Spectral studies on the cadmium-ion-binding properties of bovine brain S-100b protein

Henry DONATO, Jr.,* Rajam S. MANI and Cyril M. KAY†

Medical Research Council Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

The effect of Cd^{2+} binding on bovine brain S-100b protein was studied using c.d. u.v. difference spectroscopy and fluorescence measurements. At pH 7.5, S-100b protein binds two Cd^{2+} ions per monomer with a K_d value of 3×10^{-5} M. Addition of Cd^{2+} resulted in perturbing the single tyrosine residue (Tyr^{17}) in the protein as indicated by u.v. difference spectroscopy and aromatic c.d. measurements. In the presence of Cd^{2+} , the tyrosine residue moves to a more non-polar environment, since a red shift was observed in the u.v. difference spectrum. When the protein was excited at 278 nm, the tyrosine fluorescence emission maximum was centred at 306 nm. Cd^{2+} addition resulted in an increase in intrinsic fluorescence intensity. Fluorescence titration with Cd^{2+} indicated the protein binds Cd^{2+} with a K_d value of 3×10^{-5} M. 2-*p*-Toluidinylnaphthalene-6-sulphonate-labelled protein, when excited at 345 nm, had a fluorescence emission maximum at 440 nm. Addition of Cd^{2+} to labelled protein resulted in a 5-fold increase in fluorescence intensity accompanied by a 5 nm blue shift in the emission maximum, suggesting that the probe, in the presence of Cd^{2+} , moves to a hydrophobic domain. U.v. difference spectroscopic studies indicated a unique Cd^{2+} -binding site on the protein, since Cd^{2+} addition yielded a large positive absorption band in the 240 nm region that is not found with either Ca^{2+} or Zn^{2+} ions. Similar absorption bands have been observed in Cd-protein complexes such as Cd-metallothionein [Vasak, Kagi & Hill (1981) *Biochemistry* 20, 2852–2856] and also in model complexes of Cd^{2+} with 2-mercaptoethanol. This absorption band is believed to arise as a result of charge-transfer transitions between the thiolate and Cd^{2+} . Of the two Cd^{2+} -binding sites on the β -chain, one must be located at the *N*-terminal end near the single tyrosine residue, since Cd^{2+} and Zn^{2+} produced similar effects on the intrinsic protein fluorescence. The other Cd^{2+} site which is unique to Cd^{2+} must be Cys^{84} , located at the *C*-terminal end.

INTRODUCTION

The highly acidic S-100 proteins consist of a mixture of isoforms. Dimers formed by association of the two closely related polypeptide chains, α and β , include S-100a ($\alpha\beta$), S-100b ($\beta\beta$) and S-100a₀ ($\alpha\alpha$) (Mani & Kay, 1987; Donato, 1986). S-100b from bovine brain is a 21158 Da homodimer which represents about 50% of the S-100 fraction from the tissue. The unusual metal-ion-binding properties of S-100b have been the subject of many studies. Amino-acid-sequence studies show that the β -chain has sequence identity with the Ca^{2+} -binding proteins calmodulin, troponin C and calbindin, and possesses an 'EF hand' Ca^{2+} -binding site near the *C*-terminus and a 'pseudo EF hand' Ca^{2+} -binding site in the *N*-terminal region (Isobe & Okuyama, 1978, 1981; Szebenyi *et al.*, 1981). Ca^{2+} binding perturbs the environment around Tyr^{17} , the single tyrosine residue, decreases intrinsic protein fluorescence intensity, reduces the α -helical content by 6%, facilitates the chemical modification of Cys^{84} , and induces a 2-fold increase in the fluorescence intensity of 2-*p*-toluidinylnaphthalene-6-sulphonate (TNS), but no shift in emission maximum of the dye (Baudier & Gérard, 1983; Baudier *et al.*, 1986; Mani & Kay, 1987).

There are also several Zn^{2+} -binding sites on S-100b. Zn^{2+} binding produces perturbations of the Tyr^{17} environment, but in this case the intrinsic protein fluorescence intensity increases severalfold. The secondary structure of the protein is little affected by Zn^{2+} ions, but there is a marked exposure of a hydrophobic patch on S-100b (Baudier & Gérard, 1983; Baudier *et al.*, 1986; Mani & Kay, 1987). A notable distinction between S-100b and

S-100a is that only S-100b binds to phenyl-Sepharose in the presence of Zn^{2+} and is subsequently eluted with EDTA-containing buffers (Baudier *et al.*, 1982).

The Ca^{2+} and Zn^{2+} perturbations are additive, indicating that there are separate binding sites for each ion. Metal-ion-substitution studies are often used to gain further information about metal-ion-binding sites in proteins. In this regard, Cd^{2+} has been used as a probe ion for both Zn^{2+} and Ca^{2+} sites in proteins in n.m.r. studies. The c.d. n.m.r. spectra of liver alcohol dehydrogenase (Bobsein & Myers, 1981), superoxide dismutase (Bailey *et al.*, 1980), carboxypeptidase and carbonic anhydrase (Armitage *et al.*, 1978), where Cd^{2+} is bound in Zn^{2+} sites, have been reported. In addition, Cd^{2+} n.m.r. spectra for calmodulin and calbindin have been reported with Cd^{2+} bound in the EF hand Ca^{2+} -binding sites (Anderson *et al.*, 1983; Vogel *et al.*, 1983). The above results imply that Cd^{2+} is a potential metal-ion probe for both the Ca^{2+} and Zn^{2+} sites of S-100b. The aim of the present study was to determine the nature of the interaction of the Cd^{2+} with S-100b using spectroscopic methods known to respond uniquely to Ca^{2+} or Zn^{2+} binding.

MATERIALS AND METHODS

Protein purification

S-100b was prepared using the procedure for purifying the α -chain of S-100a (Leung *et al.*, 1986). Peak IV obtained from the DEAE-Sephadex A-25 column yielded pure S-100 β -chain. S-100b was renatured by extensive dialysis against 100 mM-Tris (pH 7.5)/100 mM-KCl/15 mM-2-mercaptoethanol/2 mM-EDTA

Abbreviations used: TNS, 2-*p*-toluidinylnaphthalene-6-sulphonate; S-100b, bovine brain S-100b protein.

* Present address: Department of Chemistry, College of Charleston, Charleston, SC 29424, U.S.A.

† To whom correspondence should be sent.

to remove the urea. Salt, 2-mercaptoethanol and EDTA were removed by extensive dialysis (at least four changes) against 100 mM-Tris, pH 7.5, which was also treated with Chelex-100 resin to remove traces of bivalent cations, in particular Ca^{2+} and Zn^{2+} .

Spectroscopy

U.v. absorption and u.v. difference spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer over the wavelength range 310–220 nm with 1-cm-path-length cells. The spectra were measured at 25 °C with a Lauda thermoregulator connected to the sample compartment. Portions of perturbant, namely Ca^{2+} , Cd^{2+} or Zn^{2+} , were added to the sample cell, and an equal volume of Chelex-100-treated water was added to the reference cell.

C.d. measurements were made on a Jasco J500C instrument fitted with a DP-500N data processor as described previously (Mani & Kay, 1983a). A mean residue weight of 116.252 was used in calculating the molar ellipticity.

Fluorescence spectra were obtained with a Perkin-Elmer model MPF-44B spectrofluorimeter, and all measurements were made at 25 °C. The instrument was operated in the radio mode, with 6 nm bandwidths for excitation and emission slits. The A_{278} of the sample was less than 0.1. Protein concentrations were determined from absorbance measurements by using a value of 2.4 as the absorption coefficient $A_{278}^{1\%}$ for this protein (Mani *et al.*, 1982).

RESULTS

The effect of Cd^{2+} and Ca^{2+} addition on the aromatic c.d. spectrum of S-100b is shown in Fig. 1. The ellipticity of the protein is negative between 250 and 300 nm. The two bands at 284 and 278 nm can be assigned to the single tyrosine residue at position 17. Cd^{2+} addition resulted in perturbation of the tyrosine residue: for example, the $[\theta]_{278}$ value decreased from -27.2 to $+5.4$ degrees $\cdot \text{dmol}^{-1}$. In the presence of Ca^{2+} , the $[\theta]_{278}$ value changed from -27.2 to -3.3 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. However, when both Cd^{2+} and Ca^{2+} were added to apoprotein, the observed $[\theta]_{278}$ value was $+14.8$ degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ and was independent of the order of addition of the two metal ions. In addition to the single tyrosine residue, one or more of the seven phenylalanine residues were also affected by the presence of Cd^{2+} , as revealed by changes in the 260–270 nm region. Since Cd^{2+} was able to induce a significant change in the $[\theta]_{278}$ value, apoprotein was titrated with Cd^{2+} by monitoring $[\theta]_{278}$ as a function of added Cd^{2+} . The titration curve levelled off when 2 mol of Cd^{2+} were added/mol of S-100b subunit, suggesting the presence of two Cd^{2+} binding sites on the β -chain of the protein (inset to Fig. 1).

In the far-u.v. region of the c.d. spectrum, addition of 4 mol of Cd^{2+} /mol of S-100b produced a nearly 10% decrease in $[\theta]_{222}$ compared with 5% observed with Ca^{2+} (Mani *et al.*, 1982). Addition of both Cd^{2+} and Ca^{2+} to S-100b caused the ellipticity at 222 nm to decrease from -16800 ± 200 to -14500 ± 200 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$, independently of the order of addition of metal ions, suggesting that Cd^{2+} and Ca^{2+} are probably binding at different sites on the protein. The results obtained from the aromatic and far-u.v. c.d. studies suggest that the order of addition of metal ions to S-100b is not significant, since in both experiments the magnitude of change induced by one metal ion was not significantly influenced by the presence of the other and the observed c.d. spectra were the essentially the same.

Fluorescence studies

The intensity of the S-100b emission at 304 nm increased dramatically upon Cd^{2+} addition (Fig. 2). When the protein was

titrated with Cd^{2+} , a monophasic titration curve was obtained, indicating the existence of one class of Cd^{2+} -binding site with a K_d value of $(3.7 \pm 0.5) \times 10^{-5}$ M, corresponding to the Cd^{2+} concentration required to induce a 50% change in the observed

Fig. 1. Aromatic c.d. spectra of 1.34×10^{-4} M-S-100b in 0.1 M-Tris, pH 7.5 (—), in 0.1 M-Tris/ 5.4×10^{-4} M- Cd^{2+} , pH 7.5 (---), in 0.1 M-Tris/2 mM- Ca^{2+} , pH 7.5 (····) and in 0.1 M-Tris/ 5.4×10^{-4} M- Cd^{2+} /2 mM- Ca^{2+} , pH 7.5 (— · — · —).

The inset shows the percentage change in the ellipticity at 278 nm as a function of the Ca^{2+} /S-100b molar ratio.

Fig. 2. Fluorimetric titration of 9.9×10^{-6} M-S-100b in 0.1 M-Tris, pH 7.5, with Cd^{2+} .

The excitation wavelength was 278 nm and the emission intensity was measured at 304 nm.

Table 1. Relative fluorescence intensity of S-100b in the presence of various concentrations and combinations of Cd²⁺, Ca²⁺ and Zn²⁺ ions

The values in parentheses indicate the order of addition of the metal ion. The concentration of S-100b was 9.9×10^{-6} M, with an absorption of 0.05 at 278 nm. The excitation wavelength was 278 nm and the emission wavelength chosen was 304 nm.

Concn. (M)			Fluorescence intensity (arbitrary units) at 304 nm
Ca ²⁺	Zn ²⁺	Cd ²⁺	
—	—	—	31.4
—	—	7.4×10^{-5}	77.4
1.8×10^{-3} (2)	—	1.7×10^{-4} (1)	34.4
1.8×10^{-3}	—	—	25.7
—	6×10^{-5}	—	64.3
—	6×10^{-5} (1)	6×10^{-5} (2)	83.0

Fig. 3. Fluorimetric titration of 9.9×10^{-6} M-S-100b plus 6×10^{-5} M-Zn²⁺ in 0.1 M-Tris, pH 7.5, with Cd²⁺

The excitation wavelength was 278 nm and the emission intensity was measured at 304 nm.

fluorescence intensity. Since the protein binds two Cd²⁺ ions per monomer, the observed K_d value conceivably represents an average value for the two sites or, alternatively, the two sites bind Cd²⁺ with similar affinity with the result that it is not possible to differentiate them by this technique. The effect of Cd²⁺, Ca²⁺ and Zn²⁺ ions on the fluorescence intensity of S-100b is indicated in Table 1. When Ca²⁺ was added to the Cd²⁺-saturated protein, the observed fluorescence intensity at 304 nm decreased by nearly 50 %, thereby bringing it back to the level of the apoprotein. However, Ca²⁺ by itself, when added to the apoprotein, produced only an 18 % decrease in fluorescence intensity, suggesting that Ca²⁺ has a more pronounced effect on the protein in the presence of Cd²⁺. Addition of Cd²⁺ to zinc-bound S-100b (Zn-S-100b) resulted in an additional 30 % increase in fluorescence intensity. In fact, one can titrate Zn-S-100b with Cd²⁺ (Fig. 3), from which a K_d value of 2×10^{-5} M was observed for Cd²⁺, indicating that the affinity of the protein for Cd²⁺ was not influenced by the presence of Zn²⁺.

TNS fluorescence

The hydrophobic probe TNS fluoresces weakly in polar solvents, but its intensity becomes enhanced in apolar solvents or when bound to a hydrophobic pocket on the protein (McClure & Edelman, 1966). TNS-labelled apoprotein, when excited at 345 nm, had an emission maximum around 438 ± 2 nm. Addition of Cd²⁺ to labelled protein resulted in a nearly 5-fold enhancement in fluorescence intensity and this was accompanied by a 5 nm blue shift in the emission maximum (Table 2), implying that the probe in the presence of Cd²⁺ occupies a more hydrophobic environment, i.e. the protein exposes a hydrophobic region in the presence of Cd²⁺. From the Cd²⁺ titration curve generated by monitoring the fluorescence intensity at 440 nm as a function of Cd²⁺ concentration, a K_d value of 3×10^{-5} M was derived, and this corresponds to the Cd²⁺ concentration required to induce a 50 % change in fluorescence intensity. Cd²⁺-induced changes in TNS fluorescence of S-100b were compared with those produced by Zn²⁺ and Ca²⁺ (Table 2). Millimolar concentrations of Ca²⁺ produced only a 3-fold increase in fluorescence intensity, with no noticeable change in emission maximum, whereas Zn²⁺ addition resulted in a 10-fold increase in fluorescence intensity, along with a 10 nm blue shift in the emission maximum. When Cd²⁺ was added to TNS-labelled S-100b protein containing Zn²⁺ (Zn-S-100b), the emission intensity increased, whereas Ca²⁺ addition to TNS-labelled Zn-S-100b protein resulted in a decrease in the fluorescence intensity at 440 nm. However, addition of Ca²⁺ to Cd²⁺ saturated S-100b (Cd-S-100b) protein produced a further

Table 2. Effect of Cd²⁺, Ca²⁺ and Zn²⁺ on the TNS fluorescence of S-100b in 100 mM-Tris, pH 7.5

The values in parentheses indicate the order of addition of the metal ion. The concentration of S-100b was 9.9×10^{-8} M and that of TNS was 1.4×10^{-5} M. The excitation wavelength was 325 nm.

Concn. (M)			Relative fluorescence intensity (arbitrary units)	Emission maximum (λ_{max})
Ca ²⁺	Zn ²⁺	Cd ²⁺		
—	—	—	31.4	436
2×10^{-3}	—	—	92.3	436
—	—	1.2×10^{-4}	155.0	430
2×10^{-3} (2)	—	1.2×10^{-4} (1)*	233.0	430
—	6×10^{-5}	—	297.0	425
2×10^{-3} (2)	6×10^{-5} (1)	—	174.0	435
—	6×10^{-5} (1)	6×10^{-5} (2)	370.0	430
2×10^{-3} (1)	6×10^{-5} (2)	—	165.0	435

increase in TNS fluorescence, suggesting that the Ca^{2+} effect on the Cd-S-100b protein is distinct from the Zn-S-100b protein.

U.v. difference spectroscopy

Fig. 4 shows the Cd^{2+} -induced difference spectrum of S-100b. There are features around 280 nm region that are probably due to perturbations in the environment of the single tyrosine residue, whereas the peak at 269 nm and a shoulder at 263 nm and probably due to perturbations in the phenylalanine residues. However, the dominant feature of the difference spectrum is the presence of a large positive absorption band below 250 nm. Fig. 5 reveals the difference spectrum in the 310–250 nm wavelength region at lower protein concentration. Even at this low protein concentration, one observes a pronounced shoulder at 240 nm with increasing absorbance at lower wavelengths. That this feature is due to Cd^{2+} interaction with S-100b is suggested by the titration of S-100b with Cd^{2+} while monitoring the ΔA_{240} value, which is shown in Fig. 6. The apparent dissociation constant, K_d , for this spectral change is 3.6×10^{-5} M, and this value is in agreement with K_d values established by using fluorescence measurements.

DISCUSSION

The amino acid sequence of S-100b protein (Fig. 7) shows identity with the Ca^{2+} -binding proteins belongs to the calmodulin family. The normal 'EF hand' Ca^{2+} -binding site is located at the C-terminus between residues 61 and 72. The second Ca^{2+} -binding site, which operates at an elevated pH of 8.4, lies between residues 18 and 31 and is referred to as the 'pseudo EF hand'. The β -subunit of S-100b protein binds two Ca^{2+} ions with dissociation constants ranging from 10^{-5} to 10^{-3} M, depending on pH, ionic strength and the presence of Zn^{2+} (Mani *et al.*, 1983; Baudier *et al.*, 1986; Baudier & Cole, 1989). S-100b protein also possesses one high and two low-affinity zinc binding sites per subunit, with K_d values in the range of 10^{-8} to 10^{-6} M (Baudier & Gérard, 1983; Mani & Kay, 1983b). As yet, models have not been proposed for the Zn^{2+} -binding sites on S-100 proteins. Nevertheless, histidine residues are known to be ubiquitous ligands for Zn^{2+} ions in proteins (Holmes & Matthews, 1981). Spectroscopic studies have shown the high-affinity Zn^{2+} -binding sites to be different from the Ca^{2+} -binding sites and located near the single tyrosine residue, Tyr¹⁷ (Baudier & Gérard, 1983; Mani & Kay, 1986). The logical ligands for co-ordinating the Zn^{2+} ion could be the histidine residues in position 15 or 25. Since Ca^{2+} antagonizes Zn^{2+} binding to low-affinity sites, it is conceivable that the two low-affinity Zn^{2+} -binding sites are located at the C-terminal end, in close proximity to the normal 'EF hand' Ca^{2+} -binding site (histidine residues 85 and 90).

Cd^{2+} ion is a useful and reliable probe in the study of Ca^{2+} -binding sites in proteins (for a review, see Vogel *et al.*, 1985), since Ca^{2+} and Cd^{2+} ions have the same charge and a very similar size (0.097 and 0.099 nm respectively). However, some Ca^{2+} -binding proteins have a different affinity for Cd^{2+} compared with Ca^{2+} , and for this reason, in the present study, the interaction of Cd^{2+} with S-100b was carried out using spectroscopic approaches in order to compare the spectral perturbations induced by this metal ion in comparison with those produced by Ca^{2+} and/or Zn^{2+} .

Addition of Cd^{2+} to S-100b protein produced marked changes in the near- and far-u.v. c.d. spectra of the protein. Fig. 1 exhibits a positive ellipticity in the 270–290 nm region when 4 equiv. of Cd^{2+} are added to S-100b. Hence Cd^{2+} , like Ca^{2+} , alters the asymmetric environment about the single tyrosine residue in the β -chain. Subsequent addition of Ca^{2+} to Cd^{2+} -containing S-100b produced a further increase in ellipticity at 278 nm (from

5.4 to 14.8 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$). Since the observed effects in the aromatic c.d. spectrum of the protein are similar and independent of the order of addition of the metal ions (Cd^{2+} and Ca^{2+}), one may conclude that Cd^{2+} and Ca^{2+} are possibly binding at different sites on the protein.

In the far-u.v. region of the c.d. spectrum of S-100b, addition of Cd^{2+} produced a nearly 10% decrease in ellipticity at 222 nm, whereas Ca^{2+} addition induced only a 6% decrease in the $[\theta]_{222}$ value. Zn^{2+} binding to S-100b resulted in minimal change (less than 1%) in the $[\theta]_{222}$ value (Mani & Kay, 1983a, b). Hence Cd^{2+} -binding results in an alteration of protein secondary structure to a much larger extent than either Ca^{2+} or Zn^{2+} .

The intrinsic protein fluorescence at 304 nm, when excited at 278 nm, decreases in intensity when Ca^{2+} is bound and increases in intensity when Zn^{2+} is bound (Baudier *et al.*, 1986; Mani & Kay, 1987). It has been suggested that Ca^{2+} binding increases static quenching, whereas Zn^{2+} binding decreases static quenching of tyrosine emission. A likely candidate for the static quenching mechanism is formation of a hydrogen bond between the hydroxy group of Tyr¹⁷ and a carboxy-ion acceptor group (Baudier & Gérard, 1983). Since Cd^{2+} binding resulted in an increase in the fluorescence intensity at 304 nm, the effect of Cd^{2+} binding is similar to that of Zn^{2+} binding. Addition of Ca^{2+} to the Cd^{2+} -loaded protein resulted in a dramatic decrease in fluorescence intensity. When Ca^{2+} was added to Zn^{2+} -loaded protein, a decrease in the fluorescence intensity was observed and interpreted as due to Ca^{2+} binding at sites distinct from the Zn^{2+} -binding sites (Baudier & Gérard, 1983). By analogy, our results suggest that Ca^{2+} and Cd^{2+} bind independently at different sites on S-100b, in agreement with our near- and far-u.v. c.d. results. The relative fluorescence intensity of Zn^{2+} -loaded S-100b (Zn-S-100b) increased from 64 to 83 arbitrary units when 6×10^{-5} M- Cd^{2+} was added to the protein. Hence the possibility exists for a Cd^{2+} -binding site on S-100b, which is distinct from the Zn^{2+} -binding site, i.e. out of the two Cd^{2+} -binding sites at least one is unique and different from both Ca^{2+} - and Zn^{2+} -binding sites on S-100b.

Although Cd^{2+} does not appear to bind to the Ca^{2+} -binding sites on the apoprotein, it is conceivable that it may bind at the Ca^{2+} sites on the Zn^{2+} -saturated protein. Baudier & Cole (1989) studied Ca^{2+} and Tb^{3+} (terbium ion) binding to S-100b. With apo-S-100b, Tb^{3+} initially went to the Zn^{2+} sites and bound to Ca^{2+} sites only when the Zn^{2+} sites were saturated. The binding of Tb^{3+} to Ca^{2+} -binding sites was monitored by measuring the decrease in fluorescence intensity of Zn-S-100b. By analogy, if Cd^{2+} were to bind to Zn-S-100b at the Ca^{2+} -binding sites, then a decrease in Zn-S-100b fluorescence intensity could be anticipated. On the contrary, the relative fluorescence intensity of Zn-S-100b increased from 64.3 to 83.0 arbitrary units when 6×10^{-5} M- Cd^{2+} was added (Table 1). To rule out the possibility that the affinity of Cd^{2+} for the Ca^{2+} sites on Zn-S-100b is very low, a titration of Zn-S-100b was carried out with Cd^{2+} to millimolar concentrations of Cd^{2+} (Fig. 3). Addition of Cd^{2+} to Zn-S-100b protein only resulted in an increase in fluorescence intensity, thereby giving no evidence for Cd^{2+} binding at the Ca^{2+} sites.

Another experimental parameter which is often used to probe the nature of metal-ion binding to S-100b is the use of the hydrophobic probe TNS. The apparent K_d value of 3×10^{-5} M obtained by titrations of TNS-labelled protein with Cd^{2+} compares well with the result obtained with the intrinsic fluorescence measurements. Experiments were designed to ascertain whether Cd^{2+} was mimicking the effects of either Ca^{2+} or Zn^{2+} , and the results are summarized in Table 2. For instance, one may compare the effects of adding Cd^{2+} and Ca^{2+} to TNS-labelled Zn-S-100b protein. Addition to Cd^{2+} to Zn^{2+} -loaded

Fig. 4. U.v. difference spectrum of 5.1×10^{-5} M-S-100b in 0.1 M-Tris, pH 7.5, containing 9.9×10^{-6} M-Cd²⁺

Results are expressed as differences in molar absorption, based on protein concentration.

Fig. 5. U.v. difference spectrum of 9.9×10^{-6} M-S-100b in 0.1 M-Tris, pH 7.5, containing 5.5×10^{-5} M-Cd²⁺

Results are expressed as differences in molar absorption, based on protein concentration.

labelled protein produced a 15% increase in TNS fluorescence intensity, whereas Ca²⁺ addition resulted in a 41% decrease in intensity. Clearly Cd²⁺ is not mimicking the effect of Ca²⁺ and presumably is not binding to the same sites. Also, addition of Ca²⁺ to Cd²⁺-saturated labelled protein induced a 50% increase in fluorescence intensity in contrast with a 41% decrease observed with Zn²⁺-labelled protein. Hence it would appear that Cd²⁺ is not bound to S-100b in exactly the same manner as Zn²⁺.

To clarify the nature of the interaction of Cd²⁺ with S-100b, u.v. difference spectroscopy was employed. As with Ca²⁺ and Zn²⁺ ions, addition of Cd²⁺ to S-100b produced a positive absorption (i.e. a red shift) in the 280 nm region, suggesting that the single tyrosine residue is moving to a more non-polar environment. The difference spectrum induced by Cd²⁺ at a lower S-100b protein concentration (Fig. 5) reveals the presence of positive bands below 250 nm. There is a prominent shoulder at 240 nm and increasing absorbance at even lower wavelengths. Difference-spectral features, such as these have been observed

Fig. 6. Response of the differential absorbance at 240 nm of S-100b in 0.1 M-Tris, pH 7.5, to increasing Cd²⁺ concentration

before in cadmium-protein complexes, such as Cd-metallothionein (Vasak *et al.*, 1981), horse liver alcohol dehydrogenase (Drum & Vallee, 1970) and also in model complexes of Cd²⁺ with 2-mercaptoethanol (Vasak *et al.*, 1981). In all these complexes, sulphur atoms are co-ordinated to Cd²⁺, and the observed bands have been assigned to charge-transfer transitions between thiolate and cadmium (Vasak *et al.*, 1981). Hence it is reasonable to assign the 240 nm-difference-spectral feature in Cd-S-100b to a Cd $\leftrightarrow$ thiolate interaction. Competition experiments were carried out by adding either Ca²⁺ or Zn²⁺ to Cd²⁺-loaded protein and observing the difference absorbance at 240 nm. Results obtained gave no evidence for either Ca²⁺ or Zn²⁺ competing with Cd²⁺ for binding at this site.

It is now possible to speculate on the sites of Cd²⁺ binding on the protein. Our spectral studies suggest that Cd²⁺ does not bind at the Ca²⁺-binding sites which are presumed to be the 'EF-hand' region, i.e. residues 61-72 and the 'pseudo EF-hand' region, residues 18-31 (Szebenyi *et al.*, 1981). For example, in the near- and far-u.v. c.d. studies the order of addition of Ca²⁺ and Cd²⁺ ions had no significant effect on the final c.d. spectrum, suggesting that the two metal ions bind at different sites. Also, the effect of Ca²⁺ and Cd²⁺ on the intrinsic fluorescence intensity of S-100b is different, again implying that the two metal ion bind at different sites. The β -chain has only two cysteine residues, Cys⁷⁰ and Cys⁸⁴, and since Cys⁷⁰ is within the 'EF hand' Ca²⁺-binding site (Isobe & Okuyama, 1981), it is unlikely to interact with Cd²⁺. Hence Cd²⁺ must be interacting with Cys⁸⁴ on the C-terminal end of the β -chain, which presumably represents a unique Cd²⁺-binding site on the protein.

Of the two Ca²⁺-binding sites on the protein, at least one is common to both Cd²⁺ and Zn²⁺, since the perturbations induced in S-100b protein fluorescence by Cd²⁺ and Zn²⁺ are very similar. Since S-100b has no tryptophan residues, the observed fluorescence must be due to Tyr¹⁷. Turner *et al.* (1989) have studied the effect of Zn²⁺ on the fluorescence decay and mean lifetimes of S-100b. Their results also suggest that Zn²⁺ binds in the N-terminal region of S-100b and Tyr¹⁷. It is reasonable to assume that a metal-binding site near Tyr¹⁷, possibly involving either His¹⁵ or His²⁵, when occupied by Zn²⁺ or Cd²⁺, results in the observed increase in protein fluorescence. In summary, then, out of the two Cd²⁺-binding sites on the β -chain, we believe one is located at the N-terminal region near Tyr¹⁷ and the other unique Cd²⁺-binding site is Cys⁸⁴ located at the C-terminal end.

Fig. 7. Amino acid sequence of the β -subunit of bovine brain S-100b protein (Isobe & Okuyama, 1981)

The putative Ca^{2+} -binding site is bracketed, with X, Y, Z, -Y, -X and -Z representing the ligands that co-ordinate the Ca^{2+} ion. A variant form of Ca^{2+} -binding site similar to the I-II domain of bovine intestinal calcium-binding protein (Szebenyi *et al.*, 1981) is found in the *N*-terminal region (underlined). Amino acid residues are identified by the single-letter notation. Ac refers to an acetyl group.

We are indebted to Mr K. Oikawa, Mr L. Hicks and Mr A. Keri for their expert technical assistance. This work was supported by the Medical Research Council of Canada and the Alberta Heart and Stroke Foundation.

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Received 21 September 1990/19 November 1990; accepted 22 November 1991