Ca²⁺ and Zn²⁺-binding properties of nitrated S-100b protein from bovine brain

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The single tyrosine residue in S-100b protein was nitrated by treatment with tetranitromethane in 0.1 M-Tris/HCl buffer, pH 8.0, containing 2 mM-EDTA. The nitrated protein did not differ significantly in secondary structure from its native unmodified counterpart, as revealed by far-u.v. c.d. measurements. The effect of Ca²⁺ on the modified protein was different from that on the native protein, e.g. addition of Ca²⁺ resulted in a loss of helical content from 55 to 47% with the native protein whereas Ca²⁺ had no significant effect on the gross conformation of the nitrated derivative. Near-u.v. c.d. studies also indicated a very minimal effect on the tyrosine residue and this was also reflected in the u.v.-absorption difference spectrum. Polyacrylamide-gel electrophoresis in the absence of SDS showed the nitrated S-100b to move faster in the presence of EDTA compared with the calcium-bound state, suggesting that the modified protein does bind Ca²⁺ although it does not undergo a major conformational change in response to Ca²⁺ addition. In contradistinction, Zn²⁺ binding was not influenced by nitration, as demonstrated by aromatic c.d. and u.v.-difference spectroscopy. It is clear from this study that the single tyrosine residue in S-100b is critical to sense the Ca²⁺-induced conformational changes in the protein.

INTRODUCTION

In recent years, we have been interested in the study of the molecular changes which occur when Ca²⁺ and Zn²⁺ ions interact with brain S-100a and S-100b proteins. The near-u.v. c.d. spectrum of S-100b protein consists of bands associated with transitions involving the amino acids phenylalanine and tyrosine and the ellipticity of the protein is negative between 250 and 300 nm (Mani *et al.*, 1982). At neutral pH, the region from 250 to 270 nm is dominated by the 'sawtooth' fine structure characteristic of the absorption spectrum of phenylalanine. The two bands at 276 and 284 nm can be assigned to the single tyrosine residue at position 17 in the primary sequence (Isobe & Okuyama, 1978). S-100b protein contains a typical EF hand type calcium-binding region located between residues 61 and 72. Previous spectroscopic studies (Mani *et al.*, 1982; Mani & Kay, 1983a; Baudier & Gérard, 1983) have shown that Ca²⁺ and Zn²⁺ binding results in the perturbation of the single tyrosine residue. Hence any modification of the tyrosine may yield further information on the nature of the metal-ion-induced conformational changes in S-100b protein.

Tetranitromethane (TNM) is a useful and, under carefully controlled conditions, specific reagent for the chemical modification of tyrosine residues in proteins (Sokolovsky *et al.*, 1966). Nitration of tyrosine residue with TNM shifts the electronic transitions of the molecule from the near u.v. to the visible spectral region. As a result, the absorption and c.d. spectra of such derivatives will be much less influenced by the contribution from other aromatic chromophores. A study of these spectra should furnish details regarding the microenvironment of tyrosine residues in the molecule and any changes in this region induced by metal ions.

In the present study, we have employed absorption and absorption difference spectra, along with c.d. measurements, on the 3-nitrotyrosyl derivative of S-100b. The Zn²⁺ binding property of the nitrated protein was similar to that of the native protein. However, nitrated S-100b behaved differently from the native molecule with regard to calcium binding, suggesting that the single tyrosine residue is essential for monitoring the Ca²⁺-induced conformational changes in the protein.

MATERIALS AND METHODS

Bovine brain S-100b protein was prepared as previously described (Mani *et al.*, 1983) with a minor modification. The solvent system used for the Sephacryl S-200 gel filtration column consisted of 0.1 M-Tris/HCl buffer, pH 7.5, containing 2 mM-EDTA, 0.4 M-NaCl and 6 M-urea. Inclusion of 6 M-urea in the solvent was necessary since in its absence a high-M_r protein of 50 kDa co-eluted with the S-100 proteins. S-100b protein was isolated by using a hydroxyapatite column. Final purification of S-100b was achieved by Zn²⁺-dependent affinity chromatography on a phenyl-Sepharose column as described by Baudier *et al.* (1982). S-100b protein used in the present study was homogeneous according to the criteria set out in Mani *et al.* (1983), which included polyacrylamide-gel electrophoresis in the presence and absence of SDS.

Nitration of S-100b was carried out by treatment of the protein in 0.1 M-Tris/HCl buffer, pH 8.0, with aliquots of TM (0.84 M solution in 95% ethanol) for 45 min at room temperature in the presence of either EDTA or Ca²⁺. The ratio of reagent to tyrosine used was 100:1. After reaction, the modified protein was initially

Abbreviation used: TNM, tetranitromethane.

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dialysed against 0.1 M-Tris/HCl buffer, pH 7.5, containing 2 mM-EDTA at 4 °C and subsequent dialysis was carried out with at least four changes against the solvent (0.1 M-Tris/HCl buffer, pH 7.5) that had been passed through a Chelex-100 column in order to remove divalent cations. Absorption and absorption difference spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer and c.d. measurements were taken on a Jasco J500C instrument fitted with a DP-500 N data processor, as described earlier (Mani & Kay, 1983b). The concentration of nitrotyrosine was estimated from the A_{381} by using an absorption coefficient of $2200 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Nitrotyrosyl S-100b concentration was measured by interference fringe counts in the ultracentrifuge, assuming 41 fringes to be equivalent to 10 mg/ml (Babul & Stellwagen, 1969). SDS/polyacrylamide-gel electrophoresis was performed according to Weber & Osborn (1969). Standard polyacrylamide-gel electrophoresis was performed at pH 8.6 with Tris/glycine buffer as described by Schaub & Perry (1969).

Ultracentrifuge studies were routinely done at 5 °C in a Beckman Spinco Model E ultracentrifuge equipped with a photoelectric scanner multiplex accessory and high-intensity light source. The Rayleigh interference optical system was also used. Low-speed sedimentation equilibrium runs were performed according to Chervenka (1969).

Ca^{2+} -binding assays were carried out as described in an earlier paper on troponin-C (McCubbin *et al.*, 1986), using the relatively new Centricon 10 microconcentrators from Amicon as ultrafiltration devices to perform rapid flow dialysis as a means of monitoring Ca^{2+} -binding to S-100b protein (Blatt *et al.*, 1968). S-100b protein was incubated for 15 min with excess calcium and was then centrifuged in a Beckman Model L centrifuge at 4000 rev./min for 30 min. Free Ca^{2+} in the supernatant (in this case the solution centrifuged across the dialysis membrane) was analysed by using the metallochromic indicator Arsenazo III (Sigma) according to Thomas (1982). S-100b protein was initially dissolved in 0.1 M-Tris/HCl buffer, pH 8.0, containing 2 mM-EDTA and was subjected to exhaustive dialysis against Chelex-100 column treated 0.1 M-Tris/HCl, pH 7.5. Protein (40–50 μmol) was placed in a prewashed Centricon 10 microconcentrator along with a known amount of Ca^{2+} (5–10-fold molar excess), incubated for 15 min and the solution was centrifuged. The supernate was assayed for Ca^{2+} with Arsenazo III by using a 20 μM solution of dye in 0.1 M-Tris/HCl, pH 7.5, and monitoring the Ca^{2+} -induced absorbance changes at 650 nm on a Perkin-Elmer Lambda 5 spectrophotometer. Standard curves (known amount of Ca^{2+} added versus A_{650}) were generated at the start and the conclusion of an experiment to ensure the reproducibility of the dye. From the amounts of free Ca^{2+} found in the supernatant (from the standard curve), along with the total Ca^{2+} added and the amount of protein used, it was possible to calculate the amount of Ca^{2+} bound to the protein.

RESULTS AND DISCUSSION

Ca^{2+} binding

The S-100b protein polypeptide chain (β subunit) consists of 91 amino acids and is characterized by a single tyrosine residue present in position 17 along the polypeptide chain (Isobe & Okuyama, 1981). Upon

binding Ca^{2+} and Zn^{2+} S-100b protein undergoes a conformational change resulting in the alteration of the environment of its single tyrosine and one or more phenylalanine residues (Mani *et al.*, 1982; 1983; Baudier & Gérard, 1983; Mani & Kay, 1983a, 1985).

TNM in the past has proved very useful for studying the effect of Ca^{2+} on the structure of calcium binding proteins such as calmodulin and troponin-C (McCubbin & Kay, 1975; Walsh *et al.*, 1979). The rate of reaction of TNM with the single tyrosine in the β -chain of S-100b protein in the presence of 1 mM- Ca^{2+} at pH 8.0 was very slow. After 45 min of incubation only 0.2–0.3 mol of TNM was bound/subunit, whereas in the presence of 2 mM-EDTA at pH 8.0 0.6–0.7 mol of TNM reacted/subunit, suggesting that the single tyrosine residue in S-100b is more accessible to react with TNM in the absence of Ca^{2+} . The degree of ionization of the single tyrosine residue in S-100b protein, as a function of pH, was studied earlier by spectrophotometric titration, u.v.-difference spectroscopy, c.d. and ^1H -n.m.r. studies. All these measurements clearly indicated that the single tyrosine residue in the presence of Ca^{2+} was buried in the interior of the protein (Mani *et al.*, 1982, 1983). Thus the observed higher reaction rate of S-100b with TNM in EDTA medium is due to the increased exposure of the tyrosine residue to solvent.

The absorption spectrum of nitrated S-100b was similar to that of native S-100b. The fine structure associated with the native protein in the 250–270 nm region, due to a high phenylalanine to tyrosine ratio (7:1), was also conserved in the nitrated sample and was apparent in the derivative plots, $-\Delta A/\Delta \lambda$ versus λ .

In any chemical modification studies it is imperative to ascertain whether or not the structure and, in this instance, also the metal-binding ability, of the protein have been altered. Treatment with TNM is also known to induce aggregation in certain proteins (Muszynska & Riordan, 1976) and for this reason low-speed sedimentation equilibrium studies were carried out with the nitrated protein in 0.1 M-Tris/HCl/1 mM-EDTA, pH 7.5. Under these conditions the weight-average M_r distribution ranged from 17000 to 22000, suggesting that nitration did not result in any aggregation of the protein, since native S-100b is known to exist as a dimer of M_r 20000 in the same solvent. Polyacrylamide-gel electrophoresis of nitrated S-100b, in the presence and the absence of Ca^{2+} , is shown in Fig. 1. The modified protein, like the native one, moved faster in the absence of Ca^{2+} , suggesting that nitrated S-100b binds Ca^{2+} ; this is a characteristic feature of calcium-binding proteins (Cox & Stein, 1981). Calcium-binding assays revealed the nitrated S-100b to bind 0.9 mol of Ca^{2+} /subunit, which is in agreement with the value obtained with the native protein, i.e. 1 mol of Ca^{2+} /subunit. Having established that the modified protein does bind Ca^{2+} similarly to the native protein, the next step was to ensure that nitration did not result in any major conformational change. For this purpose far-u.v. c.d. measurements were carried out with the modified protein. The far-u.v. c.d. spectrum of S-100b has been well documented (Mani *et al.*, 1982). For instance, in the absence of Ca^{2+} , the $[\theta]_{222}$ is $-17000 \pm 300 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$, while the addition of Ca^{2+} causes an approx. 10% decrease to $-15200 \pm 300 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. With the modified protein the $[\theta]_{222}$ value obtained was $-16600 \pm 300 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ in the absence of any Ca^{2+} ,

Fig. 1. Electrophoresis of nitrotyrosyl S-100b in Tris/glycine buffer, pH 8.6 (12.5% gels) containing 1 mM-EDTA (a) and in the presence of 1 mM- Ca^{2+} (b)

suggesting that the structure of nitrotyrosyl S-100b is indistinguishable from that of native S-100b with respect to the average secondary structure of the folded polypeptide chain. However, addition of Ca^{2+} did not induce any significant change in the $[\theta]_{222}$ value. The observed change was within the experimental error, i.e. $\pm 300 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. Hence the nitrated protein behaves differently from the native, which lost nearly $1500 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ in ellipticity in the presence of Ca^{2+} . Since the modified protein does not undergo a conformational change that can be detected by c.d., we

were unable to establish its affinity for calcium by the c.d. titration method.

Near-u.v. c.d. spectra of S-100b are shown in Fig. 2. The ellipticity of the protein is negative between 250 and 300 nm and the two bands at 284 and 278 nm can be assigned to the single tyrosine, while the phenylalanine c.d. bands are located at 268.5 and 262 nm. Binding of Ca^{2+} resulted in a perturbation of the single tyrosine and one or more phenylalanine residues and the ellipticity values become more positive, e.g., $[\theta]_{268.5}$ decreases from -55 to $-20 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ in the presence of Ca^{2+} . The aromatic c.d. spectrum of the nitrated S-100b is similar to that of the native protein (Fig. 3), but the binding of Ca^{2+} had no noticeable effect on the c.d. spectrum, again confirming that Ca^{2+} binding does not induce any significant conformational change. However, when one compares Figs. 2 and 3 it is noted that in the case of the native protein there is a slight sharpening effect at 268.5 and 262 nm and this may be as a result of a slight alteration in the microenvironment around one or more phenylalanine residues.

The difference in the absorption properties of native S-100b protein between 250 and 300 nm induced by Ca^{2+} has already been reported (Mani *et al.*, 1982). The observed difference peaks at 287 and 280 nm arise from the perturbation of the single tyrosyl chromophore. In addition one or more phenylalanine chromophores are also affected. The sign of the tyrosyl difference peak (i.e. a 'red shift') indicates that the chromophore moves to a less polar environment in the presence of Ca^{2+} (Donovan, 1969). The Ca^{2+} -induced u.v.-difference spectrum obtained with the modified protein is shown in Fig. 4. Binding of Ca^{2+} resulted in perturbation of the environment of one or more phenylalanine residues in a manner similar to that in native S-100b protein. However, Ca^{2+} binding altered the environment of the tyrosine residue differently, since now a blue shift was observed with the modified protein, implying that this chromophore is now more exposed to the solvent. Thus even though the modified protein binds Ca^{2+} it differs significantly from the native protein in the manner in which it responds to Ca^{2+} .

Zn^{2+} binding

The effect of Zn^{2+} binding on the near-u.v. c.d. spectra of native and modified S-100b protein is shown in Figs. 2 and 3, respectively. In the case of nitrated S-100b, addition of Zn^{2+} resulted in alteration of the environment of the tyrosine residue, since the ellipticity becomes more positive, i.e., from $+3$ to $+11 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ around the 280 nm region. A similar trend was noticed with the native protein and the ellipticity value at 280 nm changed from -20 to approx. $-10 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. In addition to tyrosine, phenylalanine residues are also affected in both instances to a similar extent, thus clearly establishing that modified S-100b behaves very much like native S-100b as far as Zn^{2+} binding is concerned. Addition of Zn^{2+} had no significant effect on the overall secondary structure of the nitrated S-100b as indicated by far-u.v. c.d. studies (results not shown) and, in this respect as well, the nitrated sample acted like native S-100b, since Zn^{2+} does not induce any gross conformational change with the native protein (Mani & Kay, 1983a).

Fig. 4 reveals a typical Zn^{2+} -induced u.v. absorption difference spectrum generated with the modified protein.

Fig. 2. Aromatic c.d. spectra of S-100b (1.8 mg/ml) in 0.1 M-Tris/HCl, pH 7.5 (—), and in the same buffer containing 1 mM- Ca^{2+} (.....) or 0.4 mM- Zn^{2+} (----)

Fig. 3. Aromatic c.d. spectra of nitrotyrosyl S-100b (1.2 mg/ml) in 0.1 M-Tris/HCl, pH 7.5 (—), and in the same buffer containing 1 mM- Ca^{2+} (.....) or 0.4 mM- Zn^{2+} (----)

In the presence of Zn^{2+} , the tyrosine residue was slightly blue-shifted, whereas with the native S-100b Baudier & Gérard (1983) observed a red shift, suggesting that with the introduction of the nitro group the tyrosine residue

Fig. 4. U.v. difference spectra of nitrotyrosyl S-100b produced by 1 mM- Ca^{2+} (.....) and 0.4 mM- Zn^{2+} (----)

Chelex-treated nitrotyrosyl S-100b in 0.1 M-Tris/HCl, pH 7.5, was used for these studies. The data were corrected for dilution and are expressed as the difference in molar absorption, $\Delta\epsilon$.

is slightly more accessible to solvent in the presence of Zn^{2+} . In addition to tyrosine, phenylalanine residues in the nitrated S-100b were perturbed but in a manner similar to the native protein. The observed peak around 300 nm could be due to the nitrophenolate ion (McCubbin & Kay, 1975).

Ca^{2+} and Zn^{2+} ions are believed to bind at different sites on the polypeptide chain, based on the earlier c.d. and fluorescence studies carried out with the apoprotein

(Baudier & Gérard, 1983; Mani & Kay, 1983a). However, in the present study, by modifying the single tyrosine residue in the polypeptide chain, we have been able to demonstrate that the structure evolved as a consequence of modification responds differently to Ca^{2+} and Zn^{2+} , thereby clearly establishing that the two metal binding sites must be different.

The amino acid sequence of S-100b protein (β -subunit) shows regions of strong clustering of hydrophobic, basic and acid amino acids. The acidic cluster of 12 amino acids from residues 61 to 72 exhibits a high sequence homology with the 'calcium-binding sites' proposed by Kretsinger (1976) and this site is also characterized by a single phenylalanine residue present in position 71. Hence one would anticipate addition of Ca^{2+} to alter at least the environment of phenylalanine-71 present in this calcium-binding loop region, and indeed this is the case. The single tyrosine residue at position 17 is located away from this calcium-binding site, and for this reason it probably is not perturbed. Of course one should also take into account the possible steric hindrance due to the nitro moiety.

The tyrosine residue at position 17 is in close proximity to two histidine residues at positions 15 and 25, and histidine residues are known to act as ligands for co-ordinating Zn^{2+} (Holmes & Mathews, 1981). Baudier & Gérard (1983), using a fluorescent hydrophobic probe, have shown that Zn^{2+} binding to S-100b takes place at the *N*-terminus region in close proximity to tyrosine-17. Since the Zn^{2+} -binding site, possibly involving histidine-15 or -25, is very close to tyrosine-17, we are able to monitor, with the nitrated S-100b, changes in the environment of tyrosine as a result of Zn^{2+} addition.

In conclusion, the single tyrosine residue in S-100b is critical to sense the Ca^{2+} -induced conformational changes in the protein. Since S-100b did not undergo any major alteration in secondary structure as a consequence of treatment with TNM, nitrotyrosine-17 in S-100b can be considered as a reliable and specific probe for studying the metal-binding properties of S-100b protein by employing spectroscopic techniques.

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