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STRUCTURE OF THE CALCIUM BINDING SITES IN TROPONIN-C AND CALMODULIN STUDIED BY EXAFS AND XANES(o)

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1. - INTRODUCTION.

The most important outcome of X-ray diffraction studies on calcium-binding proteins has been the uncovering in fish parvalbumins of a unique three-dimensional structure for Ca recognition, called the EF hand conformation(1). Furthermore the presence of such conformation in other Ca-modulated cytosolic proteins has been predicted from the analysis of sequence homologies(2). The use of synchrotron radiation makes it feasible the study of local structures when single crystals are not available. The interatomic calcium-oxygen distance (R) and the coordination number can be determined from EXAFS(3-5). Moreover the Ca-ligand charge transfer and coordination geometries can be determined from XANES (X-ray Absorption Near Edge Structures)(6, 7). As part of a more systematic study employing these techniques on several Ca-binding proteins and simple Ca compounds, we report here data obtained with Troponin-C and

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Calmodulin. TN-C contains two classes of Ca binding sites[8]: two sites with high affinity for Ca ($K = 10^{-8}$ M), which also bind Mg, and two sites with a lower affinity ($K = 10^{-6}$ M), specific for Ca. Calmodulin, which regulates a large number of intracellular activities, is vastly homologous to TN-C[9]. In fact it contains four calcium-binding sites ($K = 10^{-6}$ M), whose specificity, however, is not yet well established[10].

2. - EXPERIMENTAL.

TN-C was prepared from rabbit skeletal muscle by the method of Perry and Cole[11]. Calmodulin was isolated from beef brain by a method involving ammonium sulphate fractionation, DEAE chromatography and gel filtration. Precautions were taken in later stages of purification to obtain proteins with a minimal content of calcium. TN-C-metal complexes were prepared by dissolving the freezedried protein in double-distilled water and stochiometrically adding $CaCl_2$ and $MgCl_2$ to obtain the desired molar ratios. Samples containing Tb were prepared by first adding 2 moles per mole protein of $TbCl_3$. Calmodulin-metal complexes were prepared in a similar fashion to TN-C. Protein solutions were then freezedried and packed into special holders.

X-ray absorption spectra were measured at the Frascati synchrotron radiation facility "PULS" using the X-ray beam emitted by the 1.5 GeV storage ring ADONE which was operated at about 50 mA. The radiation was monochromatized by a Si (220) channel-cut crystal and the energy resolution at the Ca K-edge was about 0.4 eV, which is close to the intrinsic width of the Ca 1s level. Interatomic distances were extracted from EXAFS oscillations (Fig. 1). The experi-

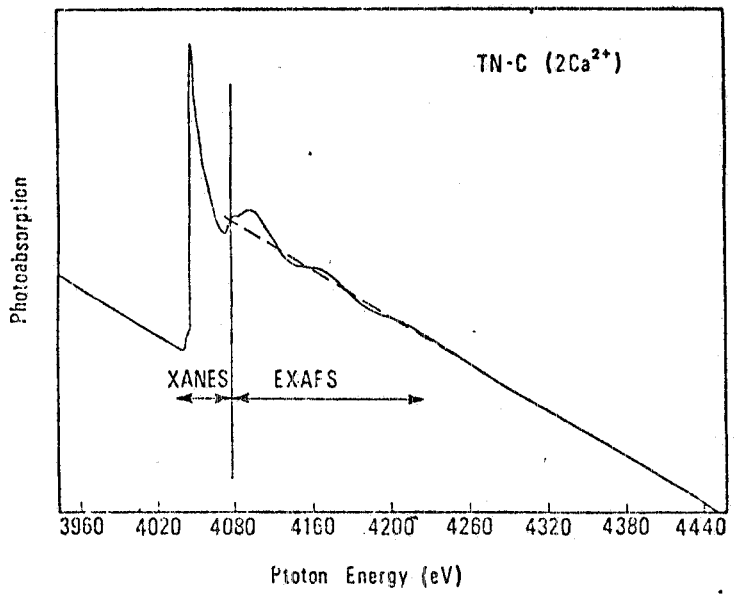

FIG. 1 - Typical X-ray absorption spectrum of Troponin-C.

mental phase ($\Phi(K)$) of the EXAFS oscillations due only to the first coordination shell was obtained by antitransform of the Fourier analysis : $K\chi(K) = A(K) \sin \Phi(K)$; from the EXAFS theory $\Phi(K)/2K = R + \varphi(K)/2K$ where R is the average interatomic distance and $\varphi(K)$ is the total phase shift of the excited photoelectron (Fig. 2).

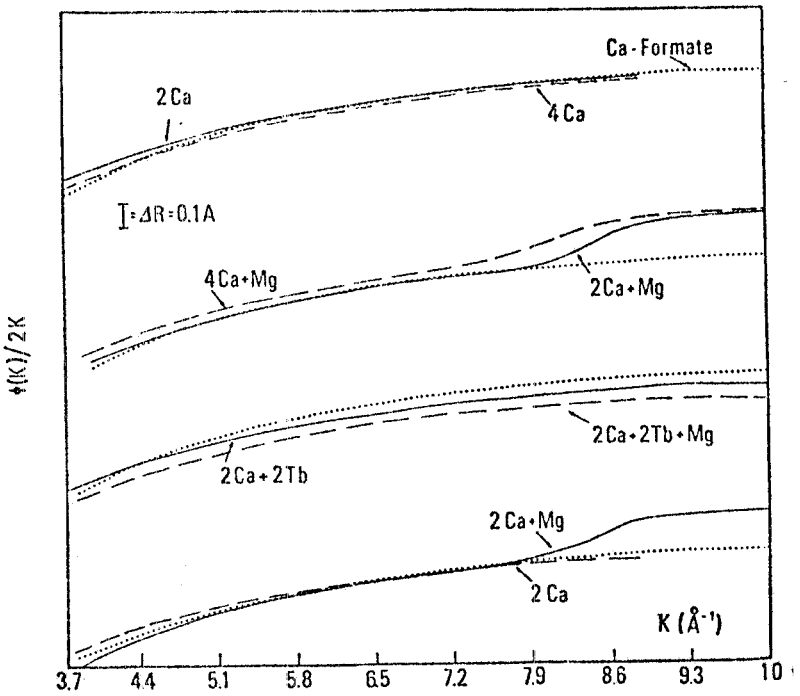

FIG. 2 - $\Phi(K)/2K$ curves of TN-C in different conditions compared with Ca-formate (·····).

The separation between the curves of the proteins and the model compound Ca-formate give directly the difference between interatomic distances. The results are summarized in Table I. The absorption

TABLE I - Calcium-Oxygen Average Interatomic Distances from EXAFS and Energies of Structure in XANES of TN-C and Calmodulin.

Sample	mol. Me/ Ca	mol. P/ Tb	Mg	R	ΔR	Absorption max. C ± 0.1 eV	Δ(B - C) ± 0.2 eV°
CaO				2.40+			
Formate				2.40	0.0	4049.2	5.8
TN-C	2	-	-	2.40	0.0	4049.2	4.6
	4	-	-	2.39	- 0.01	4049.2	4.8
	2	2	-	2.36	- 0.04	4049.2	5.0
	2	-	+	2.40	0.0° (0.15)ˣ	4049.0	5.3
	4	-	+	2.43	0.03° (0.17)ˣ	4049.0	5.3
	2	2	+	2.33	- 0.07	4049.4	5.4
Calmodulin	2	-	-	2.40	0.0	4049.6	6.0

+ Determined by crystal diffraction
° Determined before the step due to the beat (Fig. 2)
ˣ Determined after the beat

$\Delta R = R_{Sample} - R_{Formate}$ (± 0.02 A).

derivatives of the XANES of TN-C/metal complexes are reported in Fig. 3. The shift of the peak C_o is proportional to the Ca-O charge transfer and the splitting Δ (Fig. 3) gives a measure of the covalency of bonding which is strongly dependent on coordination geometry.

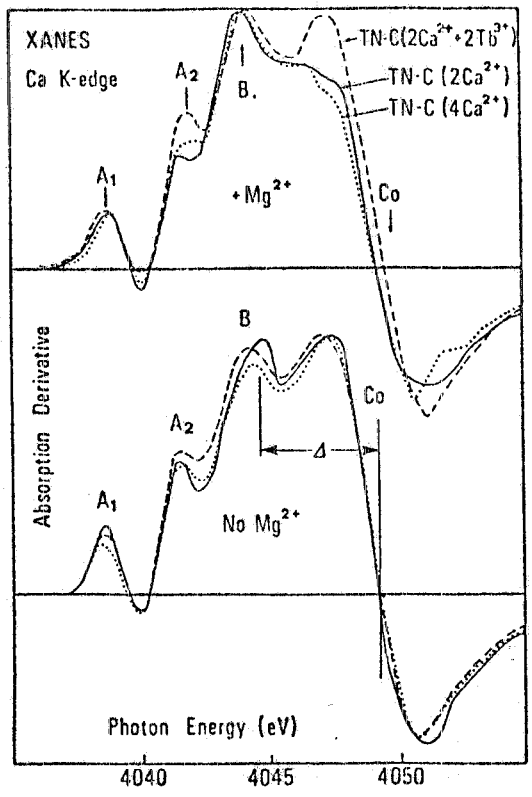

FIG. 3 - Absorption derivatives of near edge structures of TN-C.

3. - RESULTS AND CONCLUSIONS.

Calcium-binding sites in TN-C. A comparison of XANES of TN-C/2Ca and TN-C/4Ca (Fig. 3) shows a moderate increase of the splitting Δ in the fully saturated protein, which can be attributed to the low affinity sites. Moreover the EXAFS (Fig. 2) reveal only a slight decrease of the interatomic distance in low affinity sites ($\Delta R = -0.01$ Å). Altogether these results suggest a small difference in coordination geometry and average distances between the two classes of sites. The addition of 2 moles of Tb per mole of TN-C, saturating only the high affinity sites[12], permits the observation of signals originating from the low affinity ones. Under these conditions the latters appear to have a higher splitting Δ and a shorter R than the high affinity sites (see Table I).

Effect of Magnesium. The XANES spectra of the high affinity sites (TN-C/2Ca plus Mg) reported in Fig. 3 reveal a large increase in the intensity of the peak B and an increase of the splitting (Table I). These differences can be ascribed to a change in coordination geometry due to the binding of Mg. Such a change is revealed in EXAFS spectra by the appearance of a beat (Fig. 2), possibly indicating the presence of two classes of Ca-ligands distances separated by ca. 0.2 Å. In the presence of Mg TN-C/4Ca seems to undergo a change in coordination geometry similar to

that of the high affinity sites as revealed by XANES (Fig. 3). EXAFS spectra indicate a significant lenghtening of the average interatomic distances in the fully saturated protein (Fig. 2). These alterations can be explained either by an effect of Mg on the low affinity sites or by an interaction between the two classes of sites.

Calmodulin. From the EXAFS spectra of Calmodulin with 2 Ca bound, an average interatomic distance of 2.40 Å can be calculated. A preliminary analysis of XANES (see Table I) indicates that the energy position of peak C is shifted toward higher energy as compared to TN-C, and the splitting Δ is also increased (= 6.0 eV vs. 4.8). Hence the sites coordinating Ca in Calmodulin might belong to a type different from that present in parvalbumins and TN-C.

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