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Rapid Report

Secondary structure of the 33 kDa, extrinsic protein of Photosystem II: a far-UV circular dichroism study

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Abstract

The 33 kDa extrinsic protein of Photosystem II is an important component of the oxygen-evolving apparatus which functions to stabilize the manganese cluster at physiological chloride concentrations and to lower the calcium requirement for oxygen evolution. Chou-Fasman analysis of the amino-acid sequence of this protein suggests that this component contains a high proportion of α-helical structure and only relatively small amounts of β-sheet structure. A computational study using more sophisticated techniques (Beauregard, M. (1992) Environ. Exp. Bot. 32, 411–429) concluded that the protein contained little periodically ordered secondary structure. In this study, we have directly measured the relative proportions of secondary structure present in the 33 kDa protein using far-ultraviolet circular dichroism spectroscopy. Our results indicate that, in solution, this protein contains a large proportion of β-sheet structure (38%) and relatively small amounts of α-helical structure (9%). A structural model of the 33 kDa protein based on a constrained Chou-Fasman analysis (Teeter, M.M. and Whitlow, M (1988) Proteins 4, 262–273) is presented.

Keywords: Photosystem II; Circular dichroism; Protein structure; Secondary structure; Oxygen-evolving complex

PS II is a multi-subunit thylakoid membrane protein complex which catalyzes the light-driven oxidation of water to molecular oxygen and the reduction of plastoquinone to plastoquinol. This complex consists of both intrinsic and extrinsic protein subunits. Intrinsic polypeptides with apparent molecular masses of 49 (CP 47), 45 (CP 43), 34 (D1), 32 (D2), 9 and 4.5 (α and β subunits of cytochrome *b*-559), and 4 kDa (*psb*I gene product) appear to form the minimum complex capable of photosynthetic oxygen evolution [1,2]. In higher plants, three additional extrinsic protein components with apparent molecular masses of 33, 24 and 17 kDa are associated with the oxygen-evolving complex. The 24 and 17 kDa components can be removed by salt-washing (usually 1.0 M NaCl) which dramatically lowers the oxygen-evolving capacity of PS II vesicles [3] and PS II membranes [4]. This activity is recovered upon reconstitution with the 24 and 17 kDa components. These proteins are assumed to play a role in the regulation of calcium and chloride concentrations within the PS II complex.

The extrinsic 33 kDa protein is much more tightly associated with the intrinsic PS II proteins than are the 24 and 17 kDa proteins. Removal of this protein requires treatment with high concentrations of alkaline Tris [5], $CaCl_2$ [6], or NaCl-urea [7]. Treatment with alkaline Tris also leads to the loss of the manganese cluster associated with the active site of PS II [4]. This was initially taken as evidence that the manganese cluster was associated with this extrinsic protein. $CaCl_2$ and NaCl-Urea washes, however, efficiently remove the 33 kDa protein without the concomitant loss of the manganese cluster if the protein-depleted PS II preparation is maintained at a high chloride concentration (> 100 mM) [7]. At chloride concentrations below 100 mM, two of the four manganese associated with PS II

Abbreviations: Chl, chlorophyll; Mes, 2-(*N*-morpholino)ethanesulfonic acid; LDS, lithium dodecyl sulfate; PS II, Photosystem II; UV, ultraviolet.

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rapidly become paramagnetically uncoupled and then dissociate from PS II membranes [8]. These studies indicate that the extrinsic 33 kDa protein acts as a manganese-stabilizing protein for PS II. Additionally, mutants in the cyanobacterium *Synechocystis* 6803 which lack the 33 kDa protein cannot grow autotrophically at reduced calcium concentrations [9]. Biochemical removal of the 33 kDa protein from PS II membranes increases the calcium requirement for oxygen evolution [2]. These results suggest that the 33 kDa protein also modulates the calcium requirement of PS II. Recently, we have confirmed and extended previous studies [6,7,10,11] which demonstrated that significant rates of oxygen evolution can occur in the absence of the manganese-stabilizing protein. The presence of this protein is, however, required for the high rates of oxygen evolution observed in vivo and in isolated PS II preparations [1,2].

While this protein obviously is an important component of the oxygen-evolving complex, relatively little is known of its structural organization. Previous studies have demonstrated that the 33 kDa extrinsic protein is associated with CP 47 [12–14] and perhaps other intrinsic components of PS II. Studies examining the stoichiometry of this protein indicate that there are either one [15,16] or two copies [17] present per PS II reaction center. Additionally, the presence of an intramolecular disulfide bridge has been demonstrated [18,19]. Secondary structure prediction algorithms have been used to examine the structure of the 33 kDa protein [20]. This author concluded that the 33 kDa extrinsic protein contained only small amounts of periodic secondary structure (i.e. α-helix and β-sheet).

In this communication we have examined the secondary structure of the 33 kDa protein in solution by far-UV circular dichroism spectroscopy. Our results suggest that the 33 kDa protein contains a large proportion of β-sheet structure and a relatively small amount of α-helical structure. These results differ significantly from the secondary structure predicted by Beauregard [20] or from unconstrained Chou-Fasman analysis [21]. A structural model of the 33 kDa extrinsic protein based on a constrained Chou-Fasman analysis using the method of Teeter and Whitlow [22] is presented.

Chloroplasts were isolated from market spinach (*Spinacia oleracea* L.) as previously described [23]. Chl concentration was measured by the method of Arnon [24]. Oxygen-evolving PS II membranes were prepared by the method of Berthold et al. [25] with the modifications described by Ghanotakis and Babcock [26]. Typical preparations had a Chl *a*/*b* ratio of 1.9–2.0 and an oxygen evolution rate of 500–600 mmol (mg Chl)$^{-1}$ h^{-1}. The 33 kDa extrinsic protein was isolated as previously described [2,17]. The purified protein exhibited a single band on LiDS-polyacrylamide gels [17], even when overloaded and could be functionally reconstituted with $CaCl_2$-washed PS II membranes (data not shown).

We have verified the extinction coefficient of the 33 kDa extrinsic protein. The purified 33 kDa extrinsic protein was extensively dialyzed against 10 mM $NaHCO_3$ at 4°C. About 2 nmol of protein containing 9.5 nmol of a norleucine standard and 0.1% phenol were frozen in a pyrex glass tube at −70°C, 6 M HCl was added, the tube was sealed under vacuum and the protein sample was hydrolyzed at 110°C for 24 h. Amino acid analysis was performed by HPLC as described by Klotz and Higgins [27]. The amino-acid composition was essentially identical to that determined by Kuwabara and Murata [28]. The protein concentration was calculated based on the average concentration of the following amino acids: glutamic acid, alanine, isoleucine, leucine and arginine. The extinction coefficient of the 33 kDa protein was calculated and verified as being 16 mM^{-1} cm^{-1} at 276 nm. This value was identical to that previously reported [28] when the molecular mass of the protein was corrected to that deduced from its amino-acid sequence [29].

For the circular dichroism measurements the purified 33 kDa protein, following dialysis against 5 mM Mes-NaOH (pH 6.0), was further exhaustively dialyzed against a sodium-potassium phosphate buffer (pH 7.0). The concentration of the 33 kDa protein was measured using the determined extinction coefficient and adjusted to 1.0 mg ml^{-1}. The circular dichroism spectra were recorded on an Aviv 62DS circular dichroism spectrometer in a cuvette with a 0.1 mm pathlength (Hellma) at 20°C. The instrument was calibrated using a freshly made aqueous solution of (+)-10-camphorsulfonic acid [30]. The optical pathlength was verified based on the absorption spectra of 10 mM uracil on an Aviv 118DS absorbance spectrometer. The circular dichroism spectra were collected on a PC, a data point being collected every 0.5 nm with a 1.5 nm bandwidth and a 1 s time constant. The data were linearly smoothed ±2 points. The spectra are presented as the mean residue ellipticity. Data at wavelengths where the absorbance was greater than approx. 1.0 (indicated by a dynode voltage greater than 400 V) were discarded. Five repetitive scans were averaged in the ultraviolet region between 178 and 270 nm and subsequently used for secondary structure analysis based on the singular value decomposition theorem [30]. Unconstrained Chou-Fasman analysis was performed using the program MPREDICT (Crofts, T., University of Illinois). Chou-Fasman analysis which was constrained to the observed secondary structure fractions was performed using the program SEQ [22].

Fig. 1 shows the circular dichroism spectrum of the 33 kDa extrinsic protein. A strong positive band is present at 196 nm while a weak negative band is

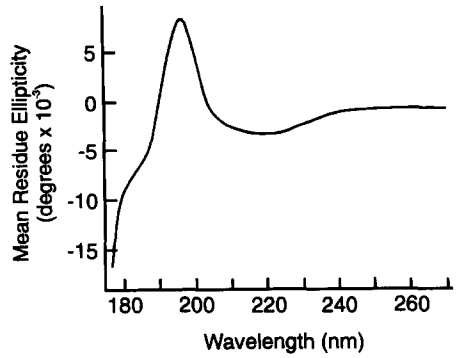

Fig. 1. Far-UV circular dichroism spectra of the spinach 33 kDa extrinsic protein. Circular dichroism data were collected from 178–270 nm at a protein concentration of 1.0 mg ml^{-1} at 20°C. A data point was collected every 0.5 nm with a 1.5 nm bandwidth and a 1 s time constant. The data were linearly smoothed ±2 points.

Table 1
Secondary structure analysis of the 33 kda extrinsic protein from spinach

Secondary structure type	Circular dichroism	Chou-Fasman	Beauregard
α-Helix	9%	28%	13%
β-Sheet	38%	25%	9%
Turn	17%	12%	–
Other	35%	35%	–

The secondary structure fractions were obtained from the far-UV circular dichroism spectra shown in Fig. 1. using singular value decomposition methods [30] and an unconstrained analysis. The Chou-Fasman prediction [21] was obtained using the program MPREDICT. The Beauregard prediction [20] was made using three complementary analysis methods for the prediction of secondary structure [34].

present in the 225–230 nm region. Previously published circular dichroism spectra of the 33 kDa protein [18,31,32] extended only to 200 nm, which did not allow these authors to provide a secondary structure analysis of this protein. It has been shown that circular dichroism data must be collected to at least 184 nm to provide reliable secondary structure estimates [33].

Table 1 shows the secondary structure fractions which were determined from the circular dichroism spectra shown in Fig. 1. Singular value decomposition methods were used to estimate the secondary structure content of this protein. The 33 kDa extrinsic protein contains a relatively large proportion of β-sheet structure (38%) and a relatively small proportion of α-helical structure (9%). Also presented are the secondary structure fractions which were predicted using either unconstrained Chou-Fasman analysis or the methods of Biou et al. [34] as implemented by Beauregard for the 33 kDa extrinsic protein [20]. Neither computational approach provides a satisfactory description of the secondary structure content. Chou-Fasman analysis seriously overestimated the amount of α-helix present while Beauregard's analysis seriously underestimated β-sheet content of this protein.

While circular dichroism results yield fairly reliable estimates of the secondary structure composition of proteins, it does not provide any information concerning the location of the secondary structure elements within the protein sequence. Unconstrained Chou-Fasman analysis does give information concerning the location of the secondary structural elements; however, for the 33 kDa extrinsic protein this approach yields a predicted structure that was not verified by the circular dichroism data (Table 1). Teeter and Whitlow [22] have presented a hybrid computational approach which utilizes a Chou-Fasman analysis which is constrained to the secondary structure fractions obtained by circular dichroism spectroscopy or other techniques. In their original study, they found good agreement in the predicted location of secondary structure elements in the protein crambin using this hybrid analysis and the X-ray crystallographic structure of this protein. This

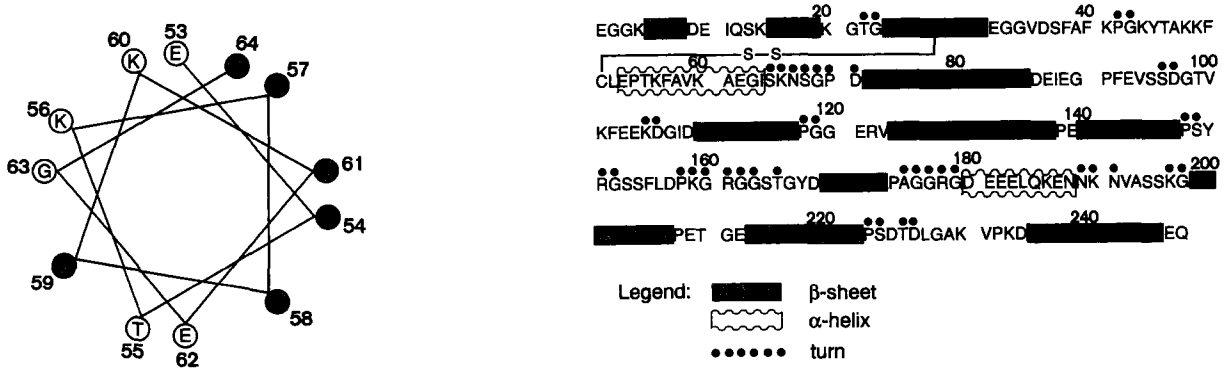

Fig. 2. Model of the secondary structure of the spinach 33 kDa extrinsic protein. β-strands, α-helixes and turns predicted by the constrained Chou-Fasman method of Teeter and Whitlow [22] are shown. Amino-acid residues are designated by their single-letter codes. Inset: helical wheel diagram for predicted α-helix I (^{53}E-^{64}I). Hydrophobic residues are shaded. Residue numbers are shown.

method has also been used to examine the apolipoproteins A-1 and E-3 [35]. We have utilized this technique of analysis to produce the model of the secondary structure of the 33 kDa extrinsic protein which is shown in Fig. 2.

This model predicts that the 33 kDa extrinsic protein contains eleven β-strands and two α-helical domains. Three of the β-strands are located to the N-terminal side of the first α-helical domain (^{53}E-^{64}I) and three additional β-strands are located to the C-terminal side of the second α-helical domain (^{181}E-^{188}N). Five β-strands are located between these two α-helical regions. Thus, the distribution of the predicted secondary structural elements in this protein are quite symmetrical. The predicted β-sheet domains of the protein are relatively rich in hydrophobic residues (48%) while being depleted in charged residues. This is consistent with a tertiary structure in which the β-sheet domains would form the hydrophobic core of the protein while the loop (and α-helical) domains which separate the β-strands would lie at the protein's surface.

The first putative α-helical domain (^{53}E-^{64}I) is amphipathic. A helical wheel for this helix is shown in Fig. 2, inset. This structure has properties which are fairly typical for class 'G' amphipathic helixes [36] which are usually found in globular proteins. An 'Eisenberg plot' of the residues predicted to lie in this domain also cluster in the globular region of the plot (data not shown) [37].

It has been speculated that the 33 kDa extrinsic protein binds manganese [29] and/or calcium [38,39]. Little direct evidence has been presented for either of these hypotheses. Oxygen evolution can clearly occur in the absence of the 33 kDa extrinsic protein either in cyanobacterial mutants which lack the *psb*O gene [1] or in PS II preparations from which this protein has been removed [2]. Since oxygen evolution clearly requires an intact tetranuclear manganese cluster, manganese-binding by the 33 kDa protein appears unlikely. In the pea 33 kDa extrinsic protein, it has been suggested that the region ^{84}T-^{114}T forms an 'EF-hand' calcium-binding site [38,39]. This hypothesis was based on sequence homology with other calcium-binding proteins. Our model would suggest that no helix-turn-helix motif exists in the spinach protein. Other types of calcium-binding sites could, however, be present [20].

It should be cautioned that our model presented in Fig. 2 is quite speculative in nature. The model is based on the circular dichroism properties of the 33 kDa extrinsic protein in solution. It is not known whether this protein undergoes structural rearrangements upon binding to PS II. Additionally, while the constrained Chou-Fasman procedure [22] appears to work well for other proteins, it is possible that this method could give aberrant results in this particular instance. Thus, we view this model as a working hypothesis which is continually being tested and refined.

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