The 33 kDa Protein of Photosystem II Is a Low-Affinity Calcium- and Lanthanide-Binding Protein†

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ABSTRACT: We have shown that the isolated 33 kDa protein of photosystem II contains one calcium and one lanthanide low-affinity binding site with binding constants (K_D) on the order of 10^{-5} M. Binding of calcium or lanthanides to this site induces conformational changes in the protein that manifest in fluorescence emission spectra of the protein, circular dichroism spectra, and calorimetric thermograms where the phase transitions are shifted to lower temperatures. The role of calcium binding to the 33 kDa protein in the attainment of its native structure and the significance of this interaction for the oxygen evolution process are discussed.

The 33 kDa protein of photosystem II (PSII)[1] recently has been studied intensively because of its important function in the process of oxygen evolution, where it plays the role of a manganese-stabilizing protein (MSP) (*1*−*3*). It can be removed from PSII by alkaline Tris treatment which leads to partial manganese release and total loss of oxygen evolution activity by PSII, but in the presence of Ca^{2+} ions and high NaCl concentrations, the oxygen evolution activity is partially preserved, indicating that the 33 kDa protein is not directly engaged in Mn binding or coordination (*1*). The real molecular mass of the spinach protein deduced from the amino acid sequence is ∼26.7 kDa (*4*). The spinach protein consists of 248 amino acids with eight Tyr residues and one Trp_{241} close to the C-terminus and shows the presence of one disulfide bridge between Cys_{28} and Cys_{51} (*4*, *5*). The protein exhibits unusual fluorescence emission dominated by tyrosine when excited at ≤285 nm (*6*−*8*). To date, the protein has been resistant to crystallization probably because of its flexible structure (*9*). The predicted structure of the 33 kDa protein, based on CD spectra, indicates the presence of a low α-helix content (3−4%) and high contents of *β*-sheet (40−47%) and unordered structure (50−56%) (*10*, *11*). On the basis of thermostability, acidic isoelectric point, anomalous electrophoretic mobility, and the high content of secondary structure elements, it was concluded that the 33 kDa protein is "natively unfolded" (*11*). However, recent analysis of structural properties of this protein showed that it has a rather compact conformation with a well-developed secondary structure; i.e., it is closer to a "molten globule" than to an unfolded state (*9*). The role of the 33 kDa protein in calcium binding is controversial, although it is not supposed to be involved in the formation of the high-affinity Ca^{2+}-binding site in PSII (*1*, *12*). Usually, two Ca atoms per PSII are detected in the isolated PSII preparations, among which one is close to the Mn cluster, is bound with high affinity, and is directly involved in water oxidation (*13*). The calcium atom at this site can be competitively substituted with several metals, but only strontium substitution supports oxygen evolution (*13*). The localization of the other, probably low-affinity Ca^{2+}-binding site(s) in PSII remains uncertain. The role of the 33 kDa protein was suggested in this respect (*1*, *12*, *14*−*18*), as well as of 17 and 23 kDa extrinsic proteins (*13*, *14*), but direct evidence is still lacking. The recent findings of Ca^{2+}-stimulated protein dimerization (*19*) and conformational change observed by FTIR (*20*) at high protein concentrations may indicate some specific interactions between calcium and the 33 kDa protein.

It is known that lanthanides, such as europium ($_{63}$Eu), dysprosium ($_{66}$Dy), terbium ($_{65}$Tb), and lanthanum ($_{57}$La), are metals that are useful as probes in measurements of Ca^{2+} binding by proteins (*14*, *21*−*23*). Having very similar ion radii and similar coordination numbers, the lanthanides substitute Ca^{2+} in proteins even though they exhibit different charges. Moreover, lanthanides exhibit higher affinities for proteins than calcium (*21*).

In the studies presented here, we used steady-state protein fluorescence, circular dichroism (CD) spectroscopy, and differential scanning calorimetry (DSC), together with metal content analysis and the ligand binding method, to examine lanthanides and calcium binding properties of the isolated 33 kDa protein and the influence of this binding on the conformation of the protein.

MATERIALS AND METHODS

The 33 kDa protein was isolated from spinach by the method of Yamamoto and Kubota (*24*) that uses alkaline Tris treatment of PSII particles instead of 1 M $CaCl_2$

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[1] Abbreviations: PSII, photosystem II; CD, circular dichroism; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy.

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treatment. This avoids isolation of the calcium-loaded 33 kDa protein. Because absorption and fluorescence spectra of the protein purified using this procedure indicated some impurities, it was additionally purified. The extract of the 33 kDa protein fraction after Tris treatment (0.8 M, pH 8.4) of PSII membranes was saturated with ammonium sulfate to 75% saturation, and the precipitated protein was centrifuged. After the protein had been dissolved in a small volume of 10 mM NaCl (pH 6.5), it was further purified by repeated fractionation with ammonium sulfate between 30−75% (I fractionation) and 45−75% (II fractionation) saturation. After the finally precipitated protein had been dissolved in 10 mM NaCl (pH 6.5), the protein solution was dialyzed overnight against 10 mM NaCl (pH 6.5) and centrifuged at 100000*g* for 30 min to remove solid impurities. Protein concentrations were estimated by the Lowry method, by ultraviolet absorption spectroscopy using a millimolar extinction coefficient of 16 at 275 nm, or directly by weighing a lyophilized aliquot of the protein solution. The protein purity was examined by SDS−PAGE and absorption and fluorescence spectroscopy.

Fluorescence spectra were recorded with a Perkin-Elmer LS 50B fluorometer using 5 nm excitation and emission slits in a 5 mm × 5 mm cuvette.

Differential scanning calorimetry studies were performed using a CSC model 6100 Nano II calorimeter (Calorimetry Sciences Corp.) with cylindrical cells. The calorimeter was equipped with the original data acquisition and analysis software. To avoid bubble formation during heating modes, the samples were degassed prior to being loaded for a period of 10−15 min. Then the sample cell was filled with ∼400 μL of protein solution, and an equal volume of buffer was used as a reference. The cells were sealed and thermally equilibrated for ∼20 min at 20 °C. The data were collected in the range of 20−120 °C at a heating scan rate of 1 °C/min and under 3 bar of pressure. Scans of the buffer as a sample and a reference were also performed to collect the apparatus baseline. The obtained baseline was then subtracted from the protein thermograms. The specific volume used to transform the calorimetric data into temperature-dependent molar heat capacity data was 0.73 mL/g.

Circular dichroism (CD) spectra were recorded on a Jasco J-710 spectropolarimeter at room temperature. The data were collected at a bandwidth of 1.0 nm, a data interval of 0.05 nm, a scan speed of 10 nm/min, and a time constant of 4 s, and five scans were automatically averaged. The CD spectra were analyzed using CONTIN/LL and CDSSTR programs from the CDPro package downloaded from the Internet (http://lamar.colostate.edu/∼sreeram/CDPro). Since only the CONTIN/LL method gave consistent and reproducible results, these data are presented. Detailed information about the methods and reference data sets can be found in ref *25*.

For the lanthanides binding studies, the protein at 0.2 mg/mL was incubated for 30 min at 4 °C with 0.5 mM $DyCl_3$ or $EuCl_3$ and then the metal ions excess was removed by dialysis against 10 mM NaCl (pH 6.5) overnight. In the experiments with EDTA, the chelator was added at a concentration of 2 mM after the incubation with a lanthanide for 10 min and then dialyzed as described above. The metal content was determined in the protein by the induction-coupled plasma method (Perkin-Elmer).

The metal binding constants (dissociation constant, K_D) were determined by the ligand binding method from Scatchard plots using a calcium and lanthanide indicator, tetramethylmurexide (*26*), at 20 ± 2 °C. The formation of the metal−indicator complex was followed spectrophotometrically in a dual-wavelength mode at 507−554 or 490−554 nm using an SLM Aminco DW2000 spectrophotometer. The measurements were performed at a protein concentration of 10 μM in 10 mM NaCl and 25 mM Mes buffer (pH 6.5), and in the presence of 0.1 mM tetramethylmurexide. The lanthanide concentration used for titration varied from 0 to 50 μM (every 2 μM in the range of 0−20 μM and every 4 μM in the range of 20−50 μM), and that of Ca^{2+} ions varied from 0 to 100 μM (every 5 μM).

Table 1: Binding of Lanthanides with the Isolated 33 kDa Protein Determined by the Induction-Coupled Plasma Method

sample treated with $EuCl_3$ or $DyCl_3$	Eu (Dy)/protein (mol/mol)	
	dialyzed	EDTA-washed
33 kDa protein	1.08 ± 0.07 Eu 1.19 ± 0.04 Dy	<0.10 <0.10

Table 2: Binding Constants (dissociation constant, K_D) of Lanthanides and Calcium with the 33 kDa Protein and the Number of Binding Sites in the Protein Determined by the Ligand Binding Method[a]

metal	K_D ($\times 10^{-5}$ M)	Me/protein (mol/mol)
Eu^{3+}	0.61 ± 0.11	1.05 ± 0.02
Eu^{3+} and 100 μM Ca^{2+}	1.30 ± 0.13	0.96 ± 0.04
Dy^{3+}	1.92 ± 0.44	1.15 ± 0.17
Ca^{2+}	5.55 ± 0.62	1.43 ± 0.06

[a] The medium consisted of 10 μM protein in 10 mM NaCl, 25 mM Mes buffer (pH 6.5), and 0.1 mM tetramethylmurexide. The lanthanide concentration used for titration varied from 0 to 50 μM, and the calcium concentration varied from 0 to 100 μM. Other details are in Materials and Methods. Means ± the standard deviations are from three replicates.

RESULTS

The results of metal content analysis of the 33 kDa protein incubated with the lanthanide ions show that approximately one Eu^{3+} ion and Dy^{3+} ion are bound per protein molecule, and these ions can be removed by EDTA washing (Table 1). This indicates that there is one binding site for lanthanides and possibly also for calcium on the 33 kDa protein.

The determined binding constants of the lanthanides and calcium with the 33 kDa protein using the ligand binding method are shown in Table 2. The obtained K_D value for Eu^{3+} is 3 times lower than the value for Dy^{3+} ions and nearly 10 times lower than that for Ca^{2+} ions. When the obtained K_D values are compared with that of typical Ca^{2+}-binding proteins, such as parvalbumin, which is on the order of 10^{-7} M (*27*), the lanthanide- and Ca^{2+}-binding site on the 33 kDa protein can be regarded as a low-affinity site. The estimated number of metal-binding sites on the 33 kDa protein from ligand binding measurements is approximately one (Table 2) for all three ions, and it is in agreement with the metal content analysis (Table 1). To determine if the lanthanides and calcium bind the same site in the 33 kDa protein, the measurements of the level of europium binding to the 33 kDa protein were performed in the presence of Ca^{2+} ions. The obtained binding constant, K_D, was at least 2 times higher than in the absence of calcium, but the number of Eu^{3+}-binding sites remained unchanged (Table 2). This indicates that Eu^{3+} and Ca^{2+} ions compete for the same, single site on the 33 kDa protein.

The fluorescence emission spectrum of the purified 33 kDa protein is dominated by tyrosine emission with the maximum

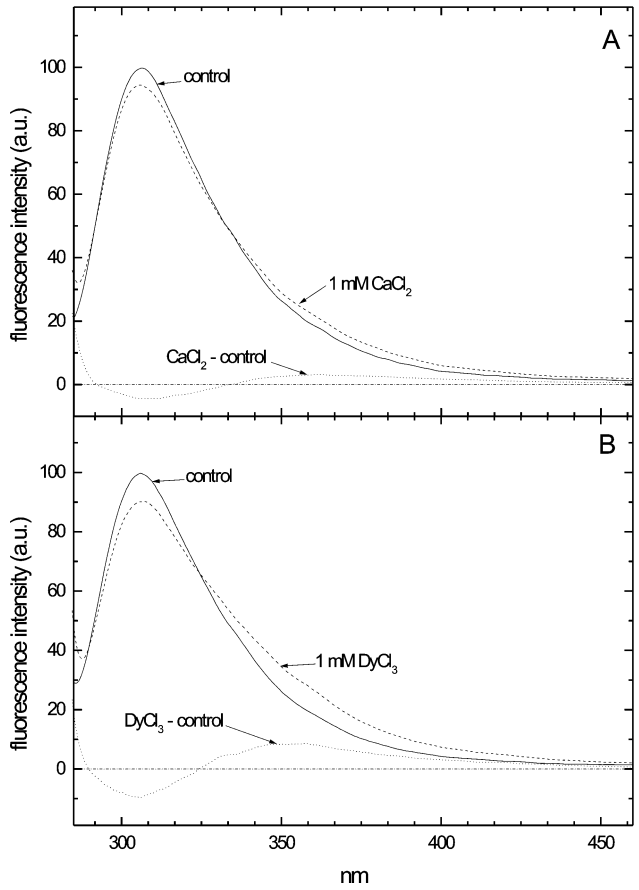

FIGURE 1: Effect of 1 mM $CaCl_2$ (A) and 1 mM $DyCl_3$ (B) on the fluorescence emission spectrum of the 33 kDa protein and the corresponding difference spectra. Excitation at 275 nm and a protein concentration of 0.1 mg/mL in 10 mM NaCl and 25 mM Mes (pH 6.5).

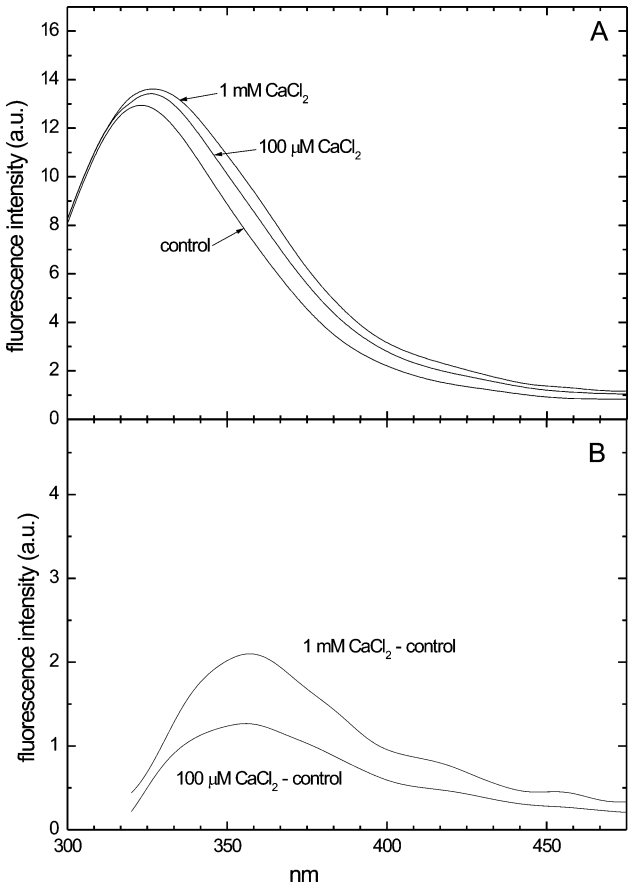

FIGURE 2: Effect of 100 μM and 1 mM $CaCl_2$ (A) on the fluorescence emission spectrum of the 33 kDa protein and the corresponding difference spectra (B). Excitation at 295 nm. Other conditions are as described in the legend of Figure 1.

at 306 nm using excitation at 275 nm (Figure 1A). In the presence of 1 mM $CaCl_2$, increased fluorescence can be observed in the range of Trp emission (~350 nm), and a decrease in the intensity of Tyr emission. This is clearly evident in the difference spectrum. Similar but considerably more pronounced effects are seen in the presence of 1 mM $DyCl_3$ (Figure 1B). These changes clearly indicate an influence of calcium and dysprosium on the protein conformation. When the protein is excited at 295 nm (Figure 2A), where only Trp emission is observed, a progressive shift of λ_{max} can be observed in the presence of increasing $CaCl_2$ concentrations from 323 nm in the absence of Ca^{2+} ions up to 326.5 nm in the presence of 1 mM $CaCl_2$ (Figure 2A). These spectral changes are caused by increased emission in the range of 350−360 nm, as revealed in the corresponding difference spectra (Figure 2B). Even more evident changes in the Trp emission region can be found in the presence of Dy^{3+} ions when only Trp is excited (Figure 3A). The addition of increasing $DyCl_3$ concentrations shifts λ_{max} of the protein emission from 323 to 332 nm for 1 mM $DyCl_3$. The difference spectra clearly show (Figure 3B) that these changes are due to increased emission with λ_{max} at 350 nm which corresponds to the fluorescence of Trp exposed to the polar surroundings. The effects of $EuCl_3$ were similar to those of $DyCl_3$ (data not shown). This indicates that binding of Ca^{2+} and lanthanides to the 33 kDa protein causes such conformational changes of the protein that lead as a consequence to Trp exposure from the hydrophobic protein interior with a λ_{max} of 323 nm to the polar environment with a λ_{max} of ~350 nm, corresponding to Trp emission in water (*28*). Similar effects of calcium and lanthanides on the Trp emission of the 33 kDa protein confirm that the lanthanides bind to the same metal-binding site as calcium in the protein but with a higher affinity.

The CD spectrum of the 33 kDa protein is characterized by the maximum around 197 nm that originates from the high β-sheet content in this protein (Figure 4A). Addition of increasing $CaCl_2$ concentrations causes progressive lowering of the ellipticity at the maximum. In the presence of Eu^{3+} and Dy^{3+} ions, even more pronounced changes than those for Ca^{2+} ions in the protein CD spectrum can be observed (Figure 4B). Lanthanides present at 100 μM caused even more significant effects on CD spectra than 1 mM calcium. These observations indicate that binding of both calcium and lanthanides to the protein causes similar structural changes. However, at the same ion concentration, the effects exerted by lanthanides are considerably more pronounced. To correlate the observed changes in the CD spectra upon the metal binding with structural changes of the protein, it seemed advisable to further analyze CD spectra using empirical methods for quantitative estimation of the secondary structure. Simulations of the CD spectra of the 33 kDa protein in the absence and presence of the investigated metal ions resulted in the secondary structure predictions, obtained using CONTIN/LL software, shown in Table 3. For the calculations, reference sets 6 and 7 were used since these sets contain CD spectra of five unfolded proteins (*25*). As

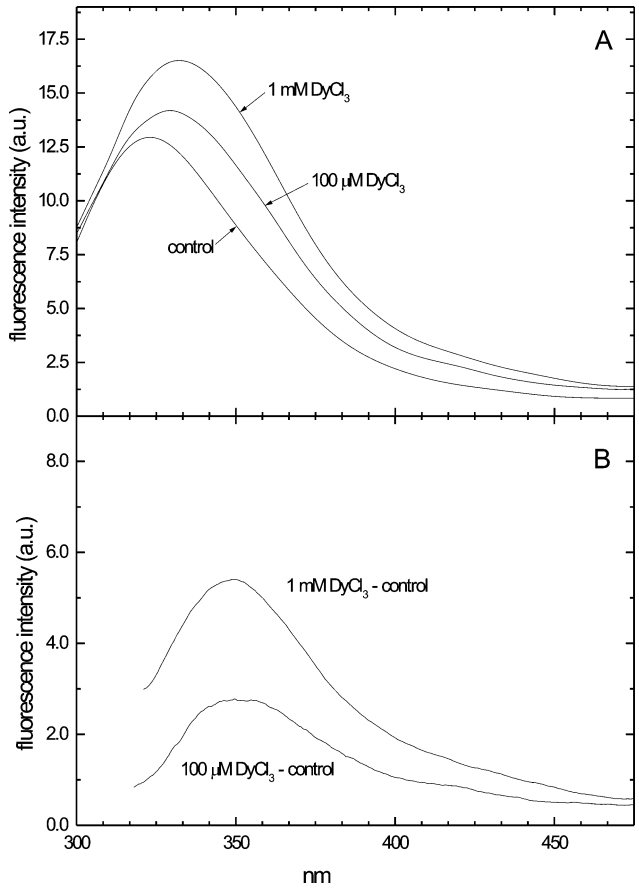

FIGURE 3: Effect of 100 μM and 1 mM $DyCl_3$ (A) on the fluorescence emission spectrum of the 33 kDa protein and the corresponding difference spectra (B). Excitation at 295 nm. Other conditions are as described in the legend of Figure 1.

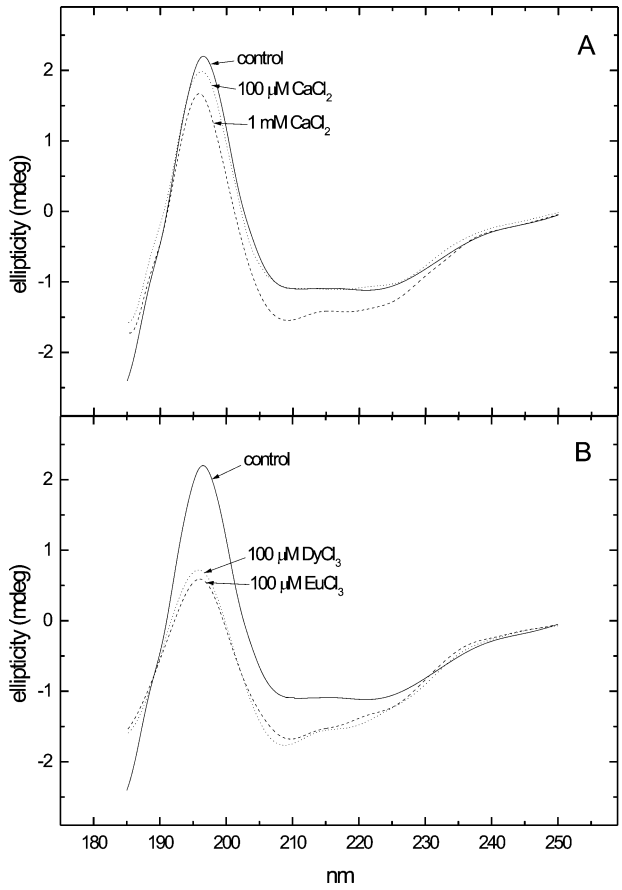

FIGURE 4: Effect of $CaCl_2$ (A) and $DyCl_3$ and $EuCl_3$ (B) on the CD spectrum of the 33 kDa protein. Protein concentration of 0.1 mg/mL in 1 mM NaCl (pH 6.5). For more details, see Materials and Methods.

can be seen, the contribution of the α-helix, β-sheet, and unordered structure of the pure protein is consistent with other previously reported values. However, there is no significant effect of any of the investigated metals on the secondary structure of the 33 kDa protein, although evident changes can be noticed in the CD spectra. The reason for this could be that we deal only with small, local changes in the protein structure which are not reflected in the total protein conformation.

Illustrated in Figure 5A, the DSC curve of the 33 kDa protein exhibits an endothermic peak with a midpoint temperature (T_m) of 63 °C and a broad transition in the range of 80−100 °C with the maximum at 97.5 °C (Figure 5A). The protein is known for its thermostability (*11*), and it is not irreversibly denatured even at the upper limit of our measurement (125 °C) so the second run on the same sample was possible. As compared to the first run, both the maxima are shifted to lower temperatures (Figure 5A) by 15−20 °C, suggesting that the protein undergoes facilitated conformational changes during the repeated heating. Apparently, the thermal behavior of the 33 kDa protein is different from that of most proteins with well-defined three-dimensional structure where one narrow maximum is observed corresponding to a highly cooperative transition usually associated with protein irreversible denaturation. On the contrary, the 33 kDa protein displays properties consistent with the molten globular state, including a compact folding intermediate with a near-native secondary structure but lacking the tertiary structure. This fact may be the reason for the low cooperativity non-two-state thermal unfolding found in our thermograms. The first maximum observed around 60 °C in the control sample corresponds well with the thermal range of the protein unfolding found in temperature-dependent CD spectra by Lydakis-Simantiris et al. (*11*). Considering the high-temperature peak, we cannot exclude the possibility that it is a result of aggregation that is however certainly not associated with the protein precipitation. Presumably, this aggregation stabilizes the molten conformation of the domains. The addition of calcium and lanthanides clearly changes the thermograms (Figure 5B). The maximum at 60 °C is not observed anymore, apart from a slight shoulder for the Ca^{2+}-treated protein. Instead, for the Ca^{2+}-treated sample, a peak at ∼42 °C appears; for the Dy^{3+}-treated sample, a shoulder at ∼51 °C can be observed, while for the Eu^{3+}-treated sample, no peaks in this region can be found. For all the metal-treated samples, a broad maximum around

Table 3: Predicted Secondary Structure Components of the 33 kDa Protein in the Absence and Presence of Calcium, Europium, and Dysprosium Ions Based on Far-UV CD Spectra[a]

sample	α-helix	β-sheet	turn and unordered	total
control	3	38.5	58.5	100
with 100 μM $EuCl_3$	3	38.5	58.5	100
with 100 μM $DyCl_3$	3.5	38	58.5	100
with 100 μM $CaCl_2$	3	39	58	100
with 1 mM $CaCl_2$	3.5	37.5	59	100

[a] The numbers are averages of results obtained from reference sets 6 and 7 using the CONTIN/LL method (*25*).

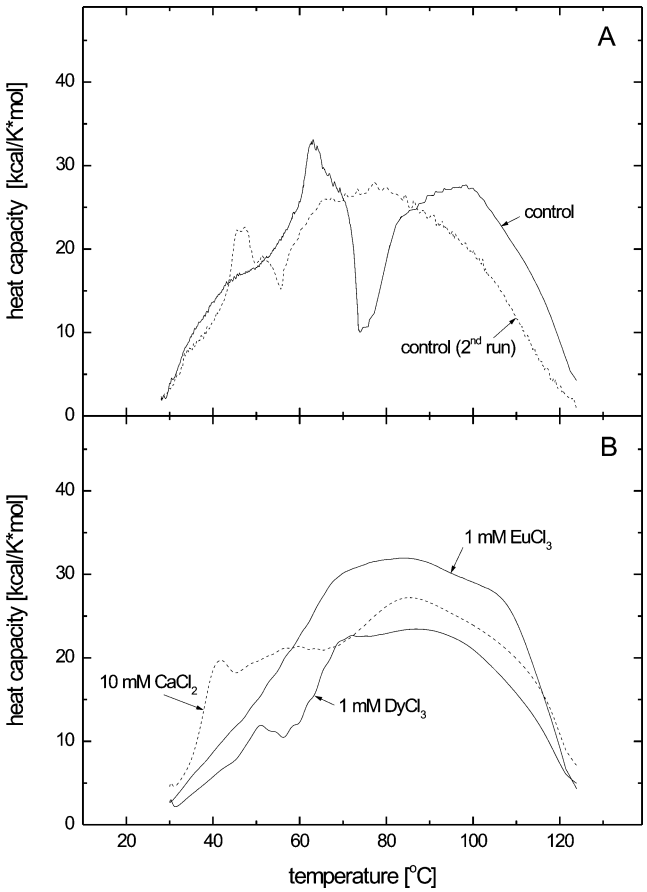

FIGURE 5: DSC thermograms of the 33 kDa protein alone (A) and in the presence of 10 mM $CaCl_2$, 1 mM $DyCl_3$, and 1 mM $EuCl_3$ (B). Protein concentration of 1 mg/mL in 10 mM NaCl and 25 mM Mes (pH 6.5). For more details, see Materials and Methods.

85 °C can be observed extending to 120 °C. Generally, the shape of the thermograms in the presence of the investigated ions resembles that of the thermally treated control protein. This points to the conclusion that calcium and especially the used lanthanides reduce the cooperativity of protein unfolding and shift its endothermic transitions to lower temperatures.

DISCUSSION

The data presented herein indicate that the 33 kDa protein exhibits calcium and lanthanide binding properties, and this binding induces protein conformational changes that manifest in the fluorescence emission spectra of the protein, CD spectra, and thermograms of calorimetric measurements.

Judging from the acidic isoelectric point (pI = 5.1) of the 33 kDa protein, we cannot exclude the possibility that it binds metals nonspecifically, as reported for PSII (*29*, *30*), but our results showing that the purified 33 kDa protein has one, homogeneous metal-biding site where lanthanides and calcium bind competitively were verified by two independent methods and point to the specific site. Moreover, stimulation of oxygen evolution in PSII membranes by low concentrations of Eu^{3+}, Dy^{3+}, and Ca^{2+} ions (*14*, *30*) may suggest that their binding site is on the 33 kDa protein and this site is functional in the process of oxygen evolution.

The effect of Ca^{2+} and lanthanides on fluorescence emission spectra of the 33 kDa protein observed in our studies using excitation at 275 nm is similar to pressure-induced changes in the emission spectra, i.e., lowering of Tyr emission and the increase in Trp emission (*6*) which is the result of protein unfolding. When Trp was preferentially excited (295 nm) under atmospheric pressure, it exhibited a maximum at 316 nm which indicates its hydrophobic surroundings (*6*). Increased pressure generated a progressive shift in Trp emission up to 352 nm, indicating Trp exposure to the polar surroundings during protein unfolding. Thus, as observed in our studies, the increased Trp emission at around 350 nm after the action of Ca^{2+} and lanthanides, when Trp is preferentially excited, also points to some unfolding effect of these metals on the protein. The effect of the investigated metal ions on the CD spectrum of the protein observed in our studies is similar to the temperature-induced changes in the 33 kDa structure (*11*). When the protein was pretreated with heat up to 90 °C and cooled to 25 °C, it exhibited a CD spectrum of a partially unfolded protein corresponding to the protein conformation at ~45−55 °C without preheating (*11*).

Although the presented analysis of CD spectra did not reveal any significant changes in the secondary structure of the protein after the action of lanthanides and calcium, it should be mentioned that even more pronounced changes in the CD spectra were observed in some mutants of the 33 kDa protein without significant alterations in the content of different secondary structure elements (*31*, *32*). It is possible that small, local changes in the protein structure upon metal binding are not reflected in the total protein conformation and the programs for structure analysis are not sensitive to such changes, although they are evident in the CD spectra (Figure 4). In contrast to our study, it has been recently shown using FTIR (*20*) that Ca^{2+} ions induced a 10% reduction in the β-sheet content in the 33 kDa protein. These differences are most likely due to the very high protein concentration (~30 mg/mL) used in the FTIR measurements. It was already shown that even at the lower concentration of the 33 kDa protein, the presence of calcium stimulates formation of dimers and aggregates (*19*). Thus, the effects observed by Heredia et al. (*20*) are probably connected with dimerization and aggregation of the protein stimulated by the presence of calcium. Nevertheless, their study also points to the interaction of Ca^{2+} ions with the 33 kDa protein. It should also be mentioned that the FTIR method is less straightforward and reliable than the CD method (*5*, *12*) for the determination of the secondary structure of proteins. As an example, the literature data about the content of α-helix in the spinach 33 kDa protein calculated from CD spectra are in the range of 4−9%, and those based on FTIR spectra vary from 2 to 27% (*2*). An effect of Ca^{2+} ions on the β-sheet content of the 33 kDa protein similar to that reported in ref *20* was already observed by Zhang et al. (*15*) using FTIR measurements, under similar experimental conditions, but the effect of Ca^{2+} ions on the α-helix content was completely different.

Our calorimetric data indicate that the endothermic peaks are shifted 15−20 °C lower in the case of the heat-pretreated protein. Both Ca^{2+} and lanthanides seem to exert an effect on the protein similar to that of heat pretreatment, i.e., facilitated protein conformational change (unfolding) during heating. It is worth mentioning that although metal ion binding involves structural changes in the protein that are local, their possible propagation to other parts of the protein molecule could be taken into account. The more pronounced effects of lanthanides than of calcium on the spectral and thermal properties of the 33 kDa protein probably originate

from the higher affinity of lanthanides for the protein. Moreover, the stronger effect of europium than of dysprosium on DSC thermograms, as well as on oxygen evolution (*14*), is probably caused by differences in the ion radii of these two lanthanides; i.e., Eu^{3+} and Dy^{3+} exhibit ion radii that are longer and shorter than that of calcium, respectively. Furthermore, the ionic strength of Eu^{3+} is higher than that of Dy^{3+} ions. Therefore, when Eu^{3+} ion occupies the Ca^{2+}-binding site in the 33 kDa protein, it affects the tertiary protein structure more strongly than Dy^{3+} ion.

The question of the possible Ca^{2+}-binding site within the protein arises. On the basis of the protein sequence, calcium-binding domains that are highly homologous to "EF hands", classical high-affinity Ca^{2+}-binding sites, were suggested to occur in the 33 kDa protein (*16*, *17*). However, the EF-hand has a typical helix−loop−helix structure that is not predicted for the 33 kDa protein (*5*, *18*). When compared with those of Ca^{2+}-binding proteins containing typical EF-hands, like parvalbumins (*27*), the obtained binding constant for calcium ($K_D \sim 10^{-5}$ M) implies that the Ca^{2+}-binding site on the 33 kDa protein is a low-affinity site. We cannot exclude the possibility that some segments of the 23 kDa protein could complement a Ca^{2+}-binding site in the 33 kDa protein and increase its protein binding affinity. Interestingly, a low-affinity Ca^{2+}-binding site was suggested to occur on the contact surface of the 23 and 33 kDa proteins, and this site was probably responsible for the stimulatory effect of Eu^{3+}, Dy^{3+}, and Ca^{2+} ions on oxygen evolution (*14*). It has been suggested that the 33 kDa protein belongs to the group of molten globule proteins that are characterized by significant flexibility and the absence of a definite tertiary structure (*9*). Therefore, binding of calcium to this protein may easily affect its tertiary structure and facilitate adoption of its definite conformation. In support of this view, it was found that the presence of calcium was necessary for the crystallization of the 33 kDa protein (*19*) probably due to stimulation of protein dimerization by calcium ions (*1*). This effect may have also some relevance for 33 kDa protein dimerization *in vivo* which was suggested in ref *33*.

Another question is whether the Ca^{2+}-binding site on the 33 kDa protein is different or the same as that involved directly in water oxidation. Although high NaCl concentrations and the addition of Ca^{2+} can partially substitute for the 33 kDa protein in preserving oxygen evolution activity of PSII, it is supposed that the 33 kDa protein is not directly involved in Mn and Ca coordination involved in water oxidation (*1*, *12*). The effect of the 33 kDa protein is rather due to stabilization of the optimal conformation of PSII reaction center proteins engaged in Mn−Ca coordination. A similar effect may exist in the case of the 23 kDa protein that was described as a "Ca^{2+} concentrator" (*34*). Therefore, it is reasonable to assume that the calcium that binds the Mn cluster is different from the calcium that is bound by the 33 kDa protein and is not directly involved in the process of oxygen evolution. However, it may also influence the conformation of the 33 kDa protein itself and/or the neighboring proteins (e.g., 23 kDa protein) and also indirectly affect the oxygen evolution activity.

In summary, we have shown that 33 kDa protein contains one calcium and lanthanides low-affinity binding site and metal binding to this site induces a partial structural change of this protein that can be followed by fluorescence spectroscopy, circular dichroism spectra, and calorimetric thermograms of this protein. Calcium-induced conformational changes in the 33 kDa protein may be important for preserving its native structure and binding to other proteins that is necessary for optimal oxygen evolution activity of PSII.

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