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Hydrophobic Environment of Cysteine Residues in Pig Brain S-100 Proteins

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The effects of metal ion binding to pig brain S-100 proteins on the hydrophobicity of their cysteine (Cys) residues were examined using the fluorescent thiol reagent, *N*-(1-anilinonaphthyl-4)maleimide (ANM). The hydrophobicity order was Ca^{2+}/S-100 < K^+/S-100 < Mg^{2+}/S-100 < S-100 < Zn^{2+}/S-100.

Pig brain S-100 proteins are Ca^{2+}-binding proteins, which have three isoforms (S-100a, S-100a′, and S-100b) with a pair of $\alpha\beta$, $\alpha'\beta$, and $\beta\beta$ subunits, respectively.[1,2] The chemical properties of S-100a and S-100a′ are very similar, so a mixture of S-100a and S-100a′, denoted as S-100a.a′, can be practically regarded as homogeneous; whereas those of S-100b are substantially different from the former two.[3] The S-100 proteins are classified in the EF-hand protein family, and each subunit (10500 molecular weight) contains two EF-hand domains, denoted as the N- and C-terminal domains. The Ca^{2+} affinity of the C-terminal domain (K_{dCa} = 20–50 μM) is greater than that of the N-terminal one (K_{dCa} = 200–500 μM).[4–6] In addition, the S-100 proteins can bind Zn^{2+}, Mg^{2+}, and K^+.[3,7] The binding sites of Zn^{2+} have been found to be the histidine (His) residues, being unique from other cation-binding sites.[8]

It is known that the binding of Ca^{2+} to bovine brain S-100 proteins significantly enhances the reactivity of the cysteine (Cys) residues in the S-100 proteins to a thiol-specific reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[3,9] This effect is due to the shift of Cys-85 in the α-subunit and Cys-84 in the β-subunit to the aqueous medium caused by Ca^{2+}-binding.[3,9–11] It has also been reported that the binding of Zn^{2+} to the S-100 proteins hardly affects the reactivity of the Cys-residues toward DTNB.[3,9] Based on the results obtained using thiol-specific fluorescent probes, Baudier and Cole showed that the binding of Zn^{2+} to S-100b caused a shift of Cys-84 to a more hydrophobic environment.[9,11] It has been found that the binding of Mg^{2+} to the S-100 proteins moderately enhances the reactivity of the Cys-residues toward DTNB.[12] The binding of K^+ to the S-100 proteins showed no effect on the reactivity of the Cys-residues toward DTNB in a manner similar to the Zn^{2+}-binding.[13] The effects of both the Mg^{2+}-binding and K^+-binding to the S-100 proteins on the hydrophobic environment around the Cys-residues have not been investigated.

In the present study, the hydrophobic environment of the Cys-residues in the S-100 proteins caused by the K^+-binding or Mg^{2+}-binding was investigated by fluorescence measurements. A thiol-specific probe, *N*-(1-anilinonaphthyl-4)maleimide (ANM), was used for this purpose.[14] The content of the ANM-labeled Cys-residues in S-100a.a′ and S-100b was estimated to be about 1.5 and 0.9 per mol of protein using DTNB.[13] These values are somewhat less than the number of Cys-residues that reacted with DTNB in Ca^{2+}/S100a,a′ and Ca^{2+}/S-100b,[12,13] suggesting the validity of the labeling of Cys-84 in the β-subunit and Cys-85 in the α-subunit. The effects of the K^+-, Mg^{2+}-, Ca^{2+}-, and Zn^{2+}-bindings to pig brain S-100 proteins on the emission spectrum were compared with those to bovine brain S-100 proteins regarding the reactivity of the Cys-residues toward DTNB. Further, the effects of Li^+-, Na^+-, Sr^{2+}-, and Ba^{2+}-bindings to pig brain S-100a.a′ on the spectrum were also investigated because these cations have properties similar to those of K^+, Ca^{2+}, and Mg^{2+}.

Figures 1a and 1b show the emission spectra of ANM-labeled S-100a.a′ and S-100b. The relative intensities and the wavelengths of their emission peaks are summarized in Table 1. The binding of Ca^{2+} to S-100a.a′ decreased the fluorescence intensity along with a red shift in the emission peak from 428 to 448 nm. This shift can be regarded as resulting from the shift of Cys-85 in the α-subunit and Cys-84 in the β-subunit to the more aqueous environment.[3,9,11] The binding of K^+ to S-100a.a′ showed a 50% decreased red shift in the emission peak compared to the shift obtained by the binding of Ca^{2+} to S-100a.a′. The conformation change around those Cys-residues caused by the K^+-binding appears to be slighter than that caused by the Ca^{2+}-binding. The binding of Mg^{2+} to S-100a.a′ produced a slighter red shift in the emission peak (Table 1). This suggests that the conformation change around these Cys-residues caused by the binding of Mg^{2+} to S-100a.a′ is slight. In contrast with the binding of Ca^{2+}, K^+, and Mg^{2+}, the binding of Zn^{2+} to S-100a.a′ enhanced the fluorescence intensity in the emission peak by 37% without a peak shift. This indicates that the Cys-residues moved to the more hydrophobic environment.

The binding of Ca^{2+}, K^+, and Mg^{2+} to S-100b produced the same qualitative phenomenon as those to S-100a.a′. The emission peak of the Ca^{2+}/S-100b, K^+/S-100b, and Mg^{2+}/S-100b shifted slightly more than those of the S-100a.a′ complexes, reflecting the difference in the tertiary structure between S-100a.a′ and S-100b. The binding of Zn^{2+} to S-100b showed an obvious blue shift in the emission peak from 425 to 422 nm, indicating the shift of Cys-84 to the more hydrophobic environment. It has been confirmed that the binding of Zn^{2+} to S-100b plays an important role in the structure formation.[11,15]

Figure 1c shows the emission spectra of ANM-labeled S-100a.a′ in the presence of alkali and alkaline earth metal ions. Three results were obtained. The first is that the binding of Ba^{2+}, Sr^{2+}, and Ca^{2+} to S-100a.a′ showed an apparent red shift in the emission peak accompanying a decrease in the fluorescence intensity in this order. The second is that those changes caused by Ba^{2+}-binding, Sr^{2+}-binding, and Ca^{2+}-binding are more definitive than that caused by Mg^{2+}-binding to S-100a.a′ (Figs. 1a and 1c). The hydrophobic environment around the Cys-residues in Mg^{2+}/S-100a.a′ seems to be different from those in Ba^{2+}/S-100a.a′, Sr^{2+}/S-100a.a′, and Ca^{2+}/S-100a.a′. The third is that the wavelength of the emission

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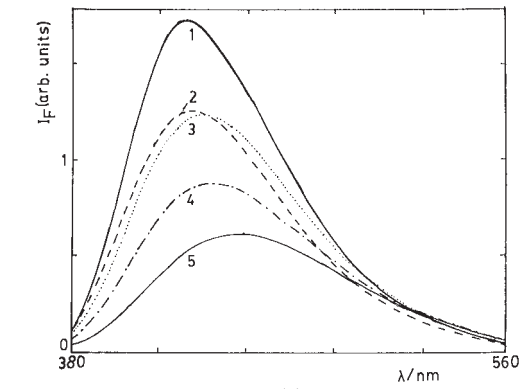
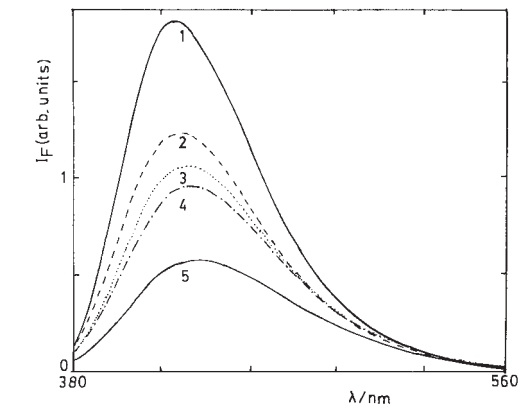
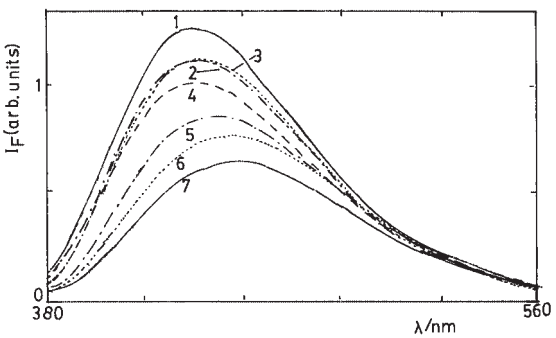


(a)



(b)



(c)

Fig. 1. The fluorescence spectra of ANM-labeled S-100a.a′ (a, c) and S-100b (b). In Figs. 1a and 1b, lines 1, 2, 3, 4, and 5 are the spectra of Zn^{2+}/S-100, S-100, Mg^{2+}/S-100, K^+/S-100, and Ca^{2+}/S-100, respectively. In Fig. 1c, lines 1, 2, 3, 4, 5, 6, and 7 show the spectra of Mg^{2+}/S-100a.a′, K^+/S-100a.a′, Na^+/S-100a.a′, Li^+/S-100a.a′, Ba^{2+}/S-100a.a′, Sr^{2+}/S-100a.a′, and Ca^{2+}/S-100a.a′, respectively. The protein concentration was 10 μM. The measurements were performed at 25 °C. The sample solution was excited with 355-nm (10-nm bandwidth) radiation.

peak of Li^+/S-100a.a′, Na^+/S-100a.a′, and K^+/S-100a.a′ was quite the same. The emission spectra of Na^+/S-100a.a′ nearly overlapped that of K^+/S-100a.a′. The hydrophobic environment around the Cys-residues in these S-100 complexes should be quite similar.

The effects of the binding of metal cations on the environment around the Cys-residues are classified into four group; Zn^{2+}, alkali cations, Mg^{2+}, and other alkaline earth metal cations. This was confirmed by the absorption difference spectrum measurements (Fig. 2). Sr^{2+}-binding to S-100a.a′ showed a negative difference absorption spectrum, in which the shape is substantially the same as that of the difference absorption spectrum caused by Ca^{2+}-binding. The molar coefficient of the former was about 40% of the latter. The conformation change in S-100a.a′ caused by Sr^{2+}-binding seems to be a degenerated form of that caused by Ca^{2+}-binding. This probably relates to the environment around the Cys-84, Cys-85 residues in S-100a.a′. The binding of Ba^{2+} to S-100a.a′ also showed a similar difference absorption spectrum to that caused by Ca^{2+}-binding except for the lack of a negative peak at 293 nm; the shift of the tryptophan (Trp)-90 residue in the α-subunit is probably slight. On the contrary, Mg^{2+}-binding to S-100a.a′ showed a very broad difference absorption spectrum without any peak.

Table 1. Wavelengths and Fluorescence Intensities at the Emission Peak of the S-100 Complexes

Condition	S-100a.a′		S-100b	
	λ_{max}/nm	I_F	λ_{max}/nm	I_F
GEDTA	428	1.26	425	1.23
Zn^{2+}	428	1.73	422	1.81
Mg^{2+}	433	1.23	428	1.06
K^+	438	0.88	428	0.96
Ca^{2+}	448	0.61	431	0.58

The binding of Li^+, Na^+, and K^+ to S-100a.a′ showed positive difference absorption spectra, which are mutually similar (Figs. 2e, 2f, and 2g). The binding of K^+ to S-100a.a′ showed an apparent positive peak at 294 nm. This suggests that K^+-binding to S-100a.a′ causes a shift of the Trp-90 residue to a more hydrophobic medium. Although the binding of Zn^{2+} to S-100a.a′ showed a negative difference absorption spectrum with several peaks, the shape of the spectrum differed from any of those caused by Ca^{2+}, Sr^{2+}, and Ba^{2+}-binding. The results obtained from the difference absorption spectrum measurements were consistent with those obtained from the ANM fluorescence spectroscopic measurements.

In summary, the hydrophobicity around the Cys-residues in S-100a.a′ and S-100b is as follows: Ca^{2+}/S-100 < K^+/S-100 < Mg^{2+}/S-100 < S-100 < Zn^{2+}/S-100. On the other hand, the order of the reactivity of their Cys-residues toward DTNB has been reported to be as follows: S-100, K^+/S-100, and Zn^{2+}/S-100 ≪ Mg^{2+}/S-100 ≪ Ca^{2+}/S-100.[9,12,13] The hydrophobicity around the Cys-residues in S-100a.a′ cannot account for the reactivity of their Cys-residues toward DTNB. The comparison of the former with the latter must be performed in a homologus series, Ba^{2+}/S-100a.a′, Sr^{2+}/S-100a.a′, and Ca^{2+}/S-100a.a′. The binding of Mg^{2+} to S-100a.a′, which causes a shift of more of the Cys-residues to the aqueous medium, differs fundamentally from the binding of Ca^{2+} to S-100a.a′. Although the rate constant of the Cys-residues in S-100a.a′ toward DTNB was little affected by the K^+-binding, the hydrophobicity of the Cys-residues in S-100a.a′ was decreased slightly by the K^+-binding. In order to obtain a definitive structural characterization around the Cys-residues in

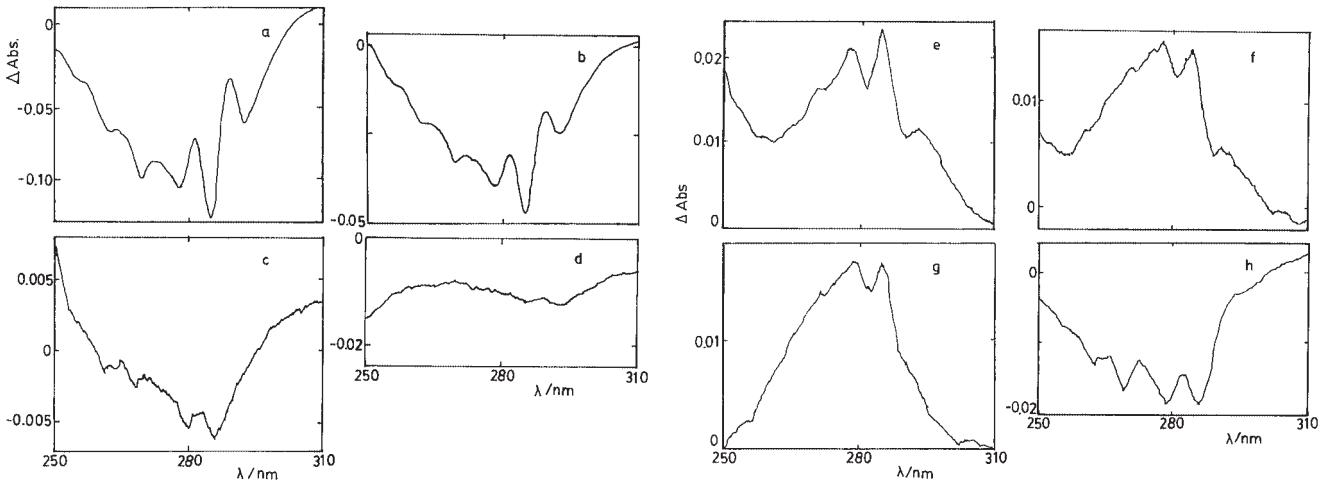


Fig. 2. The difference absorption spectra of S-100a.a′ complexes. In Fig. 2, a, b, c, d, e, f, g, and h show the difference absorption spectra of S-100a.a′ caused by Ca^{2+}-binding, Sr^{2+}-binding, Ba^{2+}-binding, Mg^{2+}-binding, K^{+}-binding, Na^{+}-binding, Li^{+}-binding, and Zn^{2+}-binding, respectively.

Mg^{2+}/S-100a.a′, K^{+}/S-100a.a′, and Ca^{2+}/S-100a.a′, the use of high-resolution NMR techniques will be needed.

Experimental

Materials. S-100a.a′ and S-100b were prepared from pig brain as described in a previous paper.[16] ANM and ethylenedioxybis(ethylamine)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Kanto Chemical Co., Inc.

Modification of Cys-Residues. After completely removing 2-mercaptoethanol by dialysis against 20 mM Tris-HCl (pH 7.15), the Cys-residues in the 150 μM S-100a.a′ and S-100b were reacted with 300 μM ANM for 1 h at 0 °C. The addition was 1 mM $CaCl_2$. The reaction was terminated by the addition of 3 mM 2-mercaptoethanol to ANM.[14] After the 2-mercaptoethanol was completely removed by dialysis, the sample solution was filtered using a 0.2-μm Millipore filter.

Fluorescence Spectroscopy. The fluorescence measurements were carried out using a Shimadzu RF-1500 spectrophotometer at 25 °C. The ANM fluorescence measurements were performed by excitation with 355-nm radiation (10-nm bandwidth). The scanning wavelength region was 380 to 560 nm (10-nm bandwidth). The experimental medium was a mixture of 10 μM S-100, 20 mM Tris-HCl (pH 7.15), and additives. The additives included 0.1 mM EGTA, 0.1 mM $ZnCl_2$, 10 mM $MgCl_2$, 0.1 M KCl, 1 mM $CaCl_2$, 1 mM $SrCl_2$, 1 mM $BaCl_2$, 0.1 M NaCl, and 0.1 M LiCl.

Difference Spectrum. The difference absorption spectra were measured at 25 °C using a recording spectrophotometer (Shimadzu UV-3100S). The difference spectrum of Ca^{2+}/S-100a.a′ vs. S-100a.a′, Sr^{2+}/S-100a.a′ vs. S-100a.a′, Ba^{2+}/S-100a.a′ vs. S-100a.a′, Mg^{2+}/S-100a.a′ vs. S-100a.a′ were obtained with 100 μM of S-100a.a′ in the presence of Ca^{2+} at 1 mM, Sr^{2+} at 1 mM, Ba^{2+} at 1 mM, and Mg^{2+} at 10 mM. Those of K^{+}/S-100a.a′ vs. S-100a.a′, Na^{+}/S-100a.a′ vs. S-100a.a′, Li^{+}/S-100a.a′ vs. S-100a.a′, Zn^{2+}/S-100a.a′ vs. S-100a.a′ were obtained in the presence of K^{+} at 0.1 M, Na^{+} at 0.1 M, Li^{+} at 0.1 M, and Zn^{2+} at 0.4 mM, respectively.

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