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Analysis of pH-Induced Structural Changes of the Isolated Extrinsic 33 Kilodalton Protein of Photosystem II†

T. Shutova,‡ K.-D. Irrgang,§ V. Shubin,|| V. V. Klimov,‡ and G. Renger*,§

Institute of Soil Science and Photosynthesis, RAS, Pushchino, Moscow Region, 142292 Russia, Max-Volmer-Institute for Biophysical Chemistry and Biochemistry, Technical University Berlin, D 10623, Berlin, Germany, and A. N. Bakh-Institute of Biochemistry, Moscow, 117071, Russia

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ABSTRACT: Structural properties of the isolated extrinsic regulatory 33 kDa protein of the water-oxidizing complex were analyzed at different pH values. It was found that (a) titrations of the buffer capacity reveal a characteristic hysteresis effect that is unique for the 33 kDa subunit and is not observed for the other extrinsic proteins, (b) changes of the emission from the fluorescence probe 1,8-ANS are indicative of an increased accessibility of the hydrophobic core of the 33 kDa protein to the dye at lower pH, (c) the near-UV circular dichroism spectrum of the polypeptide is altered owing to a pH decrease from 6.8 to 3.8 and becomes drastically changed at pH 2.8, and (d) the content of secondary structure elements remains virtually constant in the range 3.8 < pH < 6.8, with the following values gathered from far-UV CD spectra: ∼8% α-helix, ∼33% β-strand, ∼15% turns, and ∼44% random coil. Further acidification down to pH 2.8 gives rise to a decreased α-helix and increased β-strand and random coil content. A theoretical model [Ptitsyn, O., & Finkelstein, A. (1983) *Biopolymers 2*, 15−22] was used to predict the probability and location of secondary structure elements within the protein sequence. On the basis of these calculations, an extended hydrophobic β-sheet domain could exist in the center of the protein and an α-helix in the C-terminal region. From these data, the 33 kDa protein is inferred to change its tertiary structure *in vitro* upon acidification of the aqueous environment. Possible implications of these features are discussed.

Photosynthetic water oxidation to molecular oxygen takes place via a sequence of four univalent oxidation steps at a manganese-containing unit referred to as water-oxidizing complex (WOC)[1] [for reviews, see Debus (1992), Rutherford et al. (1992), and Renger (1993)]. This process is energetically driven by light-induced formation of the strongly oxidizing cation radical $P680^{+\bullet}$ [for a review, see Renger (1992)]. A redox-active tyrosine (Y_Z) acts as intermediary electron carrier. At the current state of the art, the smallest unit that can be isolated with a functionally competent WOC is a complex of about 250 kDa excluding the detergent boundary layer (Haag et al., 1990). Apart from a few smaller polypeptides (apparent molecular mass <10 kDa), it contains six intrinsic proteins (D1, D2, CP47, CP43, and the two subunits of cytochrome b_{559}) and a 33 kDa protein as the only extrinsic subunit (now often designated as PS II-O protein because of its encoding by the *psb*O gene) [for a recent review, see Vermaas et al. (1993)]. The functional redox groups P680, Y_Z, Pheo, Q_A, and Q_B are known to be located in the D1/D2 heterodimer. However, to date it is not clear which of these subunits are involved in binding of the WOC nor have specific amino acid residues been unambiguously identified as ligands of the manganese cluster. Site-directed mutagenesis experiments in the cyanobacterium *Synechocystis sp.* revealed several amino acids in D1 and D2 that are of functional and/or structural relevance in establishing a competent WOC (Boerner et al., 1992; Chu et al., 1995 a,b; Nixon & Diner, 1992; Vermaas et al., 1990). Likewise, psb B gene deletion mutants lacking regions of 3−8 amino acid residues from particular domains of the putative large hydrophilic loop E of CP47 (Eaton-Rye & Vermaas, 1991; Haag et al., 1993) markedly affect the stability of the WOC and its photoactivation, although participation of this loop in manganese ligation appears to be less likely (Gleiter et al., 1994, 1995).

With respect to a possible functional and/or structural role of PS II polypeptides in establishing a functionally component WOC, the extrinsic 33 kDa protein has been thoroughly analyzed since its discovery 15 years ago [see Seidler (1996) for a review]. Extraction of this protein by washing with 1 M $CaCl_2$ strongly suppresses the oxygen evolution, but the manganese cluster remains bound provided that a sufficiently high Cl^- concentration is maintained (Ono & Inoue, 1983; Miyao & Murata, 1984). At lower Cl^- concentrations, two of the four manganeses become paramagnetically uncoupled (Mavankal et al., 1987) and are subsequently released from PS II (Ono & Inoue, 1984). Removal of the 33 kDa protein also affects the turnover and stability of higher redox states of the WOC (Miyao et al., 1987; Vass et al., 1987; Ono & Inoue, 1985).

Studies on green algae (*Chlamydomonas reinhardtii* or *Euglena gracilis*) led to the conclusion that the 33 kDa

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* Author to whom correspondence should be addressed.
‡ Institute of Soil Science and Photosynthesis.
§ Technical University Berlin.
|| A. N. Bakh-Institute of Biochemistry.

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[1] Abbreviations: 1,8-ANS, 8-anilino-1-naphthalenesulfonate; BSA, bovine serum albumin; β, buffer capacity; DTE, 2,3-dihydroxybutane-1,4-dithiol; MES, 2-(*N*-morpholino)ethanesulfonic acid; $P680^{+\bullet}$, redox-active specially bound chlorophyll *a* within the PS II reaction center; Pheo, pheophytin; PS II, photosystem II; PS II-O, extrinsic regulatory protein encoded by the *psb O* gene; WOC, water-oxidizing complex; Y_Z, redox-active tyrosine of polypeptide D1.

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protein is required for a stable assembly of the WOC (Mayfield et al., 1987; de Vitry et al., 1989; Hiramatsu et al., 1991). Recently, however, this view has been revised on the basis of experiments performed with *psb*O deletion mutants from cyanobacteria. It was shown that these mutants are able to evolve oxygen at reduced rates and therefore the extrinsic 33 kDa protein was inferred to not be an indispensable component of a functionally competent WOC (Burnap & Sherman, 1991; Philbrick et al., 1991; Bockholt et al., 1991). On the other hand, these mutants exhibit a Ca^{2+} demand for their growth that is markedly higher than that of the wild type (Philbrick et al., 1991). The differences between green algae and cyanobacteria observed in the phenotype of mutants lacking the 33 kDa protein were explained by the nature of the other extrinsic proteins. Recently a cytochrome *c*-550 supporting oxygen evolution has been reported in a cyanobacterial mutant lacking the 33 kDa protein (Shen et al., 1995a,b). *psb*O gene deletion mutants show also a higher vulnerability to light stress than wild-type cells from *Synechocystis sp*. PCC 6803 (Mayes et al., 1991). The authors concluded that the polypeptide plays an important role in protecting against donor side photoinhibition. The latest studies revealed that the 33 kDa protein plays an essential role in the dark stability of the WOC and its photoactivation (Engels et al., 1994). As a result of the above-mentioned findings, the extrinsic 33 kDa protein is an important constituent of a fully intact WOC that is essential for the stabilization of the functional manganese cluster [for a recent review, see Seidler (1996)].

Its structural organization, however, is not well-known. Different lines of evidence indicate an intimate interaction with loop E of CP47 (Bricker & Frankel, 1987; Bricker et al., 1988; Enami et al., 1987, 1989, 1991; Odom & Bricker, 1992). Furthermore, it is known that the N-terminus of the 33 kDa protein is essential for its binding (Eaton-Rye & Murata, 1989). A structurally important element of this protein is the conserved disulfide bridge (Tanaka & Wada, 1988; Tanaka et al., 1989). Treatment of PS II membrane fragments with dithiothreitol leads to inhibition of oxygen evolution while the kinetics of $P680^{+\bullet}$ reduction by Y_Z and the manganese content remain unaffected (Irrgang et al., 1992). This inhibition is probably caused by reductive cleavage of the S−S bridge that gives rise to structural changes of functional relevance for the reaction pattern of the WOC (K.-D. Irrgang, B. Geiken, B. Lange, and G. Renger, unpublished results). The latest studies, however, indicate that the S−S bridge is not an absolutely indispensable structural element for the function of the 33 kDa protein (Betts et al., 1996).

Recently it was shown that the WOC exhibits pronounced pH-dependent structural changes (Messinger & Renger, 1994; Kebekus et al., 1995) which might be related to pH-induced effects of the 33 kDa protein. The latter idea gained support by the observation of considerable photoinduced and Mn^{2+}-induced changes of the buffer capacity of PS II membrane fragments (Opanasenko et al., 1992; Shutova et al., 1992, 1994) that are indicative of exposure or burying of protonizable groups due to structural rearrangements of PS II proteins. Titration of the isolated 33 kDa protein revealed an acid/base hysteresis of its proton-acceptor properties that is evidently related to pH-dependent transitions between two stable conformational states of the protein (Shutova et al., 1993). The present study provides a detailed investigation of pH-induced structural changes of the isolated 33 kDa protein using both experimental approaches and theoretical modeling.

MATERIALS AND METHODS

Isolation and Purification of the 33 kDa Protein. Photosystem II membrane fragments were prepared from spinach according to Berthold et al. (1981) with some modifications (Völker et al., 1985). The 33 kDa protein was isolated by salt-washing (Ono & Inoue, 1983; Irrgang et al., 1988) including proteinase inhibitors. The polypeptide was then concentrated by ultrafiltration in an Amicon cell (PM-10 membrane) and dialyzed against 20 mM MES−NaOH buffer, pH 6.5, containing 30 mM NaCl. The dialyzed sample was further purified by gel filtration chromatography on an ACA 54 column (1.7 × 30 cm). The protein homogeneity was checked via SDS/urea/PAGE and silver staining (Laemmli, 1970; Oakley et al., 1980; Haag et al., 1990). Its concentration was spectrophotometrically determined either at 205 nm (Scopes, 1974) or at 276 nm using the extinction coefficient described in Kuwabara and Murata (1984) and Jansson (1984) and in addition by the method developed by Bradford (1976).

Acid−Base Titration. Acid−base titrations were carried out at $T = 20$ °C by using a home-built automatic laboratory setup as described earlier (Opanasenko et al., 1992; Shutova at al., 1992). The titration solutions contained either 10 mM HCl or 10 mM NaOH. The titration rate was optimized, assuring that the buffer capacity maximum of a calibration curve was independent of the titration direction. Normally the overall titration time was about 1 min. For calibration of the titration curves, the same acid and base titration experiments were performed with 0.2 mM NaH_2PO_4. Before starting the titration experiment, carbon dioxide was removed by bubbling argon through the reaction medium containing 100 mM NaCl. After the pH was adjusted to the desired values, the concentrated desalted protein was added to 20 μM final concentration. After completion of the titration cycle, the obtained curve $pH = f(V)$ was transformed by a computer into the function $V = f(pH)$, where V is the volume of added titrant. Subsequently, the base line curve obtained by analogous measurements in 100 mM NaCl solution without protein was subtracted from the resulting titration curve of the sample (100 mM NaCl plus protein), and the data were smoothed and differentiated.

Spectroscopic Measurements. Room temperature absorption and fluorescence spectra were measured using a Specord-M40 spectrophotometer and a Hitachi-850 spectrofluorometer, respectively. The tryptophan fluorescence was excited at 278 nm and that of 1,8-ANS (8-anilino-1-naphthalenesulfonate) at 346 nm. CD spectra in the UV region were monitored with a Jasco J-600 spectropolarimeter at the following optical path lengths: 4 mm in the region of 310−260 nm, 1 mm in the region of 260−198 nm, and 0.2 mm in the region of 200−184 nm. Calibration was carried out using a freshly prepared solution of D-camphor-10-sulfonate. The protein concentration was 30 μM. Prior to CD measurements, the protein was dialyzed against 10 mM phosphate/10 mM NaCl buffer and concentrated in Centriprep-10 tubes (Amicon).

Secondary Structure Analysis. Two different approaches were used for the determination of secondary structure

elements: (i) the percentage of α-helix, β-sheet, turns, and random coil content was gathered from CD spectra by using the recently developed program BELOK; and (ii) the possible location of these elements within the primary structure was calculated with a theoretical model on the basis of its amino acid sequence (Ptitsyn & Finkelstein, 1983).

The program BELOK used for evaluation of CD data is based on a one-step ridge regression procedure of a data base that contains the CD spectra of 45 proteins whose secondary structure elements are known from X-ray data analysis. The procedure comprises the following steps. From the structure data base, those proteins were at first selected that are characterized by CD spectra similar to that of the 33 kDa protein. The root-mean-square deviation between the CD spectrum of the 33 kDa protein and that of a particular protein k of the data base is given by

$$\sigma_k = [\Sigma_i(\Delta\epsilon_i^{\,k} - \Delta\epsilon_i^{\,33})/n]^{1/2}$$

where $\Delta\epsilon_i^{\,k}$ = the difference of molar absorption coefficients for left and right polarized light at wavelength i of the k-th protein, $\Delta\epsilon_i^{\,33}$ = the same difference for the 33 kDa protein, and n = the number of experimental points. The magnitude of the σ_k values was used as a criterion for the similarities of the secondary structures between the 33 kDa protein and a particular protein k of the data base. The proteins with smallest σ_k volumes were grouped into a "similarity" set.

In the second step, the reliability of the secondary structure prediction was checked for each of the proteins of the "similarity" set. For this purpose, proteins from this set were independently selected and their secondary structures predicted on the basis of their CD spectra. The percentage of secondary structure elements calculated in this way was compared with that known from the X-ray structure analysis. Finally, the root-mean-square deviation, the maximum error, and the correlation coefficient for this "similarity" set were calculated. In the case of statistical reliability, the set was used to calculate the secondary structure elements of the 33 kDa protein from its CD spectrum. The correlation coefficients between the data obtained from CD spectra and X-ray analyses of the "similarity" set were as follows: 0.98, α-helix prediction; 0.96, β-strand prediction; 0.63, turns; 0.81, random coil [for a comparison of this method with other programs for secondary structure prediction from CD spectra, see Yang et al. (1986)].

In an alternative approach, the secondary structure of the isolated spinach 33 kDa protein at different pH values was predicted on the basis of theoretical considerations by using the known amino acid sequence of the 33 kDa protein (Oh-Oka et al., 1986) and the theory of molecular secondary structure presented by Ptitsyn and Finkelstein (1983). This method seems to be more appropriate than the theoretical approaches developed by Chou and Fasman (1978) and Biou et al. (1988), because it permits one to take into account effects owing to a variation of thermodynamic parameters of the protein environment, e.g., the pH value of the medium. On the basis of the computational algorithm emerging from this theoretical approach, a program was developed that is called "ALB" (A. Finkelstein, version 1983 adapted to PC by A. Badretdinov). It predicts probabilities for the existence of secondary structure elements within amino acid sequences. If the probability is high or low, the corresponding structural element will be characterized by the symbols "fixed" or "fluctuated", respectively.

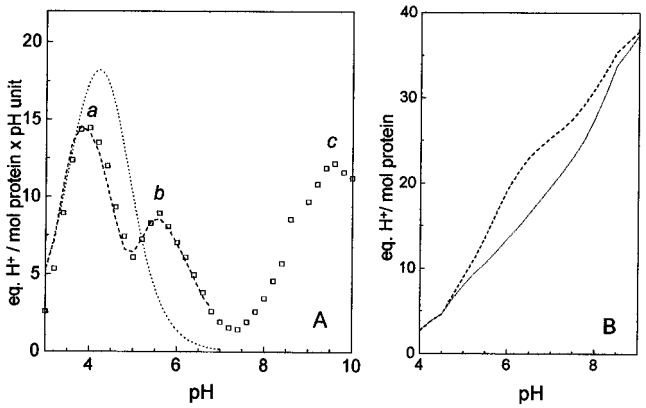

FIGURE 1: Titration of the isolated 33 kDa extrinsic protein from spinach by acid or base. (A) Experimental (open squares) buffer capacity of the 33 kDa protein as a function of pH. Titration was carried out from pH 6.8 by addition of either HCl or NaOH. The dotted and dashed curves were calculated by using the Henderson−Hasselbalch formalism with the following parameters: $N_a = 14$, $pK_a = 3.8$, $N_b = 21$, and $pK_b = 4.4$ (dotted); $N_a = 21.34$, $pK_a = 4.04$, $N_b = 10.79$, and $pK_b = 5.90$ (dashed). (B) Titration curves of the 33 kDa extrinsic protein by acid (dashed line) and base (solid line) from pH 9.0 and pH 4.0, respectively. For experimental details, see Materials and Methods.

Using the pK values of amino acids in solution (Timasheff, 1962) leads to a calculated buffer capacity curve of the protein in the acidic pH region that exhibits marked differences to that obtained by titration measurements. This effect is ascribed to pK shifts of titratable groups (peak *b*, Figure 1A). Since information is lacking on the actual pK values of each amino acid residue, a simplified method was used. In order to account for these pK shift effects, a global influence was assumed as an approximation; i.e., the calculation using program "ALB" was performed with the pK values of the amino acids in solution, but the pH of suspension was shifted by 0.6 pH unit toward the alkaline region.

RESULTS

Titration Experiments. The open square data points of Figure 1A show the buffer capacity of the isolated extrinsic 33 kDa protein as a function of pH obtained from titration experiments as outlined in a former study (Shutova et al., 1993). The buffer capacity curve exhibits two distinct peaks in the acidic range at pH 4.0 (peak *a*) and pH 5.8 (peak *b*), and a band *c* peaking above pH 9.0. An evaluation of this data reveals that within the pH range of 3.0−7.0 (peaks *a* and *b*) approximately 32 H^+ per 33 kDa protein are bound. Likewise, about 24 groups per protein are titrated in the alkaline pH range (band *c*). These findings should be compatible with the content of protonizable amino acid residues of the protein provided that all of them are accessible to the titration. The following analysis was restricted to the range $3.0 \leq pH \leq 7.0$ because the peak in the alkaline range is not well resolved.

It is known that the 33 kDa protein from spinach contains 14 aspartates and 21 glutamates (Oh-Oka et al., 1986). Therefore, in the most simple case of all 35 carboxylic groups being protonizable without any interaction, the pK values reported in the literature [see Timasheff (1962)] could be used to calculate the titration curve. This procedure leads to the dotted curve of Figure 1A. It readily shows that the

experimental results are not reconcilable with the underlying assumptions of the "theoretical curve". A comparison with the data points reveals that the discrepancy mainly originates from using inappropriate p*K* values. Recent detailed studies using reaction centers from anoxygenic purple bacteria have shown that the p*K* values of amino acid residues in proteins can markedly differ from those in solution (Beroza et al., 1995). A detailed analysis as performed by Beroza et al. (1995) is impossible because the detailed structure of the 33 kDa protein is lacking. Therefore, a simplified model was used that comprises two types of protonizable amino acid residues with pK_1 and pK_2. In this way, the experimental results can be well described by an ensemble of 21−22 carboxylic groups ($N_a = 21.34$) with p$K_1 = 4.04$ and 11 groups ($N_b = 10.79$) with p$K_2 = 5.90$. Two conclusions can be drawn from this analysis: (i) almost all of the aspartates and glutamates of the 33 kDa protein participate in protolytic equilibration with the aqueous phase; and (ii) below a fraction of about 60% of deprotonated carboxylic groups, the p*K* value is not markedly changed compared with that of solution, but a higher extent of deprotonation leads to marked shifts of the p*K* values of the remaining amino acids. An assignment to specific residues cannot be achieved. As a consequence of this limitation, a global effect will be taken into account for calculating the theoretical structure based on the ALB method as outlined under Materials and Methods.

The extrinsic 33 kDa protein from spinach does not carry any histidine residues that are capable of binding protons in the neutral pH range. This is consistent with the finding that the buffer capacity exhibits a pronounced minimum around pH 7.5. Protonizable amino acid residues in the alkaline region are tyrosine, lysine, and arginine with p*K* values of 10.1, 10.8, and 12.4, respectively (Timasheff, 1962). The extrinsic 33 kDa protein is rich in lysine (25 residues). It also contains eight tyrosines and four arginines (Oh-Oka et al., 1986). These residues are assumed to be responsible for band *c*.

In conclusion, the buffer capacity of the 33 kDa protein is inferred to be due to the carboxylic groups of dicarbonic amino acids (glutamate, aspartate) in the acidic pH range (peaks *a* and *b*) and basic amino acid residues (lysine, arginine, tyrosine) in the alkaline region (band *c*).

As was pointed out earlier (Shutova et al., 1993), a very interesting feature of the titration curve of the 33 kDa protein is its dependence on the direction of titration; i.e., the p*K* values of the same titratable groups are different in acid−base and base−acid titrations as shown in Figure 1B. This feature is a characteristic property of the 33 kDa protein because it is not observed under the same experimental conditions for the other two extrinsic proteins of 18 and 24 kDa and for the water-soluble protein BSA (data not shown). In this case, the titration curves were practically the same for both (acid and base) titration directions. It is interesting to note that a second (and subsequent) repetition of the acid−base titration cycle of a sample containing the 33 kDa protein gave similar results; i.e., the general phenomenon of a hysteresis remained unchanged (data not shown). Likewise, the acid−alkaline hysteresis in the titration curve of the 33 kDa protein is not eliminated by a 2−3-fold retardation of the titration rate either by addition of ampholytes to the medium or by dilution of the titrant (normal titration time 1 min, see Materials and Methods). Under the same conditions, the p*K* of a phosphate buffer solution used for calibration is independent of the titration direction. The above-mentioned data show that the hysteresis in the titration curve of the 33 kDa protein is a reproducible phenomenon and not related to both diffusion processes in the titration cell and the lag phase of the electrode.

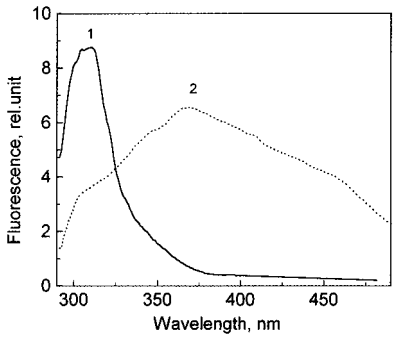

FIGURE 2: Room temperature fluorescence spectra of the 33 kDa protein from spinach. The solid curve (1) represents a set of virtually indistinguishable spectra that were obtained at pH 6.8, 3.8, and 5.2 (corresponding to the p*I* of this protein) and in the presence of 1,8-ANS. The dotted curve (2) shows the spectrum of the 33 kDa protein after modification of the protein by DTE. For experimental details, see Materials and Methods.

In summary, the titration experiments show that among the extrinsic regulatory proteins of the WOC only the manganese-stabilizing 33 kDa protein exhibits an acid−base hysteresis. This phenomenon is not due to irreversible denaturing effects of the protein. Therefore, it has to be concluded that pH-induced structural rearrangements take place within the isolated extrinsic 33 kDa protein. These changes were further analyzed in more detail by three different approaches: (a) fluorescence of endogenous (tryptophan) and exogenous (1,8-ANS) probes that give rise to structure-dependent fluorescence emissions; (b) circular dichroism measurements; and (c) a model-based theoretical structure analysis.

Fluorescence of 33 kDa Protein. An essential structural element of the 33 kDa protein is its intrinsic S−S bridge. Numerous studies have demonstrated the relevance of this S−S bridge for the conformation of the protein and its ability to restore the oxygen evolution activity (Tanaka et al., 1989; Eaton-Rye & Murata, 1989; Irrgang et al., 1992). Accordingly, at first glance it may be attractive to consider the idea that the hysteresis observed in the pH titration experiments is associated with a cleavage of this S−S bridge during the titration. However, a closer inspection reveals that this is highly unlikely (Torchinskii, 1974).

In order to exclude the possibility that the S−S bridge of the 33 kDa protein could exhibit a particular sensitivity to pH-induced cleavage, measurements of the tryptophan fluorescence were performed. According to Tanaka et al. (1989), the native conformation of the 33 kDa protein is characterized by a fluorescence spectrum of the tryptophan residue having a maximum at 310 nm. A marked structural change of this protein induced by reduction of the disulfide bridge results in a significantly wider spectrum and a pronounced shift of its maximum to 350 nm. This phenomenon has been explained by the transfer of the tryptophan residue from a hydrophobic to a hydrophilic environment (Tanaka et al., 1989). Figure 2 shows fluorescence spectra of the 33 kDa protein at different pH values and for that structurally modified by DTE. Clearly the emission spectra

are virtually independent of pH but exhibit the expected drastic changes after cleavage of the S−S bond. These data prove that the S−S bridge remains intact under the acid−base titration conditions used in this study. Furthermore, based on the tryptophan fluorescence measurements, it can be inferred that the presumed pH-dependent conformational changes of the 33 kDa protein which are responsible for the hysteresis effect do not comprise gross structural rearrangements.

Hydrophobic Fluorescence Probe 1,8-ANS. A simple fluorometric probe technique was applied for further investigations of the pH-dependent structural changes within the extrinsic 33 kDa protein. This method is based on the finding that intermediary states of a protein can be identified by monitoring the fluorescence spectrum of the hydrophobic fluorescent probe 1,8-ANS. Its emission spectrum depends on the accessibility of hydrophobic domains within the protein to the water-soluble fluorophore (Semisotnov et al., 1991). The binding of the 1,8-ANS probe could induce structural changes in the 33 kDa protein, thus giving rise to artifacts. To test this possibility, the tryptophan fluorescence of the 33 kDa protein was measured. It was found that in the presence of 1,8-ANS the maximum of the tryptophan fluorescence remained virtually unaffected (solid curve in Figure 2). This finding shows that 1,8-ANS itself does not induce significant structural changes and therefore this probe appears to be a suitable tool to monitor pH-dependent conformational changes of the 33 kDa protein.

It was shown by Semisotnov et al. (1991) that a β-sheet domain is more hydrophobic than an α-helix and therefore gives rise to a higher affinity to 1,8-ANS. At pH 6.5, the fluorescence of 1,8-ANS is virtually the same in both the presence and absence of the 33 kDa protein (data not shown). As the 33 kDa protein contains a relatively large proportion of β-structure [see Xu et al. (1994) and the results presented in the following section], it may be concluded that at neutral pH this hydrophobic β-structure is buried within the interior of the protein and therefore not accessible to ANS. However, this is obviously not the case at other pH values where significant changes of 1,8-ANS fluorescence are observed upon addition of the 33 kDa protein whereas the spectrum in protein-free buffer solutions remains invariant to pH in the range from 3 to 8 (data not shown). The spectra depicted in Figure 3A show that a shift from pH 7.0 to pH 3.8 leads to both a significant increase of the emission intensity and a blue-shift of 10−13 nm of the fluorescence maximum of 1,8-ANS (compare spectra 1 and 2 of Figure 3A). It was shown that the affinity of 1,8-ANS to proteins significantly increases when the rigidity of their tertiary structures is weakened without changing the general secondary structures of the polypeptides (Semisotnov et al., 1991); i.e., their hydrophobic cores are not destroyed but become more accessible to the fluorophore than in their native states. Taking this feature into account, it can be concluded: (i) the 33 kDa protein is characterized by the presence of a hydrophobic core that is not accessible to the aqueous phase at neutral pH; (ii) protonation of dicarbonic amino acid residues of the extrinsic 33 kDa protein influences the tertiary (and secondary?) structure of the polypeptide; and (iii) the conformation of the 33 kDa protein at pH 3.8 is characterized by a higher accessibility of the hydrophobic core to the aqueous phase.

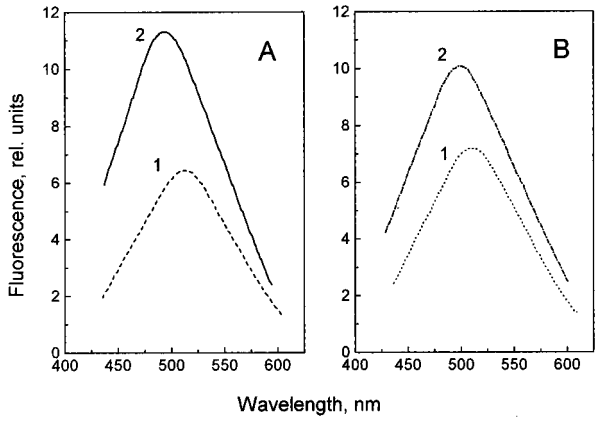

FIGURE 3: Emission spectra of the hydrophobic fluorescent probe 1,8-ANS added to the 33 kDa protein at different pH values of the medium (λ_{exc} = 346 nm). (A) Spectrum 1 measured at pH 7.0 and spectrum 2 at pH 3.8. (B) Both spectra were measured at pH 5.6 by adding either HCl to the protein kept at pH 7.0 (curve 1) or NaOH to protein kept at pH 3.8 (curve 2).

Figure 3B illustrates the difference between fluorescence spectra of 1,8-ANS in solutions of the 33 kDa extrinsic protein that were adjusted to pH 5.6 following two different protocols: spectrum 1 is obtained when the protein incubated at pH 7.0 is transferred within a few seconds to pH 5.6 by the addition of a HCl aliquot whereas spectrum 2 is obtained with a sample undergoing a similar pH change from pH 3.5 to pH 5.6 by addition of a KOH aliquot. The spectra are clearly different in both the position of their maxima and the extent of their emissions, depending on the pH used for preincubation of the protein. This result correlates with the titration experiments and also reveals an acid−base hysteresis in the structural rearrangement of the extrinsic 33 kDa protein. After incubating these two samples for 10 min at pH 5.6, the difference between their spectra disappeared (data not shown). Accordingly, after a sufficiently long incubation, a true equilibrium state of protonation is achieved that is independent of the pH at the beginning provided that irreversible changes (e.g., at pH 2.8, vide infra) can be excluded. On the other hand, the pH-induced fluorescence increase of the 1,8-ANS−protein complex at pH 3.8 remained stable even after a rather long incubation time (∼1 h).

Circular Dichroism Spectra of the 33 kDa Protein. In order to gain further information on pH-induced conformational changes of the 33 kDa protein, circular dichroism (CD) spectra were measured in the near (275−305 nm) and far (184−260 nm) UV region. Figure 4A shows CD spectra in the far-UV region of the 33 kDa protein at three different pH values. It is seen that a decrease to pH 3.8 virtually does not affect the CD spectrum while further acidification to pH 2.8 leads to significant structural changes.

Table 1 compiles the percentage of secondary structure elements at pH 6.8 and pH 3.8 calculated from the CD spectra on the basis of the program BELOK (see Materials and Methods). The results show that the secondary structure of the polypeptide is practically the same at pH 6.8 and at pH 3.8. The following values were obtained: 7−8% of α-helix, 32−33% of β-strand, 14−15% of turns, and 43−44% of random coil. These values are in good agreement with the recently reported data of Xu et al. (1994) (see Table 1). Problems, however, arise for the calculation at pH 2.8 because the data base does not contain proteins with CD

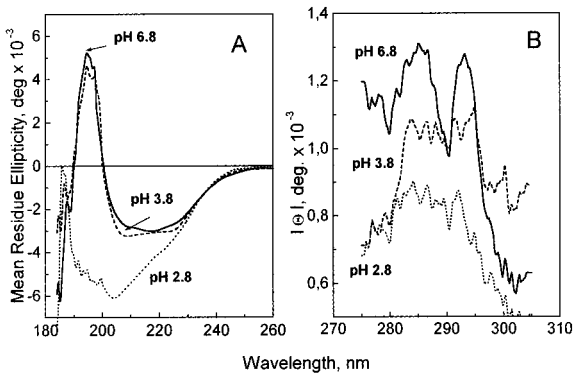

FIGURE 4: Far- (A) and near-UV (B) circular dichroism spectra of the 33 kDa protein at pH 6.8, 3.8, and 2.8. Protein concentration was 30 μM in a medium containing 10 mM phosphate buffer and 10 mM NaCl. For details, see Materials and Methods.

Table 1: Content (%) of Secondary Structure Elements of Spinach 33 Protein[a]

secondary structure elements	CD data, BELOK		CD data (Xu et al., 1994), pH 7.0	FTIR data (Ahmed et al., 1995)
	pH 6.8	pH 3.8		
α-helix	7	8	9	27
β-strand	33	32	38	36
turns	15	14	17	24
random coil	43	44	35	13

[a] Contents were obtained from the far-UV CD spectra shown in Figure 4A by using the program BELOK and comparison with data reported in the literature that were gathered either from the CD spectrum by using singular value decomposition methods and an unconstrained analysis (Xu et al., 1994) or from FTIR data (Ahmed et al., 1995).

spectra of very high similarity. Therefore, only a qualitative trend can be gathered from the CD spectrum at pH 2.8: the decrease of pH from 3.8 to 2.8 is inferred to cause a decrease in the percentage of α-helix and turns while that of β-structures and random coil increases. It has to be emphasized that the structural changes induced at pH 2.8 are reversible if the protein is rapidly transferred back to higher pH. After an incubation time of about 1 min at pH 2.8, the structure of the 33 kDa protein in solution is irreversibly modified.

Figure 4B shows the CD spectra obtained in the near-UV region at three different pH values. In this case, effects are monitored that arise from individual amino acid residues, thereby providing a sensitive probe of their local environments. The major contributions to the near-UV circular dichroism of proteins originate from the amino acids tyrosine and tryptophan, and the disulfide bridge, while that of phenylalanine is generally much weaker (Kahn, 1979; Strickland, 1976).

In its quasi-native state at pH 6.8, the 33 kDa protein exhibits two well-resolved positive CD bands with peaks at 285.5 and 293.5 nm. The CD band with a maximum at 293.5 nm is likely reflecting the long wavelength maximum of the L_b transition of the only tryptophan residue (W241) of the 33 kDa protein. This assignment is based on findings indicating that in proteins the maxima of L_b transitions of tryptophan residues are typically observed in the 288−293 nm region of the CD spectra (Goodman & Toniolo, 1968; Strickland, 1976).

The band with a maximum at 285.5 nm might also be attributed to W241, i.e., the short maximum of the L_b transition that was found to be characterized by a maximum in the range of 281−286 nm for tryptophan residues in proteins (Strickland, 1976). Alternatively, the 285.5 nm band could originate from tyrosine residues, previously shown to exhibit a CD maximum in the region of 281−289 nm (Strickland, 1976). Usually, the longer maximum in the near-UV region of the CD spectra of proteins is attributed to tryptophan residues while the band near 285 nm is ascribed to tyrosine residues (Strickland, 1976). Therefore, it is reasonable to ascribe the 285.5 nm band to one or more of the eight tyrosines of the 33 kDa protein. These two bands—at 293.5 nm and at 285.5 nm—disappear upon acidification (see Figure 4B): at pH 3.8, the ellipticity is changed and becomes significantly modified upon further acidification down to pH 2.8.

A complete decrease of all CD bands in the near-UV region was also observed by Tanaka et al. (1989) after cleavage of the S−S bridge. In this report, however, also the fluorescence attributed to W241 markedly changed while in our experiments at pH 3.8 the fluorescence maximum of W241 remained virtually unaffected (see *Fluorescence of 33 kDa Protein*). It is therefore concluded that at pH 3.8 the symmetry of the W241 environment differs from that at pH 6.8 but this alteration occurs within the nonaqueous phase rather than being caused by complete transfer from the nonaqueous to the aqueous environment.

In summary, the analysis of the CD spectra presented in this study leads to the conclusion that a transition from pH 6.8 to 3.8 causes an increase of the rotational freedom of aromatic residues buried in the interior (near-UV CD) without markedly affecting the secondary structure of the polypeptide chain in the 33 kDa protein (far-UV CD). Further acidification down to pH 2.8 induces drastic structural changes. In extending these studies, we attempted to localize the secondary structure elements of these pH-induced changes within the amino acid sequence by model calculations.

Modeling of the 33 kDa Protein Secondary Structure. The results of the analysis for the 33 kDa protein at three different pH values are depicted in Figure 5. It shows different regions of the polypeptide chain predicted to form α-helices, β-sheets, or turns. In order to test the reliability of these predictions, the overall content of the secondary structure elements calculated by the ALB program was compared with the corresponding values gathered from evaluations of CD spectra. Table 2 compiles the data obtained by the two different approaches and in addition compares our results with theoretical predictions reported recently by Beauregard (1992) and Xu et al. (1994).

An inspection of these data reveals that according to the theory of Ptitsyn and Finkelstein (1983) the extrinsic 33 kDa protein from spinach is expected to contain a total content of 8% and 20% of "fixed" α-helix and β-strands, respectively, at pH 6.8. In addition, this method predicts 13% and 28% of "fluctuated" α-helix and β-strand domains, respectively. A value of 8% for the α-helix content is in perfect agreement with the conclusions gathered from CD data (see Table 1). Therefore, the four "fixed" α-helices predicted by the "ALB" algorithm and marked in Figure 5 by α1−α4 are considered to be real secondary structure elements while the "fluctuated" helices of markedly lower probability (see Materials and Methods) are unlikely to exist. The situation is somewhat different for the predicted β-strands. The calculated total content of 20% "fixed" β-strands is markedly

Table 2: Contents (%) of Secondary Structure Elements of the 33 kDa Protein[a]

secondary structure elements	far-UV CD data analysis CD data, BELOK (pH 6.8)	theoretical prediction based on protein sequence			
		Beauregard (1992)	Chou−Fasman (Xu et al., 1994)	"fixed" ALB prediction	"fluctuating" ALB prediction
α-helix	7	13	28	8	13
β-strand	33	9	25	20	28
turns	15		12	16	
random coil	43		35		

[a] Gathered from the CD spectrum at pH 6.8 (Figure 4A) by data evaluation with the program BELOK compared with values calculated on the basis of its amino acid sequence by using a theoretical model (Ptitsyn & Finkelstein, 1983) and other theoretical predictions reported in the literature (Beauregard, 1992; Xu et al., 1994).

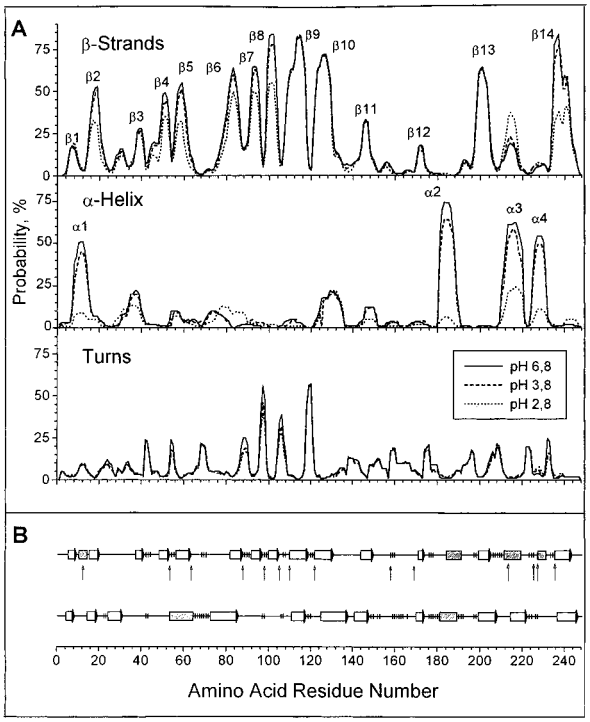

FIGURE 5: (A) Probability of secondary structure formation along the polypeptide chain of the extrinsic 33 kDa protein from spinach at different pH values: pH 6.8, pH 3.8, and pH 2.8. (B) Prediction of secondary structure elements in the 33 kDa protein from spinach (at pH 6.8) (upper part). For comparison, the recent data of Xu et al. (1994) are presented (lower part). Shaded box, α-helix; arrow, *β*-strands; vertical line, turns. Conserved amino acids with carboxylic groups are marked by vertical arrows.

lower than that of corresponding values derived from CD data (see Table 1). On the other hand, the sum of the percentages of "fixed" plus "fluctuated" *β*-strands is unrealistically large. Accordingly, based on a comparison with the spectral data compiled in Table 1, it appears reasonable to assume that only a fraction of the "fluctuated" *β*-strands is really existing. The most likely candidates are those marked by *β*1, *β*3, *β*11, and *β*12 while another one near the C-terminus of the protein (T210−F217) is highly unlikely because the model predicts a "fixed" α-helix (α3) for the same region. Based on these considerations, the 33 kDa protein is expected to form 14 *β*-strands at physiological pH values. A cluster of five *β*-strands (*β*6−*β*10) is located in the middle part of the protein sequence. The prediction of a loop between these adjacent and buried *β*-strands suggests that they are antiparallel, stabilize each other, and very likely form a long *β*-sheet domain. A comparison between the predicted secondary structure and the hydrophobicity properties (data not shown) reveals that in the central *β*-sheet domain a hydrophobic region exists around *β*9 and *β*10. It is possible that the compact hydrophobic *β*-sheet domain comprises also three additional *β*-fragments: *β*3, *β*4, and *β*5, together with turns. The last two *β*-strands (*β*13 and *β*14) are localized at the C-terminal side which also contains three α-helices (α2, α3, and α4). It has been speculated that region T84−T114 forms a calcium-binding site (Wales at al., 1989; Webber & Gray, 1989). The C-terminal side is predicted to be comparatively rich in α-helices. Helix α2 is located in the area of maximum hydrophilicity of the primary structure of the 33 kDa protein and forms a compact arrangement of Asp and Glu residues with high negative charge. As the locus K159−K186 including helix α2 is not accessible to the bulk solvent when the protein is associated with PS II (Frankel & Bricker, 1995), it is assumed that the 33 kDa protein forms a hydrophilic pocket enriched in carboxylic groups near helix α2.

A comparison of the secondary structure elements which are predicted at pH 6.8 and at pH 2.8 reveals that an almost complete protonation of all dicarbonic amino acid residues leads to a marked decrease of the α-helix content. According to the calculation, there is virtually no fixed helix content and only a small content (∼5%) of fluctuating α-helix structure at pH 2.8. In contrast to the α-helix content, the probability of *β*-structure domains is predicted to be less sensitive to proton concentration: a decrease is expected for the *β*-strands near the N-terminus, i.e., *β*2, *β*4, *β*5, and *β*14 near the C-terminus and in the center *β*-sheets: *β*6, *β*7, and *β*8. The α-helix in the region T210−L220 is almost completely converted into a *β*-structure at pH 2.8.

A comparison with the results obtained by another theoretical approach (Xu et al., 1994) is shown in Figure 5B. An inspection of the data reveals striking similarities with respect to the existence of an extended *β*-strand domain in the middle of the protein and the location of α-helix α2. On the other hand, different predictions were made for the location of additional α-helices. At present, it is difficult to find criteria in favor of one of the theoretical approaches, but it has to be emphasized that the "ALB" method correctly predicts that only minor changes of the secondary structure take place when the 33 kDa protein is transferred from pH 6.8 to 3.8 while drastic effects arise when going further down to pH 2.8. In an attempt to gather further information, the "ALB" algorithm was applied to the sequences of 33 kDa proteins from four different species. A very interesting feature emerges from this comparative analysis: the hydrophilic helix α2 (E181−N188) and the extended *β*-sheet core in the middle of the protein chain are conserved in all species that were analyzed (Shutova et al., 1995). Furthermore, these structural elements are also predicted by the theoretical approach of Chou and Fasman as outlined by Xu et al. (1994)

(see Figure 5B). Based on these findings, the α-helix in the hydrophilic region (E181−N188) and the extended β-sheet domain are assumed to be of structural and/or functional relevance for the 33 kDa protein.

DISCUSSION

The experimental data and theoretical modeling analysis of the present study led to the following findings and conclusions on the 33 kDa protein in solution: (i) binding and release of protons within the range $7.0 \leq pH \leq 3.8$ exhibit a characteristic reversible acid−alkaline hysteresis which is specific for the 33 kDa protein and not found for the other extrinsic proteins of 17 and 23 kDa or soluble proteins like BSA; (ii) the secondary structure of the polypeptide chain remains virtually unaffected in this pH range whereas the preserved hydrophobic core becomes more accessible to the aqueous phase and the rotational freedom of aromatic amino acid residues is increased upon acidification to pH 3.8; a further decrease of the pH down to 2.8 causes dramatic changes of the secondary and tertiary structure; (iii) there exist two highly conserved secondary structure domains: an α-helix in the hydrophilic region (E181−N188) near the C-terminus of the 33 kDa protein and an extended β-sheet core in the middle of the protein chain (Shutova et al., 1995).

The possible relevance of these features for the 33 kDa protein in its native membrane-bound form remains to be discussed. The acid−base hysteresis is assumed to be of structural and/or functional relevance because this property was found to be unique for the 33 kDa protein. In this respect, it has to be kept in mind that *in vivo* the pH of the lumen decreases upon illumination [down to values below pH 5 under saturating light; see Siggel (1975)]. This process can be comparatively rapid (Tiemann et al., 1979), and the protonation of the 33 kDa protein would follow the "acidic" titration of Figure 1B (dashed curve). On the other hand, in the dark the light-induced ΔpH disappears and the deprotonation should occur according to the "alkaline" titration curve of Figure 1B (dotted curve). This phenomenon could be of physiological relevance in regulating the adaptation of the WOC to different pH values of the lumen. In this respect, it is interesting to note that based on recent studies structural changes were inferred to take place in the WOC at pH 5.3−5.6 and 6.2−6.5 which are independent of the redox state S_i (Messinger & Renger, 1994) and affect the accessibility to exogenous reductants (Kebekus et al., 1995). It is therefore attractive to speculate that the 33 kDa protein and its structural changes unraveled in the present study are involved in these phenomena.

Another functionally important point should be briefly considered. It is now well established that proteins play an essential role for protolytic reactions of the WOC [for the latest review, see Haumann and Junge (1996)] and the 33 kDa protein is a very likely candidate to participate in the proton transport pathway into the lumen. In this respect, the predicted conserved secondary structure elements α-helix (E181−N188) and the extended β-sheet domain are of particular interest because both contain conserved protonizable amino acids (Asp, Glu) (see Figure 5B). It is therefore reasonable to assume that these parts of the protein could form hydrogen bond networks that function as proton wires between the catalytic site of water oxidation and the lumen. Further experiments are required to test this idea.

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