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The Ca^{2+}-binding sequence in bovine brain S100b protein β-subunit

A spectroscopic study

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Conformational changes in the β-subunit of the bovine brain Ca^{2+}-binding protein S100b (S100-β) accompanying Ca^{2+} binding were investigated by analysis of the spectroscopic properties of the single tyrosine residue ($Tyr^{17\beta}$) and flow-dialysis binding experiments. S100-β binds Ca^{2+} sequentially at two sites to change the conformation of the protein. The first Ca^{2+} ion binds to site IIβ, a typical Ca^{2+}-binding site in the *C*-terminal region, and it does not significantly perturb the proximal environment of $Tyr^{17\beta}$. After the first site is occupied, another Ca^{2+} ion binds to the *N*-terminal Ca^{2+}-binding site, Iβ, and strengthens a hydrogen bond between $Tyr^{17\beta}$ and a neighbouring carboxylate acceptor group, which results in a large increase in the $Tyr^{17\beta}$ fluorescence spectrum half-width and a positive absorption and c.d. signal between 290 and 275 nm. Ca^{2+} binding to the S100b·$Zn^{2+}{}_6$ complex, studied by flow-dialysis and fluorescence measurements showed that, although Zn^{2+} ions increase the affinity of S100b protein for Ca^{2+}, the Ca^{2+}-binding sequence was not changed. Tb^{3+} (terbium ion) binding studies on the S100b·$Zn^{2+}{}_6$ complex proved that Tb^{3+} antagonizes only Ca^{2+} binding site IIβ and confirmed the sequential occupation of Ca^{2+}-binding sites on the S100b·$Zn^{2+}{}_6$ complex.

INTRODUCTION

The family of intracellular calcium (Ca^{2+})-binding proteins (CaBPs) seem to have evolved from a four-domain Ca^{2+}-binding ancestor (Demaille, 1982). Calmodulin and troponin C have retained this type of structure, and they bind four Ca^{2+} ions per protein molecule. Despite differences in their primary structures and biological functions, they have similar crystal structures (Babu *et al.*, 1985; Herzberg & James, 1985; Sundaralingam *et al.*, 1985). Other members of the CaBP family have lost the Ca^{2+}-binding ability of one or more of the domains, and in some cases they have lost the domains themselves. The β-subunit of the bovine brain CaBP S100b (S100-β) and the 9 kDa intestinal calcium-binding protein (ICaBP) belong to the class of CaBPs that have two Ca^{2+}-binding domains, and they show high sequence similarity in their primary structures (Isobe & Okuyama, 1978; Desplan *et al.*, 1985). [For a review on S100 proteins, see Kligman & Hilt (1988).] They are characterized by one 'typical' Ca^{2+}-binding domain (site II) in the *C*-terminal part of the molecule and one 'putative' domain (site I) in the *N*-terminal part (Szebenyi *et al.*, 1981). The 'putative' Ca^{2+}-binding domain is characterized by insertion of an additional residue between the third and fourth Ca^{2+} ligand of the Ca^{2+}-binding loop.

Purified S100b(ββ) is a homodimer in native solvents. The β-subunits are held together by non-covalent forces and are symmetrically positioned [^{1}H-n.m.r. spectra of the protein dimer correspond to that of a single subunit (Mani *et al.*, 1983; Ångstrom & Baudier, 1985)]. Conformational effectors such as Zn^{2+} ions or alkylation of $Cys^{84\beta}$ were proved to destabilize the quaternary dimer protein structure and to favour the dissociation of subunits at low protein concentrations (Baudier *et al.*, 1986*b*; Baudier & Cole, 1988). Destabilization of the S100b quaternary protein structure resulted in a large increase in the β-subunit affinity for Ca^{2+}. The 9 kDa ICaBP is a monomeric protein which may also aggregate in concentrated protein solutions (Dorrington *et al.*, 1974, 1978). There have been several studies of the interactions of ICaBP with Ca^{2+} and lanthanide ions, and these have revealed the sequence of ion binding and provided detailed analysis of the accompanying Ca^{2+}-induced conformational changes in the protein (Chiba *et al.*, 1983, 1984; O'Neil *et al.*, 1984; Shelling *et al.*, 1983, 1985; Chiba & Mohri, 1987). Although the Ca^{2+}-dependent conformation changes in S100-β have also been studied (Mani *et al.*, 1982, 1983; Baudier & Gerard, 1983; Baudier *et al.*, 1986*a*), the relationship of conformational changes to saturation of S100-β by Ca^{2+} was not fully characterized. We therefore decided to extend the earlier studies to a level comparable with those reported for ICaBP, establishing the sequence in which the two domains of S100-β bind Ca^{2+} by correlation with conformational changes.

Abbreviations used: S100-β, β-subunit of bovine brain Ca^{2+}-binding protein S100b; CaBP, calcium (Ca^{2+})-binding protein; ICaBP, intestinal calcium-binding protein.

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MATERIALS AND METHODS

S100-β was prepared by zinc-dependent affinity chromatography on phenyl-Sepharose (Baudier *et al.*, 1983) and treated as previously described to remove contaminant bivalent ions (Baudier *et al.*, 1986*a*). The S100b protein used for spectroscopic studies was chromatographed twice on the phenyl-Sepharose column and finally chromatographed on a Mono Q column, which is likely to remove a possible tryptophan-containing S100a protein contaminant and oxidized S100b species (Baudier *et al.*, 1986*a*).

Since protein concentration affects the dimer–monomer equilibrium of apo-S100 proteins (Baudier *et al.*, 1986*b*) and the quaternary structure affects the Ca^{2+} affinity of S100 (Baudier & Gerard, 1986; Baudier *et al.*, 1986*b*), studies on the relationship between conformational changes and saturation of S100 protein by Ca^{2+} are invalid unless the experimental conditions for investigations of the conformational changes are identical with those of the Ca^{2+} binding studies. Therefore all the experiments described in the present work on S100-β were performed under similar conditions, i.e. 60 μM-S100b($\beta\beta$) in 20 mM-Tris/HCl buffer, pH 7.5, at 20 °C.

The fluorescence spectra were obtained on a Perkin–Elmer MPF-44A spectrofluorimeter equipped with a Perkin–Elmer 7500 professional computer. Protein solutions were excited at 275 nm, and the fluorescence emission between 280 and 440 nm was measured. Each protein fluorescence spectrum was corrected automatically for the Raman light-scattering spectrum of the buffer. U.v. absorption and difference absorption were measured on a Cary 219 spectrophotometer. If necessary, correction for scattered light was made as indicated by Gerard *et al.* (1975).

Flow-dialysis experiments were performed as previously described (Baudier *et al.*, 1986*a,b*). The binding data were plotted by the Scatchard method to estimate the number of Ca^{2+}-binding sites on the protein or as binding isotherms that represented the number of Ca^{2+} ions bound/molecule of protein as a function of the negative logarithm of the total Ca^{2+} concentration. The binding isotherms were computer-fitted and were used to determine graphically the correspondence between mol of Ca^{2+} bound/mol of S100b and the total Ca^{2+} concentration.

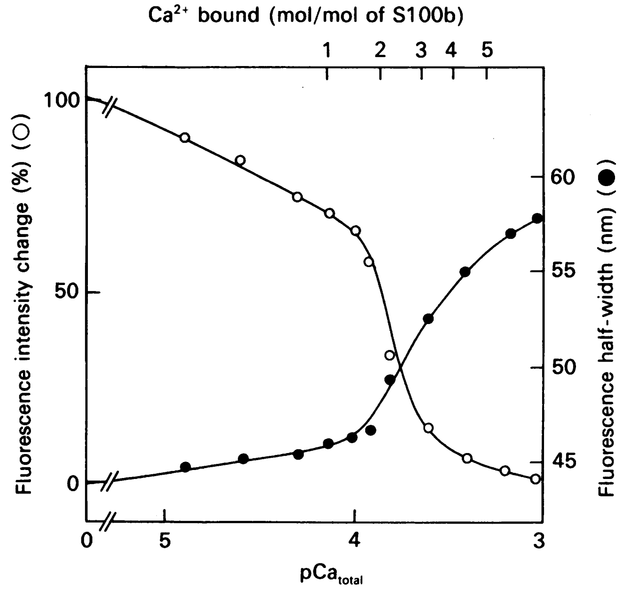

Fig. 1. Tyrosine fluorescence of S100b

Titration profiles for percentage changes in tyrosine fluorescence intensity (○) and changes in tyrosine fluorescence half-width (●) upon Ca^{2+} binding. The percentage changes in tyrosine fluorescence were calculated by normalizing the highest tyrosine fluorescence intensity to 100% and the lowest to 0%. The lower part of the Figure gives the negative logarithm of the total Ca^{2+} added (pCa_{total}). The upper part gives the corresponding amount of Ca^{2+} bound/mol of S100b dimer determined from flow-dialysis experiments (see the Materials and methods section).

RESULTS

Conformational changes during titration of S100b with Ca^{2+}

Effect of Ca^{2+} on Tyr(17β) fluorescence. S100b-β contains a single tyrosine residue in position 17β which constitutes a sensitive indicator of conformational changes in the *N*-terminal part of the polypeptide chain where Ca^{2+}-binding site Iβ is located. The spectral properties of this residue in S100b have already been described (Mani *et al.* 1982, 1983; Baudier & Gerard, 1983; Lux *et al.*, 1985; Baudier *et al.*, 1986*a*).

Ca^{2+} binding to S100b decreases the tyrosine fluorescence intensity by a factor of 2, and this decrease in intensity is associated with a large increase of the fluorescence-spectrum half-width ($\Delta\lambda$) from 46 $\pm$ 1 nm to 57 $\pm$ 1 nm (Baudier *et al.*, 1986*a*). Note that in a previous report the spectral half-widths for apo- and Ca^{2+}-bound S100b were reported to be 38 nm and 45 nm respectively (Lux *et al.*, 1985). These lower values presumably arose because no care was taken to avoid contamination of the native reduced form of S100b with its oxidized counterpart. Oxidized S100b has a spectral half-width of 36 $\pm$ 1 nm, which does not vary upon Ca^{2+} binding, and may represent up to 50 % of the purified S100b if no reducing agent is included in the buffer during preparation and storage (Baudier *et al.*, 1986*a,b*). In the present work the changes in tyrosine fluorescence intensity (ΔF) and spectral half-width as a function of increasing amount of Ca^{2+} (Fig. 1) showed that, upon binding of the two first Ca^{2+} ions to each S100b dimer, fluorescence intensity was decreased by nearly 45% without noticeable change in fluorescence half-width. By contrast, saturation of the protein with Ca^{2+} produced the maximal decrease of tyrosine fluorescence intensity and a sharp increase in the fluorescence half-width to 57 $\pm$ 1 nm. These results on fluorescence were the same whether Ca^{2+} levels were increased by progressive titration of S100b or stepwise (0.2 mM- followed by 1 mM-Ca^{2+}). Changing the pH from 7.5 to 8.3 was without appreciable effect on either the fluorescence parameters or the Ca^{2+} titration curves.

Effects of Ca^{2+} on u.v. absorption. Fig. 2(*a*) shows the u.v. differential absorption spectra for S100b in the presence of 120 μM-Ca^{2+} and 1 mM-Ca^{2+} with reference to the protein in Ca^{2+}-free buffer. The S100b·Ca^{2+}_2 complex (S100b plus 120 μM-Ca^{2+}, curve ----) showed a significant negative differential absorption in the tyrosine

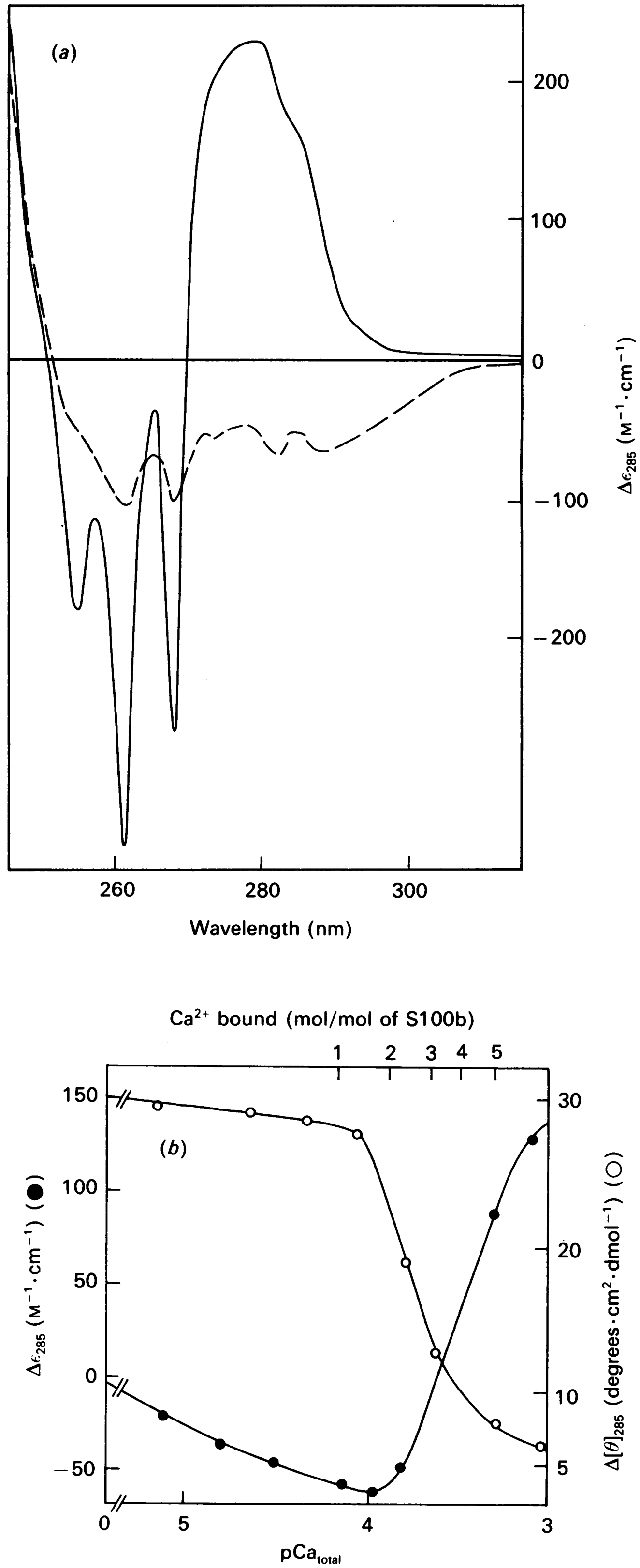

Fig. 2. U.v. absorption and c.d. spectra of S100b

(*a*) U.v. difference spectra of S100b (60 μM) plus 120 μM-Ca^{2+} (broken line), plus 1 mM-Ca^{2+} (continuous line). (*b*) Titration profiles of the u.v. differential absorption (●) and c.d. (○) changes at 285 nm upon Ca^{2-} binding to S100b dimer. The representation of the Ca^{2+} binding data is the same as that used in Fig. 1.

absorption range between 290 and 275 nm as well as in the vibronic phenylalanine absorption bands between 275 and 250 nm; both of these observations indicate increased exposure of the aromatic amino acids to more polar environment. Saturation of S100b with Ca^{2+} (1 mM; curve ——) abruptly reversed the negative differential signal to a positive one between 290 and 275 nm, and the negative signals for phenylalanine residues were even more negative. The two-step saturation of the protein with Ca^{2+} did not produce any increase in light-scattering of the protein solution, which rules out the possibility that the spectral changes result from protein aggregation. However, in the progressive Ca^{2+} titration there was a slight increase in light-scattering of the protein sample above 200 μM total calcium, and therefore the final titration points (Fig. 2*b*) for the changes in tyrosine absorption at 285 nm were corrected for the light-scattering contribution. Note also that the shape of the differential absorption spectrum after subtracting the light-scattering contribution was identical with that reported in Fig. 2(*a*). Although the protein solution showed no apparent turbidity during the Ca^{2+} titration, the slight increase in light scattering is suggestive of Ca^{2+}-induced protein aggregation. Such a hypothesis was ruled out by gel filtration of S100b on a fast-protein-liquid-chromatography Superose 12 column equilibrated with 1 mM-Ca^{2+} or 1 mM-EDTA. Indeed, no difference in the elution profile between the Ca^{2+}-bound and Ca^{2+}-free proteins was observed. Therefore the slight change in light-scattering observed during the progressive Ca^{2+} titration possibly results from subtle time-dependent changes in subunit–subunit interaction within a single S100b dimer. Time-dependent conformational changes have previously been reported for the S100a-$\alpha\beta$ heterodimer, which resulted in a destabilization of the quaternary protein structure (Baudier & Gerard, 1986). Nevertheless, time-dependent conformational changes of the S100b protein dimer probably do not significantly affect S100-β conformation, since the effects of Ca^{2+} on fluorescence, absorption and c.d. spectra were identical after progressive Ca^{2+} titration or stepwise (0.2 mM followed by 1 mM-Ca^{2+}). Changing the pH of the buffer from 7.5 to 8.3 had no appreciable effect on the differential spectra or Ca^{2+} titration curves.

Effect of Ca^{2+} on the $Tyr^{17\beta}$ spectrum. The near-u.v. c.d. spectrum of S100b (Mani *et al.*, 1983; Baudier *et al.*, 1986*a*) is characterized by a rather high negative ellipticity signal in the tyrosine absorption range ($[\theta]_{285} = -30$ degrees·cm^2·dmol^{-1}) which increases (to -5 degrees·cm^2·dmol^{-1}) upon the binding of Ca^{2+} to the protein. Fig. 2(*b*) shows the calcium titration of the tyrosine c.d. signal at 285 nm as a function of the fractional Ca^{2+} occupancy. Up to 2 mol equiv. of Ca^{2+} bound per mol of S100b dimer (S100b·$Ca^{2+}{}_2$) no significant changes in tyrosine ellipticity occurred, and it is the binding of the additional mol equiv. of Ca^{2+} that induced most of the ellipticity increase. There was no difference on final c.d. spectra whether Ca^{2+} levels were increased by progressive titration of S100b or stepwise (0.2 mM- followed by 1 mM-Ca^{2+}).

Conformational transitions during titration of the S100b·$Zn^{2+}{}_6$ complex with Ca^{2+}

Since Zn^{2+} binding to S100b markedly increases the

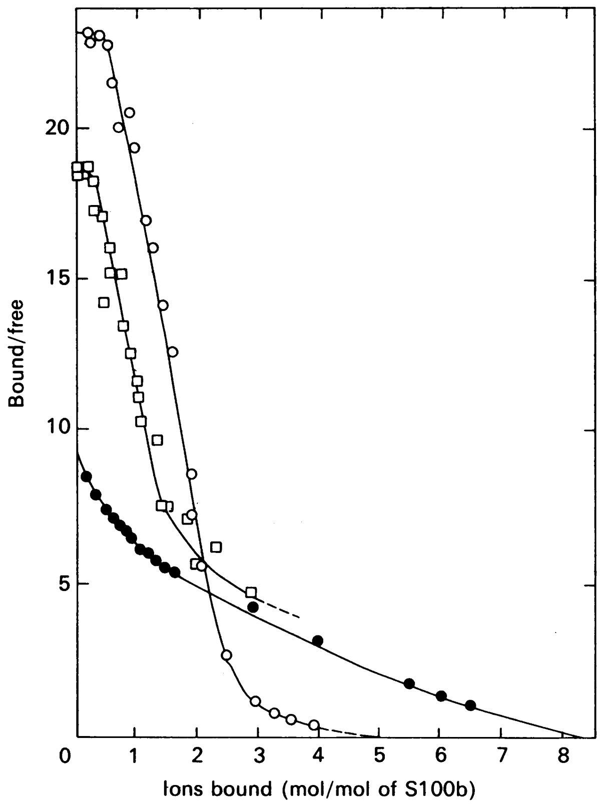

Fig. 3. Scatchard-plot representation of Ca^{2+} and Tb^{3+} binding to S100b

Flow-dialysis experiments were conducted as described in the Materials and methods section. Ca^{2+} binding to S100b protein dimer (●, ○) was studied in the absence (●) or in the presence of 6 mol equiv. of Zn^{2+}/mol of S100b (○). Tb^{3+} binding (□) was studied in the presence of 6 mol equiv. of Zn^{2+}/mol of S100b, using $^{45}Ca^{2+}$ as a probe for the occupation of the Ca^{2+} sites by Tb^{3+}.

affinity of the protein for Ca^{2+} (Baudier *et al.*, 1986*a*), possibly resulting in a change in the Ca^{2+}-binding sequence, we titrated the zinc complex of S100b with Ca^{2+} to saturation. In Fig. 3 is shown a Scatchard plot of the Ca^{2+} binding data for the S100b·$Zn^{2+}{}_6$ complex compared with that for the protein in the absence of Zn^{2+}. It confirms the dramatic increase in the affinity of the first 2 mol equiv. of Ca^{2+} bound (K_d 2 μM) and shows a decrease in non-specific Ca^{2+} binding. The addition of 6 mol equiv. of Zn^{2+} to S100b induced a marked increase in tyrosine fluorescence intensity associated with a decrease of the spectral half-width to 33 nm (Baudier *et al.*, 1986*a*; Lux *et al.*, 1985). Subsequent addition of Ca^{2+} to the S100b·$Zn^{2+}{}_6$ complex returned the tyrosine fluorescence intensity to its initial value (Baudier & Gerard, 1983) and increased the tyrosine spectral half-width to 55 ± 1 nm, as observed in the absence of Zn^{2+}. The change in tyrosine fluorescence intensity (ΔF) and spectral half-width ($\Delta\lambda$) during Ca^{2+} titration (Fig. 4) showed that, when 2 mol equiv. of Ca^{2+} were bound/mol of S100b·$Zn^{2+}{}_6$ complex, 75 % of the total fluorescence had decreased without significant change in $\Delta\lambda$. The increase in the half-width fluorescence was associated with the decrease of the last 25 % fluorescence intensity and resulted from the saturation of the protein with Ca^{2+}.

Conformational changes during titration of S100b·$Zn^{2+}{}_6$ complex with Tb^{3+}

In the interests of finding other ionic probes to characterize the Ca^{2+}-binding sites of S100b, we turned to Tb^{3+}. Tb^{3+} binding to apo-S100b at the level of 2 mol equiv. of Tb^{3+}/mol. of S100b induced changes in tyrosine absorption and fluorescence properties that do not mimic those induced by Ca^{2+}. Further studies, by flow dialysis and ^{1}H-n.m.r., on the binding of Tb^{3+} to apo-S100-β proved that Tb^{3+} antagonized two particular Zn^{2+}-binding sites when added to apo-S100-β and did not antagonize Ca^{2+} binding sites (J. Ångstrom, J. F. Lefevre & J. Baudier, unpublished work). Therefore it was expected that, only after the Zn^{2+}-binding sites were saturated with Zn^{2+}, would Tb^{3+} compete with Ca^{2+} binding. Fig. 3 shows the Scatchard plot for Tb^{3+} binding to the S100b·$Zn^{2+}{}_6$ complex obtained by flow dialysis using $^{45}Ca^{2+}$ as probe for the occupation of the Ca^{2+} sites by Tb^{3+}. $^{45}Ca^{2+}$ was used to confirm that the Tb^{3+} added ions to the S100b·$Zn^{2+}{}_6$ complex would indeed antagonize Ca^{2+}-binding sites. The Scatchard plot was linear for the first 2 mol equiv. Tb^{3+} bound/mol of S100b dimer. Above 2 mol equiv. of Tb^{3+} added/mol of S100b the protein solution became turbid, reflecting protein aggregation. These results indicate that at least two Ca^{2+} sites can be antagonized by Tb^{3+}. This was confirmed by the observation that the binding of 2 mol. equiv. of Tb^{3+} induced fluorescence changes in the S100b·$Zn^{2+}{}_6$ complex identical with those induced by the first two Ca^{2+} ions, i.e. a 75 % tyrosine fluorescence decrease (Fig. 4). Subsequent addition of Ca^{2+} to the S100b·$Zn^{2+}{}_6$·$Tb^{3+}{}_2$ complex again decreased the tyrosine fluorescence intensity, by nearly 25 %, and increased the half-width fluorescence spectrum to 51 ± 1 nm over Ca^{2+} concentration ranges that corresponded to the binding of the third and fourth Ca^{2+} ion to the S100b·Zn^{2+} complex.

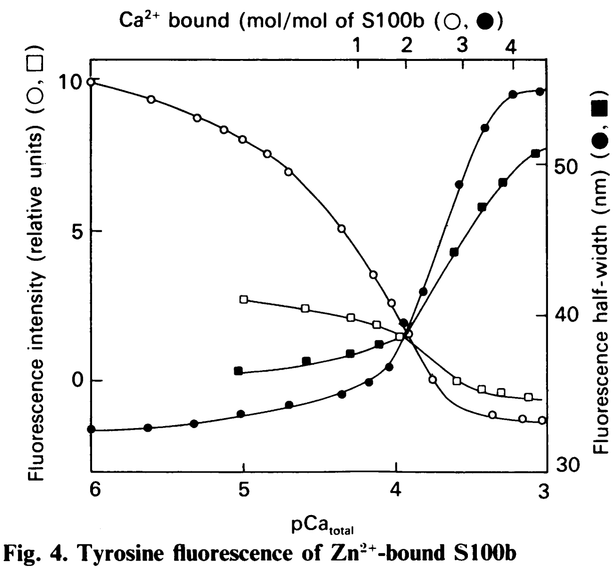

Fig. 4. Tyrosine fluorescence of Zn^{2+}-bound S100b

Titration profiles for the changes in tyrosine fluorescence intensity (○, □) and changes in tyrosine fluorescence half-width (●, ■) of S100b·$Zn^{2+}{}_6$ complex (○, ●) or S100b·$Zn^{2+}{}_6$·$Tb^{3+}{}_2$ complex (□, ■) upon Ca^{2+} binding. The changes in tyrosine fluorescence intensity are relative to the fluorescence of apo-S100b, noted as zero on the scale. The representation is the same as that used in Fig. 1 for the S100b·$Zn^{2+}{}_6$-complex titrations.

DISCUSSION

The purpose of the present work was to establish the order in which the two kinds of Ca^{2+}-binding domains in S100-β bind Ca^{2+} and to correlate the sequence of binding with changes in the conformation of the protein. To do so it is helpful to consider first the effects of Ca^{2+} binding on the environment of the single tyrosine residue of S100-β, namely $Tyr^{17\beta}$, to develop a broader picture of conformational changes in the domain of the *N*-terminal Ca^{2+}-binding site Iβ.

Conformational changes in the *N*-terminal part of S100-β as reflected in the spectral properties of $Tyr^{17\beta}$

The *N*-terminal part of S100-β contains the single tyrosine residue ($Tyr^{17\beta}$) which constitutes a sensitive indicator of conformational changes in the vicinity of the Ca^{2+}-binding site Iβ. The tyrosine fluorescence spectrum of apo-S100b is characterized by a rather high maximum spectral half-width ($\Delta\lambda$) (46 ± 1 nm) (Fig. 1). Ca^{2+} binding to S100b increased the tyrosine fluorescence spectrum half-width (56 ± 1 nm) (Fig. 1). Such change in tyrosine fluorescence half-width can be explained by the occurrence of a stronger hydrogen bond involving the hydroxy group of tyrosine residues (Moreno & Weber, 1982; Mani *et al.*, 1983; Lux *et al.*, 1985).

Hydrogen bonds involving $Tyr^{17\beta}$ residues might also explain the unusual pre-eminent absorption band at 285 nm that characterizes the S100b absorption spectrum. The red shift in the absorption spectra of tyrosine derivatives above 285 nm has been attributed to changes in hydrogen bonds (Strickland *et al.*, 1972). It is therefore significant that, at the acidic pH of 2.8, the S100b protein expressed a negative differential absorption spectrum compared with apo-S100b protein at pH 7.5 with a minimum at 285 nm (result not shown), confirming that, at neutral pH, hydrogen bonds occur between $Tyr^{17\beta}$ residues and carboxylate acceptor groups (Lux *et al.*, 1985). In the presence of saturating amounts of Ca^{2+}, the hydrogen bond between $Tyr^{17\beta}$ and the carboxylate acceptor group is strengthened, and the S100b expresses a positive differential absorption spectrum at 285 nm. The decrease in tyrosine ellipticity at 285 nm observed for S100b in the presence of Ca^{2+} (Fig. 2*b*; see also Mani *et al.*, 1982) is also consistent with the notion that the strength of the hydrogen bond was affected by Ca^{2+}. Hydrogen-bonding agents acting on tyrosine derivatives caused red shifts of the c.d. spectra to the same extent as the affected absorption spectra (Strickland *et al.*, 1972).

It is interesting at this stage to mention that the three-dimensional structure of S100-β is predicted to resemble that of ICaBP and that conformational similarity may exist between the *N*-terminal halves of the two proteins. S100-β and 9 kDa ICaBP are both characterized by the presence of a single tyrosine residue ($Tyr^{17\beta}$ and Tyr-13 respectively) in identical positions in α-helix I flanking the 'putative' calcium-binding site I in the *N*-terminal domain. Tyr^{13} of ICaBP partially interacts with a neighbouring carboxylate group in the Ca^{2+}-free state of the protein (Shelling *et al.*, 1983; O'Neil & Hofmann, 1987), and a stronger hydrogen bond is formed between the hydroxy group of the Tyr-13 and Glu-35 of ICaBP in the presence of Ca^{2+} (Szebenyi *et al.*, 1981; Shelling *et al.*, 1983). The situation of $Tyr^{17\beta}$ is thus identical with that observed for Tyr^{13} of ICaBP. The similarity of the environment of tyrosine residues in S100b and ICaBP is also reflected in the resemblance of their fluorescence and absorption spectra as well as in the positive differential spectra between 290 nm and 270 nm in the presence of Ca^{2+} that characterize both proteins (Dorrington *et al.*, 1974; Chiba & Mohri, 1987; O'Neil & Hofmann, 1987).

The order in which sites I and II bind Ca^{2+} is revealed by a conformational change in S100b

One major factor which must be taken into account in considering the properties of the S100b protein is that it is a dimer under our experimental conditions. However, in the following discussion we will assume that the spectral as well as the Ca^{2+}-binding properties of S100-β can be analysed independently from its association as a dimer. Indeed, ^{1}H-n.m.r. studies on apo- and Ca^{2+}-saturated S100b protein revealed that the two β-subunits have identical environments which result in ^{1}H-n.m.r. spectra for the protein that correspond to that of a single β-subunit (Mani *et al.*, 1983; Angstrom & Baudier, 1985). Furthermore, the sequential Ca^{2+} binding model for S100b developed below supports such a postulation.

In 20 mM-Tris, pH 8.3, flow-dialysis binding studies revealed that S100b($\beta\beta$) protein dimer binds specifically 4 mol. equiv. of Ca^{2+}/mol of protein, which are responsible for conformational changes in the protein structure. At pH 7.5, additional Ca^{2+}-binding sites are titrated. The lower-affinity sites titrated at pH 7.5 probably result from non-specific Ca^{2+} binding involving charged chemical groups of the protein which are sensitive to pH change (Baudier *et al.*, 1986*a*). We have indeed observed that the number of low-affinity Ca^{2+}-binding sites progressively decreased when the pH increased from near neutral to basic (pH 8.3) (J. Baudier, unpublished work). However, saturation of S100b protein with Ca^{2+} induced similar conformational changes at pH 7.5 and 8.3 that must therefore have resulted from the occupation of identical specific Ca^{2+}-binding sites. These sites have been previously assigned to typical amino acid sequences on the β-subunits and named site Iβ and site IIβ (Baudier *et al.*, 1986*a*).

In the present study we demonstrated that, at physiological pH (i.e. 7.5), Ca^{2+} binding to S100b protein is sequential. The first two Ca^{2+} ions have only a small effect on the optical properties of $Tyr^{17\beta}$, but saturation of the protein with Ca^{2+} dramatically changed the close environment of this residue. $Tyr^{17\beta}$ fluorescence titration, which is sensitive to small conformational changes in the overall protein structure, therefore shows that each $S100b \cdot Ca^{2+}{}_{n}$ species ($1 < n < 5$) contributes to the changes in tyrosine fluorescence intensity, in agreement with our previous data obtained at pH 8.3 (Baudier *et al.*, 1986*a*). However, only the $S100b \cdot Ca^{2+}{}_{>2}$ complexes increased the strength of hydrogen bond between $Tyr_{17\beta}$ and the carboxylate acceptor group, as revealed by the increase in tyrosine fluorescence half-width. Sequential Ca^{2+}-binding was confirmed by the u.v.-absorption and c.d. changes at 285 nm. The titration curves proved that only the $S100b \cdot Ca^{2+}{}_{>2}$ complexes significantly modified the close environment of $Tyr^{17\beta}$. $Tyr^{17\beta}$ is located in the α-helix that flanks the Ca^{2+}-binding site Iβ, and it is likely that saturation of this site with Ca^{2+} is responsible for major changes in spectral properties of this residue. If we assume that the spectral changes of $Tyr^{17\beta}$ can be analysed independently of the association of the β-subunit as a dimer, these data indicate that Ca^{2+} binds first to the *C*-terminal site, IIβ, and then to the *N*-terminal site, Iβ, to strengthen the hydrogen bond between $Tyr^{17\beta}$ and the carboxylate group. A similar Ca^{2+}-binding sequence was suggested for the S100-α subunit in S100αα protein, on the bases of fluorescence titrations of the single tryptophan in position 90α (Baudier *et al.*, 1986*a*). Note that the Ca^{2+} titration curves for S100b show no real plateau at the theoretical 4 Ca^{2+} bound/β_2, but this is not inconsistent with the presence of only four specific Ca^{2+}-binding sites. Indeed, at pH 7.5, the non-specific Ca^{2+} binding that occurs simultaneously with the saturation of the lower-affinity specific sites (sites Iβ) interferes in the analysis of the correspondence between mol of Ca^{2+} bound/mol of S100b and the spectroscopic signals resulting from the occupation of the lower-affinity-specific sites (Iβ). It is also interesting to note that an identical binding sequence was demonstrated for the binding of the Ca^{2+} analogue Tb^{3+} on the Ca^{2+}-binding sites of ICaBP and that the Ca^{2+} saturation curve for the changes in $Tyr^{17\beta}$ absorption at 285 nm is identical with the Tb^{3+} titration curve for changes in the absorption at 287 nm of Tyr^{13} of ICaBP (Chiba & Mohri, 1987).

The sequence of Ca^{2+} binding to S100b is apparently not changed in the presence of Zn^{2+}, since Ca^{2+} titration curves for the changes in $Tyr^{17\beta}$ fluorescence parameters (ΔF, $\Delta\lambda$) were similar to those observed in the absence of Zn^{2+}. The first two Ca^{2+} ions bound to the $S100b \cdot Zn^{2+}{}_6$ dimer only produced a monotonous $Tyr^{17\beta}$ fluorescence intensity decrease, but it required the binding of the third and fourth Ca^{2+} ions to $S100b \cdot Zn^{2+}{}_6$ to strengthen the hydrogen bond between $Tyr^{17\beta}$ and the carboxylate acceptor group responsible for the increase in the tyrosine fluorescence half-width. Studies of Tb^{3+} binding to the $S100b \cdot Zn^{2+}{}_6$ complex also confirmed the sequential filling of site IIβ and site Iβ. Indeed, the $S100b \cdot Zn^{2+}{}_6$ complex specifically bound 2 mol equiv. of Tb^{3+}/mol of S100b dimer, mimicking the effect of the first two Ca^{2+} ions bound on tyrosine fluorescence. Owing to the restricted flexibility of 'putative' EF-hand sites (Szebenyi & Moffat, 1986) and the high content of positively charged residues in the loop of the 'putative' site, Iβ, Tb^{3+} undoubtedly preferentially binds at the 'typical' EF-hand site, IIβ. If the Tb^{3+} binding sequence occurred in the opposite way, energy transfer between $Tyr^{17\beta}$ and Tb^{3+} would have been expected, as is the case between Tyr^{13} of ICaBP and Tb^{3+} in ICaBP (Chiba *et al.*, 1984; O'Neil *et al.*, 1984), but no Tb^{3+} luminescence was detected up to 2 mol equiv. of Tb^{3+} bound/mol of S100b dimer.

Conclusion

In solution, at concentrations > 10 μM, S100b exists as a dimer with Ca^{2+} affinity ranging between 10 and 50 μM for the *C*-terminal site, IIβ, and between 200 and 500 μM for the *N*-terminal site, Iβ. When Zn^{2+} is bound to S100b, the quaternary structure becomes less stable (Baudier & Cole, 1988) and site-IIβ affinity for Ca^{2+} increases to micromolar range. It is likely that this change in Ca^{2+} affinity results from an increase of accessibility of site IIβ to Ca^{2+}. The fact that the Ca^{2+} affinity of site IIβ is dependent on its accessibility also explains a part of the strong antagonistic effect of KCl on Ca^{2+} binding to S100b proteins (Baudier *et al.*, 1986*c*). When KCl (or ionic strength in general) increases, the hydrophobic interactions between subunits increase, rendering the S100b proteins more compact (Mani & Kay, 1984). Under these conditions the IIβ Ca^{2+}-binding sites become less accessible to solvent, and the apparent protein affinity for Ca^{2+} markedly decreases. If the physiological significance of the putative *N*-terminal lower-affinity Ca^{2+}-binding site, Iβ, remains to be demonstrated, it is, however, possible now to propose that site IIβ may have a regulatory function for S100b (S100-β?). Indeed, the possibility of this site having its affinity regulated by conformational effectors such as Zn^{2+} ions or proteins like mellitin (Baudier *et al.*, 1987), suggests that, *in vivo*, the protein might have its Ca^{2+} affinity regulated upon interaction with target proteins or other cellular components.

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