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Biochimica et Biophysica Acta 1604 (2003) 95–104
BIOCHIMICA ET BIOPHYSICA ACTA
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Comparative studies on the properties of the extrinsic manganese-stabilizing protein from higher plants and of a synthetic peptide of its C-terminus ☆

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Received 18 December 2002; received in revised form 26 February 2003; accepted 19 March 2003

Abstract

The present study describes a comparative analysis on the fluorescence properties of the manganese-stabilizing protein (MSP), a synthetic peptide corresponding to its C terminus and a 7:1 (molar ratio) mixture of *N*-acetyl-tyrosine and *N*-acetyl-tryptophan, respectively, together with reconstitution experiments of oxygen evolution in MSP-depleted photosystem II (PS II) membrane fragments. It is found: (i) at neutral pH, the fluorescence from Trp^{241} is strongly diminished in MSP solutions, whereas it highly dominates the overall emission from the C-terminus peptide; (ii) at alkaline pH, the emission of Tyr and Trp is quenched in both, MSP and C-terminus peptide, with increasing pH but the decline curve is shifted by about two pH units towards the alkaline region in MSP; (iii) a drastically different pattern emerges in the 7:1 mixture where the Trp emission even slightly increases at high pH; (iv) the anisotropy of the fluorescence emission is wavelength-independent (310–395 nm) and indicative of one emitter type (Trp) in the C-terminus peptide and of two emitter types (Tyr, Trp) in MSP; and (v) in MSP-depleted PS II membrane fragments the oxygen evolution is restored (up to 85% of untreated control) by rebinding of MSP but not by the C-terminus peptide, however, the presence of the latter diminishes the restoration effect of MSP. A quenching mechanism of Trp fluorescence by a next neighbored tyrosinate in the peptide chain is proposed and the relevance of the C terminus of MSP briefly discussed.

Keywords: Photosystem II; MSP; C-terminus synthetic peptide; Reconstitution of oxygen evolution; Tyrosine and tryptophan fluorescence

1. Introduction

Photosynthetic water oxidation to molecular oxygen takes place at a manganese-containing unit, the water oxidizing complex (WOC) that is incorporated into the membrane integral protein matrix of photosystem II (PS II) (for a review see Ref. [1] and references therein). Extrinsic proteins bound to the lumenal side of PS II are of physiological relevance as stabilizing and regulatory subunits. Among these the protein encoded by the *psbO* gene and an apparent mol. wt. of 33 kDa is unique because it is the constituent of PS II in all oxygen evolving organisms, in marked contrast to other extrinsic polypeptides that were entirely changed during evolutionary development from cyanobacteria to plants (see Ref. [2] and references therein). The PsbO protein is essential for stabilizing the manganese cluster and therefore often referred to as "manganese-stabilizing protein" (MSP). Extraction of this protein strongly diminishes the rate of oxygen evolution under saturating continuous wave (CW) illumination and affects the turnover and stability of the higher redox states of the WOC [3–5]. Recently, based on different susceptibilities to digestion by trypsin, the structure of the bound MSP was inferred to depend on the redox state of the manganese

Abbreviations: CW, continuous wave; PS II, photosystem II; MSP, manganese-stabilizing protein; PsbO, PS II product of *psbO* gene=MSP; CP47, chlorophyll-*a* containing 47-kDa polypeptide; Trp, tryptophan; Tyr, tyrosine; WOC, water oxidizing complex

☆ Part of this work has been presented at the XII International Congress on Photosynthesis in Brisbane (Australia).

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doi:10.1016/S0005-2728(03)00025-2

cluster [6]. In addition to the stabilizing effect the MSP is also known to regulate the Cl^- and Ca^{2+} demand of the WOC (see Refs. [7,8] and references therein). These findings reveal that the MSP is of special relevance for a functionally competent WOC. However, the structural determinants for MSP binding and functioning are not yet clarified. At present, even the stoichiometry of the MSP copies per PS II is a matter of controversial discussion [9–12]. In a recent study [13] evidence has been presented for the existence of two domains in the N terminus of MSP from algae and higher plants that are involved in subunit binding to PS II. Both domains are required for binding of two MSP/PS II and maximum reconstitution of the oxygen evolution capacity, whereas only one domain (^{15}T–^{18}E) is sufficient to bind one copy concomitant with 50% reconstitution of WOC activity. Based on sequence alignment, the MSPs from cyanobacteria were inferred to contain only the latter type of domain and consequently these organisms bind only one copy per PS II [13], in line with recent structural analysis from X-ray crystallographic data [14]. Differences among the PsbO proteins have been reported previously [8,15] together with the finding that in green algae the PsbO protein is required for stable assembly of the WOC [16–18] whereas *psbO* deletion mutants of cyanobacteria are still able to evolve oxygen [19–22]. The characteristics of *psbO* genes can be successfully applied for tracing the evolution from prokaryotic to eukaryotic species [23] and to construct phylogenetic trees for plastid development as outlined recently [24].

In spite of these striking differences, it was shown that in MSP depleted PS II samples from plants the O_2 evolution can be restored with the PsbO protein from cyanobacteria and vice versa [2]. Interestingly, an even higher thermal stability of O_2 evolution activity is achieved in PS II membrane fragments from spinach when via reconstitution experiments the original MSP is replaced by a recombinant MSP from *Thermosynechococcus elongatus* [25]. This finding is indicative of conserved structural determinants for the interaction between the PS II integral part and MSP. Several lines of evidence indicate that the N terminus interacts with a domain in the large lumenal loop E of the integral component CP47 of the PS II core complex [26–28]. Recently, however, also the C terminus was shown to be critical for binding to PS II and restoration of O_2 evolution [29]. This conclusion is supported by the finding that chemical modification of amino acid residues in the C terminus, i.e. in the regions of D_{157}–D_{168} and E_{212}–Q_{247}, is only observed for the MSP in solution [30]. Furthermore, cross-linking between Lys^{48} and Glu^{246} revealed that N- and C-terminus domains are close together [31] so that both can be involved in MSP binding to PS II. In a recent study it was convincingly shown that also the domain Val_{148}–Gly_{163} containing several key charged residues is of relevance for functional interaction of MSP with PS II [32]. A very clear illustration of structural constraints for functional competence of the MSP has been obtained in *Arabidopsis thaliana* that contains two genes (*psbO* and *psbO2*) for encoding the proteins that differ in the mature form by only about 10 amino acid residues. Although most characteristics like the conserved Cys residues giving rise to an S–S bridge and of a Val residue which is important for β-sheet formation are retained, only the *psbO* gene product can provide full functional competence [33].

One way to gather information on the pecularities of the MSP is a detailed analysis of the properties of the isolated protein in solution. Using this approach, the secondary structure was found to be characterized by a high content of antiparallel β-sheets and turns together with a low fraction of α-helices [34–37]. Based on the observation of a high stability to thermal degradation, the MSP was inferred to attain a natively unfolded structure in solution [38]. On the other hand, an analysis of the hydrodynamic and optical properties favors the idea of a molten globule structure [39]. Apart from this particular question, the MSP exhibits unique properties. Striking features are the content of several carboxylic groups with unusually high pK_a values of about 5.7 and the pronounced hysteresis in pH titration of opposite direction [35,36,40]. As outlined by Shutova et al. [35,39], these properties suggest that the MSP might participate in proton transfer from the WOC into the lumen through a hydrogen bond network. In addition, it might exert a regulatory role when the lumen becomes acidified in the light down to pH of about 5 [41] and re-equilibrated in the dark.

The PsbO protein of higher plants contains a single tryptophan (Trp) residue that is strictly conserved in the MSP of all green algae and higher plants (10 different species) reported so far (see Ref. [32]). Since the tryptophan fluorescence is sensitive to changes of its microenvironment, this property is one of the most widely used tools to probe the structure, dynamics and folding/unfolding of proteins (for a review see Ref. [42]). It is therefore worth to analyze the fluorescence properties of isolated MSP from higher plants that contains a single Trp residue and a next neighbor Tyr residue (conserved in all MSP sequences analyzed so far, see Ref. [32]) located in the C-terminus domain. In a recent study the fluorescence spectrum of MSP from spinach in solution was inferred to be the composite of two emitters: tyrosine(s) and W241 with the latter exhibiting an unusual strong quenching [43]. The origin of this feature is not clarified. The present communication describes comparative studies of the properties of the MSP and a synthetic peptide corresponding to that of the C terminus of MSP. It is shown that the neutral tyrosine Y242 exerts at most only weak quenching efficiency on the Trp W241 emission whereas the tyrosinate anion gives rise to rather strong effects. The implications of these findings are discussed.

2. Materials and methods

The MSP was isolated by a salt-washing of PS II membrane fragments as described in Shutova et al. [43].

In order to avoid loss of reconstituted oxygen evolution, the protein samples were not lyophilized but further purified by chromatography on a DEAE-Sepharose 6-B. The homogeneity of the protein was checked via SDS/urea/PAGE and the protein concentration spectrophotometrically determined at 276 nm by using an extinction coefficient of 16 mM^{-1} cm^{-1} as outlined in Ref. [9]. Prior to the measurements, the protein was additionally dialyzed against phosphate/NaCl buffer, pH = 7.2 and concentrated. The synthetic peptide was obtained from INNOVAGEN, Lund, Sweden. The molecular weight of the peptide was confirmed by Mass Spectral Analysis (MALDI.TOF VOYAGER). Spectroscopic measurements were corrected for spectral sensitivity of the instruments. The band width did not exceed 2 nm. The sample concentration was adjusted by using the optical density. Anisotropy measurements were performed as described in Ref. [44] and calculated according to the equation:

$$A(\lambda) = \frac{F_{VV}(\lambda) - G \times F_{VH}(\lambda)}{F_{VV}(\lambda) + G \times F_{VH}(\lambda)}$$

where $F_{VV}(\lambda)$ and $F_{VH}(\lambda)$ are fluorescence intensities at wavelength λ when the polarizers are either both vertically oriented (index VV) or when the excitation polarizer is vertically and the emission horizontally oriented (index VH) (the polarizers are located in front of and behind the cuvette). The correction factor $G = F_{HV}/F_{HH}$ (F_{HH} is measured when both polarizers are of horizontal orientation) accounts for different instrument responses for vertically and horizontally polarized light.

The reactivation of the oxygen evolution in salt-washed PS II membranes with freshly prepared MSP was performed according to the procedure described by Lydakis-Simantiris et al. [38] and the oxygen evolution rate measured with a Clark-type electrode (HANSATECH, Norfolk, UK). Values of about 600 μmol O_2/mg Chl*h were monitored for untreated control samples with 1 mM $K_3[Fe(CN)_6]$ and 0.5 mM 2.6-dichloro-1.4-benzoquinone (DCBQ) as electron acceptor. In $CaCl_2$-washed samples, about 25% of the original activity was retained but this value markedly dropped down after 1 min of illumination with saturating light while the rate of the untreated control remained virtually constant over several minutes. After incubation with MSP at saturating concentration, about 85% of the original oxygen evolution capacity was restored.

3. Results and discussion

3.1. Fluorescence spectra

In our former report [43] it was inferred that the overall fluorescence spectrum of the native MSP in solution strongly depends on the excitation wavelength and reflects the superposition of the emission from two types of emitters: the buried W241 giving rise to a fluorescence band peaking at 329.5 nm, and tyrosine(s) leading to a band with a maximum at 306 nm. Selective excitation of the tryptophan emission by 297-nm light causes comparatively weak fluorescence emission centered at 330 nm. The extent of Trp fluorescence is markedly enhanced (the quantum yield increased by a factor of about seven) and its peak position gradually shifts towards longer wavelengths when MSP is incubated with the denaturing agent guanidinium hydrochloride (Gua-HCl) (data not shown). This phenomenon is indicative of a strong quenching of W241 emission located in a hydrophobic microenvironment in native MSP in solution. In a recent study it was postulated that Trp^{510} in the globular "head" domain of the myosin molecule (from rabbit skeletal muscle) is strongly statically quenched by electronic coupling with Tyr^{503} and that this quenching decreases owing to structural changes induced by nucleotide binding [45]. Accordingly, this type of Trp quenching might also exist in MSP.

In order to obtain further information, comparative measurements of the fluorescence properties were performed with buffer solutions containing either a mixture of *N*-acetyl-tyrosine-amide and *N*-acetyl-tryptophan-amide (for the sake of abbreviation in the following referred to as Tyr/Trp mixture) or a synthetic peptide corresponding to the C terminus of the MSP including W241 and Y242 and the native MSP.

Fig. 1 shows the fluorescence spectra of solubilized MSP (full-lined curve 1), of the synthetic C-terminus peptide KDVKIQGVWYAQLES (dash-lined curve 2) and of a 7:1 molar Tyr/Trp mixture in aqueous solution (dot-lined curve 3), excited either at 275 nm (Panel A) or at 297 nm (panel B). For the sake of comparability, nearly the same content of the tryptophan chromophore was used in the three sample types. An inspection of this data readily shows drastic spectral differences between the systems. The most remarkable features are: (i) at both excitation wavelengths the relative contribution of the Trp fluorescence to the overall emission is markedly diminished in the native MSP in solution compared with that of the 7:1 Tyr/Trp mixture and the C-terminus peptide (compare curves 2 and 3 with curve 1); and (ii) regardless of the excitation wavelength λ_{ex}, the fluorescence spectra of the synthetic peptide (which contains one tyrosine and one tryptophan residue) are highly dominated by the tryptophan emission, in sharp contrast to the MSP spectra and their strong dependence on λ_{ex} (compare curves 1 and 2 in panels A and B). These findings indicate that at neutral pH a Tyr residue next to Trp within a peptide does not lead to significant quenching of the fluorescence emission of the latter in a hydrophilic environment. If one assumes that this phenomenon is not markedly dependent on the polarity of the microenvironment, it can be concluded that W241 quenching in the native MSP is not caused by the neighbored protonated Y242, but probably arises from interaction with another quencher, with the S–S bridge being a potential candidate as outlined by Shutova et

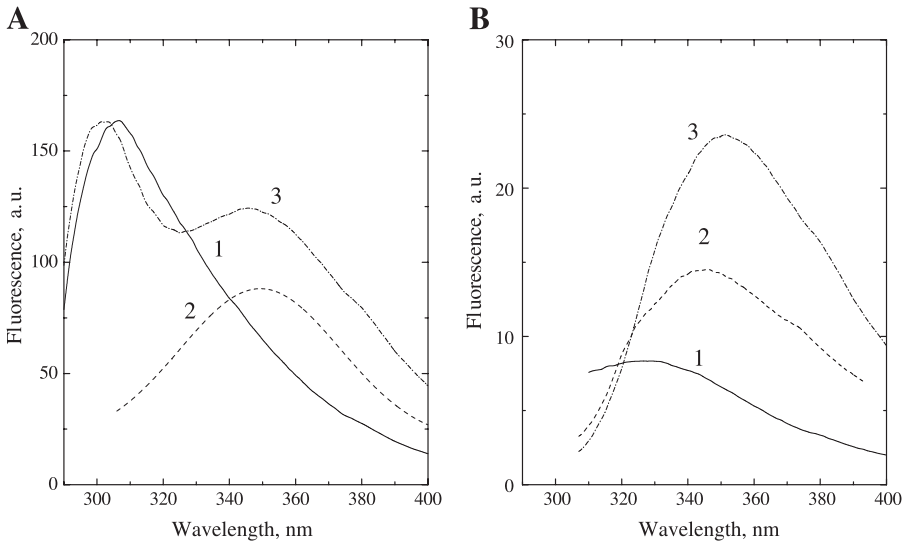

Fig. 1. Fluorescence emission spectra of the native MSP (curve 1), a synthetic C-terminus peptide (curve 2) and a 7:1 (molar ratio) mixture of *N*-acetyl-Tyr amide and *N*-acetyl-Trp amide, respectively (curve 3). The samples were excited at 275 nm (panel A) or at 297 nm (panel B). The concentrations of all the samples were adjusted to optical density of 0.2.

al. [43]. The latter idea is highly supported by recent studies on the quenching of 3-methylindole fluorescence by amino acid side chains with the S–S bridge acting as most powerful species [46].

3.2. pH dependence of fluorescence emission

Compared with the findings at neutral pH, a totally different pattern emerges at alkaline pH as is illustrated in Fig. 2. The left panel (A) shows that the Trp fluorescence of the C-terminus peptide becomes drastically diminished at pH 12.3. To check for the trivial effect of fluorescence suppression of isolated Trp at high pH, experiments were performed with solutions of *N*-acetyl-tryptophan-amide. The data obtained and depicted in the right panel (B) of Fig. 2 reveal that the emission exhibits even a slight increase at high pH. Therefore, the most likely explanation for the C-terminus peptide feature is a quenching effect by the neighbored tyrosinate anion that is formed at high pH.

In order to analyze this possibility in more detail, pH-titration studies were performed in different sample types. Fig. 3 summarizes the results obtained for the pH-dependencies of the normalized amplitudes of Tyr and Trp fluorescence in solutions of the C-terminus peptide and the 7:1 Tyr/Trp mixture. A comparison of this data reveals: (a) the normalized extent of the bands owing to both Tyr (measured at 305 nm, open circles) and Trp (measured at 360 nm, filled circles) emission of the C-terminus peptide exhibits a

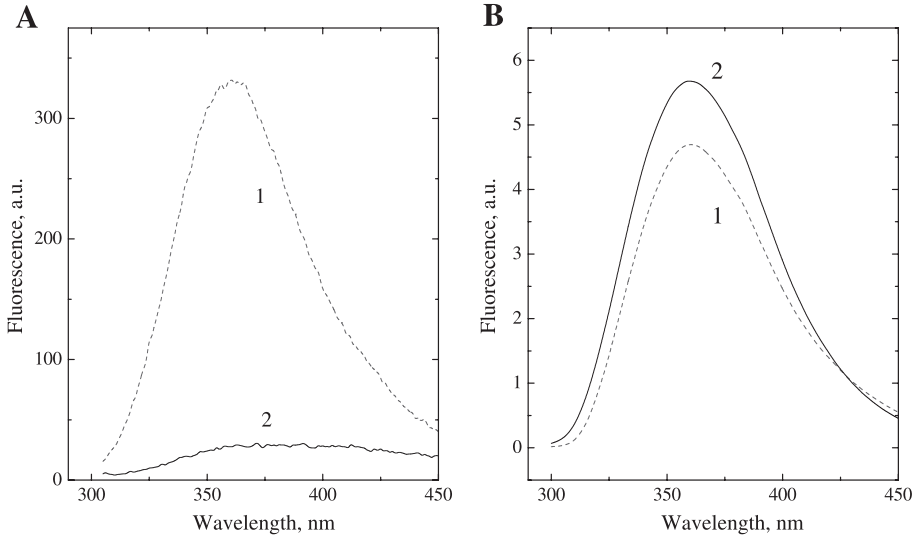

Fig. 2. Fluorescence spectra of the C-terminus peptide (panel A) and of *N*-acetyl-Trp amide in solution (panel B) at pH = 7.2 (curve 3) and at pH = 12.4 (curve 2), respectively.

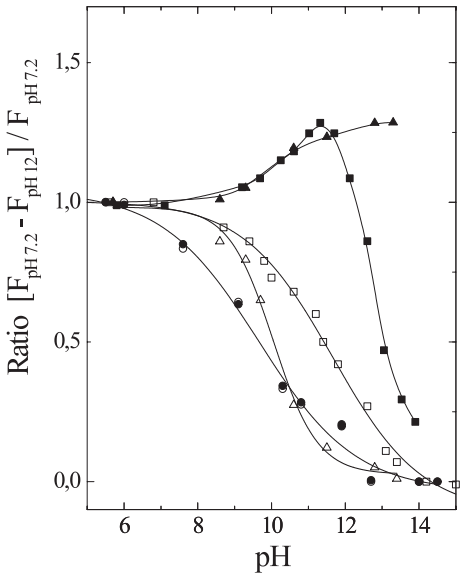

Fig. 3. Normalized fluorescence emission of Tyr (open symbols) and Trp (closed symbols) of 7:1 mixture of *N*-acetyl-Tyr amide and *N*-acetyl-Trp amide (triangles), synthetic C-terminus peptide (circles) and MSP (squares). Proton titration curves with pK_a = 9.7 (C-terminus peptide), 10.1 (7:1 Tyr/Trp mixture) and 11.7 (MSP). The data of the MSP were redrawn from Shutova et al. [43].

virtually identical decrease with progressing pH that can be described by deprotonation of a single group with a p*K* value of about 9.7; (b) the decrease of Tyr emission in the 7:1 Tyr/Trp mixture (open triangles) is characterized by a similar p*K* of 10.1 but a much steeper decline that is indicative of the involvement of two protons; and (c) in sharp contrast to the C-terminus peptide, the Trp emission (filled triangles) in the 7:1 Tyr/Trp mixture even increases at alkaline pH.

In spite of the different shape of the titration curves, the Tyr is quenched with nearly the same p*K* (9.7 versus 10.1) in both sample types while the Trp emission exhibit opposite pH dependencies. Since the tyrosinate is almost non-fluorescent, this data strongly supports the idea that the anion acts as quencher of the Trp emission only if both amino acids are neighbours as in the case of the C-terminus peptide.

If this type of quenching by tyrosinate is a general phenomenon, the residue Y242 in its deprotonated form is also expected to suppress the W241 fluorescence of the MSP. The data for the pH dependence of the emission from Tyr and Trp in the native MSP were reported recently [43] and are redrawn in Fig. 3. We have checked that high pH does not lead to irreversible structural changes of the MSP in solution. It was found that MSP incubated at pH = 12.3 retained the full capacity of restoration of the oxygen evolution activity in the reconstitution assay at pH = 6.5 (data not shown). A comparison with the fraction of tyrosinate gathered from the pH dependence of the 300-nm absorption that is characteristic for the tyrosine anion form in the MSP [43] confirms that the decrease of the "306-nm band" originates from a deprotonation of Tyr residues but its p*K* is markedly shifted by 1.5–2 pH units towards the alkaline region (open squares). The amplitude of the emission ascribed to W241 is characterized by a peculiar shape: a slight increase in the range from pH 9 to 11 is followed by a steep decline above pH 11.3 (filled squares). A comparison of the data readily shows that in contrast to the C-terminus peptide, the suppressions of Tyr and Trp emissions at alkaline pH do not coincide. This feature indicates that at least two effects contribute to the overall pH dependency of Trp fluorescence. Although rather steep, the flank of the decrease at alkaline pH is in line with the idea that also in the native MSP in solution the tyrosine anion form exerts a strong quenching effect on the W241 emission.

Since the spinach MSP contains seven Tyr residues, it is most likely that not all of these are in the same dielectric environment and that the titration curve represents an average with slight variations of the p*K* values of individual groups. However, since the titration curve can be satisfactorily described by a single p*K*, the deviations are rather small, i.e. Y242 is characterized by a value of 11.7 ± 0.3. The idea of a comparatively narrow distribution of the environmental p*K* shift of tyrosine is supported by theoretical model predictions based on an algorithm developed by Ptitsyn and Finkelstein [47] and the amino acid sequence of MSP. These calculations predict that the majority of the tyrosine residues are located in regions of β-strands as is illustrated in Fig. 4 and therefore most likely somewhat "shielded" from the aqueous bulk phase.

The increase of the W241 fluorescence between pH 8 and 11 can be explained by disappearance of another type of quenching, depending on its total efficiency at neutral pH. This effect could be either caused by a modification of the interaction with the S–S bridge owing to slight structural changes or originate from the presence of an additional quencher. In an attempt to obtain further information, CD spectra were measured in the near-UV region (270–310 nm). The spectra obtained at pH = 11.8 and 6.7 exhibit a

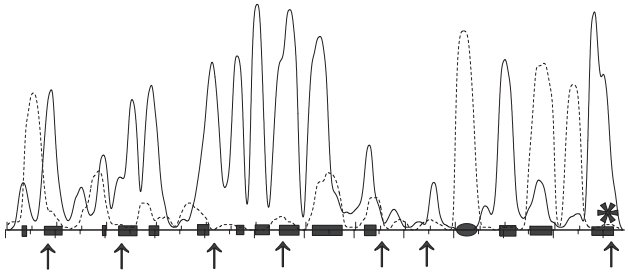

Fig. 4. Prediction of secondary elements in MSP based on the Ptitsyn–Finkelstein algorithm. The secondary structural elements β-strands and α-helices are symbolized by full- and dash-lined curves, respectively; the locations of Tyr and Trp in the sequence is indicated by arrows and a star, respectively. For comparison, the prediction of Pazos et al. [51] based on a threading structural model is shown (β-strands as black boxes and α-helix as a grey oval).

similar shape but the ellipticity is changed. The amplitude of the 293-nm band that is characteristic for Trp is diminished at pH = 11.8 but becomes fully restored after subsequent pH shift to 7.3 (data not shown). The results closely resemble our previous findings at pH = 3.8 [36]. It is therefore assumed that also in the alkaline the rotational freedom increases of aromatic acid residues buried in the interior. At present, however, an unambiguous distinction between the abovementioned alternatives of diminished quenching is impossible.

The marked shift by about two pH units towards the alkaline region of the Tyr fluorescence decrease of the MSP compared with that of the C-terminus peptide is ascribed to an increase of the p*K* value of the OH group of Tyr residues that are buried into an environment with low dielectric constant within the MSP [43]. Based on analogous conclusions for the p*K* shift of tyrosines in Δ^5-3-ketosteroid-isomerase [48], the local dielectric constant of the hydrophobic domain around W241 is estimated to be 10–20 as compared with about 80 of aqueous bulk phase. Since the Tyr residues are distributed among the whole sequence (Refs. [7,8,36] and Fig. 4), this result provides further evidence for a rather compact structure of large domain(s) with comparatively low dielectric constant within the MSP (in solution). The most likely candidates are secondary structure elements (α-helices, β-sheets) that are also typical for a molten globule structure [49,50]. This conclusion is highly supported by a recent threading structural model that shows the MSP as an all-β-protein with two homologous β domains of approximately 120 amino acids. These domains containing eight to nine and six to seven β-strands, respectively, are connected by a flexible Pro–Gly–Gly motif [51]. It is interesting to note that the results of our theoretical structure analysis with the Ptitsyn–Finkelstein algorithm (see Fig. 4) are fully consistent with the existence of a compact β-strand domain in the N-terminal half of the protein. For the sake of direct comparability, the predicted location of β-strands in the MSP sequence according to the threading model [51] is indicated by black bars at the bottom line. In this respect, it appears noteworthy that both methods predict a pronounced β-strand in a domain that has been recently discovered in the Δ18M mutant of plant MSP to be important for its functional interaction with the intrinsic core of PS II [13]. An inspection of the C-terminal part (above residue 150) reveals that the location of one α-helix and three β-strands are predicted by both methods, while within the domain between residues 205 and 230, according to the Ptitsyn–Finkelstein algorithm, two additional α-helices are expected but not within the framework of the threading model. The absence of these two α-helices is in line with the structural model presented in Ref. [8]. The striking similarity of structural modeling by different methods in favor of compact β-structures for the N-terminus part of the protein but with conflicting results for C-terminal domain could reflect structural features in the latter part which are specific for MSP. They might be relevant for its structural/functional role in the intact WOC (proton-induced conformational changes in the C terminus [36]).

3.3. Mechanism of Trp fluorescence quenching by tyrosinate

Regardless of the difference in the pH dependence of emission between the C-terminus peptide and MSP, the results of both sample types strongly support the idea that Trp fluorescence is efficiently quenched by the adjacent tyrosinate. Therefore, questions arise on the mechanism of this effect. In general, two modes of quenching can be considered: excited state electron or excited state proton transfer. Usually the electronically excited Trp acts as electron donor in the former and as hydrogen acceptor in the latter type of quenching [52,53]. Based on the less stringent structural requirements, it has been proposed that excited state electron transfer is probably the dominating quenching mechanism of Trp fluorescence in peptides and proteins [46]. Therefore, it appears most likely that a rapid light-induced electron transfer from tyrosinate (YO^-) to electronically excited tryptophan (Trp*) takes place, leading to biradical formation and the subsequent radiationless recombination to the ground state:

$$Trp^* - YO^- \rightarrow Trp^{-\bullet} - YO^{\bullet} \quad (1a)$$

$$Trp^{-\bullet} - YO^{\bullet} \rightarrow Trp - YO^- \quad (2a)$$

The light-induced interaction of Trp residues with other redox active species is well known. It was recently shown for the opposite direction that a photo-excited electron transfer between riboflavin and Trp in a riboflavin binding protein can occur in 100 fs [54] and a similar reaction with an anthraquinone type drug is also fast (1 and 6 ps, depending on protein conformation) [55]. It is therefore conceivable that an electron transfer reaction from tyrosinate to the next neighbored photo-excited Trp could also take place in the picosecond time domain thus giving rise to strong quenching of Trp fluorescence. From a thermodynamic point of view the tyrosinate is much easier oxidizable than neutral protonated Tyr [56] and therefore the quenching effect arises only when Tyr is deprotonated.

3.4. Fluorescence anisotropy

In order to gather further information on the properties of the fluorescence emitters in the MSP, anisotropy spectra were monitored and compared with those of the C-terminus peptide. The results for the wavelength dependence of emission anisotropy are depicted in Fig. 5. The left panel (A) shows that the emission of the C-terminus peptide which is dominated by the Trp fluorescence exhibits a wavelength independent anisotropy in the whole region of the emission band (310–395 nm) regardless of the excitation wavelength (275 or 295 nm). This feature is indicative of a single emitter Trp. A more complex pattern emerges for the native MSP in solution as illustrated in panel B. In this

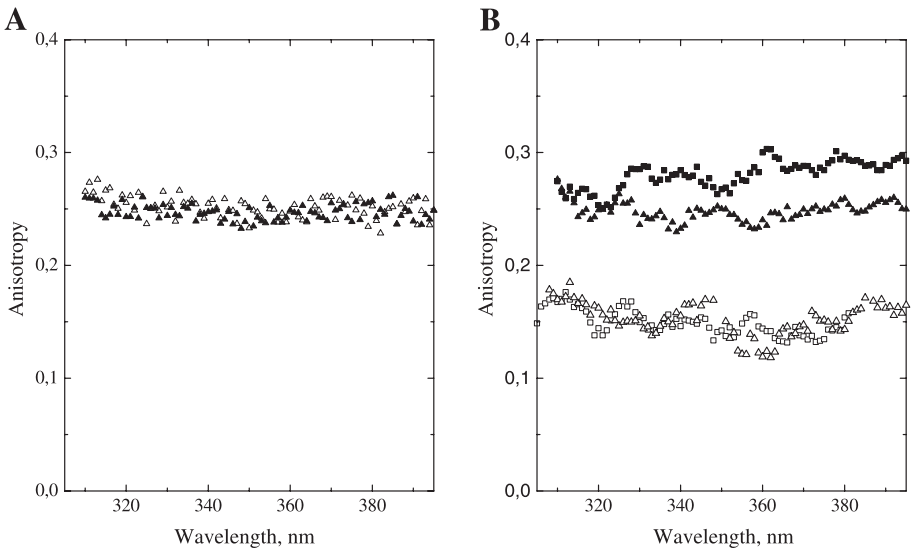

Fig. 5. Wavelength dependence of fluorescence anisotropy of C-terminus peptide (panel A) and MSP (panel B) excited either at 275 nm (open symbols) or 297 nm (filled symbols). In panel B, triangles and squares symbolize results obtained at 5 and −27 °C, respectively.

case, the extent of the anisotropy markedly depends on the excitation wavelength, i.e. it is significantly smaller when Tyr residues are predominantly excited at 275 nm compared with that of Trp fluorescence (excited at 297 nm). This finding is an independent line of strong evidence for the existence of two different types of emitters in MSP, i.e. Tyr and Trp. With respect to the wavelength dependence of the anisotropy, both emitters exhibit hardly any variation and resemble the property of the C-terminus peptide. The wavelength-independent anisotropy would also be in perfect agreement with the generally held belief that among the two lowest excited electronic states of Trp, ^1L_a and ^1L_b, the former is the fluorescing state in all proteins [57]. However, since the internal conversion between states ^1L_a and ^1L_b was recently reported to take place within 100 fs, the reported anisotropy value for Trp is actually the average after internal state mixing [58]. Regardless of this particular problem, the absolute values of the Trp emission anisotropy are virtually the same in both the C-terminus peptide and MSP. As expected for another emitter, the Tyr emission anisotropy differs from that of Trp (it is smaller).

In an attempt to obtain further information on possible orientation effects, the anisotropy measurements of the MSP were performed at two different temperatures. An inspection of the data depicted in panel B of Fig. 5 reveals that the temperature effect significantly differs between Trp and Tyr fluorescence anisotropy. The anisotropy value of the Trp emitter increases when the sample is frozen by temperature decrease from +5 °C down to −27 °C. An analogous feature is not observed for the Tyr emission. This difference can be explained by the presence of seven Tyr residues and only one Trp in the spinach MSP. Accordingly, when the sample is frozen the mobility of the MSP is highly restricted. As a consequence, the anisotropy of each individual emitter state is expected to increase drastically, provided that rapid excitation energy transfer (EET) can be excluded. In frozen MSP solution, with a single Trp in each protein, the latter effect can be excluded and the increase of Trp emission anisotropy is indicative of a small orientation effect of the complexes in the frozen state. At present, the origin of this effect is not clear but is specific for the MSP, because a similar feature of anisotropy increase due to temperature decrease from +5 °C to −27 °C is not observed in solutions of the C-terminus peptide (data not shown). In marked contrast to the Trp emission anisotropy increase, a corresponding effect is lacking for the Tyr emission. This feature is not surprising since the transition dipole moments of the seven Tyr emitters in each MSP can cover a wide range of orientation angles and, in addition, EET between these species cannot be excluded.

3.5. Reconstitution of oxygen evolution by MSP rebinding and effects of the C-terminus peptide

In order to study a possible functional role of the C terminus in situ, reconstitution experiments of the oxygen evolving capacity were performed with PS II membrane fragments that are deprived of the MSP by washing with $CaCl_2$ [3,4,59]. Fig. 6 shows typical curves for the restoration of oxygen evolution capacity as a function of the MSP/PS II ratio in the assay medium. The maximum extent of reconstitution is achieved at about 2 MSP/PS II. This 100% value corresponds to about 85% of the rate of oxygen evolution in untreated PS II membrane fragments (see Materials and methods). The dependence on the MSP concentration in Fig. 6 closely resembles the findings reported in the literature [13,60,61]. In a previous study, somewhat higher MSP concentrations were reported to be required for full reconstitution [62], but this effect turned out to originate from using MSP that was lyophylized before

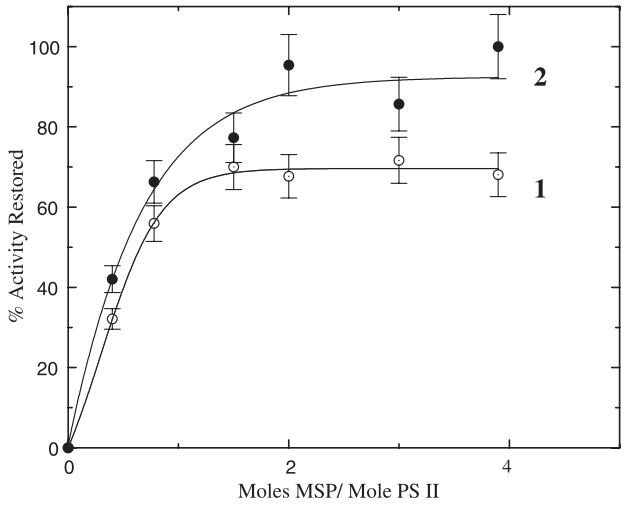

Fig. 6. Restoration of O_2 evolution in the salt-treated PS II membrane fragments as a function of MSP concentration in the presence (open circles) and absence (filled circles) of 10 C-terminus peptide per PS II in the reconstitution assay (for experimental details, see Materials and methods; each point of the open and filled circles represents the average of three and four measurements, respectively).

use. This phenomenon indicates that structural motif(s) of functional relevance for reconstitution become(s) modified upon lyophilization and subsequent solvation in the assay medium. The underlying mechanism of this effect is not yet clarified. In this respect, it is interesting to note that two forms of MSP are encoded in *A. thaliana* (genes *psbO* and *psbO2*) but only one form (encoded by *psbO*) is able to support full functional competence although both copies exhibit very similar structural characteristics and differ by only about 10 amino acid residues [33].

If the same type of reconstitution experiment is performed in the presence of the C-terminus peptide instead of MSP, virtually no reconstitution of oxygen evolution can be observed (data not shown). An interesting feature emerges when the restoration assay contains both the C-terminus peptide and freshly prepared MSP. In this case, the full extent of reconstitution of the oxygen evolution cannot be achieved, i.e. the maximum level drops down. In order to illustrate this effect, experiments were performed at 0.4 mol MSP/mol PS II and varying amounts of C-terminus peptide. The results depicted in Fig. 7 reveal that the extent of inhibition of the reconstitution saturates at about 10 copies of C-terminus peptide.

A double reciprocal plot $(v_{O_2})^{-1}$ versus $(\text{MSP/PS II})^{-1}$ of the data of Fig. 6 leads to virtually parallel lines (not shown). This feature might be indicative of an uncompetitive inhibition of MSP binding by the C-terminus peptide. Additional experiments performed at subsaturating concentrations of the C-terminus peptide did not provide further information owing to the scatter of the experimental data. Although the inhibition mechanism is not yet clarified, it seems reasonable to assume that the C-terminus peptide binds to PS II, thus impairing the reconstitution of oxygen evolution by MSP. Interestingly, in untreated PS II membrane fragments the addition of the C-terminus peptide does not diminish the maximum rate of oxygen evolution (data not shown). Two step reconstitution experiments were performed to illustrate the mutual interaction. It was found that the extent of reconstituted oxygen evolution rate reached only a level of 66% when the C-terminus peptide was added (10 peptides/PS II) at first to the assay medium (40 min preincubation) followed by the MSP, compared with that of 100% achieved at an opposite order of MSP and peptide addition (data not shown). This difference corresponds exactly with the inhibition of the reconstitution at 10 peptides/PS II (see Fig. 6) and clearly shows that the C-terminal peptide does not affect the MSP reconstitution when added after MSP to the assay medium. Gel filtration experiments did not reveal C-terminus peptide binding to MSP in solution. Accordingly, all of the above findings are in line with the conclusion that the C terminus of the MSP plays an important role for the interaction with the integral part of the PS II core [30,31,63].

In this respect, it is interesting to note that the C terminus of the MSP exhibits a strikingly high conservation of Trp which is present in all MSPs of green algae and higher plants sequenced so far [32]. The Trp is characterized by the largest hydrophibic surface of all natural amino residues and can interact with many partners. A thorough search of proteins reveals that the distribution number of partners interacting with Trp peaks at 6 (including protein residues only) and 8 (including water and substrate molecules) [64].

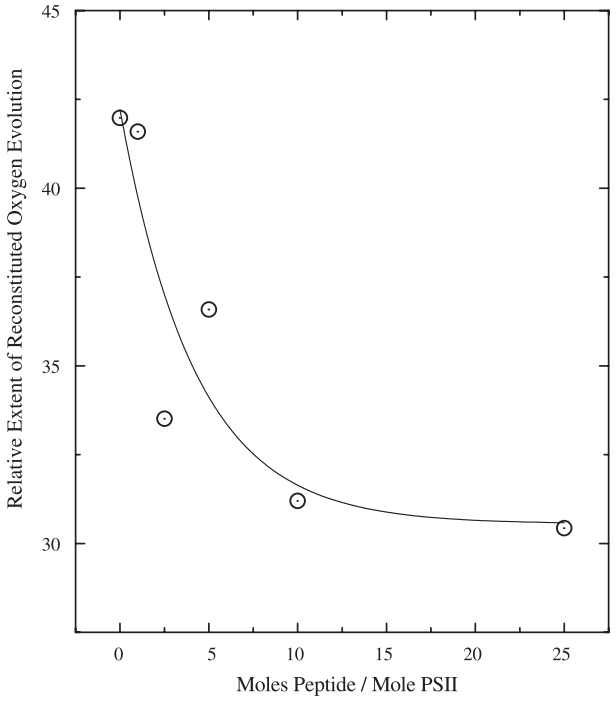

Fig. 7. Restoration of O_2 evolution by 0.4 MSP/PS II as a function of the number of C-terminus peptide per PS II in the assay medium (for experimental details, see Materials and methods).

It is therefore conceivable that W241 might play an important structural role for the C-terminus structure possibly in connection with the next neighbor Y242, which is also fully conserved in MSP of green algae and higher plants [32]. It remains to be unraveled why W241 has been inserted into the MSP during the evolutionaly development from cyanobacteria to higher plants.

The present results also show that the small C-terminus peptide does not satisfy the structural/functional requirements for a reconstitution of the full WOC function. In this respect, it is interesting to mention that in a previous study we found that after mild trypsin treatment of PS II membrane fragments, giving rise to proteolytic cleavage of the MSP, a 15-kDa part of this protein remains bound and retains the oxygen evolution activity [65]. The size of this fragment is much larger than the synthetic C-terminus peptide used in the present study. Furthermore, the SDS-PAGE analysis in our former study did not permit the decision whether or not small fragments of the MSP remain still bound to PS II in addition to the detected 15-kDa fragment. This particular problem will be addressed in forthcoming studies. Since recent cross-linking studies revealed proximity of C-terminus and N-terminus regions [31], it is likely that both domains are important for MSP binding to PS II. The recently gathered data from X-ray crystallographic structure analysis of a PS II complex from *Thermosynechococcus vulcanus* [66] reveal that parts of the N and C termini are indeed close together, but at the present resolution of 3.7 Å, the details of the interaction(s) are not yet clarified (J.-R. Shen, personal communication).

4. Concluding remarks

The present analysis of the fluorescence properties provides additional strong support for the existence of two types of emitters in the MSP from spinach: tyrosines and the single tryptophan W241. It also shows that a protonated Tyr residue next to Trp does not significantly affect the fluorescence emission but acts as powerful quencher in its deprotonated tyrosinate form in both MSP and a small synthetic C-terminus peptide. Based on the pH dependence of the Tyr emission and model simulations, it is concluded that a large fraction of the MSP in solution forms a compact structure with a marked extent of hydrophobic domain(s), possibly in a molten globule state [39]. The latter idea is supported by a recent detailed analysis on structural features of proteins in solution [67]. This form is assumed to be important for its interaction with the membrane-bound PS II core. The data also support the idea that the C terminus is of relevance for the specific binding of the MSP to the intrinsic PS II core. It is assumed that the MSP is not only an important structural element for stabilizing the manganese cluster of the WOC but is also important for its functional competence by actively participating in proton transport and adaptation to lumenal acidification under illumination. The remaining O_2 evolution capacity in *psbO* deletion mutants of cyanobacteria [19–22] is explainable by establishing alternative proton transfer routes. This mode of switching to ''crutch'' proton pathways is well known from mutants of anoxygenic purple bacteria [68]. A possible role for substrate water accessibility to the catalytic site remains to be clarified because the $H_2^{18}O/H_2^{16}O$ isotope exchange was recently found to be invariant to lack of the MSP or even becoming retarded after its removal by $CaCl_2$ washing [69].

Acknowledgements

The authors would like to thank Dr. J.-R. Shen for invaluable comments and structural information on the bound MSP. They gratefully acknowledge the financial support by the Deutsche Forschungsgemeinschaft to G.R. (Sfb 498 TP C4 and 436 RUS 113/635) and by the Swedish National Research Council, the Kempe Foundation, the Swedish Academy of Science to G.S., STINT and the Russian Foundation of Basic Research.

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