

Conformation Changes in S-100 Proteins Caused by K^+-Binding

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The conformation changes in S-100a.a′ and S-100b caused by K^+-binding were determined by several methods in the apo-state and in the Ca^{2+}-bound state. 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 not affected by the K^+-binding. On the contrary, the reactivities of those in Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100b toward DTNB were enhanced or decreased, respectively, by the K^+-binding. The conformation changes in S-100b, Ca^{2+}/S-100a.a′, and Ca^{2+}/S-100b caused by the K^+-binding were also detected using a fluorescence environmental probe, 2-*p*-toluidinonaphthalene-6-sulfonate (TNS). A clear difference in the decrease of the TNS fluorescence intensity obtained by the K^+-binding between S-100a.a′ and S-100b was demonstrated in the Ca^{2+}-bound state. The measurements of the difference absorption spectrum of S-100a.a′ obtained by the K^+-binding showed a positive spectrum, while those of S-100b showed a negative spectrum, regardless of the Ca^{2+}-binding.

Bovine brain S-100 proteins are Ca^{2+}-binding proteins existing as a mixture of 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 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 substantially differ from the former two.[3] S-100 proteins belong to the EF-hand protein family, and each subunit (molar mass $\fallingdotseq$ 10.5 kg mol^{-1}) contains two EF-hand domains, denoted as the N- and C-terminal ones. Their Ca^{2+} dissociation constants (K_{dCa}) are 200–500 μM for the former and 20–50 μM for the latter.[4–6]

It has been reported that Ca^{2+}-binding to S-100 proteins is weakened by K^+-binding or Mg^{2+}-binding.[6,7] Measurements of the difference and the native fluorescence spectra show that K^+ did not significantly affect the conformation of the S-100 proteins.[6,7] However, Mani and Kay have shown that K^+ causes the Stokes' radius of S-100a.a′ and S-100b to decrease.[8] Conclusive proof has not been obtained with regard to the degree of the conformation change. Furthermore, the properties of the conformation changes in S-100a.a′ and S-100b caused by the K^+-binding have not been compared with those caused by the Mg^{2+}-binding.

In the present study, in order to detect the conformation change in the S-100 proteins caused by the K^+-binding, four methods were employed in the apo-state and in the Ca^{2+}-bound state. One involved testing the reactivity of the cysteine (Cys) residues to a thiol-specific reagent, 2,2′-dinitro-5,5′-dithiodibenzoic acid (DTNB). The second involved the use of a fluorescence environmental probe, the 2-*p*-toluidinonaphthalene-6-sulfonic acid potassium salt (TNS). These methods have been found to be sensitive enough for the detection of a conformation change in S-100a.a′ and S-100b caused by Mg^{2+}-binding.[9] This conformation change had previously been assumed not to occur.[6,7] The third involves the fluorescence spectrophotometry of the tryptophan (Trp) residue in S-100a.a′. The fourth utilizes the difference absorption spectrum method of each of the isoforms.

Experimental

Materials. A sample was prepared from bovine brain as previously reported.[10] DTNB and TNS were purchased from Nakarai Co., Ltd.

Reaction of Cys Residues. After completely removing the 2-mercaptoethanol by dialysis, the Cys residues in the 20 μM S-100a.a′ and S-100b were reacted with 400 μM DTNB in 20 mM Tris-HCl (pH = 7.15) at 25 °C. The additives were 1 mM $CaCl_2$ and/or 0.1 M KCl. The rate constants of the Cys residues with DTNB were determined from the increase in the absorbance at 412 nm. The number of Cys residues that reacted in Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100a.a′/K^+ was calculated on the basis of the molar extinction coefficient of 5-mercapto-2-nitrobenzoate (1.36×10^4 M^{-1} cm^{-1}), and were 1.7 and 1.9 per mol of protein, respectively. The corresponding values of Ca^{2+}/S-100b and Ca^{2+}/S-100b/K^+ were both 1.0 per mol of protein. A Shimadzu UV-3100 spectrophotometer was used for these measurements.

Fluorescence Spectroscopy. Fluorescence measurements were carried out with a Shimadzu RF-1500 spectrophotometer at room temperature. The TNS fluorescence measurements were performed by excitation with 330-nm radiation (band width 10 nm). The scanning wavelength region was from 380 to 580 nm (band width 10 nm). The experimental medium was a mixture of 10 μM S-100, 20 mM Tris-HCl (pH = 7.15), 1 mM $CaCl_2$, 0.01–0.4 M KCl, 10 μM TNS, and 2 mM 2-mercaptoethanol.

The native fluorescence measurements of S-100a.a′ were performed by excitation with 290-nm radiation (band width 10 nm). The scanning wavelength region was from 310 to 450 nm (band width 10 nm). The additives were 1 mM Ca^{2+} and/or 0.1 M KCl.

Difference Spectrum. The difference spectra were measured at 25 °C using a recording spectrophotometer (Shimadzu 3100-S). The difference spectrum of K^+/S-100 vs S-100 was obtained with 100 μM of each isoform in the presence of K^+ at 0.1 M. That of

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Ca^{2+}/S-100/K^+ vs Ca^{2+}/S-100 was obtained in the presence of Ca^{2+} at 1 mM.

Results and Discussion

Figure 1 shows the reaction of DTNB with Cys-85 in the α-subunit and Cys-84 in the β-subunit of the Ca^{2+} and/or K^+-bound S-100 complexes. The rate constants of S-100a.a′, K^+/S-100a.a′, Ca^{2+}/S-100a.a′, and Ca^{2+}/S-100a.a′/K^+ were 4×10^{-3} min^{-1}, 4×10^{-3} min^{-1}, 0.36 min^{-1}, and 0.47 min^{-1}, respectively. The corresponding results for S-100b, K^+/S-100b, Ca^{2+}/S-100b, and Ca^{2+}/S-100b/K^+ were 5×10^{-3} min^{-1}, 5×10^{-3} min^{-1}, 0.21 min^{-1}, and 5.3×10^{-2} min^{-1}, respectively. It was found that the binding of K^+ to S-100a.a′ and S-100b did not cause a significant shift in these Cys residues in aqueous medium. In contrast to the binding of K^+, it has been found that the binding of Mg^{2+} to S-100a.a′ and S-100b caused a shift in the Cys residues in aqueous medium.[9] The Cys residues that reacted to DTNB in the Mg^{2+}-bound S-100 proteins were 30% greater than those of the Ca^{2+}-bound S-100 proteins.[9] It is probable that the K^+-binding to the S-100 proteins, which differs from the Ca^{2+}-binding and the Mg^{2+}-binding to them,[9,11] hardly affects their conformation in the C-terminal of each subunit.

The binding of K^+ to Ca^{2+}/S-100a.a′ increased the rate constant 30% with a 12% increase in the content of the Cys residues that reacted to DTNB. On the other hand, the binding of K^+ to Ca^{2+}/S-100b decreased the rate constant by 75% with no variation in their content. The conformation change in Ca^{2+}/S-100a.a′ caused by the K^+-binding appears to cause a shift in the Cys residues in aqueous medium, while that in Ca^{2+}/S-100b caused by K^+-binding appears to cause a reverse shift of the Cys residues. These results would reflect the difference in the tertiary structure between Ca^{2+}/S-100a.a′/K^+ and Ca^{2+}/S-100b/K^+. The binding of K^+ to S-100 proteins, in vivo, may have a role in modulating their tertiary structure. As previously reported,[9] the binding of Mg^{2+} to Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100b increased the rate constant five-fold and two-fold, respectively. The reactivities of the Cys residues in the Ca^{2+}/S-100 proteins toward DTNB were more affected by the Mg^{2+}-binding than by the K^+-binding. Irrespective of the Ca^{2+}-binding, the conformation changes in the S-100 proteins caused by the K^+-binding seem to be less appreciable than those caused by the Mg^{2+}-binding.

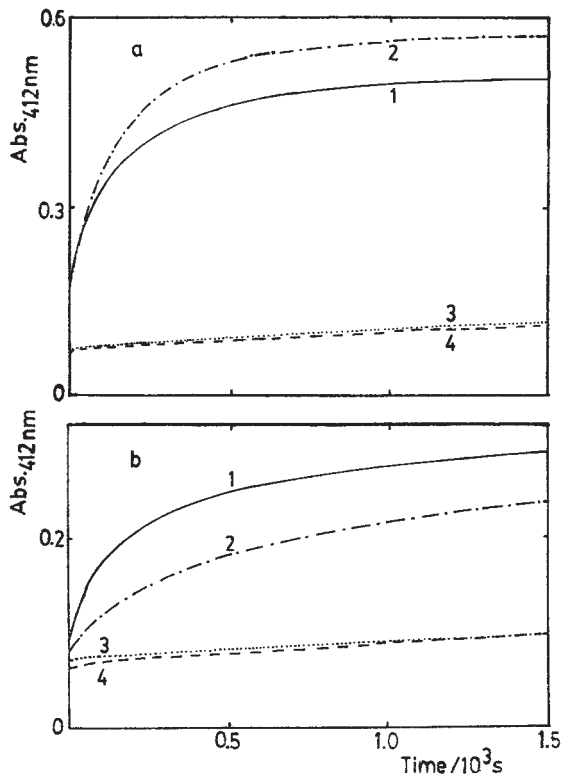

Fig. 1. The reaction of Cys residues in S-100a.a′ (a) and S-100b (b) toward DTNB. Lines 1, 2, 3, and 4 are the reaction curves of Ca^{2+}/S-100, Ca^{2+}/S-100/K^+, K^+/S-100, and S-100, respectively.

The effects of K^+-binding on the conformation of the S-100 proteins were also examined by TNS fluorescence measurements in the apo-state and Ca^{2+}-bound state. Figure 2A shows the fluorescence spectra of the TNS bound S-100a.a′ complexes and S-100b complexes. The TNS fluorescence spectrum of S-100a.a′ and that of K^+/S-100a.a′ substantially agreed, while the K^+-binding to S-100b produced a slight increase in the fluorescence intensity around 390–460 nm, accompanied by a blue shift in the emission peak from 460 to 440 nm. This indicates that the TNS-binding site in S-100b shifts to the hydrophobic medium. The detection of the conformation change in S-100b alone confirmed that the K^+-binding to the S-100 proteins does not significantly affect the conformation of each isoform in the apo-state. As previously reported,[9,11] Ca^{2+}-binding to the S-100a.a′ and S-100b remarkably enhanced the fluorescence intensity. The addition of K^+ to Ca^{2+}/S-100a.a′ caused a 25% decrease in the intensity of the emission peak, while the corresponding decrease for Ca^{2+}/S-100b was 53%. As elucidated in Fig. 1, the binding of K^+ to Ca^{2+}/S-100a.a′ and Ca^{2+}/S-100b affected the conformation of Cys-85 and that of Cys-84 in the C-terminal, respectively. The binding of K^+ to Ca^{2+}/S-100 also affected the conformation of the TNS-binding site on each isoform, because the C-terminal region includes the TNS-binding site.[3] The difference in the effect of the decrease suggests that the conformation change in Ca^{2+}/S-100a.a′ caused by the K^+-binding is less appreciable than that of Ca^{2+}/S-100b. This order is the opposite of the effect of the Mg^{2+}-binding to Ca^{2+}/S-100 on the conformation change.[9] The effect of K^+-binding to the S-100 proteins on the conformation change is more obvious in the Ca^{2+}-bound state than in the apo-state. This result is consistent with the data in Fig. 1.

The fluorescence intensity at 450 nm for Ca^{2+}/S-100a.a′ and that at 440 nm for Ca^{2+}/S-100b were plotted versus the added K^+ concentration (Fig. 2B). That at 440 nm for S-100b was similarly plotted (Fig. 2C). The data in Fig. 2B showed that at least one of the K^+-binding sites differed from the Ca^{2+}-binding sites. The shape of the titration curve of Ca^{2+}/S-100a.a′ appeared to differ from that of Ca^{2+}/S-100b. This suggests that the properties of the K^+-binding to Ca^{2+}/S-100a.a′ differ from those of the K^+-binding to Ca^{2+}/S-100b. The titration curve of S-100b showed a sigmoid shape, and did not reach saturation at 0.4 M KCl.

The foregoing results (Figs. 1A and 2A) showed that the

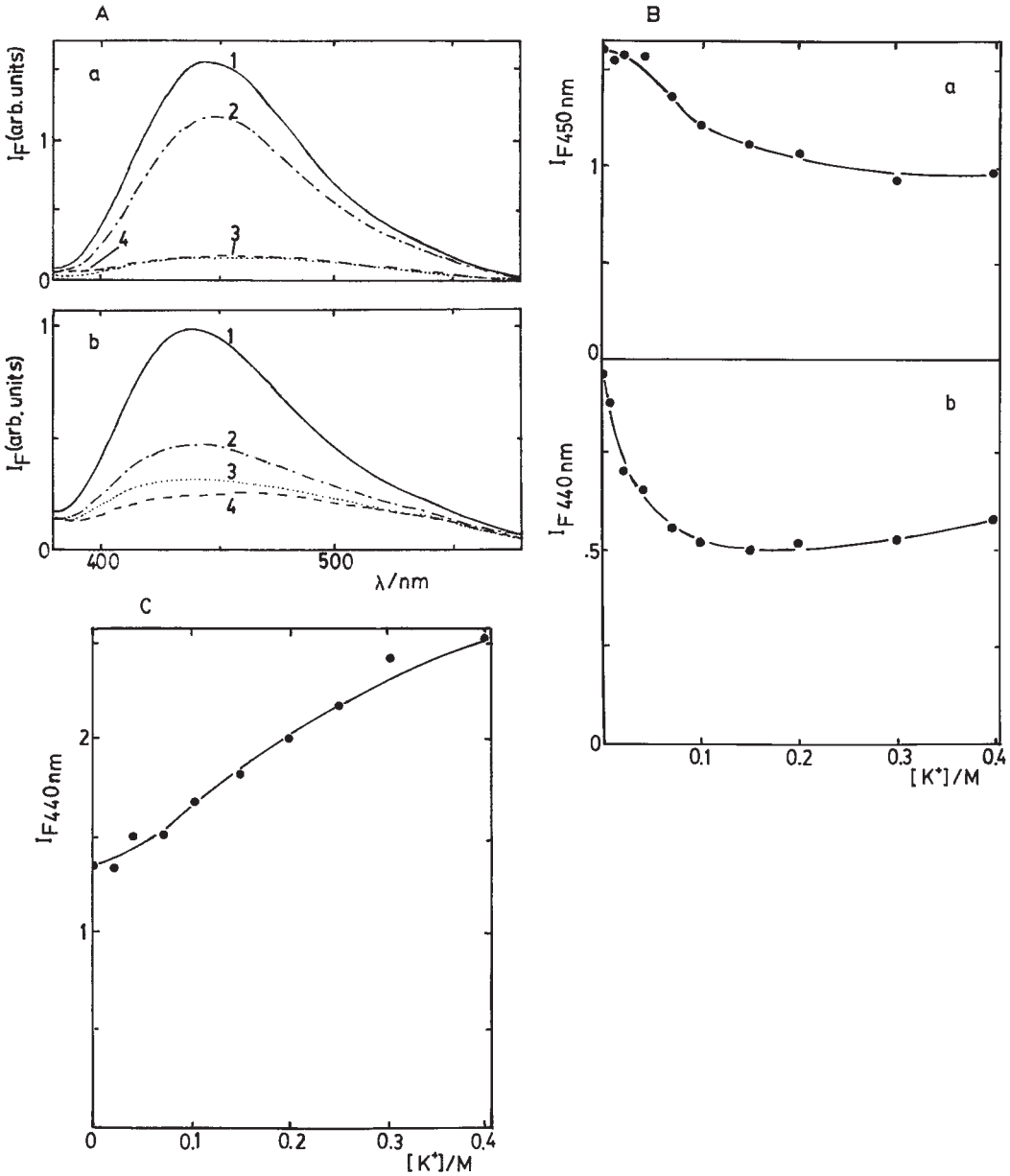

Fig. 2. The fluorescence spectra of TNS-bound S-100a.a′ (Aa) and S-100b (Ab), and the K^+ dependence of the TNS fluorescence intensity of Ca^{2+}/S-100a.a′ (Ba), Ca^{2+}/S-100b (Bb), and S-100b (C). In Fig. 2A, lines 1, 2, 3, and 4 are the spectra of Ca^{2+}/S-100, Ca^{2+}/S-100/K^+, K^+/S-100, and S-100, respectively. Ten μM TNS was added to 10 μM S-100. The concentrations of Ca^{2+} and K^+ were 1 mM and 0.1 M, respectively. The medium contains 20 mM Tris-HCl (pH = 7.15) and 2 mM 2-mercaptoethanol. The measurements were performed at room temperature. The sample solution was excited with 330 nm (band width 10 nm) radiation.

K^+-binding to apo-S-100a.a′ hardly caused any conformation changes, particularly in the C-terminal region of the α,β-subunit. This was also confirmed by a fluorescence measurement. Figure 3 shows the emission spectra of S-100a.a′. The binding of Ca^{2+} to S-100a.a′ increased the fluorescence intensity, accompanied by a red shift in the emission peak from 344 to 348 nm. This shift has been regarded as resulting from the shift in Trp-90 to the more aqueous environment.[3] The addition of K^+ alone to S-100a.a′ decreased the fluorescence intensity very little, but caused a very slight blue shift in the emission peak. A similar phenomenon was observed for the addition of K^+ to Ca^{2+}/S-100a.a′. The K^+-binding to Ca^{2+}/S-100a.a′ caused the shift in Trp-90 to the more hydrophobic environment. The conformation change around Trp-90 caused by the K^+-binding appears to be slight. The reason for this is assumed as follows. Since the electric field of K^+ is appreciably weaker than that of Ca^{2+} or Mg^{2+}, the conformation change caused by the K^+-binding to S-100 is smaller than that caused by Ca^{2+}-binding or Mg^{2+}-binding. Furthermore, Trp-90, located near the C-terminal (Cys-93), is spatially far from the K^+-binding site, e.g., the loop in the C-terminal EF-hand structure.[12–15]

The effects of the K^+-binding to S-100a.a′ and S-100b on the conformation changes in them were further investigated using the difference spectrum method. Figure 4 shows the differ-

ence spectra of K^+/S-100a.a′ vs S-100a.a′ (a1), Ca^{2+}/S-100a.a′/K^+ vs Ca^{2+}/S-100a.a′ (a2), K^+/S-100b vs S-100b (b1), and Ca^{2+}/S-100b/K^+ vs Ca^{2+}/S-100b (b2). The first spectrum showed positive peaks at 277, 284, and 295 nm. The former two peaks indicate a red shift in the absorptions of the Tyrosine (Tyr) residues: Tyr-26, Tyr-74 in the α-subunit, and Tyr-17 in the β-subunit. The positive peak at 295 nm indicates a red shift in the absorption of Trp-90. The $\Delta\varepsilon_{277}$ value, $\Delta\varepsilon_{284}$ value, and $\Delta\varepsilon_{295}$ value in the difference spectrum obtained with K^+ were 130 $M^{-1}\,cm^{-1}$, 130 $M^{-1}\,cm^{-1}$, and 40 $M^{-1}\,cm^{-1}$, respectively. These values are low compared with the $\Delta\varepsilon_{278}$ value (-930 $M^{-1}\,cm^{-1}$), $\Delta\varepsilon_{285}$ value (-1300 $M^{-1}\,cm^{-1}$), and $\Delta\varepsilon_{293}$ value (-740 $M^{-1}\,cm^{-1}$), in the difference spectrum obtained with Ca^{2+}-binding.[6,9,11] For the Ca^{2+}-binding to S-100a.a′, the above negative $\Delta\varepsilon$ values have been attributed to the shifts in the Tyr-residues and Trp-90 to the aqueous medium.[3,6] The small positive $\Delta\varepsilon$ values imply that the Trp and Tyr residues of S-100a.a′ shift to the more slightly hydrophobic medium by the K^+-binding. The above results are consistent with those of Fig. 3. In contrast to the difference spectrum of S-100a.a′, that of S-100b obtained with K^+ showed sharp negative peaks at 281 nm and 289 nm. These peaks indicate a blue shift in the absorption of Tyr-17. Both the $\Delta\varepsilon$ values were low, 60 $M^{-1}\,cm^{-1}$; so the conformation change around Tyr-17 seems to be slight. This result is consistent with the results in Figs. 1 and 2.

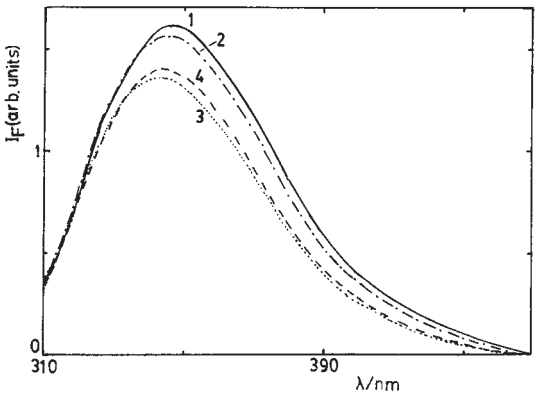

Fig. 3. The fluorescence spectra of Ca^{2+}/S-100a.a′ (1), Ca^{2+}/S-100a.a′/K^+ (2), K^+/S-100a.a′ (3), and S-100a.a′ (4). The concentrations of S-100a.a′, Ca^{2+}, and K^+ were the same as those described in Fig. 2A. The sample solution was excited with 290 nm (band width 10 nm) radiation.

The binding of K^+ to Ca^{2+}/S-100a.a′ showed a positive difference spectrum containing sharp peaks. The peaks at 278 and 284 nm are due to the red shift in the absorptions of the Tyr residues and the peak at 293 nm is due to a red shift in the absorption of Trp-90. The peaks between 250 and 270 nm correspond to the perturbation arising from the phenylalanine (Phe) residues. The values of $\Delta\varepsilon_{278}$, $\Delta\varepsilon_{284}$, and $\Delta\varepsilon_{293}$ were 360 $M^{-1}\,cm^{-1}$, 370 $M^{-1}\,cm^{-1}$, and 200 $M^{-1}\,cm^{-1}$, respectively. The $\Delta\varepsilon$ values obtained with K^+ in the Ca^{2+}-bound state were several times larger than those obtained in the apo-state. In other words, the conformation change in Ca^{2+}/S-100a.a′ caused by the K^+-binding is more appreciable than that in S-100a.a′. The positive $\Delta\varepsilon$ values show that the K^+-binding to Ca^{2+}/S-100a.a′ caused the shifts in Phe-, Tyr-residues and Trp-90 to the more hydrophobic environment. This is consistent with the result in Fig. 3. The effect of the K^+-binding on the conformation of Ca^{2+}/S-100b was examined in a similar manner. The negative peaks at 282 and 288 nm are due to the blue shift in the absorption of Tyr-17, and the corresponding $\Delta\varepsilon$ values were -160 $M^{-1}\,cm^{-1}$ and -110 $M^{-1}\,cm^{-1}$, respectively. These values are two to three times those of apo-S-100b obtained with the K^+-binding. The effect of K^+-binding

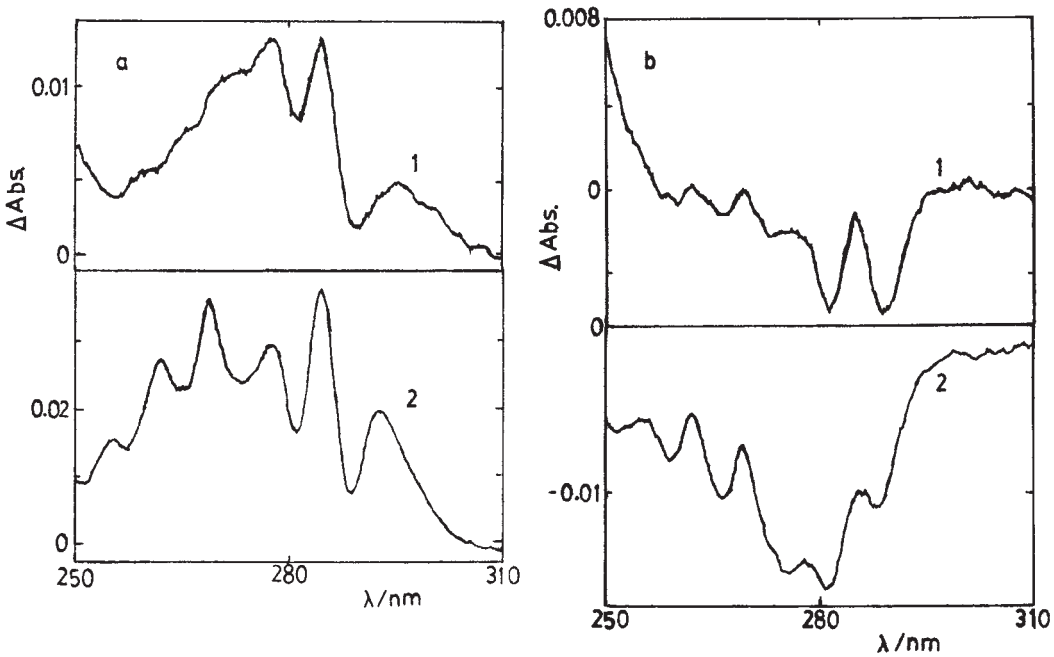

Fig. 4. The difference absorption spectra of S-100a.a′ (a) and S-100b (b) caused by K^+-binding. The upper one shows the difference absorption spectrum of K^+/S-100 vs S-100, and the lower shows that of Ca^{2+}/S-100/K^+ vs Ca^{2+}/S-100.

on the conformation of Ca^{2+}/S-100b was greater than that on the conformation of S-100b. The results in Figs. 1 and 2 were also confirmed by the difference spectrum measurements.

In summary, the conformation changes around the Cys residues and the TNS-binding site in the S-100 proteins caused by K^+-binding are slight in the apo-state; this especially applies to S-100a.a′. These observations make the hypothesis of Mani et al. unlikely. In the Ca^{2+}-bound state, these changes caused by the K^+-binding are quite obvious. Although the binding of K^+ to Ca^{2+}/S-100a.a′ enhanced the reactivity of the Cys residues toward DTNB, that of K^+ to Ca^{2+}/S-100b showed the opposite effect. The difference in the effect of K^+-binding between S-100a.a′ and S-100b was also demonstrated by the difference absorption spectroscopy, regardless of the Ca^{2+}-binding.

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