THE INFLUENCE OF CALCIUM ON THE SPECTRAL PROPERTIES OF THE NITROTYROSYL CHROMOPHORE IN TROPONIN C

William D. McCUBBIN and Cyril M. KAY

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

Received 20 May 1975

1. Introduction

The near ultraviolet circular dichroism spectrum of TN-C* consists of bands associated with transitions involving the amino acids phenylalanine and tyrosine. There are no contributions from tryptophan or cystine since these residues are absent from the molecule. At pH 8, the region from 250–270 nm is dominated by the 'sawtooth' fine structure characteristic of the absorption spectrum of phenylalanine. From 270–300 nm a single broad weak trough is found which may be assigned to tyrosine. In the presence of Ca^{2+}, there is a sharpening of the bands assigned to phenylalanine, and the tyrosine ellipticity becomes slightly more positive. There are two tyrosine residues in TN-C at positions 10 and 108 in the primary sequence [1]. In the nomenclature of Weeds and McLachlan [2], tyrosine-108 is at Ca^{2+} co-ordinating positions $S_{(7)}$ (-Y direction) in binding site S_3 and, hence, any modification of this residue might yield further information on the nature of the Ca^{2+}-induced conformational change in TN-C. Nitration of tyrosine residues with TNM shifts the electronic transitions of the molecule from the near ultra-violet to the visible spectral region. Consequently, the absorption and CD spectra of such derivatives will be much less influenced by the contribution from other aromatic chromophores. A study of these spectra should give details regarding the microenvironment of these two possible sites in the molecule and any changes in these regions induced by Ca^{2+}.

This communication describes briefly some absorption and absorption difference spectra, along with CD measurements, on 3-nitrotyrosyl derivatives of TN-C. It was found that derivatives with both tyrosine residues modified underwent the Ca^{2+} induced conformational change in an identical manner to native TN-C, suggesting that in the native molecule the state of ionization of the tyrosine residues does not seem to be involved in the mechansim of the Ca^{2+} interaction.

2. Materials and methods

Troponin-C was isolated and purified from rabbit skeletal muscle by an initial Ebashi type extraction [3] with lithium chloride, followed by chromatography on DEAE Sephadex and Sephadex G-200 in the presence of 8 M urea and EGTA. The product was homogenous by the criterion of SDS polyacrylamide gel electrophoresis. Formation of 3-nitrotyrosyl TN-C was achieved by treatment of the protein at pH 8 with aliquots of TNM (0.84 M solution in 95% ethanol) for one hour at 20°C with either EGTA or Ca^{2+} present. Two separate series of experiments employed reagent to tyrosine ratios of 4:1 and 60:1 respectively. After reaction the modified protein was separated from nitroformate ion side product by passage through a column of Bio Gel P-2, eluted with 5 mM Tris–HCl buffer at pH 8. The product was dialysed versus water and lyophilised. Absorption and absorption difference spectra were recorded on a

* *Abbreviations:* CD, circular dichroism; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic; TN-C, calcium binding protein; TNM, tetranitromethane.

Cary 118C spectrophotometer and CD measurements were taken on a Cary 60 with attached model 6001 CD accessory in accordance with previously described methodology [4]. The concentration of nitrotyrosine was estimated from the absorption at 381 nm using an extinction coefficient of 2200 M^{-1} cm^{-1}. Nitrotyrosyl TN-C concentration was measured by interference fringe counts in the ultracentrifuge, assuming 41 fringes equivalent to 10 mg/ml [5]. Ca^{2+} concentrations were adjusted by means of a Ca^{2+} buffer consisting of Ca^{2+} and 1 mM EGTA in 0.15 M KCl 50 mM Tris–HCl at pH 8 as described earlier [6].

3. Results and discussion

3.1. *Absorption and absorption difference spectra*

At a sixty-fold ratio of TNM to tyrosine, either in the absence or presence of Ca^{2+} ion, both tyrosine-10 and tyrosine-108 were nitrated. The absorption spectrum of this derivative, along with that of unmodified TN-C for comparison, is shown in fig.1. The spectrum of nitrotyrosyl TN-C was measured at pH 9 so the nitrotyrosyl residues, which have apparent pK values of approx. 7, will be in the phenolate ion form. The absorption spectrum is characterized by a broad band at 422 nm (not shown) and the spectrum from 320–250 nm is very similar to native TN-C, but exhibits a pronounced hyperchromicity throughout the region. This increase in absorptivity arises from the more intense electronic transitions that occur in the nitrotyrosyl residues compared with tyrosine ones [7]. To emphasize the fine structure characteristic of the absorption spectrum of phenylalanine residues, particularly in the case of nitrotyrosyl TN-C, derivative plots, $-\frac{\Delta\epsilon}{\Delta\gamma}$ versus λ, were computed over the range 250–275 nm. These are shown as the inset in fig.1 and clearly demonstrate the constancy in wavelength position, of these fine structure bands in the derivative.

The u.v. absorption spectrum of TN-C is known to be sensitive to the presence of Ca^{2+} ions and difference spectra produced by this cation have already been reported [8]. This experiment was repeated and extended down to 220 nm. The resulting spectrum is shown in fig.2. The maxima at 285 nm and 277 nm

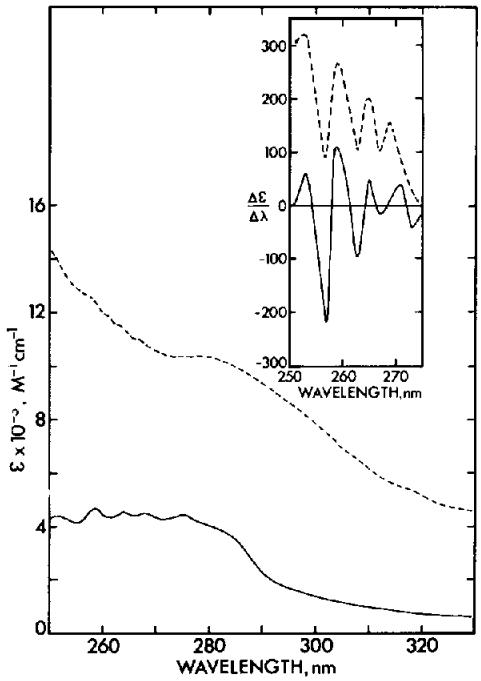

Fig.1. U.v. absorption spectra of TN-C (——) and nitrotyrosyl TN-C (------). The insert is a first derivative plot of the absorption spectra of TN-C (——) and nitrotyrosyl TN-C (------), calculated manually at 1 nm intervals from 275 nm to 250 nm. Proteins were dissolved in 0.15 M KCl, 1 mM EGTA, 50 mM Tris–HCl at pH 8.0 for TN-C and pH 9 for nitrotyrosyl TN-C, at concentrations of 1 mg/ml.

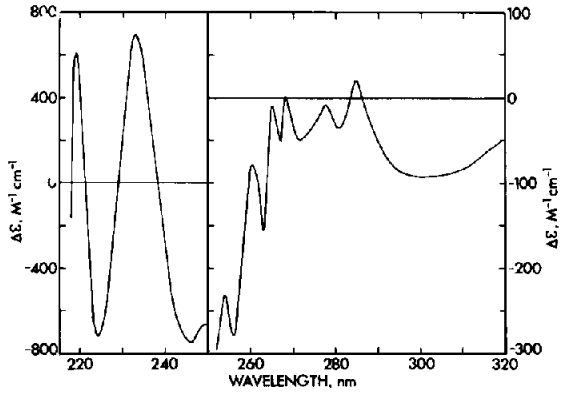

Fig.2. Difference spectrum of TN-C on addition of Ca^{2+} to the calcium-free protein. In the reference cell (1 cm path length down to 250 nm, 1 mm to 215 nm) was TN-C (1.5 mg/ml) in 0.15 M KCl, 1 mM EGTA, 50 mM Tris–HCl at pH 8. The sample cell contained the same consitiuents with 2 mM free Ca^{2+} present as $CaCl_2$.

presumably arise from perturbations of the environment of tyrosine [9] while the peaks at 268 nm, 265 nm and 254 nm arise from increases in fine structure of the phenylalanine absorption spectrum. The peak at 233 nm presumably again arises from tyrosine while the 219 nm maximum probably originates from phenylalanine. The chromophore or perturbation responsible for the broad minima around 300 nm remains unknown. It is as if there had been a baseline shift but the effect is quite reproducible, which essentially precludes this possibility. The abnormal absorption of TN-C in the 290–320 nm region has already been described [10] but these authors were also unaware of the nature of the chromophore responsible, although the suggestion was made that light scattering was unlikely.

The absorption spectrum of nitrotyrosyl TN-C is also sensitive to Ca^{2+}, as illustrated by the difference spectrum for this derivative (fig.3). The increase in fine structure of the absorption of the phenylalanine residues is again quite clear but the spectrum is dominated by a large maximum centred around 300 nm with the suggestion of a shoulder between 280 and 290 nm. Compared with the native protein the spectrum in this region has essentially undergone a complete 'flip over', again no doubt due to the more intense transitions occurring in the nitrophenolate ion compared to tyrosine.

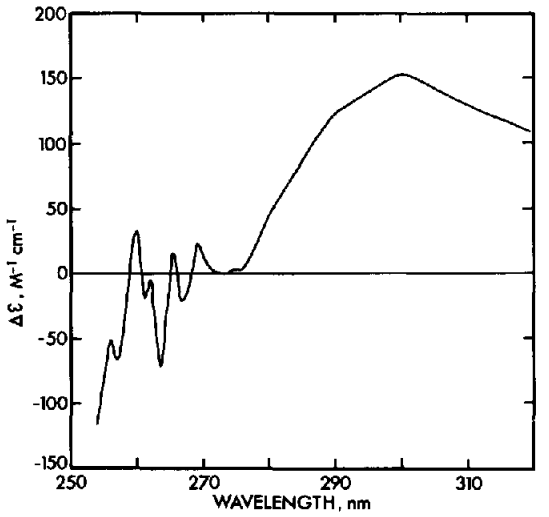

Fig.3. Difference spectrum of nitrotyrosyl TN-C on addition of Ca^{2+} to the calcium-free protein. In the reference cell (1 cm path length) was nitrotyrosyl TN-C (1.5 mg/ml) in 0.15 M KCl, 1 mM EGTA, 50 mM Tris–HCl at pH 8. The sample cell contained the same constituents with 2 mM free Ca^{2+} present as $CaCl_2$.

3.2. *Circular dichroism spectra*

Whenever a protein has been chemically modified it is imperative to ascertain whether or not the structure or the functional integrity of the protein have been altered. Consequently, in this nitration study the derivatives were examined for any signs of abnormal behaviour. The far u.v. CD spectrum of TN-C has been well documented [11,12], and the dramatic change in the value of $[\theta]_{221\ nm}$ noted as a function of the free Ca^{2+} ion concentration. This is quite reproducible. The value of $[\theta]_{221\ nm}$ is $-10\,000$ deg cm^2 dmol^{-1} in the absence of Ca^{2+}, and becomes $-14\,500$ deg cm^2 dmol^{-1} in the presence of this cation. Samples of nitrated TN-C with both tyrosines modified gave $[\theta]_{221\ nm}$ values of $-10\,200$ deg cm^2 dmol^{-1} and $-14\,500$ deg cm^2 dmol^{-1}, in the absence and presence of Ca^{2+}, implying that the structure of nitrotyrosyl TN-C is indistinguishable from that of native TN-C with respect to the average secondary structure of the folded polypeptide chain, and in its response to Ca^{2+} ions. The molecular weight behaviour of nitrotyrosyl TN-C, as determined by sedimentation equilibrum studies, did not differ significantly from the native molecule under similar conditions. Derivatives with only a single tyrosine modified behaved in an analagous manner. Thus the modified protein resembles the native protein in both its structural and functional properties. The nitrotyrosyl chromophore can therefore be considered a reliable probe for structural properties which also exist in the native protein. Furthermore, it is another important example in the growing list of the spectral properties of the nitrotyrosyl chromophore in a globular protein.

The near u.v. CD spectrum of TN-C in the absence and presence of Ca^{2+} has already been reported [11]. The ellipticity below 270 nm appears to be due essentially to phenylalanine. Ca^{2+} ions cause only a slight sharpening of these ellipticity bands with no alteration in their wavelength position. The small contribution from the tyrosine residues is slightly affected by Ca^{2+} and appears as the shallow through above 270 nm.

The CD spectra between 250 and 360 nm for nitro-

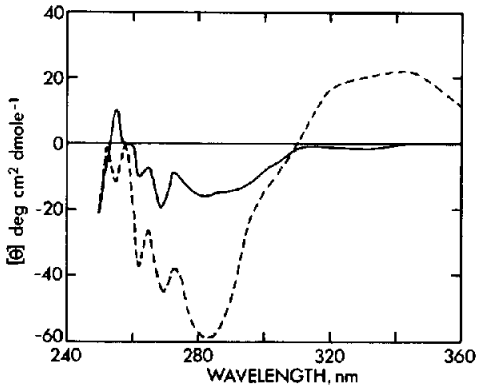

Fig.4. Circular dichroism spectra of nitrotyrosyl TN-C in 0.15 M KCl, 1 mM EGTA, 50 mM Tris–HCl at pH 8 (——) and in the same solvent containing 2 mM free Ca^{2+} (——).

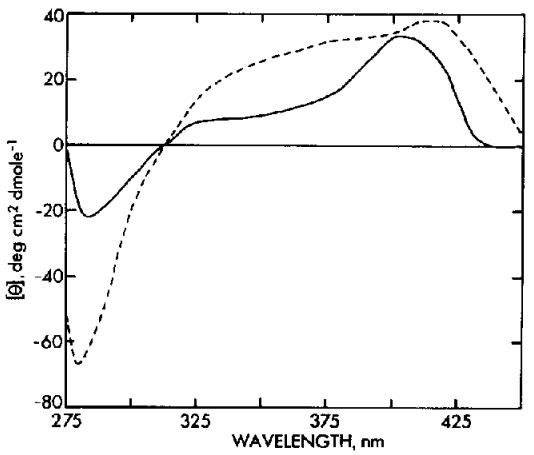

Fig.5. Circular dichroism spectra of nitrotyrosyl TN-C in 0.15 M KCl, 1 mM EGTA, 50 mM Tris–HCl at pH 9 (——) and in the same solvent containing 2 mM free Ca^{2+} (——).

tyrosyl TN-C, at pH 8, in the absence and presence of Ca^{2+} is shown in fig.4. In the absence of Ca^{2+} the spectrum is characterized by the typical band pattern of phenylalanine residues, with a weak trough near 280 nm. From 300 nm to 360 nm the optical activity is essentially zero. In the presence of Ca^{2+}, a sharp negative band occurs at 282 nm while a weak broad positive band with a maximum around 340 nm is also seen. These bands may be satisfactorily assigned to the nitrotyrosyl residues since native TN-C does not have significant optical activity in this region. The nitrotyrosyl residues are presumably in the ionized state at pH 8 and thus the effect noted should be ascribed to the phenolate anion. Solubility problems near pH 5 (to produce satisfactorily the unionized phenol form) precluded very exact CD measurements, however it does appear that the optical activity is now much less than at pH 8. A similar situation has already been described in the staphylococcal nuclease system [7]. The CD measurements on the modified protein were extended to 450 nm at pH 9. As can be seen in fig.5, the nitrotyrosyl chromophore generates a positive band near 400 nm in the absence of Ca^{2+} which increases slightly and broadens in the presence of Ca^{2+}. The broadness of this band, compared to the sharp one noted at 282 nm, will tend to limit its usefulness somewhat.

CD spectra in the region 240–300 nm arise from aromatic amino acid chromophores which are either asymmetric per se or because they are located in an asymmetric environment in the protein molecule. Thus CD spectra should be a sensitive probe to monitor changes in the conformation, or the micro-environment of the chromophores.

In the particular case of TN-C, the picture is fairly straightforward as the only contributing side chains are those of phenylalanine and tyrosine. Although on an individual basis the electronic transitions responsible for the CD bands of phenylalanine residues are extremely weak, when there is a high concentration of these residues suitable disposed near asymmetric centres, then their contribution becomes quite appreciable. The sharpening of the fine structure noted in the presence of Ca^{2+} is a result of the appearance of more ordered structure, created by the induced conformational change, resulting in an increase in spectral detail. The unionized tyrosine residues would on a theoretical basis give rise to a weak band at 275 nm with a shoulder near 282 nm; this is consistent with experimental observation.

The near u.v. CD spectrum of nitrotyrosyl TN-C may now be interpreted in close detail. The region from 265–250 nm would not be expected to differ significantly from native TN-C as the phenylalanine residues are not being affected and the average degree of secondary structure is the same in the derivative as in unmodified TN-C. The basic band shapes and wavelengths would be expected to remain the same

although minor alterations in amplitude might occur. This is essentially what is observed.

There are two major points of difference in the nitrotyrosyl group vis-a-vis tyrosine. Firstly, at physiological pH, these groups will be in the ionized or phenolate form and secondly, the steric hindrance of the nitro moiety must be borne in mind. These two differences by themselves would not necessarily be expected to alter the optical activity near 280 nm, especially in the loose structure prevalent in the absence of Ca^{2+}. However in the presence of Ca^{2+}, as a result of the large increase in order generated from the conformational change in TN-C, steric hinderance and rigidity of structure may well be significant, and the suggestion is made that the considerable increase in magnitude and sharpness of the 282 nm bands, and the magnitude of the 340 nm band observed with Ca^{2+}, must be due mainly to this effect.

In conclusion, the sensitivity of the nitrotyrosyl chromophore to subtle changes in its microenvironment makes it a useful conformational probe for the continuing study of the molecular changes occurring when Ca^{2+} binds to TN-C. It will be of great interest when looking at derivatives with only a single tyrosine reacted to elucidate which residue has been modified: tyrosine-10 near the N terminus or tyrosine-108 in the third Ca^{2+} binding site.

Acknowledgements

The authors are indebted to A. Keri and K. Oikawa for their excellent technical assistance.

References

[1] Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G. and Jackman, N. (1973) FEBS Lett. 36, 268–272.
[2] Weeds, A. G. and McLachlan, A. D. (1974) Nature 252, 646–649.
[3] Ebashi, S., Wakabayashi, T. and Ebashi, F. (1971) Biochem. 69, 441–445.
[4] Oikawa, K., Kay, C. M. and McCubbin, W. D. (1968) Biochim. Biophys. Acta 168, 164–167.
[5] Babul, J. and Stellwagen, E. (1969) Anal. Biochem. 28, 216–221.
[6] McCubbin, W. D., Mani, R. S. and Kay, C. M. (1974) Biochemistry 13, 2689–2694.
[7] Di Bello, C. and Griffin, J. H. (1975) J. Biol. Chem. 250, 1445–1450.
[8] Head, J. F. and Perry, S. V. (1974) Biochem. J. 137, 145–154.
[9] Herskovits, T. T. (1967) Methods Enzymol. 11, 748–775.
[10] Greaser, M. L. and Gergely, J. (1973) J. Biol. Chem. 248, 2125–2133.
[11] Murray, A. C. and Kay, C. M. (1972) Biochemistry 11, 2622–2627.
[12] McCubbin, W. D. and Kay, C. M. (1973) Biochemistry 12, 4228–4232.