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Origin and properties of fluorescence emission from the extrinsic 33 kDa manganese stabilizing protein of higher plant water oxidizing complex

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Abstract

The fluorescence properties of the isolated extrinsic 33 kDa subunit acting as 'manganese stabilizing protein' (MSP) of the water oxidizing complex in photosynthesis was analyzed in buffer solution. Measurements of the emission spectra as a function of excitation wavelength, pH and temperature led to the following results: (a) under all experimental conditions the spectra monitored were found to be the composite of two contributions referred to as '306 nm band' and 'long-wavelength band', (b) the excitation spectra of these two bands closely resemble those of tyrosine and tryptophan in solution, respectively, (c) the spectral shape of the '306 nm band' is virtually independent on pH but its amplitude drastically decreases in the alkaline with a p*K* of 11.7, (d) the amplitude of the 'long-wavelength' emission band at alkaline pH slightly increases when the pH rises from 7.2 to about 11.3 followed by a sharp decline at higher pH, and (e) the shape of the overall spectrum at pH 7.2 is only slightly changed upon heating to 90°C whereas the amplitude significantly declines. Based on these findings the two distinct fluorescence bands are ascribed to tyrosine(s) ('306 nm band') and the only tryptophan residue W241 of MSP from higher plants ('long-wavelength band') as emitters which are both embedded into a rather hydrophobic environment.

Keywords: Photosystem II; Manganese stabilizing protein; Fluorescence; pH dependence

1. Introduction

Photosynthetic water oxidation to molecular oxygen takes place via a sequence of four one-electron oxidation steps at a manganese containing unit, the water oxidizing complex (WOC) [1–3]. Among the proteins that are of physiological relevance for the stability and activity of the WOC, the extrinsic 33 kDa protein is of special interest. It is a constituent of all oxygen evolving photosynthesizing organisms (for recent reviews, see [4,5]). Extraction of this protein strongly diminishes the oxygen evolution rate

Abbreviations: CP47, chlorophyll-containing 47 kDa protein; MES, 2-morpholinoethane sulfonic acid; MSP, manganese stabilizing protein; P_{680}, redox active specially bound chlorophyll *a* within the PS II reaction center; Pheo, pheophytin; PS II, Photosystem II; WOC, water-oxidizing complex; Y_Z, redox active tyrosine of polypeptide D1

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and affects turnover and stability of the higher redox states of the WOC and of manganese binding [6–8]. Therefore the extrinsic 33 kDa protein is often referred to as manganese stabilizing protein (MSP) [4,5]. In marked contrast to the conserved MSP, additional regulatory extrinsic subunits were replaced by other subunits during the evolution [4,9] without significantly affecting the reaction coordinate of the redox steps in the WOC [10].

The structural characteristics of the MSP that are of relevance for its regulatory and stabilizing function of the WOC are not yet clarified. One possibility to address this problem are detailed analyses of the isolated protein. The results so far obtained reveal that the MSP with a p*I* value of 5.2 in buffer solution exhibits several unusual properties like thermostability [11] with a single Leu residue (L245) as an essential constituent for maintaining the solution structure [12], a comparatively high content of antiparallel β-sheets and turns together with a low fraction of α-helices [13–15] and a marked hysteresis of the titration curves in acidic or alkaline direction [13]. Recently the MSP was inferred to attain a definite 'molten globule' [16] rather than a 'natively unfolded' structure [12]. These peculiarities of the MSP suggest that its structural dynamics are of functional relevance. Spectroscopic probes offer a possibility to investigate the topology of proteins and monitoring conformational changes. In this respect tryptophan residues were found to provide a most suitable intrinsic indicator via the properties of their fluorescence emission (for a review, see [17]) as illustrated by studies on actinomyosin [18], apomyoglobins [19], glutamic phosphoribosylpyrophosphate aminotransferase [20], cytochrome b_5 [21] and ribonuclease A [22]. The MSP of higher plants contains a single tryptophan residue (W241) which is assumed to be embedded into a hydrophobic environment of β-sheets located near the C-terminus [13,15]. It was found that the fluorescence of the MSP markedly changes when structural changes are induced by a reductive break of the conserved S–S bridge [23]. Likewise effects were observed owing to interaction of MSP with Ca^{2+} [24]. It is tempting to ascribe the fluorescence emission to W241 and to use its variation as indicator for structural changes in the microenvironment of this residue. However, other aromatic amino residues like tyrosines cannot be ignored as emitters. Therefore the present study was performed to analyze origin and properties of MSP fluorescence. The results obtained reveal that – depending on the experimental conditions – the tyrosine emission can contribute significantly to the overall fluorescence spectrum of MSP. The implications of these findings are discussed.

2. Materials and methods

2.1. Isolation and purification of the 33 kDa protein

Photosystem II membrane fragments were prepared from spinach or pea leaves according to [25] with some modifications outlined in [26]. The 33 kDa protein was isolated by salt-washing as described in [27,28]. The polypeptide was dialyzed against 10 mM Mes–NaOH buffer (or in 5 mM ammonium acetate buffer) (pH 6.5), containing 10 mM NaCl, and then lyophilized.

The lyophilized sample was dissolved in distilled water and further purified by chromatography on a DEAE–Sepharose 6-B column. The S–S bridge in 33 kDa protein was reduced by using a 1000-fold (mol/mol) excess of DTT. All procedures were performed in the cold room ($\sim$4°C) as fast as possible to exclude protein degradation.

The protein homogeneity was checked via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [29] in the presence of 5 M urea at a 10-fold higher protein concentration in order to detect possible impurities. The protein concentration was spectrophotometrically determined either at 276 nm using an extinction coefficient of 16 $mM^{-1}\,cm^{-1}$ or after staining with Coomassie blue G250 as described by Bradford [30].

2.2. Spectroscopic measurements

Fluorescence excitation spectra of the MSP were recorded by using a Hitachi-850 spectrofluorimeter. Fluorescence steady-state measurements were performed on a home-built spectrofluorimeter described earlier [31]. All spectra were corrected for spectral sensitivity of the instruments. The bandwidth for both excitation and emission light did not exceed 2–3 nm. Protein concentration in fluorescence experi-

ments was 3–5 μM. Fluorescence spectra of MSP measured in alkaline region were corrected for reabsorption of tyrosinate as described in [32]. To avoid a tyrosinate-based inner filter effect the fluorescence of MSP was excited at 280 nm, where the extinction coefficient of tyrosinate is equal to that of tyrosine [33,34].

Absorption spectra of protein samples were recorded using a Specord-M40 spectrophotometer (Carl-Zeiss, Jena). Prior to the spectroscopic measurements the protein was additionally dialyzed against 10 mM phosphate/10 mM NaCl buffer (pH 7.2) and concentrated in Centriprep-10 tubes (Amicon). Insoluble material was removed from the sample by centrifugation (30 000 × *g*, 15 min). The cuvette was kept at constant temperature (25°C or 90°C) by using a thermostat. The temperature inside the sample cell was monitored with a copper–constantan thermocouple.

2.3. KOH titration

The KOH titration was performed by addition of very small aliquots of a highly concentrated base solution. After attaining the deprotonation equilibrium (depending on pH, the time required varies from a few seconds to minutes) absorption and emission spectra of the same MSP sample were immediately recorded.

3. Results and discussion

3.1. Fluorescence spectra of MSP at different excitation wavelength

Fig. 1 shows room temperature spectra at pH 7.2 measured by excitation at 276 nm (full-lined curve) and 297 nm (dashed curve). For the sake of direct comparability both spectra are normalized at the long-wavelength edge. The spectrum obtained with 276 nm excitation is significantly blue-shifted with a pronounced peak at around 308 nm compared with the broader spectrum measured with 297 nm excitation which is centered around 329 nm. The illumination with 297 nm light exclusively excites tryptophan residues [33] and therefore this spectrum is ascribed to W241 tryptophan. On the other hand, excitation

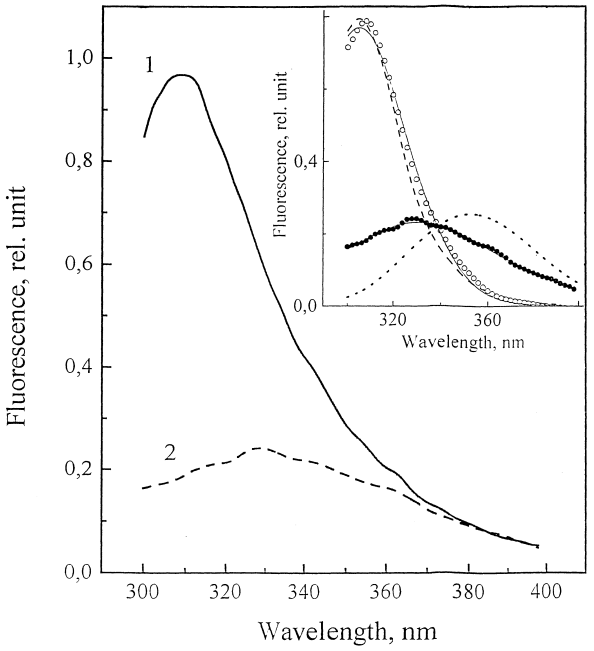

Fig. 1. Room temperature fluorescence spectra of the MSP measured in buffer solution at pH 7.2 after excitation at wavelengths of 276 nm (curve 1) and at 297 nm (curve 2). The inset presents a fit of spectrum 2 (filled circles) by a 'log-normal' curve of the form $F(\lambda) = F(\lambda_m)\exp[-(\ln^2(\lambda/\lambda_{max})/2w^2]$ (full-lined curve) and of the difference between curves 1 and 2 (open circles) together with its fit by a 'log-normal' curve. For comparison the emission spectra of L-tyrosine (dashed curve) and L-tryptophan (dotted curve) in solution are presented under the same experimental conditions as the MSP fluorescence.

at the maximum of MSP absorption at 276 nm results in an emission spectrum that is typical for a superposition of Tyr and Trp emission [17,35].

The spectral shape has been analyzed by curve fitting based on the idea that the slightly asymmetric emission spectra of aromatic amino acid residues in proteins are best described by curves of the form

$$F(\lambda) = F(\lambda_m)\exp\left[\frac{-(\ln^2(\lambda/\lambda_{max})}{2w^2}\right]$$

referred to as 'log-normal shaped' [34]. The inset of Fig. 1 shows that the experimental data of the dashed-lined spectrum (curve 2) ascribed to emission of W241 is satisfactorily described by a 'log-normal curve' with a peak of $\lambda_{max} = 329.5$ nm and a half-width of about 52.5 nm. The spectrum exhibits a marked blue shift by more than 20 nm compared with that of tryptophan in solution (dotted curve in the inset). This feature is indicative of a hydrophobic

environment of the tryptophan residue [17,36], in line with theoretical predictions of a location of W241 in a shielded β-structure domain [13,37] and with recent in vitro studies where a single tryptophan incorporated into the α-helix of a synthetic maquette was shown to be characterized by an emission maximum of 324 nm [38].

In order to analyze the origin of the spectrum emerging from 276 nm excitation, the normalized spectrum of curve 2 was subtracted from the normalized full-lined spectrum, curve 1. The data obtained and depicted in the inset of Fig. 1 (open circles) exhibits also a 'log-normal' shape (see dashed curve) characterized by a peak at around 306 nm and a markedly narrower halfwidth of about 27 nm. An assignment of this '306 nm band' also to tryptophan is highly unlikely for two reasons: (i) since MSP contains only a single tryptophan residue (W241) the existence of two emission spectra with clearly distinguishable characteristics would necessarily imply that the MSP in solution exists in two populations with distinct conformations of markedly different microenvironments of W241, and (ii) although the tryptophan fluorescence of the Cu containing azurin with an emission peak at 308 nm [39] exhibits a maximum comparable with that of the '306 nm band' of MSP, the former emission spectrum is characterized by a fine structure that is typical for indole in frozen solutions with an apolar solvent [34]. This striking feature is also observed in other proteins with markedly blue shifted Tryp emission [40–42] but does not appear in the '306 nm band' of Fig. 1 (full-lined curve in the inset). There exists no experimental precedent for such a peculiar phenomenon. Accordingly, the '306 nm band' has to originate from emitters other than W241. Tyrosine residues are the most likely candidates because their extinction at 276 nm and quantum yield are much higher than those of the fluorophore phenylalanine [17,34,43]. A comparison with the emission spectrum of L-tyrosine in solution (dashed curve in the inset) highly supports this idea.

In conclusion, the data of Fig. 1 suggest that the fluorescence spectrum of the MSP in solution is the composite of two types of emitters: the buried W241 giving rise to the '329.5 nm band' and tyrosine(s) leading to the '306 nm band'. This proposal also readily explains the dependence of the overall MSP emission spectrum on the excitation wavelength. As a consequence, the excitation spectra of the two types of emission should closely resemble the corresponding and markedly different spectra of tyrosine and tryptophan, respectively.

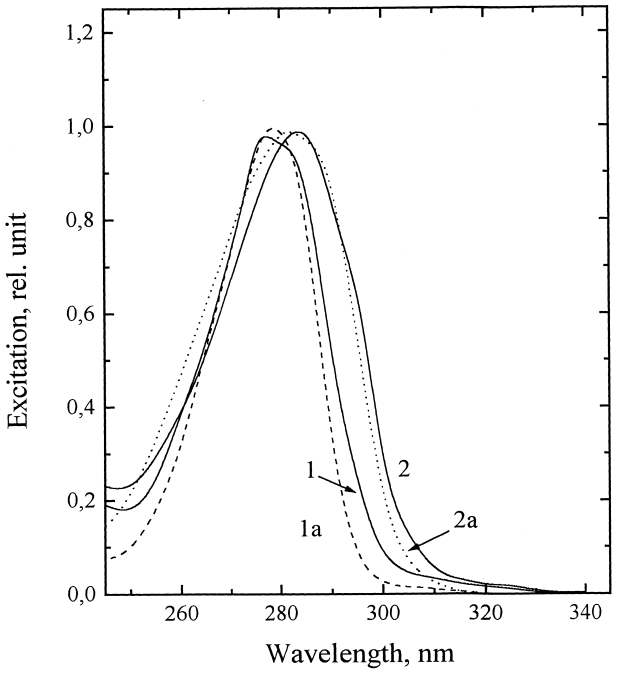

Fig. 2. Normalized excitation spectra of the fluorescence emission at 308 nm and 365 nm (full-lined curves 1 and 2, respectively) and the corresponding excitation spectra of L-tyrosine (dashed curve 1a) and L-tryptophan (dotted curve 2a).

The excitation spectra measured at 306 nm and at 360 nm are depicted in Fig. 2 and compiled with the corresponding normalized spectra of tyrosine and tryptophan in solution.

An inspection of these data shows that the excitation spectrum of the 306 nm emission closely resembles that of L-tyrosine in solution except of a red-shift by a about 5 nm in the former case. Likewise, the excitation spectrum of the 360 nm emission exhibits striking similarities with that of L-tryptophan in solution in the long wavelengths' part while for the 'blue' region the spectrum is broader. The small deviations are explainable by an overlap of tyrosine and tryptophan fluorescence in MSP. Interestingly, the same excitation spectra of these two emitters were observed in glutamine synthetase [44] with excitation maxima for tyrosine and tryptophan fluorescence at 282 nm (emission band at 303 nm) and 287 nm (λ_{em} = 338 nm), respectively.

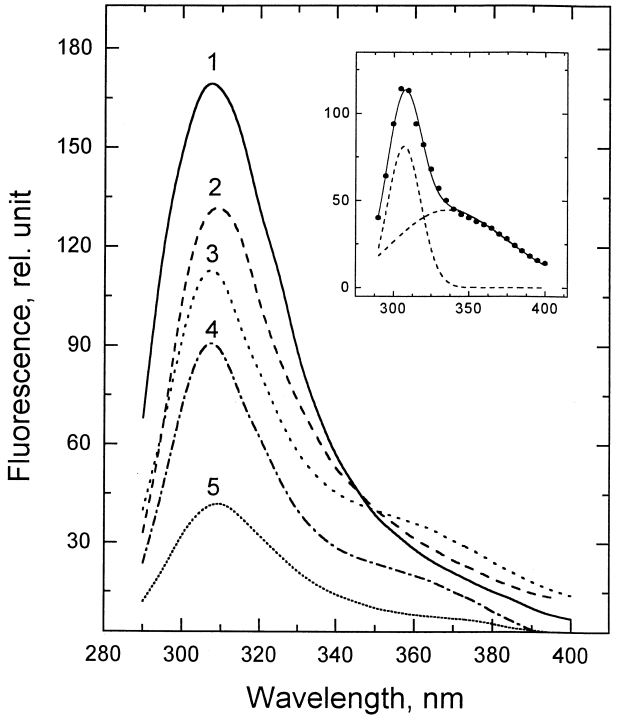

Fig. 3. Fluorescence spectra of the MSP in buffer solution at different pH values: solid curve (1), pH 7.2; dashed curve (2), pH 10.2; dotted curve (3), pH 11.3; dashed-dotted curve (4), pH 12.6; dotted-dotted curve (5), pH 13.5. Excitation wavelength 276 nm; the pH was changed by addition of KOH as described in Section 2. The inset shows the deconvolution of spectrum 3 (pH 11.3).

3.2. Fluorescence spectra of MSP as a function of pH

A further differentiation of the two different emitters is expected for the dependence of fluorescence on pH in the alkaline region [17,32]. The pK value of tyrosine in solution is 10.1 (see textbooks of Biochemistry), and the tyrosinate anion that has a very weak fluorescence leads to a red shift of the absorption band to 293 nm [45] whereas tryptophan does not change its protonation state.

Fig. 3 shows fluorescence spectra of the MSP measured at different pH values. The most striking feature of these data is the anticipated drastic decrease of the fluorescence peak at around 306 nm with increasing pH. A less pronounced but opposite effect emerges for the emission around 360 nm where a slight increase is observed upon alkalinization in the range from pH 7.2 to 11.3. At higher pH values this emission also starts to decline. If the overall spectrum is the composite of the fluorescence of two emitters all traces of Fig. 3 should be described by the superposition of two bands and their individual pH dependence analyzed. A fit of the data reveals that emission spectra measured at different pH values are indeed the sum of two bands with 'log-normal' shape. The inset of Fig. 3 shows as an example the deconvolution of the spectrum measured at pH 11.3. Two features emerge from data inspection: (i) the peak position of the '306 nm band' is virtually invariant to pH but its amplitude drastically decreases with increasing pH, and (ii) the amplitude of the 'long-wavelength' emission band at alkaline pH slightly increases when the pH rises from 7.2 to about 11.3 followed by a sharp decline at higher pH (vide infra).

The amplitude of the separated '306 nm band' as a function of pH is depicted in Fig. 4, top panel, together with the extent of absorption at 300 nm where tyrosinate predominantly absorbs [45]. The pH dependence of both parameters can be described by titration curves with a single pK_a value of 11.7. A titration curve of tyrosine fluorescence with a single pK_a value is not unique for MSP as illustrated for glutamine synthetase with 17 tyrosine and two tryptophan residues [44]. Surprisingly, the pK_a value of 11.7 in the titration curve of MSP is markedly higher than that of 10.1 for L-tyrosine in solution and of about 10 for glutamine synthetase [44]. An increase of the pK_a by 1.6 units is most likely reflecting buried tyrosine residue(s) as deduced from a comparison with a recent report on a pK_a value of about 11.3 for tyrosine incorporated into an α-helical structure of synthetic maquettes [38]. A pK_a value of 11.6 gathered from the titration curve of Δ^5-3-ketosteroid isomerase [46] was recently used to estimate the local dielectric constant ε_{local} of residue Y14 in its active site and a value of 18 obtained. In line with this data and the in vitro experiments of [38], most of the tyrosine residues in MSP are predicted to be located within β-strands, forming the hydrophobic compact region of this protein in solution [13,16]. At present it cannot be ruled out that few tyrosines residues of the MSP are characterized by normal pK_a values around 10 and simultaneously exhibit a very low fluorescence yield, e.g. by forming hydrogen bonds [47]. This possibility, however, does not affect the general conclusion on a rather hydrophobic microenvironment of the majority of tyrosines in the MSP. In this respect

it is interesting to note that pK shifts were observed also for the carboxylic groups of Asp and Glu residues in MSP [13]. In general, the localization of protonizable groups in hydrophobic regions can create specific pathways for proton transport. This process might be of functional relevance for the processes at the catalytic site of water oxidation because this reaction is most likely comprising concerted electron/proton transfer steps (see [48] and references therein).

Fig. 4, bottom panel, shows the pH dependence of

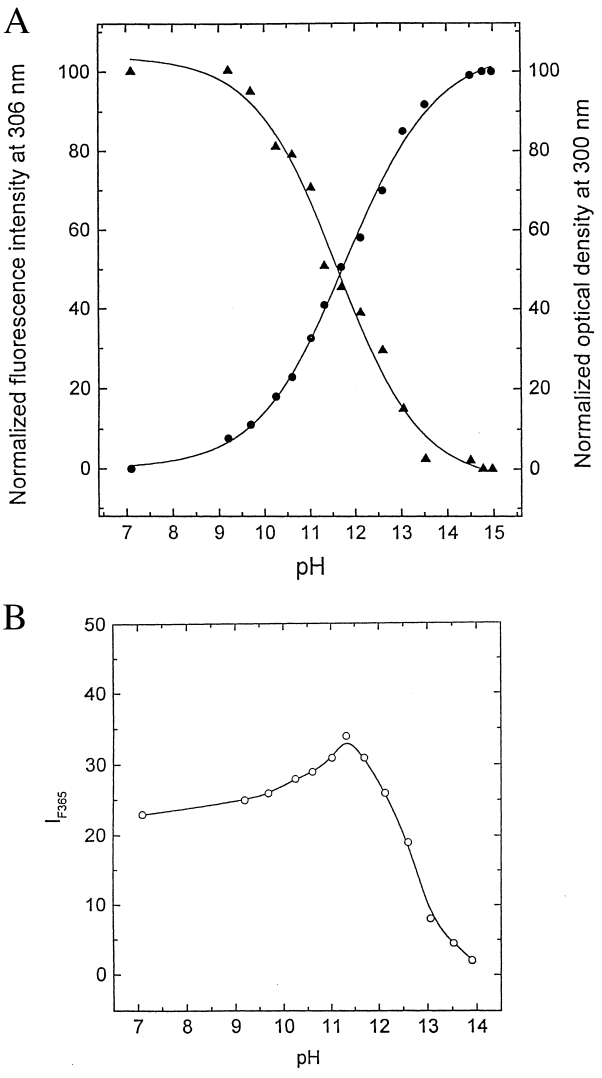

Fig. 4. Normalized fluorescence intensity at 306 nm of the separated '306 nm band' (solid triangles) and optical density at 300 nm (solid circles) of the MSP (A) and amplitude of fluorescence at 365 nm (B) as a function of pH. The '306 nm band' was gathered by a numerical fit of the data in Fig. 3 to the sum of two 'log-normal' bands. The curves represent calculated titration curves with a single pK of 11.7 in both cases.

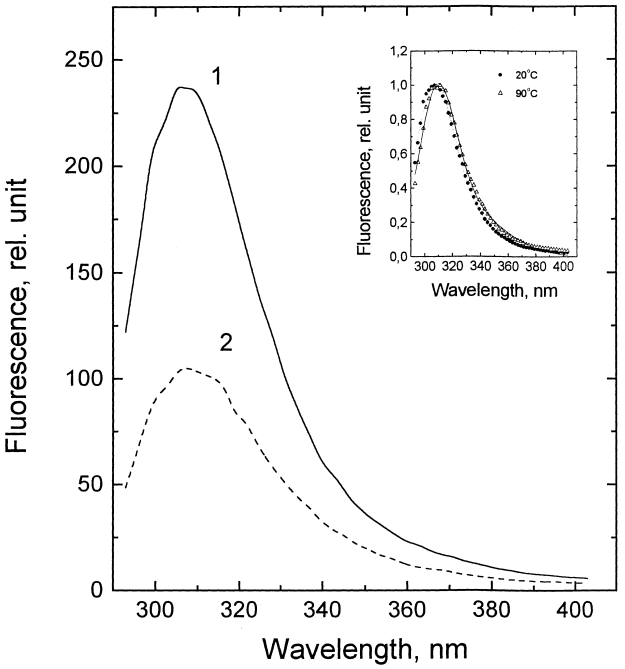

Fig. 5. Fluorescence spectra of the MSP in buffer solution of pH 7.2 and at temperatures of 20°C (curve 1) and 90°C (curve 2). The inset shows the experimental data normalized to the peak value and the fit by the sum of two 'log-normal' bands (full-lined and dotted curves).

the intensity at 365 nm, I_{F365}, where practically only tryptophan emits and reabsorption by tyrosinates can be excluded. The value of I_{F365} slightly increases in the pH region 7.5–11.3 followed by a sharp decline at higher pH. The most plausible explanation for this feature is a marked conformational change near W241 of the C-terminus of the MSP in the alkaline region of pH > 11.3. The nature of this change remains to be clarified.

3.3. *Fluorescence spectra of the MSP at high temperature*

It was recently found that heat treatment of the MSP in solution retains its capability of binding to PS II and restoring the oxygen evolution capacity of $CaCl_2$ washed PS II membrane fragments [11]. Based on these properties and data from UV-CD and FTIR spectroscopy this protein was inferred to be thermostable [11] and to attain a 'natively unfolded' structure but recent studies let us to favor the idea of a 'molten globule' structure of MSP in solution [16]. Therefore it is interesting to analyze the response of

the fluorescence emitters to heating. Fig. 5 shows the emission spectra of MSP in buffer solution (pH 7.2) at 20°C (curve 1) and 90°C (curve 2) excited with light of 276 nm. Both spectra exhibit a very similar shape as illustrated in the inset of Fig. 5, but the fluorescence yield is reduced at higher temperatures. The marked decrease of fluorescence intensity at 90°C can be readily explained by a more pronounced radiationless decay via vibrational modes but more interesting is the finding that both bands seem to be affected in a very similar way. A deconvolution of the experimental data into the sum of two 'log-normal' bands reveals that the '306 nm band' undergoes a small red shift by about 2 nm of its peak position upon heating, but its spectral shape remains almost invariant with a bandwidth of 45 ± 3 nm. The 'long-wavelength band' exhibits virtually no changes.

The striking invariance of the spectral shape to heating indicates that the microenvironment of both emitters is not disturbed at high temperatures, especially the tryptophan residue W241 as a sensitive probe to changes in its surrounding remains unaffected in its peak position. This is in perfect agreement with recent data reported in [11].

3.4. Quenching of tryptophan fluorescence in MSP

In many proteins the fluorescence properties are dominated by a pronounced emission from tryptophan residues (for reviews, see [32,34,40]). In marked contrast, at neutral pH the relative contribution of the '329 nm band' ascribed to W241 to the overall emission is comparatively small in MSP. This finding suggests that the tryptophan fluorescence in MSP is specifically diminished. The S–S bridge appears to be a potential candidate because this species was found to act as a strong quencher of the fluorescence of indole in model systems [34] and of tryptophan in α-lactalbumin [49] and hen egg-white lysozyme [50].

The S–S bridge of the MSP can be in the vicinity of W241 in the folded protein as deduced from cross-linking studies and therefore able to act as a quencher. This idea is supported by the finding that reductive opening of this bridge leads to drastic alteration of the MSP fluorescence [23] and an increase of its quantum yield by a factor of about seven after MSP treatment with DTT (data not shown).

In addition, the residue Y242 can act via its scavenger proton as quencher of electronically excited W241. This process is probable because both residues are buried in a highly hydrophobic environment [13,15] and the diffusion distance is short enough between Y242 and W241. The phenomenon still persists at high temperature because the distance between emitter and quencher remains virtually unaffected at this temperature.

4. Concluding remarks

The results of the present study unambiguously show that under all experimental conditions the overall fluorescence spectrum of MSP in solution is the composite of independent contributions originating from two different types of emitters: (i) a 'short-wavelength band' with a peak at around 306 nm ascribed to emission from excited tyrosine(s), and (ii) a 'long-wavelength band' inferred to originate from the only tryptophan residue W241. Both bands exhibit characteristic features. The titration curve of the 'short-wavelength band' with its unusually high pK_a value of 11.7 indicates that the vast majority of the tyrosine residues in MSP is embedded into a hydrophobic microenvironment. Likewise the 'long-wavelength band' with its comparatively low fluorescence yield reflects a strong quenching of W241 emission, most likely by the conserved disulfide bridge and the next neighbored Y242 residue. The results obtained imply that detailed analysis of the fluorescence properties indispensably require a deconvolution of the emission spectrum into its two major components. The characteristic features of these bands provide a useful tool to investigate structural properties of the MSP.

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