ}-(Carboxymethyl)lysine (CML) has been identified as a product of oxidation of fructoselysine (FL) in glycated (nonenzymatically glycosylated) proteins in vitro and has also been detected in human tissues and urine [Ahmed et al. (1986) *J. Biol. Chem. 261*, 4889-4894]. In this study, we compare the amounts of CML and FL in normal human lens proteins, aged 0-79 years, using specific and sensitive assays based on selected ion monitoring gas chromatography-mass spectrometry. Our results indicate that the lens content of FL increases significantly between infancy and about age 5 but that there is only a slight, statistically insignificant increase in FL between age 5 and 80 (mean ± SD = 1.4 ± 0.4 mmol of FL/mol of Lys). In contrast, the lens content of the oxidation product, CML, increased linearly with age, ranging from trace levels at infancy up to 8 mmol of CML/mol of lysine at age 79. The ratio of CML to FL also increased linearly from 0.5 to 5 mol of CML/mol of FL between age 1 and 79, respectively. These results indicate that CML, rather than FL, is the major product of glycation detectable in adult human lens protein. The age-dependent accumulation of CML in lens protein indicates that products of both glycation and oxidation accumulate in the lens with age, while the constant rate of accumulation of CML in lens with age argues against an age-dependent decline in free radical defense mechanisms in this tissue.

Glycation (nonenzymatic glycosylation) is a common posttranslational modification of proteins in vivo, resulting from reaction between glucose and amino groups on protein (Baynes et al., 1989). The adduct formed by glycation of lysine residues in protein in termed fructoselysine (FL)[1] (Figure 1), and levels of FL in hemoglobin, plasma proteins, collagen, hair, lens, and numerous other proteins in the body are known to increase in proportion to the degree of hyperglycemia in diabetes (Kennedy & Baynes, 1984). We recently described two products of oxidation of FL, N^{ϵ}-(carboxymethyl)lysine (CML) (Figure 1) and 3-(N^{ϵ}-lysino)lactic acid, and showed that these compounds were also detectable in human lens protein, collagen, and urine (Baynes et al., 1986; Ahmed et al., 1986, 1988). The present study was undertaken to compare the amounts of FL and its major oxidation product, CML, in proteins in vivo. Human lens proteins were chosen for initial studies because these proteins are among the longest lived, most slowly turned over proteins in the body (Harding & Dilley, 1976; Zigler & Goosey, 1981) and thus have the longest time to accumulate glucose adducts and their oxidation products. Because of the hypothesized role of both glycation (Cerami, 1985; Monnier, 1989) and oxidation (Cutler, 1984; Mehlhorn & Cole, 1985; Harman, 1987) in the aging of proteins in vivo, we also explored the relationship between age and the absolute and relative amounts of CML and FL in lens proteins. The results of these studies indicate that CML, rather than FL, is the major product of glycation present in adult lens proteins and also provide insight into the role of glycation and oxidation in the aging of lens proteins in vivo.

Experimental Procedures

Materials. Unless otherwise indicated, reagents were of the highest quality obtainable from Sigma or Aldrich Chemical Co. CML was prepared from reaction of glyoxalic acid with N^{α}-acetyllysine in the presence of sodium cyanoborohydride, followed by ion-exchange purification as described previously (Ahmed et al., 1986, 1988). N^{α}-Formyl-N^{ϵ}-fructoselysine (fFL) was synthesized from N^{α}-formyllysine and glucose, as described by Finot and Mauron (1969). The reaction mixture was concentrated by rotary evaporation, reconstituted in 0.2 M ammonium acetate, pH 9, and applied to a column of phenylboronic acid affinity resin (Amicon Matrex-Gel PBA-60). The fFL was eluted with 0.3 M acetic acid, concentrated by rotary evaporation, reconstituted in deionized water, and stored frozen at −70 °C.

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[1] Abbreviations: CML, N^{ϵ}-(carboxymethyl)lysine; GC/MS, gas chromatography-mass spectrometry; fFL, N^{α}-formyl-N^{ϵ}-fructoselysine; FL, fructoselysine; SIM, selected ion monitoring; TFAME, trifluoroacetyl methyl ester.

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