Verification of Conformation Change in Ca^{2+}-Bound S-100 Proteins Caused by Mg^{2+}-Binding

Sadayuki Matsuda

Hokkaido University of Education at Asahikawa, 9, Hokumon-cho, Asahikawa, Hokkaido 070-0825

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The conformation changes of S-100a.a′ and S-100b caused by Mg^{2+}-binding were determined by several methods. The fluorescence intensity of the Ca^{2+}-bound S-100a.a′ was enhanced by the Mg^{2+}-binding. The conformation changes in Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100b caused by Mg^{2+}-binding were also detected using a fluorescence environmental probe, 2-*p*-toluidinonaphthalene-6-sulfonate (TNS). The reactivities of the cysteine (Cys) residues in S-100a.a′ and S-100b to a thiol-specific reagent, 2,2′-dinitro-5,5′-dithiodibenzoic acid (DTNB), were increased by the Mg^{2+}-binding, regardless of the Ca^{2+}-binding.

Bovine brain S-100 proteins are Ca^{2+}-binding proteins (molar mass ≒ 21 kg/mol), consisting of S-100a($\alpha\alpha$), S-100a′($\alpha'\beta$), and S-100b($\beta\beta$).[1] 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; those of S-100b substantially differ from the former two.[2] The S-100 proteins belong to the EF-hand protein family, as does calmodulin, and each subunit contains two EF-hand domains, denoted as the C- and N-terminal ones. Their Ca^{2+} dissociation constants (K_{dCa}) are 20–50 μM for the former and 200–500 μM for the latter.[3–5]

It has been reported that Mg^{2+} does not produce a conformation change in the S-100 proteins.[2,3,6–8] However, Ogoma et al. directly observed Mg^{2+}-binding to S-100 proteins using the ^{25}Mg NMR spectroscopy technique.[9] The nondetection of the conformation change of the S-100 proteins caused by Mg^{2+}-binding seems to be unnatural, and the discrepancy remained. In previous studies[2,3,6–8] the effects of Mg^{2+} addition to S-100 proteins were investigated in the apo-state, and not in the Ca^{2+}-bound state.

In the present study, in order to detect the conformation change of Ca^{2+}-bound S-100 proteins caused by Mg^{2+}-binding, five methods were employed. One is fluorescence spectrophotometry of the tryptophan (Trp) residue of Ca^{2+}-bound S-100a.a′. The second is the use of a fluorescence environmental probe, the 2-*p*-toluidinonapthalene-6-sulfonic acid potassium salt (TNS) to Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100b. The third is the difference absorption spectrum method of Ca^{2+}/S-100a.a′. The fourth is the adsorption measurements of the above isoforms on phenyl-Sepharose gel. The fifth is to test the reactivity of cysteine (Cys) residues to a thiol-specific reagent, 2.2′-dinitro-5.5′-dithiodibenzoic acid (DTNB).

Experimental

Materials. S-100a.a′ and S-100b were prepared from bovine brain as previously reported.[10] TNS and DTNB were purchased from Nakarai Co., Ltd. All other reagents were of analytical grade and were used without further purification.

Fluorescence Spectroscopy. Fluorescence measurements were carried out with a Hitachi MPF-4 spectrophotometer at room temperature, the wavelength of the exciting light being 290 nm (bandwidth 10 nm). The experimental medium was a mixture of 10 μM S-100a.a′, 20 mM Tris-HCl (pH = 7.15), 1 mM $CaCl_2$, 1–15 mM $MgCl_2$, and 2 mM 2-mercaptoethanol.

The TNS fluorescence measurements were performed by excitation with 330 nm radiation (band width 5 nm). The scanning wavelength region was from 380 to 540 nm (band width 8 nm). Ten μM TNS was added to the S-100a.a′ and S-100b solution in the mole ratio of 1:1. The other conditions were the same as the fluorescence measurements.

Difference Spectrum. The difference absorption spectra were measured at 25 °C using a recording spectrophotometer (Shimadzu 3100-S). The difference spectra of Ca^{2+}/S-100a,a′ vs S-100a.a′, Mg^{2+}/S-100a.a′ vs S-100a.a′, and Ca^{2+}/S-100a.a′/Mg^{2+} vs Ca^{2+}/S-100a.a′ were obtained with 100 μM of S-100a.a′ in the presence of Ca^{2+} at 1 mM, and/or Mg^{2+} at 10 mM.

Adsorption on Gel. Adsorption of the S-100 proteins on the Phenyl-Sepharose CL-4B gel was analyzed using a previously reported procedure.[11] Briefly, 3 mL of a gel suspension equilibrated with 0.3 M NaCl, 20 mM Tris-HCl, and 2 mM 2-mercaptoethanol was mixed with 3 mL of the S-100 proteins sample solution containing 1 mM Ca^{2+} 10 mM Mg^{2+}. After 30 minutes, the concentration of S-100a.a′ and S-100b in the supernatant was determined from the absorbance at 278 nm.

Reaction of Cys Residues. After the 2-mercaptoethanol was completely removed by dialysis, the Cys-residues in the 20 μM S-100a.a′ and S-100b were reacted with 400 μM DTNB at 25 °C. The additives were 1 mM Ca^{2+} and/or 10 mM Mg^{2+}. The rate constants of the Cys-residues with DTNB were determined from the increase in the absorbance at 412 nm. A Shimadzu UV-3100S spectrophotometer was used for these measurements. The numbers of Cys-residues that reacted in Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100b were calculated, based on a molar extinction coefficient of 5-mercapto-2-nitrobenzoate (1.36×10^4 M^{-1} cm^{-1}), to be 1.9 and

1.0 per mol of protein, respectively. The corresponding values of Mg^{2+}/S-100a.a′ and Mg^{2+}/S-100b were 2.5 and 1.3 per mol of protein, respectively.

Results and Discussion

Figure 1a shows the emission fluorescence spectra of the S-100a.a′. The binding of Ca^{2+} to S-100a.a′ increased the fluorescence intensity, accompanied by a red shift of the emission peak from 344 nm to 348 nm. This shift has been regarded as a result of the shift of tryptophan (Trp)-90 in the α-subunit to the polar medium.[2] The addition of Mg^{2+} alone to S-100a,a′ increased the fluorescence intensity very little, with no shift in the emission peak. Because of the very slight increase (ca. 4%), verification of the conformation change of S-100a.a′ caused by Mg^{2+} -binding was difficult for the above method. The addition of Mg^{2+} to Ca^{2+}/S-100a.a′ increased the fluorescence intensity about 22% at 350 nm. The emission peak showed a very slight shift from 348 nm to 346 nm. In the Ca^{2+}-bound state, the conformation change of S-100a.a′ was clearly demonstrated. The fluorescence intensity at 350 nm was plotted versus the Mg^{2+} concentration (Fig. 1b). The Mg^{2+}-binding to Ca^{2+}-S-100a.a′ appears not to compete with the Ca^{2+}-binding to S-100a.a′ because the decrease in the fluorescence intensity at the emission peak does not occur. From this titration curve, the dissociation constant (K_{dMg}) was estimated to be 3×10^{-3} M. This value substantially agreed with that reported by Ogoma et al.[9]

The effect of Mg^{2+}-binding on the conformation of Ca^{2+}/S-100a.a′ was also examined by TNS fluorescence measurements. Figure 2a shows the fluorescence spectra of the TNS-bound S-100a.a′ complexes. The TNS fluorescence spectrum of S-100a.a′ and that of Mg^{2+}/S-100a.a′ slightly differed around 400-480 nm. This difference was clearly demonstrated under magnification (Fig. 2c). The emission peak in the fluorescence spectrum of TNS/S-100a.a′ shifted from 460 to 440 nm with the addition of Mg^{2+}. The Mg^{2+}-binding to S-100a.a′ increased the fluorescence intensity about 28% at 440 nm. The conformation change caused by Mg^{2+}-binding to S-100a.a′ was verified by the TNS fluorescence measurements.

As reported by Ogoma et al.,[12] Ca^{2+}-binding to the S-100a.a′ remarkably enhanced the fluorescence intensity. The addition of Mg^{2+} to Ca^{2+}/S-100a.a′ caused an increase in the fluorescence intensity accompanied by a slight shift in the emission peak from 444 nm to 440 nm. The relative intensities of the TNS fluorescence at 440 nm of S-100a.a′, Mg^{2+}/S-100a.a′, Ca^{2+}/S-100a.a′, and Ca^{2+}/S-100a.a′/Mg^{2+} were 1, 1.3, 7.9, and 11, respectively. The conformation change of Ca^{2+}/S-100a.a′ caused by Mg^{2+}-binding was confirmed. The fluorescence intensities at 440 nm of Ca^{2+}/S-100a.a′ and S-100a.a′ were plotted versus the added Mg^{2+} concentration (Fig. 2b and 2d). From the titration curve, K_{dMg} of Ca^{2+}/S-100a.a′ and that of S-100a.a′ were estimated to be 2×10^{-3} M and 2.5×10^{-3} M, respectively. These values were nearly identical with the value obtained in Fig. 1b.

The effect of Mg^{2+}-binding on the conformation of S-100b and Ca^{2+}/S-100b were examined in a similar manner to that described above. The addition of Mg^{2+} to S-100b also increased the fluorescence intensity, accompanied by a blue shift in the emission peak from 460 to 440 nm. The increase in the fluorescence intensity at 440 nm was 45%. The Mg^{2+}-binding

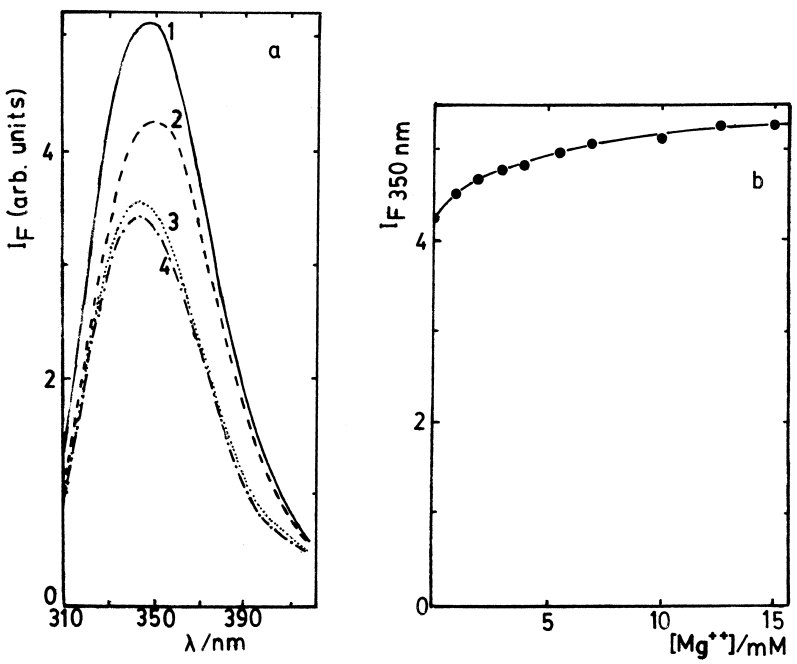

Fig. 1. The fluorescence spectra of S-100a.a′ (a) and the Mg^{2+} dependence of the fluorescence intensity (b). Lines 1, 2, 3, and 4 show the spectrum of Ca^{2+}/S-100a.a′/Mg^{2+}, Ca^{2+}/S-100a.a′, Mg^{2+}/S-100a.a′, and S-100a.a′, respectively. The concentration of S-100a.a′, Ca^{2+}, and Mg^{2+} were 10 μM, 1 mM , and 10 mM, respectively. The medium contains 20 mM Tris-HCl (pH = 7.15) and 2 mM 2-mercaptoethanol. A sample solution was excited with 290 nm (band width 10 nm) radiation. The measurements were performed at room temperature.

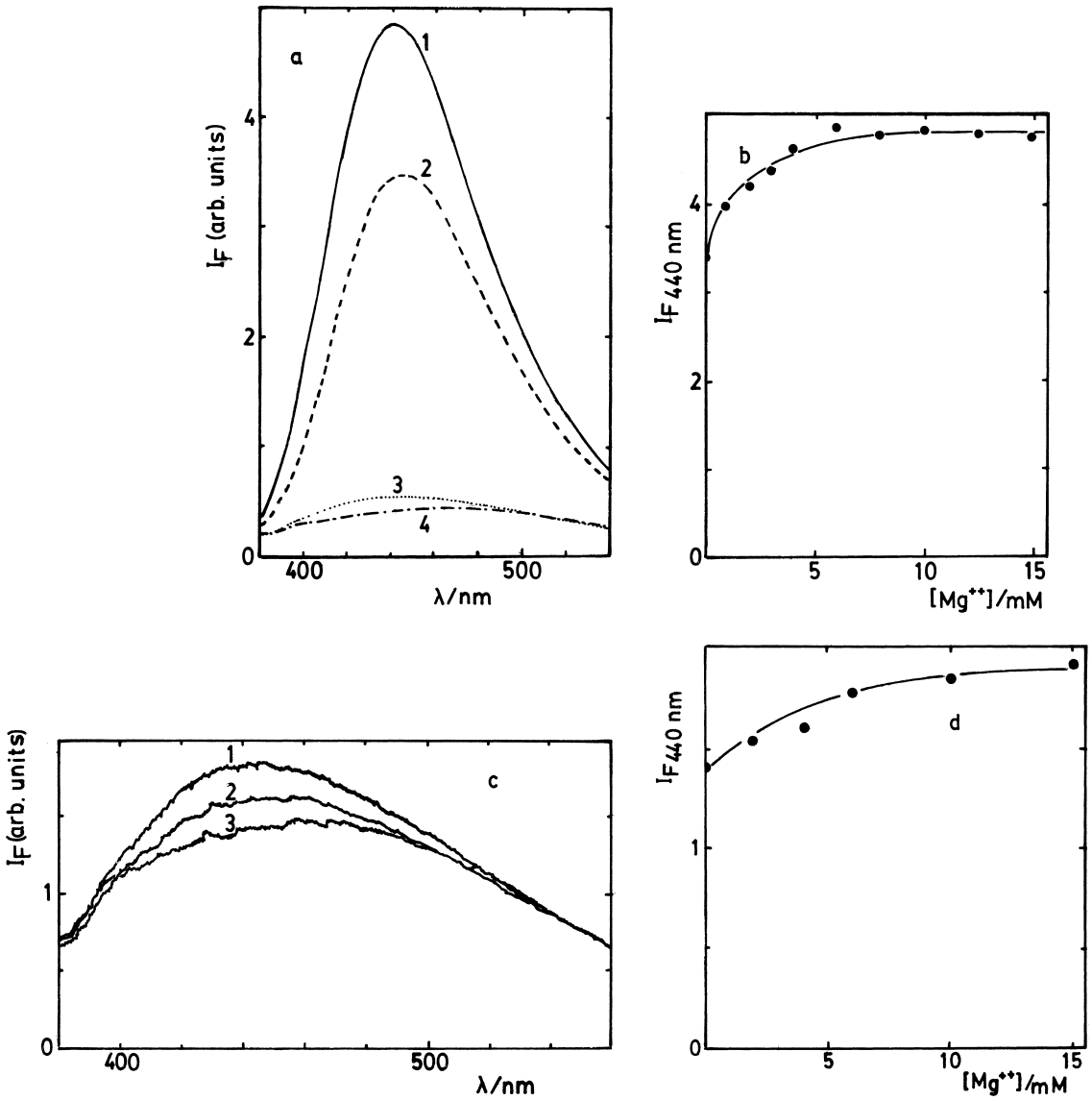

Fig. 2. The fluorescence spectra of TNS-bound S-100a.a′ (a) and the Mg^{2+} dependence of the fluorescence intensity of TNS/Ca^{2+}/S-100a.a, (b), the effect of Mg^{2+} on the fluorescence spectrum of TNS/S-100a.a′ (c), and the Mg^{2+} dependence of the fluorescence intensity of TNS/S-100a.a′ (d). In Fig. 2a, lines 1, 2, 3, and 4 show the spectrum of Ca^{2+}/S-100a.a′/Mg^{2+}, Ca^{2+}/S-100a.a′, Mg^{2+}/S-100a.a′, and S-100a.a′, respectively. Ten μM TNS was added to 10 μM S-100a.a′. The other contents of the medium are the same as described for Fig. 1. In Fig. 2c, lines 1, 2, and 3 indicate the fluorescence spectra of TNS-bound S-100a.a′ in the presence of 10, 4, and 0 mM Mg^{2+}, respectively. A sample solution was excited with 330 nm (band width 5 nm) radiation.

to Ca^{2+}/S-100b caused a slight increase in the TNS fluorescence intensity, i.e., about 11% increase at 440 nm. The relatively small increase in the TNS fluorescence intensity may be attributed to the difference in the tertiary structure between Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100b. The K_{dMg} values of Ca^{2+}/S-100b and S-100b were estimated to be 1×10^{-3} M and 3×10^{-3} M, respectively. These values substantially agreed with that reported by Ogoma et al.[9]

To verify the conformation change of the S-100 proteins caused by Mg^{2+}-binding, several methods were further employed. Figure 3 shows the results of the difference absorption spectrum of S-100a.a′. As already reported,[2,11] Ca^{2+}-binding to S-100a.a′ showed the apparent negative difference spectrum containing peaks at 278, 285, and 293 nm (Fig. 3a). Negative peaks at the former two indicate a blue shift of the absorptions of the Tyrosine (Tyr) residues: Tyr-26, Tyr-74 in the α-subunit, and Tyr-17 in the β-subunit. The Negative peak at 293 nm indicates a blue shift of the absorption of Trp-90. The value of $\Delta\varepsilon_{285}$ was -1300 M^{-1} cm^{-1}. On the contrary, Mg^{2+}-binding to S-100a.a′ showed a slightly positive broad spectrum; No sharp peaks were observed. The presence of the conformation change of S-100a.a′ caused by Mg^{2+}-binding was uncertain for this measurement. Mg^{2+}-binding to Ca^{2+}/S-100a.a′ showed a negative spectrum containing peaks at 282 and 289 nm (Fig. 3b). These negative peaks indicate a blue shift of the absorption of the Tyr residues. The contribution of Trp-90 to the difference spectrum appears to be negligible. Both the values of $\Delta\varepsilon_{282}$ and $\Delta\varepsilon_{289}$ were -120 M^{-1} cm^{-1}. The difference spec-

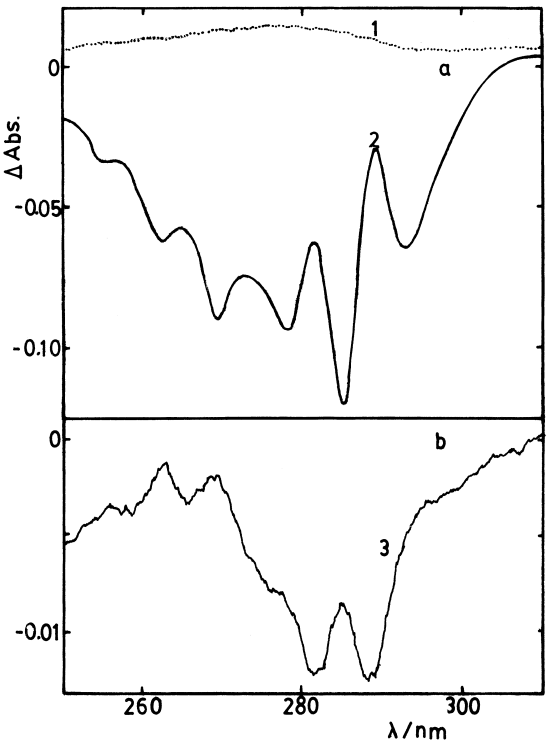

Fig. 3. The difference absorption spectra of the S-100a.a′ complexes. The upper one shows the difference absorption spectrum of S-100a.a′ caused by Mg^{2+} (1) and that of caused by Ca^{2+} (2), and the lower shows that of Ca^{2+}/S-100a.a′ caused by Mg^{2+}.

trum between 250 and 270 nm corresponds to the perturbation arising from the phenylalanine residues. These results confirmed the conformation change of Ca^{2+}/S-100a.a′ caused by Mg^{2+}-binding.

The conformation change of S-100a.a′ and S-100b caused by Mg^{2+}-binding is related to the change in their surface character, e.g., hydrophobicity. In order to detect the change in hydrophobicity, the adsorption of the S-100 proteins on the Phenyl-Sepharose CL-4B gel was examined in the presence of Ca^{2+} and Mg^{2+}. These results are summarized in Table 1. The addition of Mg^{2+} alone to S-100a.a′ did not affect the hydrophobicity, and this is also the case for S-100b. The addition of Mg^{2+} to Ca^{2+}/S-100a.a′ somewhat increased the hydrophobicity, reflecting the conformation change caused by the Mg^{2+}-binding. On the contrary, the addition of Mg^{2+} to Ca^{2+}/S-100b did not affect the hydrophobicity at all. This result suggests that the conformation change of Ca^{2+}/S-100b caused by Mg^{2+}-binding is small, compared to the case for Ca^{2+}/S-100a.a′. This idea coincides with the results obtained in Fig. 2.

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 ± 0.5
Ca^{2+} *	7.3 ± 0.1	15.2 ± 0.3
Mg^{2+}	17.3 ± 0.3	19.0 ± 0.6
Ca^{2+}, Mg^{2+}	6.1 ± 0.2	14.9 ± 0.5

The data are the mean ±S.D. of four runs. *:These are cited from a previous report.[11]

As elucidated in Fig. 2 and Table 1, the conformation change of Ca^{2+}/S-100b caused by Mg^{2+}-binding is small. Therefore, explicit evidence for the conformation change is needed. It has been found that the reactivity of Cys-residues in S-100a.a′ and in S-100b toward DTNB were remarkably increased by the binding of Ca^{2+}, while Zn^{2+} suppressed the effect of Ca^{2+} to some extent.[11] Thus the reactivity of Cys residues in S-100 proteins is sensitive to the metal ion binding. Figure 4 shows the reaction of DTNB with Cys-85 in the α-subunit and Cys-84 in the β-subunit in the Ca^{2+} and/or Mg^{2+} bound S-100 complexes. The rate constants of S-100a.a′, Mg^{2+}/S-100a.a′, Ca^{2+}/S-100a.a′, and Ca^{2+}/S-100a.a′/Mg^{2+} were 3×10^{-3} min^{-1}, 2.9×10^{-2} min^{-1}, 3.8×10^{-1} min^{-1}, and 1.9 min^{-1}, respectively. The corresponding results for S-100b, Mg^{2+}/S-100b, Ca^{2+}/S-100b, and Ca^{2+}/S-100b/ Mg^{2+} were 6×10^{-3} min^{-1}, 4.3×10^{-2} min^{-1}, 2.1×10^{-1} min^{-1}, and 4.7×10^{-1} min^{-1}, respectively. It was found that the binding of Mg^{2+} to S-100a.a′ and S-100b caused the shift of the Cys-residues in the aqueous medium. As previously described in the experimental section, the contents of the Cys residues that reacted toward DTNB in the Mg^{2+}-bound S-100 proteins were 30% greater than those of the Ca^{2+}-bound S-100

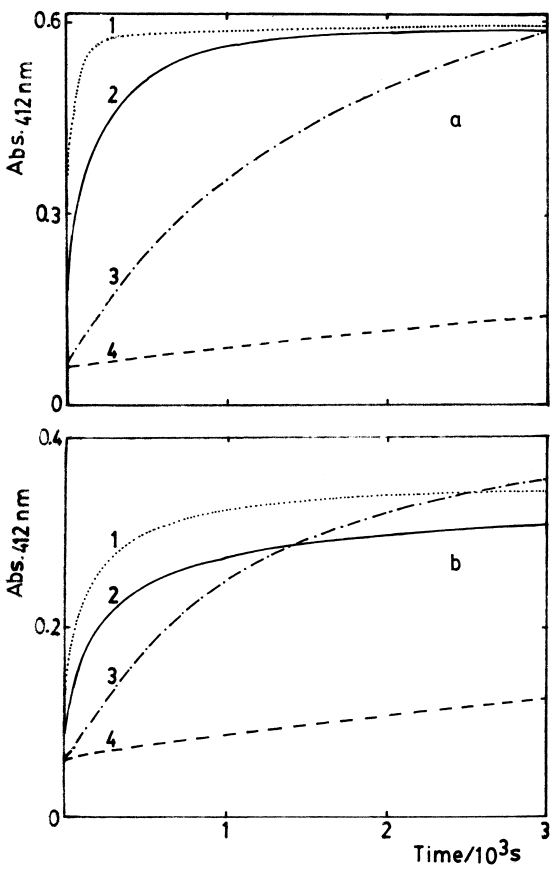

Fig. 4. The reaction of Cys-residues in S-100a.a′ (a) and S-100b (b) toward DTNB. Lines 1, 2, 3, and 4 show the reaction curves of Ca^{2+}/S-100/Mg^{2+}, Ca^{2+}/S-100, Mg^{2+}/S-100, and S-100, respectively.

proteins. This also supports the idea that Mg^{2+}- binding to S-100a.a′ causes the conformation change. In contrast with the binding of Zn^{2+} to Ca^{2+}/S-100, the binding of Mg^{2+} to Ca^{2+}/S-100 caused an increase in the rate constant.[11] The binding of Mg^{2+} to Ca^{2+}/S-100a.a′ increased the rate constant five-fold. The corresponding increase in the rate constant for Ca^{2+}/S-100b was about two-fold. The difference in the effect of the increase would suggest that the conformation change of Ca^{2+}/S-100a.a′ caused by Mg^{2+}-binding is more appreciable than that of Ca^{2+}/S-100b caused by Mg^{2+}-binding. These results are consistent with the data in Fig. 2 and in Table 1.

In summary, the conformation changes in the S-100 proteins caused by Mg^{2+}-binding have been missed so far by many authors.[2,3,6–8] However, their spectroscopic measurements were performed in the apo-state of the S-100 proteins. In the Ca^{2+}-bound state, the conformation change of the S-100 proteins caused by Mg^{2+}-binding was clearly found as previously described. The conformation change of Ca^{2+}/S-100a.a′ was more obvious than that of Ca^{2+}/S-100b. A difference between the Ca^{2+} bound S-100a.a′ and S-100b was demonstrated. The binding of Mg^{2+} alone to S-100 a.a′ and S-100b was found to cause a conformation change in them through the measurements of the reactivity of the Cys-residues toward DTNB. The TNS fluorescence measurements of each isoform in the apo-state also showed the presence of a conformation change caused by Mg^{2+}- binding.

References

1 T. Isobe and T. Okuyama, *Eur. J. Biochem.*, **116**, 79 (1981).
2 J. Baudier and D. Gerard, *Biochemistry*, **22**, 3360 (1983).
3 R. S. Mani, B. E. Boyes, and C. M. Kay, *Biochemistry*, **21**, 2607 (1982).
4 J. K. Hurley, M. F. Fillat, C. Gómez-Moreno, and G. Tollin, *Biochemie*, **77**, 539 (1995).
5 J. Baudier, N. Glasser, and D. Gerard, *J. Biol. Chem.*, **261**, 8192 (1986).
6 R. S. Mani, J. D. Shelling, B. D. Sykes, and C. M. Kay, *Biochemistry*, **22**, 1734 (1983).
7 Y. Ogoma, T. Miwa, T. Fujii, Y. Kondo, A. Hachimori, T. Shimizu, and M. Hatano, *Inorg. Chim. Acta*, **138**, 145 (1987).
8 P. L. Pingerelli, H. Mizukami, A. S. Wagner, D. E. Bartnicki, and J. P. Oliver, *J. Prot. Chem.*, **9**, 169 (1990).
9 Y. Ogoma, H. Kobayashi, T. Fujii, Y. Kondo, A. Hachimori, T. Shimizu, and M. Hatano, *Int. J. Biol. Macromol.*, **14**, 279 (1992).
10 S. Matsuda, *Bull. Chem. Soc. Jpn.*, **67**, 888 (1994).
11 S. Matsuda, *Bull. Chem. Soc. Jpn.*, **74**, 1083 (2001).
12 Y. Ogoma, T. Shimizu, H. Kobayashi, T. Fujii, A. Hachimori, Y. Kondo, T. Shimizu, and M. Hatano, *Biochem. Biophys. Acta*, **997**, 188 (1989).