In Support of the Trap Hypothesis. Chymotrypsin Is Not Rigidly Held in Its Complex with Human α_2-Macroglobulin†

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ABSTRACT: Complexes (2:1) of chymotrypsin with human α_2-macroglobulin have been prepared in the presence of 200 mM methylamine such that 90% of the chymotrypsin remains noncovalently bound to the α_2-macroglobulin. Reaction of this complex with the active-site-directed spin-labeling reagent 4-[(ethoxyfluorophosphinyl)oxy]-2,2,6,6-tetramethylpiperidinyl-1-oxy results in nitroxide labeling of the active-site serine residue of the complexed chymotrypsin. Electron spin resonance (ESR) spectra of this complex were recorded at 275 K in buffer and at 263 K in 50% glycerol. At 263 K in 50% glycerol the spectrum is that expected for a rigid glass, whereas at room temperature the ESR spectrum shows that the chymotrypsin is only slightly immobilized compared with free spin-labeled chymotrypsin. These findings are discussed in relation to possible models of inhibition of protease activity by α_2-macroglobulin. It is concluded that the trap mechanism of Barrett and Starkey [Barrett, A. J., & Starkey, P. M. (1973) *Biochem. J. 133*, 709–724] is the only model currently considered that can account for the present findings.

The abundant plasma protease inhibitor α_2M[1] reacts with proteases in a unique way such that the proteases retain proteolytic activity against low molecular weight substrates (Haverback et al., 1962; Karic & Glaser, 1981). High molecular weight substrates, however, are no longer susceptible to attack by proteases complexed to α_2M (Bieth et al., 1970; Rinderknecht et al., 1970; Harpel & Mosseson, 1973; Barrett & Starkey, 1973), nor are the proteases bound by high molecular weight inhibitors or antibodies (Barrett & Starkey, 1973; Harpel, 1976). Largely on the basis of these findings Barrett and Starkey (1973) proposed a mechanism of action for α_2M in which the protease becomes physically sequestered by the inhibitor without any direct blocking of the enzyme active site. This is referred to as the trap hypothesis. Feldman and co-workers (1985) have presented a model for α_2M that is based on this trapping mechanism of action and incorporates data from hydrodynamic (Gonias et al., 1982), small angle X-ray scattering (Österberg & Pap, 1983), and electron microscopic studies (Schramm & Schramm, 1982). In this model two subunits of the tetramer form the walls of a hollow trap into which the protease can diffuse. Subsequent to cleavage of α_2M at a site within a highly susceptible region of the polypeptide termed the bait region, the inhibitor undergoes a conformational change that prevents exit of the protease. Although covalent cross-linking between glutamic acid residue 952 of α_2M and lysine residues on the protease is known also to occur, to an extent dependent on the particular protease (Salvesen & Barrett, 1980; Wu et al., 1981), it does not seem to be a prerequisite for the nondissociability of the α_2M–protease complex (Salvesen et al., 1981; van Leuven et al., 1981).

The trap hypothesis is indeed a very attractive mechanism by which to explain many experimental findings on the accessibility of complexed proteases to substrates and inhibitors [see Travis and Salvesen (1983) for a review]. However, while there are many data *consistent* with an internalization of proteases by α_2M (vide supra), there has been no definitive demonstration that such a complete ingestion *must* occur. In this paper ESR studies of spin-labeled chymotrypsin complexed with human α_2M are reported that show that chymotrypsin, noncovalently bound to α_2M, is free to rotate relative to the α_2M molecule. This finding is only explicable by a cage-like trapping of the protease by α_2M.

Materials and Methods

Purification of α_2M. Human α_2M was purified from human plasma by zinc chelate chromatography and gel chromatography, as described previously (Dangott & Cunningham, 1982). The purity of each preparation was checked by electrophoresis under nondenaturing conditions and also in the presence of SDS. Nondenaturing polyacrylamide gel electrophoresis was performed in 5% acrylamide slabs (Davis, 1964). SDS–polyacrylamide gel electrophoresis was carried out in 7.5% slab gels according to the procedure of Laemmli (1970). α_2M concentrations were determined spectrophotometrically by using $E^{1\%}_{280} = 8.9$ (Hall & Roberts, 1978; Barrett et al., 1979) and a molecular weight of 716 000 based on the primary structure (Kristensen et al., 1984).

Iodination of Chymotrypsin. Iodinated chymotrypsin was prepared as described by Sottrup-Jensen et al. (1981) modified as in the procedure of Carpenter and Cohen (1976). Chymotrypsin, 300 μM, in 50 mM phosphate buffer at pH 7.5 was reacted with $Na^{125}I$ at a slight molar excess of iodide in the presence of a 5-fold molar excess of chloramine T (Hunter & Greenwood, 1962). The reaction was allowed to proceed for 30 s at room temperature and then stopped with the addition of an excess of sodium metabisulfite, and the pH was

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[1] Abbreviations: α_2M, α_2-macroglobulin; BTEE, N^{α}-benzoyl-L-tyrosine ethyl ester; DOXYL, 2,2-dimethyloxazolidinyl-1-oxy; PROXYL, 2,2-dimethylpyrrolidinyl-1-oxy; SDS, sodium dodecyl sulfate; SL, derivative spin-labeled with 4-[(ethoxyfluorophosphinyl)oxy]-TEMPO; TEMPO, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

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adjusted to 3.9 with acetic acid. Unreacted iodide was separated from the protein on a G25 column. The mole ratio of iodine incorporated per mole of chymotrypsin was 0.20.

Preparation of α_2M–Chymotrypsin Complex. Optimal conditions were sought for the formation of the α_2M–chymotrypsin complex in which little or no covalent cross-linking between glutamic acid-952 of α_2M and lysine residues of chymotrypsin took place. It is known that methylamine will compete with proteases for covalent attachment to α_2M (Sottrup-Jensen et al., 1981). By using ^{125}I-labeled chymotrypsin, it was possible to determine the degree of covalent and noncovalent trapping of chymotrypsin under different reaction conditions. For the concentration of α_2M used, 12 μM, it was found that methylamine at 200 mM during reaction with chymotrypsin resulted in only 10% covalent cross-linking between the protease and α_2M. The relative proportions of covalently and noncovalently bound chymotrypsin were determined from polyacrylamide gels run under native and reduced conditions. From the former it was possible to determine the proportion of ^{125}I-labeled chymotrypsin associated with α_2M. Using this value, it was then possible to calculate the fraction of covalently bound versus noncovalently bound chymotrypsin. Band intensities were determined both by densitometry from an autoradiograph of the gels and also by counting the excised bands in a γ counter. The two methods gave comparable results. Thus in the presence of 200 mM methylamine percentages of covalent cross-linking of 10.3 and 9.8, respectively, were determined for the same sample. In the absence of methylamine 32% covalent cross-linking occurred, whereas in the presence of 100 mM methylamine 13% covalent binding resulted.

For the preparation of the α_2M–chymotrypsin complex for subsequent spin-labeling, the same optimal reaction conditions as described above were used except that noniodinated chymotrypsin was used. The calculation of the stoichiometry of complexed chymotrypsin is based upon the rate of hydrolysis of a low molecular weight substrate. Since iodinated chymotrypsin and normal chymotrypsin have different reactivities toward this substrate and may also react differentially with α_2M, measurement of the activity of a mixture of iodinated and noniodinated complexed chymotrypsins would not give a unique value for the total amount of chymotrypsin trapped.

α_2M, 12 μM, in 0.1 M sodium phosphate buffer at pH 8.0 was reacted with 4 equiv of chymotrypsin in the presence of 200 mM methylamine at room temperature for 10 min. An excess of soybean trypsin inhibitor was added to complex any unreacted chymotrypsin. Excess methylamine was then removed by dialysis against four 1-L portions of 0.1 M sodium phosphate buffer at pH 8.0. The extent of reaction of chymotrypsin with α_2M was determined from chymotrypsin activity toward the low molecular weight substrate BTEE. In this way the α_2M–chymotrypsin complex containing 1.96 chymotrypsin molecules per α_2M tetramer was prepared.

Preparation of Spin-Labeled Species. (A) α_2M–SL-Chymotrypsin. Spin-labeling was performed by a modification of the procedure of Morrisett and Broomfield (1972), using pH 8.0 instead of pH 5.5 and a higher molar excess of reagent. The α_2M–chymotrypsin complex, prepared as described above, was added to a tube containing a 20-fold molar excess of the spin-labeling reagent 4-[(ethoxyfluorophosphinyl)oxy]-TEMPO (Aldrich), previously dried onto the walls of the tube from a solution in benzene. Aliquots were removed at various time points and assayed for chymotrypsin activity with the substrate BTEE; 90% inhibition was achieved after 40 min of reaction at 4 °C. The reaction was terminated after 5 h and the sample dialyzed against 0.1 M sodium phosphate buffer at pH 7.8 with three subsequent changes of dialysate.

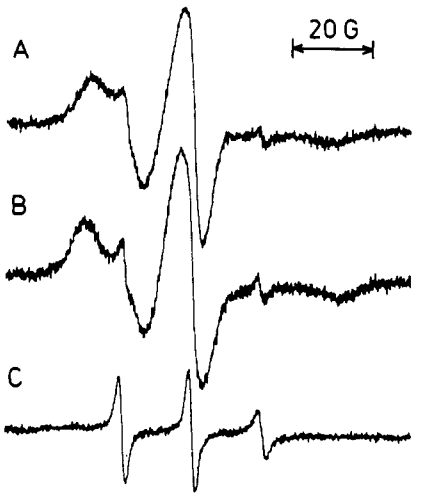

FIGURE 1: 9.4-GHz nitroxide ESR spectra recorded at 275 K in 0.1 M phosphate buffer at pH 7.8. Spectra are not normalized. The same gain was used in each case. (A) SL-chymotrypsin, 22.8 μM, six scans; (B) α_2M–SL-chymotrypsin complex, 12.2 μM α_2M (23.9 μM SL-chymotrypsin), six scans; (C) α_2M treated with spin-labeling reagent, 9.8 μM, eight scans at one-fourth the field sweep rate for the same digitization rate used for (A) and (B).

(B) SL-Chymotrypsin. This species was prepared analogously by reaction of chymotrypsin in 0.1 M phosphate buffer at pH 8.0 with a 20-fold excess of spin-labeling reagent dried on the walls of the reaction tube. The enzyme activity assay indicated complete reaction with the active site after 40 min. Excess reagent was removed by dialysis (4 × 1 L) against 0.1 M phosphate buffer at pH 7.8.

(C) SL-α_2M. A control for nonspecific reaction of the active-site-directed spin-labeling reagent with α_2M was also prepared in analogous fashion to the α_2M–SL-chymotrypsin complex. The reaction was allowed to proceed for 5 h prior to dialysis.

ESR Spectra. Spectra were recorded on a Varian E-line ESR spectrometer operating at 9.41 GHz. The parameters employed were a field set of 3350 G, sweep range of 100 G, modulation frequency of 100 kHz, modulation amplitude of 1 G, time constant of 0.032 s, and microwave power of 10 mW. Samples of approximately 0.5 mL were contained in a quartz flat cell (Wilmad, WG-813). The temperature of the E-238 cavity was regulated to within ±0.5 °C by an E-257 VT unit by passing precooled air or nitrogen into the cavity through the optical port in the front. Signal averaging by an online PDP 11/73 microcomputer was necessary for most spectra. The numbers of scans is indicated in the figure captions.

RESULTS

The reaction of serine proteases with dialkyl fluorophosphate type spin-labeling reagents results in the highly specific covalent attachment of the spin-label to the active-site serine residue (Morrisett et al., 1969; Hsia et al., 1969). The ESR spectrum of chymotrypsin labeled at the active site with 4-[(ethoxyfluorophosphinyl)oxy]-TEMPO is shown in Figure 1A. The spectrum is a superpositioning of signals from two species with very different mobilities, as judged by the separation between high- and low-field peaks and by the line shapes. From a double integration of the first derivative spectrum it was estimated that the more mobile component constitutes only 0.7% of the total spin-labeled species present. This mobile component is nondialyzable and arises either from nonspecific labeling at another site or else from active-site-

Table I: Splitting Constants for Spin-Labeled Chymotrypsin Species

			ΔS (G)[a]	
	T (K)	splitting (G)	low field	high field
SL-chymotrypsin	275	61.3	2.9	5.8
α_2M–SL-chymotrypsin	275	63.5	2.1	4.6
SL-chymotrypsin	263	70.0		
α_2M–SL-chymotrypsin	263	70.0		

[a] ΔS is the shift in the high- or low-field line position relative to the rigid limit value.

labeled chymotrypsin that has been autolyzed. Such autolysis, resulting in a two-component ESR spectrum, has been seen for trypsin (Morrisett & Broomfield, 1972) and explained by the work of Berliner and Wong (1973).

The major species gives a much broader spectrum similar to previously published spectra of trypsin labeled with the same spin-label reagent (Berliner & Wong, 1973) and of chymotrypsin labeled with 3-[(4-nitrophenoxy)carbonyl]-PROXYL- or 3-DOXYL-17β-[(methylfluorophosphinyl)oxy]-5α-androstane (Berliner & McConnell, 1971; Shimshick & McConnell, 1972). In the last two examples of labeled chymotrypsin the spin probe is rigidly held in the enzyme's active site. This permitted Shimshick and McConnell (1972) to determine the rotational correlation time of the enzyme from the variation in the high-field line position as a function of solvent viscosity. The spectrum reported here is consistent with that of nearly complete immobilization of the spin-label in the active site of the enzyme.

The spectrum of spin-labeled chymotrypsin complexed with α_2M is shown in Figure 1B. As with free chymotrypsin, there are two species present, a minor mobile component (0.9%) and a major more immobile component. The control sample of α_2M reacted with spin-labeling reagent shows a very small degree of covalent reaction with a resulting bound nitroxide that has mobility comparable to the mobile component in the chymotrypsin–α_2M complex (Figure 1C). Complexation of chymotrypsin with α_2M results in an increase in separation of the high- and low-field peaks from 61.3 to 63.5 G (Table I).

To determine the appearance of the nitroxide spectrum under rigid-limit conditions, both SL-chymotrypsin and α_2M–SL-chymotrypsin were examined in 50% glycerol at 263 K. These spectra are shown in Figure 2. They are identical in both peak position and line shape and have a separation between high- and low-field peaks (70.0 G) that is considerably greater than for the α_2M–SL-chymotrypsin complex in buffer at 275 K (Table I).

Two other control experiments were performed. In the first experiment chymotrypsin spin-labeled at the active site with 4-[(ethoxyfluorophosphinyl)oxy]-TEMPO was precipitated with 95% saturated ammonium sulfate. The ESR spectrum of the precipitated protein gave a separation between high- and low-field lines of 70.5 G at 275 K (spectrum not shown). After the protein was redissolved to give an ammonium sulfate concentration of approximately 50% saturated, the splitting returned to 63 G at 275 K. In the second experiment an attempt was made to obtain a nitroxide label rigidly attached to α_2M to demonstrate that the motion of α_2M itself results in a rigid glass spectrum. This was achieved by reacting α_2M with chymotrypsin and labeling the cysteine SH groups, newly formed by thiol ester cleavage, with 4-(2-acetamido)-TEMPO. The nitroxide ESR spectrum at 275 K gave a high-field–low-field line separation of 70.5 G.[2]

[2] L. W. Cunningham, A. H. Beth, and P. Gettins, unpublished results.

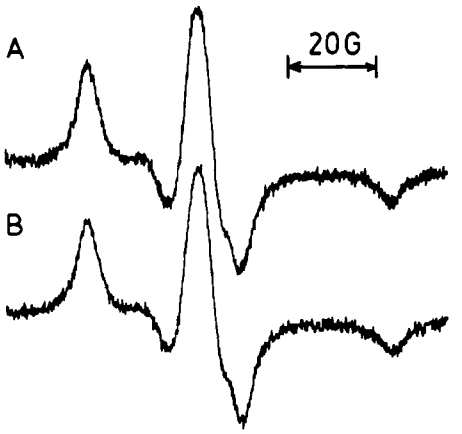

FIGURE 2: Rigid-limit nitroxide ESR spectra obtained in 50% w/w glycerol at 263 K. Spectra are not normalized. The same gain was used in each case. (A) SL-chymotrypsin, 10.4 μM, eight scans; (B) α_2M–SL-chymotrypsin complex, 5.6 μM α_2M, eight scans.

Discussion

The line shape and peak positions of the ESR spectrum of chymotrypsin labeled at the active site with 4-[(ethoxyfluorophosphinyl)oxy]-TEMPO are as expected for a spin-label held but not totally immobilized by the enzyme. According to the analysis of McCalley et al. (1972), which relates the shift in high- and low-field peak positions relative to those in the rigid glass spectrum to estimate correlation time, a correlation time of 9 ns was determined for chymotrypsin. This is less than the value of 15 ns expected for a protein of this size, estimated from the Stokes–Einstein relationship, and indicates some motion of the label within the active site. Precipitation of the sample with ammonium sulfate does, however, give the rigid glass spectrum, with a high-field–low-field line separation of 70.5 G, demonstrating that immobilization of the chymotrypsin is indeed reflected by the label in the active site, despite the latter's own limited motion.

For the complex of chymotrypsin with human α_2M, spin-labeled at the enzyme's active site, the ESR spectrum shows evidence for reduced motional freedom of the spin-label and consequently of the chymotrypsin molecule for which it acts as a reporter group. By the same analysis as above (McCalley et al., 1972), a change in effective correlation time of chymotrypsin from 9 to 13 ns is required to account for the observed spectrum. However, it is known from triplet fluorescence depolarization studies (Pochon et al., 1978) that human α_2M has a rotational correlation time of 720 ns, a value that is supported by small-angle X-ray scattering data that give a radius of gyration of 7.2 nm (Österberg & Malmensten, 1984), from which a minimum rotational correlation time of 400 ns can be calculated from the Stokes–Einstein relationship. ^{1}H NMR data on α_2M also indicate that all but bait region residues must have a correlation time in excess of 100 ns (Gettins & Cunningham, 1986). This expectation is substantiated by the ESR spectrum of the complex of α_2M with chymotrypsin in which a TEMPO-iodoacetate label was introduced on α_2M via the SH group of the thiol ester. The splitting of high- and low-field lines of 70.5 G confirms that α_2M has a correlation time of several hundred nanoseconds.

If the spin-labeled chymotrypsin molecule were rigidly held by α_2M and had a correlation time of 720 ns, its ESR spectrum should resemble the rigid-limit spectrum (Figure 2), which it clearly does not. A possible explanation for this might be as a result of a conformational change of the active site of chymotrypsin upon interaction with α_2M, producing a local "wobble". This is considered unlikely for two reasons. First,

complete immobilization is seen in 50% w/w glycerol at 263 K, and second, chymotrypsin retains normal activity toward low molecular weight substrates, thus arguing against a conformational change at the active site in the complex with α_2M. It can therefore be concluded that the spin-label and thus chymotrypsin have considerable rotational freedom relative to α_2M in the α_2M–protease complex.

This conclusion has important implications for the structure of the α_2M–chymotrypsin complex. There are three basic models for complex formation between proteases and α_2M: (1) covalent binding between the two species; (2) high-affinity noncovalent binding; (3) internalization through a conformational change of α_2M, i.e., the trap hypothesis of Barrett and Starkey (1973). Model 1 has been shown previously to be an insufficient explanation for the stability of α_2M complexes, since there is no correlation between the percentage of covalent binding and the nondissociability of the complexed protease (Salvesen et al., 1981; van Leuven et al., 1981). In the present study, moreover, conditions were chosen to minimize covalent binding of protease, with the result that 90% was noncovalently bound. For the noncovalent binding of model 2 to be the basis for complex formation, there needs to be a high binding constant to account for the nondissociability of the resultant complex. This could reasonably only be achieved by multiple contacts between protease and α_2M. For a single domain globular protein such as chymotrypsin this would effectively anchor the two molecules together and result in a correlation time for chymotrypsin that is the same as for the whole α_2M molecule, unless the binding region on the latter were a very flexible structure. Previous NMR studies have shown the bait region of α_2M to be the only highly flexible region of α_2M (Gettins & Cunningham, 1986; Arakawa et al., 1986). Upon reaction with chymotrypsin this region becomes even more mobile, to such an extent that the tyrosine at the site of proteolytic cleavage gives ^{1}H resonances that clearly exhibit spin–spin coupling (Gettins & Cunningham, 1986). It is thus not possible that this is a site for high-affinity noncovalent binding of chymotrypsin, since much broader ^{1}H resonances would be seen from the bait region residues. It is also difficult to imagine the existence of a series of such high-affinity sites, one for each of the very wide range of proteases that react with α_2M. In contrast, model 3 requires no covalent binding, nor does it necessitate specific high-affinity binding sites for each reactive protease. Within the general framework of the model one can envisage various degrees of internalization through conformational change that range from a vice-like holding of the protease, with perhaps much of the protease still protruding into solution, to a complete internalization. The present ESR findings are only consistent with model 3 generally and specifically with a conformational change that completely ingests the protease into a cavity significantly larger than the dimensions of the protease. Only in this way can the protease retain much of its rotational freedom and yet be noncovalently and effectively irreversibly associated with α_2M. The conformational change that α_2M undergoes upon reaction with protease is thus more like the closing of the door of a cage than the clamping of the jaws of a trap.

Chymotrypsin approximates to a prolate ellipsoid with axes of 20 and 15 Å (Sigler et al., 1968). Minimum dimensions for the cage in α_2M to permit rotation of chymotrypsin must thus be greater than 40 Å. Such a cavity, with radius of approximately 30 Å, can be accommodated by the hypothetical model of Feldman et al. (1985), which is thus strengthened and refined by the present findings.

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Structure of the Manganese Complex of Photosystem II upon Removal of the 33-Kilodalton Extrinsic Protein: An X-ray Absorption Spectroscopy Study†

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ABSTRACT: The structure of the Mn complex of photosystem II (PSII) was studied by X-ray absorption spectroscopy. Oxygen-evolving spinach PSII membranes containing 4–5 Mn/PSII were treated with 0.8 M $CaCl_2$ to extract the 33-, 24-, and 16-kilodalton (kDa) extrinsic membrane proteins. Mn was not released by this treatment, but subsequent incubation at low Cl^- concentration generated preparations containing 2 Mn/PSII. The Mn X-ray absorption K-edge spectrum of the $CaCl_2$-washed preparation containing 4 Mn/PSII is very similar to the spectrum of native PSII, indicating that the oxidation states and ligand symmetry of the Mn complex in these preparations are not significantly different. The Mn extended X-ray absorption fine structure (EXAFS) of $CaCl_2$-washed PSII fits to a Mn neighbor at ~2.75 Å and two shells of N or O at ~1.78 and ~1.92 Å. These distances are similar to those we have previously reported for native PSII preparations [Yachandra, V. K., Guiles, R. D., McDermott, A. E., Cole, J. L., Britt, R. D., Dexheimer, S. L., Sauer, K., & Klein, M. P. (1987) *Biochemistry* (following paper in this issue)] and are indicative of an oxo-bridged Mn complex. Our results demonstrate that the structure of the Mn complex is largely unaffected by removal of 33-, 24-, and 16-kDa extrinsic proteins, and thus these proteins do not provide ligands to Mn. The Mn K-edge spectrum of the $CaCl_2$-washed sample containing 2 Mn/PSII has a dramatically altered shape, and the edge inflection point is shifted to lower energy. The position of the edge is consistent with a Mn oxidation state of +3. The Mn EXAFS of this preparation is also quite different and cannot be simulated by using the parameters for the native Mn complex. Thus the structure of the Mn complex is disrupted upon depletion of half of the Mn.

Photosystem II (PSII)[1] catalyzes the light-driven oxidation of water to O_2 and the reduction of plastoquinone to plastoquinol. To accomplish the four-electron oxidation of water with one-electron photochemistry, PSII is thought to cycle through five successive intermediate states, S_i (i = 0, ..., 4), the S_4 state spontaneously decaying to produce S_0 and O_2 (Kok et al., 1970). A Mn-containing complex participates in the storage of oxidative equivalents and is thought to be the catalytic site for water oxidation [for reviews see Amesz (1983), Dismukes (1986), and Babcock (1987)]. The structure and oxidation states of this Mn complex in the various S states have been the subject of intense interest; EPR and X-ray absorption spectroscopies have proven to be particularly fruitful approaches.

PSII exists within the membrane as a complex of several hydrophobic intrinsic proteins and several hydrophilic extrinsic proteins. Despite considerable progress in our understanding of the structure of PSII, a clear picture of the Mn binding site has not yet emerged. It is generally assumed that the Mn complex is directly ligated to one or more PSII proteins. Although it is known from EXAFS studies that Mn is bound to terminal N, O ligands (Kirby et al., 1981a; Yachandra et al., 1987), the nature of the terminal ligands and the identity of proteins that constitute the binding site are not known. There are several papers claiming the isolation of a Mn-containing protein involved in O_2 evolution (Okada & Asada, 1983; Abramowicz & Dismukes, 1984). Biochemical studies of a non-O_2-evolving mutant of *Scenedesmus* suggest that the D1 protein, a 34-kilodalton (kDa) intrinsic protein associated with the reducing side of PSII, plays some role in Mn binding (Metz & Bishop, 1980; Metz et al., 1986).

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[1] Abbreviations: Chl, chlorophyll; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; FT, Fourier transform; MES, 4-morpholineethanesulfonic acid; 2 Mn/PSII, $CaCl_2$-washed PSII sample containing two Mn per reaction center; 4 Mn/PSII, $CaCl_2$-washed PSII sample containing four Mn per reaction center; PSII, photosystem II; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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