

Hydrophobic Character of Ca^{2+}-bound S-100 Complexes

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The hydrophobic character of S-100 complexes was investigated in terms of their adsorbability on phenyl-Sepharose gel in the presence of several co-adsorptive reagents. The following results were obtained: The hydrophobicity of S-100a.a′ was obviously increased by Ca^{2+}-binding, and that of Ca^{2+}-bound S-100a.a′ had practically no further increase upon mastoparan (MP)- or Zn^{2+}-binding. In the case of S-100b, on the contrary, the increase in the hydrophobicity by Ca^{2+}-binding was less than that for the former, and that of the Ca^{2+}-bound sample showed a further increase by MP-binding, or especially by Zn^{2+}-binding.

The hydrophobicity was also investigated with 2-*p*-toluidinonaphthalene-6-sulfonate (TNS). The adsorbability of the TNS-bound area was evaluated using fluorescence spectrophotometry. The hydrophobicity of Ca^{2+}-bound S-100a.a′ was enhanced only by MP-binding. In the case of Ca^{2+}-bound S-100b, both MP- and Zn^{2+}-binding had appreciable effects on the hydrophobicity, and their co-existence had a greater effect than the additive of each one.

S-100 proteins are Ca^{2+}-binding proteins existing in the bovine brain as a mixture of the three isoforms (S-100a, S-100a′, and S-100b) with a couple of subunits, respectively, of $\alpha\beta$, $\alpha'\beta$, and $\beta\beta$.[1,2] The chemical properties of subunits α and α' are so similar that a mixture of S-100a and S-100a′ (denoted as S-100a.a′) can be practically regarded as homogeneous; those of S-100b differ substantially from the former two.[3] S-100 proteins belong to the EF-hand protein family, as does calmodulin, and each subunit (molar mass $\fallingdotseq$ 10.5 kg mol^{-1}) contains two EF-hand domains, denoted as the C- and N-terminal ones. Their affinity to Ca^{2+} is given in terms of the dissociation constant, as K_{dCa} = 20–50 μM for the former and k_{dCa} = 200–500 μM for the latter.[4–6]

It is known that in vivo, like calmodulin,[7–9] the hydrophobic interactions of S-100 proteins with other proteins have a regulative role.[10,11] These interactions are enhanced by Ca^{2+}-binding to S-100 proteins, and their affinity to Ca^{2+} are increased by complexation with other proteins, e.g., Mastoparan (MP), a wasp toxin peptide,[12,13] with which an enhancement effect has been observed.

On the other hand, S-100 proteins show higher affinity to Zn^{2+} (K_{dZn} = 2 μM for S-100a.a′ and 0.16 μM for S-100b) than to Ca^{2+}.[6,14] The competitive effect of the co-existing Zn^{2+} was examined.[15] It has been shown that Zn^{2+} enhances the Ca^{2+} affinity of S-100b to a similar extent as MP, with practically no effect on S-100a.a′ being observed. For a further understanding of these results, it is desired to conduct a structural investigation on the characteristics of those complexes. The surface of the S-100 complex molecule should be surrounded by hydrophobic amino residues to some extent, depending on the degree of hydrophobicity. Those residues show affinity for aromatic compounds. S-100a.a′ and S-100b are separable by means of affinity-chromatography with phenyl-Sepharose gel in a zinc-dependent manner.[16] The term "affinity" means here a hydrophobic interaction between S-100 proteins and the phenyl residues of the gel.

In the present study, the hydrophobic character of S-100 complexes was investigated from four points of view. One is the hydrophobic character of the S-100 complexes, in terms of their adsorbability on phenyl-Sepharose gel. The second is the behavior of the characteristic hydrophobic amino residues, e.g., the tyrosine (Tyr) residues and tryptophan (Trp) residue of Ca^{2+}-bound S-100a.a′. These were investigated by fluorescence spectrophotometry and the difference spectrum method. The third is the reactivity of cysteine (Cys) residues to a thiol-specific reagent, 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB). The fourth is the appearence of a hydrophobic probe binding area on the surface on the S-100 complexes. A fluorescence environmental probe, *2-p*-toluidinonaphthalene-6-sulfonic acid potassium salt (TNS), was used for the above purpose.

Experimental

Materials. A sample was prepared from bovine brain as previously reported.[17] TNS and DTNB were purchased from Nakarai Co., Ltd., and Phenyl-Sepharose CL-4B gel from Pharmacia.

Adsorption on Gel. The gel was equilibrated with a solution of 0.3 M NaCl (1 M = 1 mol dm^{-3}), 20 mM Tris-HCl (pH = 7.15), and 2 mM 2-mercaptoethanol, added with the same volume of buffer. Three mL of the thus-prepared gel suspension was mixed with 3 mL of S-100 protein sample solutions of the following components: 30 μM S-100a.a′ in a buffer containing 3 mM EDTA, S-100a.a′ in a buffer containing 1 mM $CaCl_2$, S-100a.a′ in a buffer containing 1 mM $CaCl_2$ and 0.2 mM $ZnSO_4$, S-100a.a′ in a buffer containing 1 mM $CaCl_2$ and 60 μM MP, S-100a.a′ in a buffer containing 1 mM $CaCl_2$, 0.2 mM $ZnSO_4$, and 60 μM MP. The same components were also used for S-100b.

After mixing, the solution was allowed to stand for 30 minutes at room temperature, and the absorbance of the supernatant at 278 nm was measured with a Shimadzu UV-120 spectrophotometer.

The quantities of S-100a.a′ and S-100b remaining were determined from the molar-extinction coefficients of 1.22×10^4 M^{-1} cm^{-1} and 3.82×10^3 M^{-1} cm^{-1}, respectively.[1]

Difference Spectrum. Difference absorption spectra were measured at 25 °C with a Shimadzu 3100-S recording spectrophotometer. The experimental medium was a mixture of 20 mM Tris-HCl (pH = 7.15) and 2 mM 2-mercaptoethanol. The difference spectrum of Ca^{2+}/S-100a.a′ vs. S-100a.a′ was obtained with 100 μM of S-100a.a′ in the presence of Ca^{2+} at 1 mM. That of Ca^{2+}/S-100a.a′/MP vs. Ca^{2+}/S-100a.a′ was obtained with 20 μM of S-100a.a′ in the presence of MP at 25 μM. That of Ca^{2+}/S-100a.a′/Zn^{2+} vs. Ca^{2+}/S-100a.a′ was obtained with 100 μM of S-100a.a′ in the presence of Zn^{2+} at 0.2 mM.

Fluorescence Spectroscopy. Fluorescence measurements were carried out with a Hitachi MPF-4 spectrophotometer. A solution of 10 μM S-100a.a′ was excited with radiation of 275 nm (bandwidth 5 nm). The additives were 1 mM Ca^{2+}, 20 μM MP, and 0.1 mM Zn^{2+}, respectively.

TNS fluorescence measurements were performed by excitation with radiation of 330 nm (bandwidth 5 nm). The scanning wavelength region was from 380 to 540 nm (bandwidth 8 nm). The effect of TNS was observed with 10 μM S-100 complexes in a mole ratio of 1:1 under the same condition as the fluorescence measurements. The binding ratio of TNS to Ca^{2+}/S-100/Zn^{2+}/MP was obtained to be 1:1 using the continuous variation method.

Reaction of Cys-Residues. After completely removing 2-mercaptoethanol by dialysis, the Cys-residues in 30 μM S-100a.a′ complexes and in 20 μM S-100b complexes were reacted with 400 μM DTNB at 25 °C. The additives were 1 mM Ca^{2+}, 0.2 mM Zn^{2+}, and 60 μM MP for S-100a.a′, 40 μM MP for S-100b. The rate constants of Cys-Residues with DTNB were determined from the increase in the absorbance at 412 nm. The number of Cys-residues reacted in S-100a.a′ and S-100b were calculated, based on the coefficient of 5-nitro-2-thiobenzoate (1.36×10^4 M^{-1} cm^{-1}), to be 1.3 and 1.4 per mole of protein, respectively.

Results and Discussion

The results of an adsorption experiment on the S-100 complexes are summarized in Table 1. The decrease in the supernatant of the S-100 protein concentration reflects its increase in the adsorption to phenyl-Sepharose gel. Apparently, the binding of Ca^{2+} to S-100a.a′ caused an increase in the adsorption. In other words, the hydrophobicity of S-100a.a′ increased. The binding of Zn^{2+} or MP to S-100a.a′ also increased the adsorption slightly (Table 1), while the degrees of adsorption caused by them were 21% and 16%, respectevely, compared with that caused by the binding of Ca^{2+}. Once Ca^{2+}-binding sites in S-100a.a′ were saturated, the adsorption was not influenced by the addition of MP or Zn^{2+}. The surface of Ca^{2+}/S-100a.a′, Ca^{2+}/S-100a.a′/Zn^{2+}, and Ca^{2+}/S-100a.a′/MP would have a similar hydrophobicity.

Table 1. Concentrations of S-100 Proteins in the Supernatant Mixed with Phenyl-Sepharose Gel

Condition	[S-100a.a′]/μM	[S-100b]/μM
EDTA	17.9 ± 0.3	19.4 ± 0.8
Ca^{2+}	7.3 ± 0.1	15.2 ± 0.3
Zn^{2+}	15.7 ± 0.5	12.4 ± 0.2
MP	16.2 ± 0.2	18.5 ± 0.2
Ca^{2+}, Zn^{2+}	7.4 ± 0.2	8.1 ± 0.3
Ca^{2+}, MP	7.0 ± 0.1	12.5 ± 0.5
Ca^{2+}, Zn^{2+}, MP	7.1 ± 0.1	8.5 ± 0.2

The data are the mean ± S.D. of four runs.

The binding of Ca^{2+} to S-100b also caused an increase in the adsorption, and the degree of adsorption was between the effect caused by the binding of Zn^{2+} and that by the binding of MP. It is noted that the adsorption of Ca^{2+}-bound S-100b was further enhanced by the binding of Zn^{2+}, and MP did not increase the adsorption of Ca^{2+}-bound S-100b, as did Zn^{2+}. These effects show the characteristic differences between S-100a.a′ and S-100b. The adsorption of Ca^{2+}/S-100b/Zn^{2+}/MP was nearly equal to that of Ca^{2+}/S-100b/Zn^{2+}. In other words, the conformation change caused by MP-binding to Ca^{2+}/S-100b and that caused by Zn^{2+}-binding are not additive. In summary, the hydrophobicity of Ca^{2+}/S-100a.a′ was not affected by the binding of MP or Zn^{2+}. On the contrary, that of Ca^{2+}/S-100b was apparently increased by them, especially by the binding of Zn^{2+}.

In adsorption measurements of Ca^{2+}-bound S-100a.a′ complexes, no difference was found. This, however, necessarily denies the explicit presence of their conformation difference. Thus, this fact is shown by direct measurements, i.e., the difference absorption spectrum method and fluorescence spectrophotometry. Figure 1 shows the difference spectra of Ca^{2+}/S-100a.a′ vs. S-100a.a′ (a), Ca^{2+}/S-100a.a′/MP vs. Ca^{2+}/S-100a.a′ (b) and Ca^{2+}/S-100a.a′/Zn^{2+} vs. Ca^{2+}/S-100a.a′ (c). The first spectrum showed negative peaks at 278, 285, and 293 nm. Baudier and Gerard reported that the negative peaks at 278 and 285 nm appeared as a blue shift of the Tyr absorption band and the negative peak at 293 nm is a blue shift of the Trp absorption band.[3] Tyrosine residues are included in the α-subunit (Tyr-26, Tyr-74) and in the β-subunit (Tyr-17). The tryptophan residue (Trp-90) is located at the C-terminal of the α-subunit. Those shifts are regarded as resulting from a shift of their residues to the aqueous medium.[3] The shifts of the aromatic residues are consistent with the increase in the hydrophobicity of S-100a.a′ (Table 1). The spectrum in the presence of MP showed the same negative peaks at 285 and 293 nm as in the case of the difference spectrum obtained with Ca^{2+}, but the shape of the negative bands between 250 and 270 nm of phenylalanine residues differed from that obtained with Ca^{2+}. This means that the conformation of Ca^{2+}/S-100a.a′ differed from that of Ca^{2+}/S-100a.a′/MP.

The $\Delta\varepsilon_{285}$ value and $\Delta\varepsilon_{293}$ value in the difference spectrum obtained with Ca^{2+} were about -1300 M^{-1} cm^{-1} and -740 M^{-1} cm^{-1}, respectively. The corresponding values obtained with MP were -320 M^{-1} cm^{-1} and -150 M^{-1} cm^{-1}, respectively. The small $\Delta\varepsilon$ values suggest that the environment around the Trp and Tyr residues of Ca^{2+}/S-100a.a′/MP is appreciably similar to that of Ca^{2+}/S-100a.a′. The difference spectrum obtained with Zn^{2+} showed a positive shape, while the $\Delta\varepsilon$ values were also appreciably small compared with those in the difference spectrum obtained with Ca^{2+}. The $\Delta\varepsilon_{285}$ value and the $\Delta\varepsilon_{293}$ value in this spectrum were 190 M^{-1} cm^{-1} and 145 M^{-1} cm^{-1}, respectively, comparable to those obtained with MP, except for their sign. The small $\Delta\varepsilon$ values would imply that the change in the hydrophobicity around the Trp and Tyr residues would be small. In brief, the hydrophobicity in the area around the Trp and Tyr residues of Ca^{2+}/S-100a.a′ was

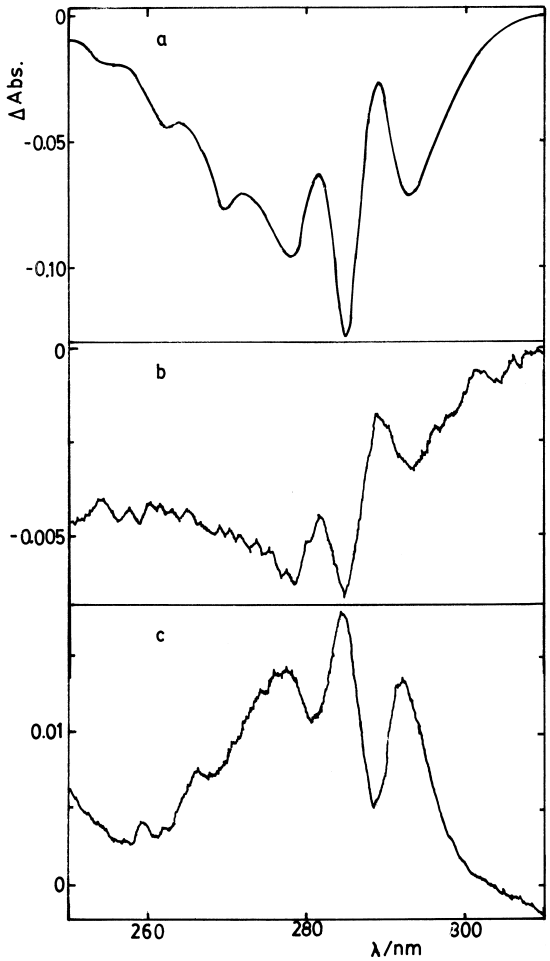

Fig. 1. The difference absorption spectrum of S-100a.a′ complexes. The upper spectrum shows the difference absorption spectrum of S-100a.a′ caused by Ca^{2+}, the middle one shows that of Ca^{2+}/S-100a.a′ caused by MP, and the lower shows that of Ca^{2+}/S-100a.a′ caused by Zn^{2+}, respectively. The contents of the medium are described in the text.

hardly affected by the binding of Zn^{2+} or MP.

The foregoing results were also confirmed by a fluorescence measurement. Figure 2 shows the emission fluorescence spectra of the S-100a.a′ and Ca^{2+}-bound S-100a.a′ complexes. The binding of Ca^{2+} to S-100a.a′ increased the fluorescence intensity accompanying a red shift of the emission peak from 340 nm to 345 nm. This shift has been regarded as resulting from the shift of Trp-90 to the aqueous medium.[3] Although the binding of Zn^{2+} and/or MP to the Ca^{2+}-bound S-100a.a′ caused an increase in the fluorescence intensity, these bindings were not coupled to the shift of the emission peak. No shift of the emission peak suggests that the Trp-90 environment in Ca^{2+}/S-100a.a′ is affected very little by the binding of Zn^{2+} and/or MP. In the difference spectrum method and fluorescence spectrophotometry, the surface hydrophobicity of Ca^{2+}-bound S-100a.a′ was confirmed to be similar to that of the Zn^{2+} and/or MP-bound Ca^{2+}/S-100a.a′ complexes.

As ellucidated in Table 1, the hydrophobicity of Ca^{2+}-bound S-100a.a′ complexes was not affected by the binding of MP or

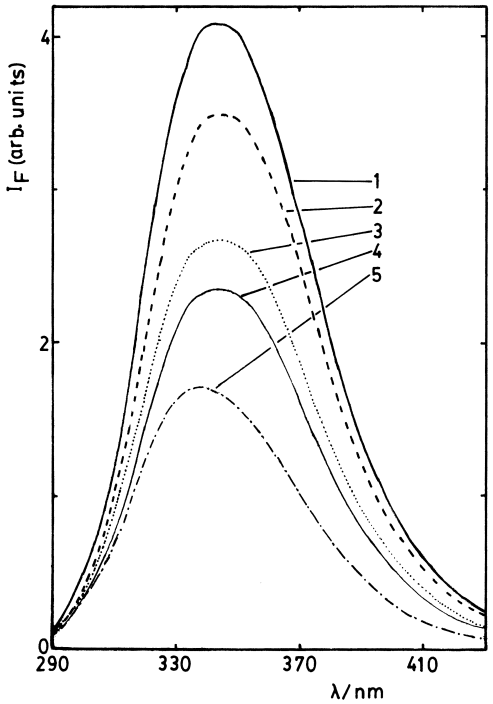

Fig. 2. The fluorescence spectra of Ca^{2+}/S-100a.a′/MP (1), Ca^{2+}/S-100a.a′/MP/Zn^{2+} (2), Ca^{2+}/S-100a.a′/Zn^{2+} (3), Ca^{2+}/S-100a.a′ (4), and S-100a.a′ (5).

Zn^{2+}. On the contrary, that of Ca^{2+}-bound S-100b was increased by them, especially by Zn^{2+}. This suggests that the reactivities of Cys-residues on the surface of Ca^{2+}-bound S-100a.a′ and S-100b complexes are different in the presence of the above-mentioned additives. Figure. 3 shows the reaction of DTNB with Cys-85 in α-subunit and Cys-84 in β-subunit in the S-100 complexes. The rate constants of S-100a.a′, Ca^{2+}/S-100a.a′, Ca^{2+}/S-100a.a′/MP, and Ca^{2+}/S-100a.a′/Zn^{2+} were 3×10^{-3} min^{-1}, 2.8×10^{-1} min^{-1}, 2.3×10^{-1} min^{-1}, and 9.8×10^{-2} min^{-1}, respectively. The corresponding results for S-100b, Ca^{2+}/S-100b, Ca^{2+}/S-100b/MP, and Ca^{2+}/S-100b/Zn^{2+} were 9×10^{-3} min^{-1}, 5.0×10^{-1} min^{-1}, 1.4×10^{-1} min^{-1}, and 1.5×10^{-2} min^{-1}, respectively. It was found that the foregoing Cys-residues shifted to the aqueous medium due to a conformational change caused by the binding of Ca^{2+}. The addition of MP or Zn^{2+} to Ca^{2+}/S-100a.a′ decreased the rate constant to 82% and 35%, respectively, compared with that of Ca^{2+}/S-100a.a′. In other words, Cys-residues became less accessible to the solvent medium. This phenomenon was also observed for the case of Ca^{2+}-bound S-100b complexes. The corresponding decreases of the rate constants for Ca^{2+}/S-100b/MP and Ca^{2+}/S-100b/Zn^{2+} were 28% and 3%, respectively. The remarkable decrease would show that the conformational change of Ca^{2+}/S-100b caused by MP or Zn^{2+} binding is more appreciable than those of Ca^{2+}/S-100a.a′ caused by them. These results are consistent with the data given in Table 1.

Information on the hydrophobicity of S-100 complexes, other than the Cys, Trp and Tyr residues, is thus needed. TNS, an environment indicator, has been used to examine the hydrophobicity of S-100 proteins.[18,19] Figure 4 shows the fluorescence spectra of TNS-bound S-100 complexes. As reported by

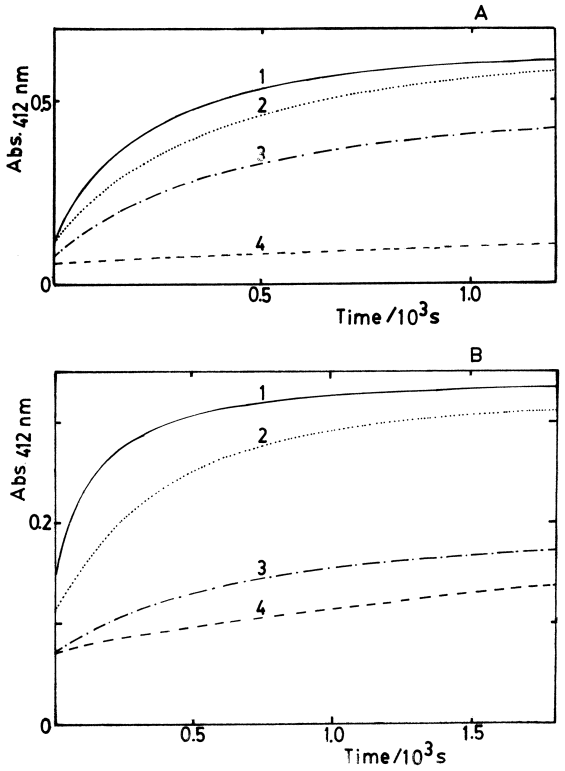

Fig. 3. The reaction of Cys-residues in S-100a.a′ complexes (A) and S-100b complexes (B) toward DTNB. Lines 1, 2, 3, and 4 show the reaction curve of Ca^{2+}/S-100, Ca^{2+}/S-100/MP, Ca^{2+}/S-100/Zn^{2+}, and S-100, respectively.

Ogoma et al.,[8] Ca^{2+}-binding to the S-100 protein caused an increase in the fluorescence intensity and the addition of Zn^{2+} to S-100b enhanced the above-mentioned effect accompanying a blue shift of the emission peak from 440 to 430 nm. The MP-binding to Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100b significantly increased the TNS fluorescence intensity accompanying a similar blue shift, and the addition of Zn^{2+} to the MP-bound complexes, especially to Ca^{2+}/S-100b/MP, further increased the TNS fluorescence intensity at 430 nm. The relative intensities of TNS fluorescence at 440 nm of Ca^{2+}/S-100a.a′, Ca^{2+}/S-100a.a′/Zn^{2+}, Ca^{2+}/S-100a.a′/MP, and Ca^{2+}/S-100a.a′/Zn^{2+}/MP were 1, 1, 18, and 19, respectively. The corresponding results for Ca^{2+}/S-100b, Ca^{2+}/S-100b/Zn^{2+}, Ca^{2+}/S-100b/MP, and Ca^{2+}/S-100b/Zn^{2+}/MP were 1, 1.8, 12, and 25, respectively. This order substantially coinsides with the order of the Ca^{2+} affinity of S-100a.a′ and S-100b.[15] The increase in hydrophobicity of TNS-bound area on the S-100 complexes would correlate with their Ca^{2+} affinity.

Because TNS does not compete with MP for binding to S-100 complexes, the TNS-binding site and the MP-binding site on S-100 protein differ from each other. On the contrary, Substance P, another target model peptide, competes with TNS for the hydrophobic site on S-100 complexes.[20] Dukhanina et al. have also found a similar phenomenon for other proteins of the S-100 family and their target peptides.[19] Therefore, the lack of competition of MP with TNS for the binding to S-100 protein would be a special example. Recently, the protein-protein interaction sites of the S-100 protein have been presumed by several authors[21,22] to be the C-terminal region of the subunit and the regions connecting the EF-hand structure, the so-called hinge regions. Kathryn et al. have also concluded, based on experiments involving NMR spectroscopy, that Ca^{2+}/S-100b shows a large localized hydrophobic surface including Phe-43, Ala-83, Phe-87, and Phe-88.[23] The foregoing regions would correspond to the MP-binding site and the TNS-binding site of S-100 complexes. A clear account for the mechanism of the increase in hydrophobicity regarding S-100 proteins has not

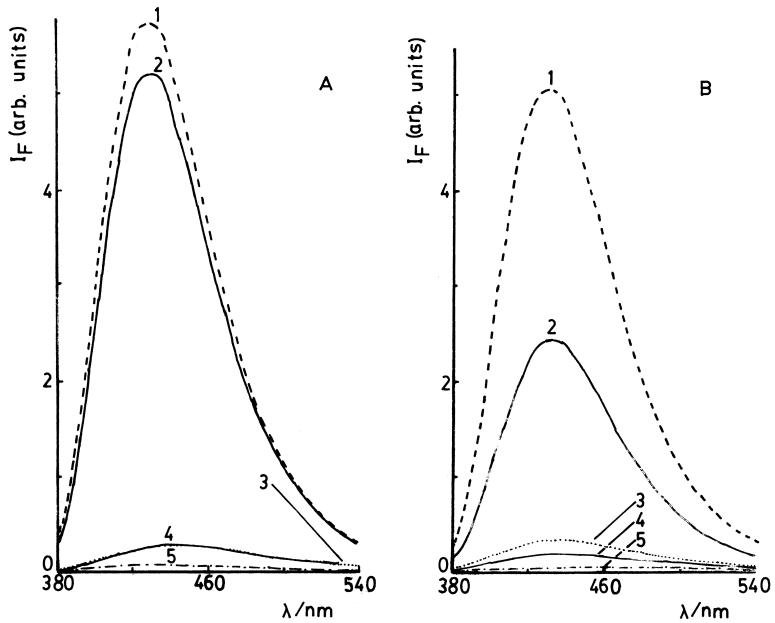

Fig. 4. The fluorescence spectrum of TNS-bound S-100a.a′ complexes (A) and S-100b complexes (B). Lines 1, 2, 3, 4, and 5 show the spectrum of Ca^{2+}/S-100/MP/Zn^{2+}, Ca^{2+}/S-100/MP, Ca^{2+}/S-100/Zn^{2+}, Ca^{2+}/S-100, and S-100, respectively.

yet been made.[24]

In conclusion, the hydrophobicity of Ca^{2+}-bound S-100a.a′ complexes was not affected by the binding of MP or Zn^{2+}. On the contrary, that of Ca^{2+}-bound S-100b was increased by them, especially by the binding of Zn^{2+}. The influence of those bindings on the hydrophobicity around the Tyr and Trp residues of S-100a.a′ was qualitatively consistent with the results of hydrophobicity. The highest hydrophobicity in TNS-bound area on Ca^{2+}-bound S-100b complex was achieved by the cooperative interaction of MP and Zn^{2+}. Thus, the presence of Zn^{2+} was essential for the formation of the foregoing area in the case of Ca^{2+}-bound S-100b, which suggests that zinc cations play an important role in the structure formation of the S-100b complex. A clear difference between Ca^{2+}-bound S-100a.a′ and S-100b comlexes was demonstrated.

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