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SUMMARY

Using synchrotron radiation at Frascati Storage ring ADONE, the X-ray Absorption Near Edge Structure (XANES) has been applied to determine homologies and modifications of the local structure of the calcium binding sites of troponin-C (TnC). In all four calcium binding sites Ca^{2+} appears to be coordinated to carboxyl and carbonyl groups in a characteristic configuration. No structural difference has been found between high and low affinity sites. A distortion of the Ca^{2+} site geometry by binding of Mg^{2+} has been observed.

The XANES of parvalbumin has been measured and found to be different from troponin C. A tentative identification of the characteristic XANES spectra of the two different Ca^{2+} sites in this protein is reported.

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INTRODUCTION

Several biological functions are regulated by changes in the concentrations of cytosolic calcium. Ca^{2+} sensitivity is conferred by specialized, sequence-homologous proteins such as troponin C (TnC), calmodulin and parvalbumin (Kretsinger, 1980). In carp parvalbumin (Moews & Kretsinger, 1975) and recently in the vitamin D-dependent calcium-binding protein, (Szebenyi et al., 1981) the only two Ca^{2+}-modulated proteins whose crystal structure has been determined, a unique configuration called "EF hand" has been identified in the region of the two Ca^{2+}-binding sites. In this configuration Ca^{2+} is bound mainly to carboxyl and carbonyl groups in a loop between two α-helices. A comparison of amino acid sequences of different Ca^{2+}-binding proteins with that of parvalbumin indicates that homologous, helix-loop-helix domains are present in many proteins of this class (Kretsinger, 1980) like TnC (Kretsinger & Barry, 1975).

Calcium ion, having the electronic configuration of a rare gas atom, is devoid of magnetic and optical response and is silent to spectroscopical analysis such as EPR or optical absorption. An additional difficulty in the study of the binding of calcium in inorganic and organic systems arises from the large variety of possible configurations and the asymmetry of the binding sites. For instance, a large variety of Ca^{2+}-ligand distances has been generally observed in the first coordination shell (Williams, 1977).

X-ray Absorption Spectroscopy (XAS), using synchrotron radiation, (Doniach et al., 1980) is a powerful emerging technique for high resolution studies of metal binding sites in proteins because it provides a probe for a specific atom in a complex system and it gives information on the local structure of the metal binding site, being sensitive to short-range order in atomic arrangements rather than to long range order. Thus XAS makes feasible the study of local structures when single crystal are not available and appears particularly suitable for the investigation of Ca^{2+}-binding sites in biological system (Bianconi et al., 1978 & 1980; Powers et al., 1978; Miller et al., 1980).

Three parts of an X-ray absorption spectrum can be distinguished on the basis of both structural information and physical photoionization process:

1) the "edge region", extending over ~ 8 eV at the absorption threshold,
2) the "XANES" (X-ray Absorption Near Edge Structure), (Bianconi, 1981) in energy range of ~ 40 eV above the edge and
3) the "EXAFS" (Extend X-ray Absorption Fine Structure (Doniach et al., 1980) at higher energy.

The edge region is determined by the first allowed transitions from the core to empty molecular orbitals. It is therefore sensitive to the covalency of metal-ligand bonds and to the effective charge of the absorbers (Bianconi, 1981). EXAFS has been widely used as a structural technique for proteins both to determine completely unknown local structures like in citochrome oxidase (Powers et al., 1981) and to obtain more accurate interatomic distances in proteins already well studied by X-ray diffraction like in haemoglobin (Eisenberger et al., 1978). The advantageous simple characteristics of EXAFS are also its limitations. Generally no information on coordination geometry (bonding angles) can be extracted and serious limitations appear in non-ordered structures (Eisenberger & Lengeler, 1980). Only in the presence of first and second neighbours in collinear fashion bonding angles can be determined by EXAFS through the "focussing" effect (Teo, 1981). In the EXAFS region the wavefunction of the excited photoelectron can be described by a simple theory, that is the high kinetic energy photoelectron, extracted from the absorber (the central atom), is weakly back-scattered by one of the neighbour atoms in a single-scattering process. This gives information about local structures only in terms of atomic radial distribution (distances) around the central atom within only ~ 4 Å (short-range).

The XANES (Belli, et al., 1980; Bianconi, 1981) contains information on the stereochemical details (coordination geometry and bond angles) which are particularly important for complex systems such as proteins,

characterized by weak order and low symmetry. In the photoionization process the low kinetic energy (10-40 eV) excited photoelectron is strongly backscattered by neighbour atoms generating a multiple-scattering process. It is for such multiple-scattering involving several atoms that XANES is informative on the relative positions of the neighbour atoms. Recent theoretical progress on XANES (Durham et al., 1981, Kutzler at al., 1980; Bianconi et al., 1982) shows that XANES is determined by higher-order pair-correlation functions of neighbour atom distribution while EXAFS gives only the first-order pair-correlation-function. Because the XANES is a structural probe of a cluster of 15 to 30 atoms including the second shell of neighbour atoms the proposed experimental approach (Bianconi et al., 1978) to the study of local structures by XANES can be used.

In this paper we have used XANES spectroscopy to study the Ca^{2+}-binding sites of TnC and parvalbumin. TnC is the Ca^{2+}-binding subunit of the troponin complex which is part of the regulatory system of muscle contraction. It binds four Ca^{2+}: two at high affinity which also bind Mg^{2+} and two at low affinity, specific for Ca^{2+} (Potter et al., 1975). The presence of Mg^{2+} appears to equalize the affinity of the four binding sites. We have found that all four Ca^{2+}-binding sites have the same coordination number and similar structure up to the second shell of neighbour atoms. Mg^{2+} induces a modification of the Ca^{2+} binding. The XANES spectrum of the two Ca^{2+}, Mg^{2+}-binding sites of carp parvalbumin is different from that of TnC and is consistent with the presence of two different local coordinations (Moews & Kretsinger, 1975).

MATERIALS AND METHODS

Materials

Salts in crystalline form were Suprapur products from Merck. Water, double distilled and deionized, was further filtered through a Chelex-100 resin to reduce metal contamination. Plastic ware was used during the preparation of proteins and samples to avoid calcium contamination from glass.

Proteins

TnC was prepared from rabbit skeletal muscle as described by Perry and Cole (1974). Parvalbumins were isolated from carp white muscle according to the methods of Pechère et al. (1971) and Kretsinger and Nockolds (1973). The pI. 4.25 component was used in this study. The purity of each preparation was checked by SDS polyacrylamide gel electrophorésis (Laemnli, 1970). The biological activity of TnC was tested by ATPase measurements in a reconstructed acto-Sl system (Castellani et al., 1980).

Sample preparation

Protein-metal complexes were prepared by dissolving freeze-dried proteins in Ca^{2+}-free water (Ca^{2+} concentration $\sim 10^{-6}$M) and by adding $CaCl_2$ or $TbCl_2$ to obtain the desired protein-metal stoichiometric ratio. Mg^{2+} was added in molar excess (2 to 4 mM). Protein-metal complexes in solution were then freezedried and the powder was placed into plastic holders mounted with parafilm windows. The concentration of Ca^{2+} in the samples was determined by atomic absorption before and after the addition of calcium. Protein concentration was quantified by optical absorption measurements using molar extinction coefficient values of 2670 for TnC and 2000 for carp parvalbumin. Serial recordings of XANES spectra of the same samples were very similar, indicating that no major changes occurred during the exposure to the X-ray beam. TnC samples were further checked for denaturation and capability to respond to binding of Ca^{2+}. Intrinsic protein fluorescence measurements and circular dichroism spectra indicated no major differences in TnC before and after exposure to radiation.

Methods

The storage ring ADONE at the synchrotron radiation facility PULS at Frascati was used as X-ray source. It was operated at 1.5 GeV and I=60 mA. The X-ray beam was monochromatized by a Si(220) channel-cut single crystal at about 17m from the source. The X-ray absorption data were obtained by transmission measurements. The high stability, high collimation ($\Delta\theta=5x5^{-5}$ rad) and the high intensity of the X-ray beam gave spectra of high resolution ($\Delta E/E \sim 10^{-4}$) and good signal to noise ratio. XANES spectra were recorded in the energy range 4000-4150 eV with steps of 0.1 eV. The energy scale was calibrated at each electron beam injection in the storage ring using calcium formate as a reference sample. The electron beam position in ADONE storage ring at the emission point was directly controlled so that no changes in the energy positions of absorption peaks larger than $\pm$0.1 eV were observed during the lifetime of the electron beam in storage ring. The electron beam size at the emission point was 0.7 mm and an entrance slit of the same size was used in the X ray monochromator. The angle of the X-ray monochromator is measured by an absolute encoder independent on the stepping motor drive. Data analysis of XANES spectra was carried out by plotting the relative absorption $\alpha_M(\hbar\omega)/\alpha_A$ where α_M is the measured absorption coefficient and α_A is the value of the high energy Ca^{2+} atomic X-ray absorption, obtained by fitting the EXAFS oscillations. This procedure allows the normalization of the spectra of different Ca^{2+} compounds to the same α_A. Different spectra of the same sample were averaged to improve the signal to noise ratio. EXAFS data analysis was carried out using the EXAFS set of programs developed at the facility (S. Mobilio et al., 1977).

RESULTS

a) EXAFS and XANES of TnC

Fig. 1 shows the EXAFS and XANES of TnC and of the model compound Ca^{2+}-formate. The separation

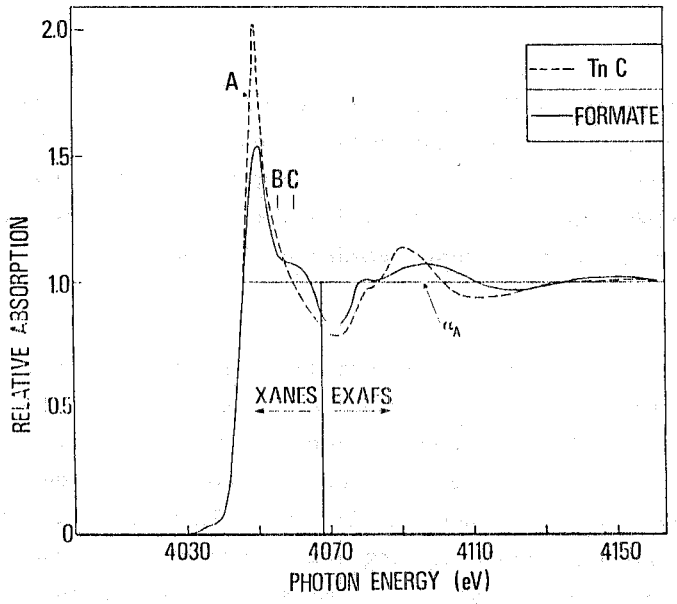

FIG. 1 - X-ray absorption spectra of TnC (TnC+2CA^{2+} and TnC+4Ca^{2+}) and Ca-formate. The EXAFS and XANES regions are indicated. The relative absorption $\alpha_M(\hbar\omega)\alpha_A$ is plotted, where $\alpha_M(\hbar\omega)$ is the measured absorption, after subtraction of the pre-edge continuum background, and α_A is the value of the high energy Ca^{2+} atomic X-ray absorption continuum obtained by fitting the measured spectrum in the EXAFS region.

between the XANES and EXAFS region has been fixed at the photoelectron kinetic energy $\hbar\omega - E_o$(eV) = $151/d^2(\text{Å})$, as discussed by Bianconi, 1981), where E_o is the absorption threshold and d the Ca^{2+}-oxygen interatomic distance. This criterion fixed the threshold of EXAFS oscillations above the value of 2.6 Å^{-1} of the photoelectron wavevector.

The analysis of EXAFS gives an "average" Ca^{2+}-oxygen interatomic distance of 2.4 Å for all the Ca^{2+}-modulated proteins we have studied. Ca^{2+}-binding proteins belong to special case where the EXAFS analysis is strongly limited by many largely scattered distances in the first coordination shell. It is characteristic of Ca^{2+} bonding in Ca^{2+} complexes and proteins that many (more than four) Ca oxygen distances are spread over a 0.3-0.6 Å range (Moews & Kretsinger, 1975) (Einsphar & Bugg, 1977; Burger et al., 1977; Einsphar & Bugg, 1974). In this case a single "average" distance is determined by EXAFS analysis coming from the phase determination of the oscillations due to the first neighbour atoms, obtained by Fourier filtering the measured spectrum. This single oscillation is given by (Doniach et al., 1980)

$$\chi(k) = \sum_{i=1}^{C.N.} \frac{A(k)}{k.R_i^2} \sin(2kR_i + \phi(k))$$

where C.N. is the coordination number (from 6 to 8 in calcium-binding proteins) and R_i are the distances of oxygen atoms from the calcium atom. Since only oxygen atoms are expected to be coordinated by Ca^{2+}, A(k) and $\phi(k)$, characteristic of the Ca^{2+}-O^{2-} pairs, can be easily determined from model compounds. In the case of 6 or 8 different distances R_i distributed in a complex way, it is clear that EXAFS gives an "average" distance which is not directly related to the arithmetic mean distance. The analysis of the low energy range of EXAFS (see Fig. 1) can give information on the interatomic distances (and their modifications) of a larger cluster (including the second and possibly the third shell) of the same size as that determining the XANES (Belli et al., 1980) but its analysis is not simple in these complex structures.

In order to avoid the limitations of EXAFS applied to strongly asymmetric Ca^{2+}-binding sites, we have analyzed the XANES region of the absorption spectrum. The multiple scattering resonances, which determine the features of XANES of Ca complexes are determined by the spatial arrangements of the first and second neighbours around the central atom Ca^{2+}(Durham et al., 1980; Bianconi et al., 1982). Since in Ca-modulated proteins Ca^{2+} is coordinated by carboxyl groups of aspartic and glutamic residues and carbonyl groups of the main chain, the XANES are determined both by the coordination geometry of oxygen first neighbours and by the carbon second neighbours. It has been recently pointed out (Bianconi et al., 1982) that if the central atom is coordinated by molecular groups with multipole bonds like COO^- and CO, the XANES are strongly determined by "shape resonances" of the excited photoelectron within the neighbour molecular groups. The characteristic multiple scattering resonances A, B and C of calcium-binding proteins and model compounds, shown in Fig. 1, should be derived by the π and σ-type "shape resonances" in scattering of low kinetic energy electrons (4-20 eV) observed in CO_2 and CO (Lynch et al., 1979). Therefore the XANES of Ca^{2+} coordinated by carboxyl and carbonyl groups should depend, through the variation of intensity and energy shift, on the coordination number, C.N., on the Ca-oxygen distances and on the Ca-O-bonding angles.

In order to find out experimentally the effect of local structure on XANES we have studied some Ca-complexes where Ca^{2+} is coordinated by carboxyl group, such as Ca-formate (Burger et al., 1977) Ca-glutamate (Einsphar & Bugg, 1974), Ca-EDTA (Ethylenediamine tetraacetic acid) (Weaklien & Hoard, 1959 and Smith & Hoard, 1959) and Ca-EGTA (Ethylene glycol bis (β-aminoethyl N, N'-tetracetic acid). The energy position of the main resonance A shifts toward higher energy by about 1 eV by increasing C.N. from 6 to 8, in agreement with the finding of Powers et al (1978). The splitting between peaks A and B goes from 6 eV in 6-fold coordinated sites of EDTA to 7.5 eV for the 7-fold coordinated site of Ca-formate. In the 6 fold coordination, where COO^- is

generally in the unidentale configuration with Ca-O-C bonding angle of about 120-150° (Einsphar & Bugg, 1977), the peak B is stronger than for the 7- or 8-fold coordination, with at least one carboxylate in the bidentate configuration with the bonding angle θ=90°.

The comparison of XANES spectra of TnC with the model compound Ca^{2+}-formate (Fig. 1) shows a general feature of XANES of calcium-binding proteins: peak A is stronger in proteins (about factor 2 larger than the high energy atomic continuum α_A) than in simple compounds. In TnC the energy of peak A is at lower energy than in Ca^{2+}-formate and the two spectra are quite different. In Fig. 2 the XANES spectrum of Ca^{2+}-EGTA complex

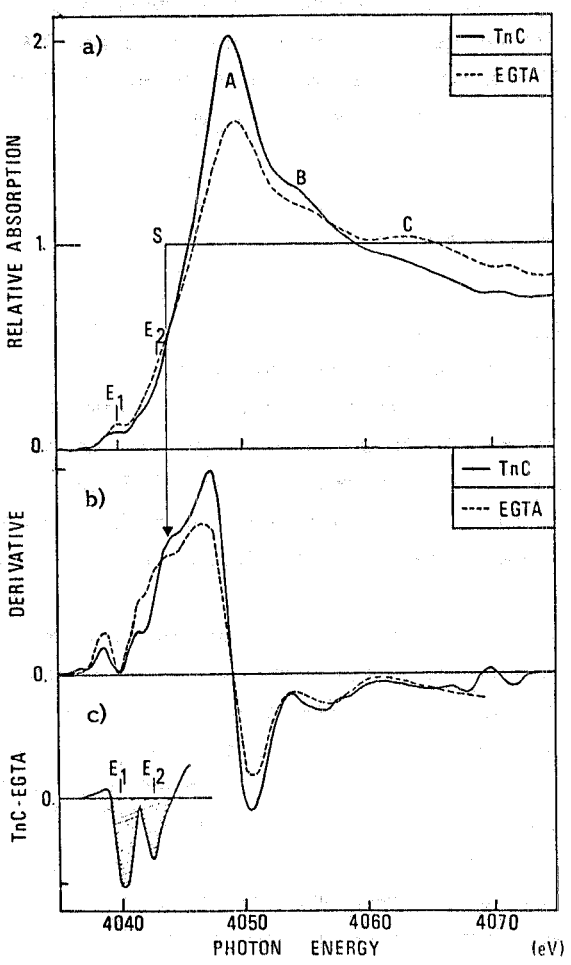

FIG. 2 - XANES spectra of TnC (TnC-2Ca^{2+} and TnC-4Ca^{2+}) and Ca-EGTA complex. a) Absorption spectra, b) derivative of absorption spectra and c) difference between the absorption spectra in the "edge region" of TnC-2Ca and Ca-EGTA.

shows a close resemblance to that of TnC. In the lower part of the figure the derivative functions of the spectra show that the energies of the A, B and C structures are the same in TnC and EGTA. The energy position of the peak A at 10.5±0.2 eV above the first structure E_1, is in the range of 6-fold coordinated Ca-complexes like EDTA and Ca^{2+} in aqueous solution (Licheri et al., 1976; Cummings et al., 1980), as well as the energy splitting between A and B (5.5 eV). We assign this similarity to the same number of oxygen atoms in the first coordination shell (C.N.=6) and to a similar geometry of the second coordination shell in the two systems, where Ca^{2+} is coordinated by the same number of COO^- group (four).

The difference between these two Ca^{2+}-binding structures can be further analyzed in the "edge region" of the absorption spectrum below the structure S. We assign the maximum of derivative S, corresponding to the rising absorption threshold in calcium binding proteins, to the threshold of allowed dipole transitions 1s $\rightarrow$ εp (Bianconi, 1981). The intensity of the peaks E_1 and E_2 is due both to quadrupole transitions and to the p-like

components of the t_{2g} and e_g molecular orbitals of the distorted octahedral cluster CaO_6. The difference in absorption between TnC and Ca^{2+}-EGTA plotted in Fig. 2 shows two large minima separated by 2.5 eV which is related with the crystal field splitting. The negative value of the difference spectrum indicates that the p-like components of the empty 3d-derived orbitals in the protein are lower. However the similar geometry is indicated by the same relative variation of both peaks.

b) High and low affinity sites of TnC

We have measured the XANES of nCa^{2+} moles per mole of TnC for the values of n=2, 4, 6. No difference within the noice level, between the spectra of TnC-2Ca and TnC-4Ca, have been found as is shown in Fig. 3. A large change has been found for n=6. In the case n=2 only the high affinity Ca-Mg sites III and IV of TnC should be occupied by Ca^{2+}, while in the case n=4 both the high affinity and low affinity, Ca-specific I and II sites are occupied (Kretsinger & Barry, 1975; Potter & Gergeley, 1975). The identity between the two spectra suggests that the structure of the high and low affinity sites with Ca^{2+} bound to the protein is the same. We have also measured the XANES of TnC-2Tb^{3+}-2 Ca^{2+}. Tb^{3+} has a larger affinity for the high affinity sites than Ca^{2+} and removes Ca^{2+} from the sites III and IV (Levis et al., 1980). If this is the case, measuring the x-ray absorption at the energy of the Ca-edge we do not see Tb^{3+} and therefore the measured XANES should be assigned to Ca^{2+} bound to sites II and I of TnC. The measured TnC-2Ca^{2+}-2Tb^{3+} and TnC-2Ca^{2+} spectra are identical within the noise level as shown in Fig. 3. This result confirms that the structure of high and low affinity sites are the same within the sensitivity of XANES to local structure. Our results are in agreement with unpublished results by Powers et al. on EXAFS and "edge" of TnC-2Ca^{2+} and TnC-4Ca^{2+} in solution which do not reveal differences. The XANES region, studied here, has been found in our study to be the most sensitive part of the calcium K absorption spectrum of calcium proteins and complexes to small differences in local structure, however one should remind that XANES is determined by a cluster around the calcium ion formed mainly by the first and the second shell therefore long range differences between sites are not detected by this method.

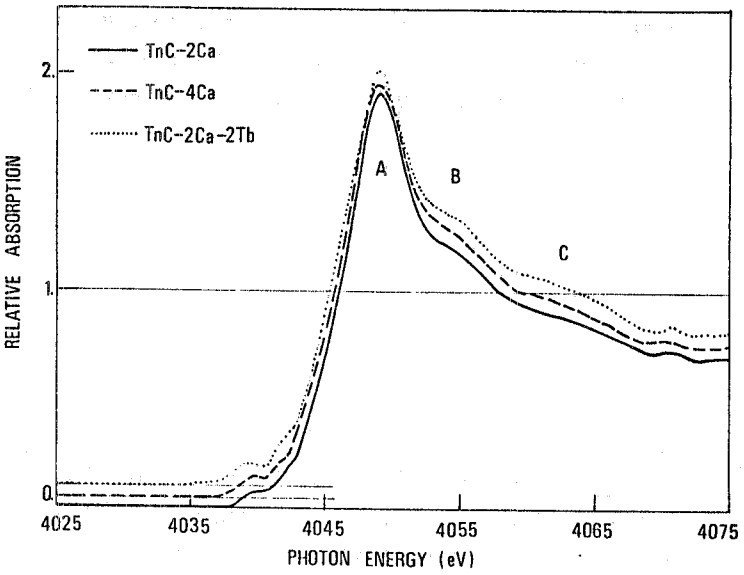

FIG. 3 - XANES spectra of TnC-2Ca^{2+}; TnC-4Ca^{2+} and TnC-2Ca^{2+}-2Tb^{3+}. The spectra coincide within the experimental errors.

A large change of the XANES spectrum has been found for TnC-6 Ca^{2+} (not shown). The measured spectrum is the sum of the absorption spectra of Ca^{2+} bound to sites I, II, III and IV and of two Ca^{2+} possibility bound with very low affinity to some external sites of this largely acidic protein. Whatever the nature of the two latter sites it is interesting to note that they seem to have quite different geometries.

c) Effect of Mg^{2+} on TnC

The effect of Mg^{2+} on the structure of Ca^{2+}-binding sites is shown in Fig. 4. The same effect has been observed both in TnC-4Ca^{2+} and TnC-2Ca^{2+}. The intensity of peak B decreases and it is hard to distinguish the peaks B and C, as can be seen from the derivative spectrum in Fig. 4. The energy shift (0.4 eV) is close to the shift between Ca-EGTA and Ca-formate where Ca^{2+} is seven-fold coordinated (0.6 eV). This effect is specific of Mg^{2+}. In order to test whether a positive charge can modify the Ca^{2+}-binding site, we have added Na^{+} to TnC as a probe. No effect on the XANES of TnC has been observed when Na^{+} is present.

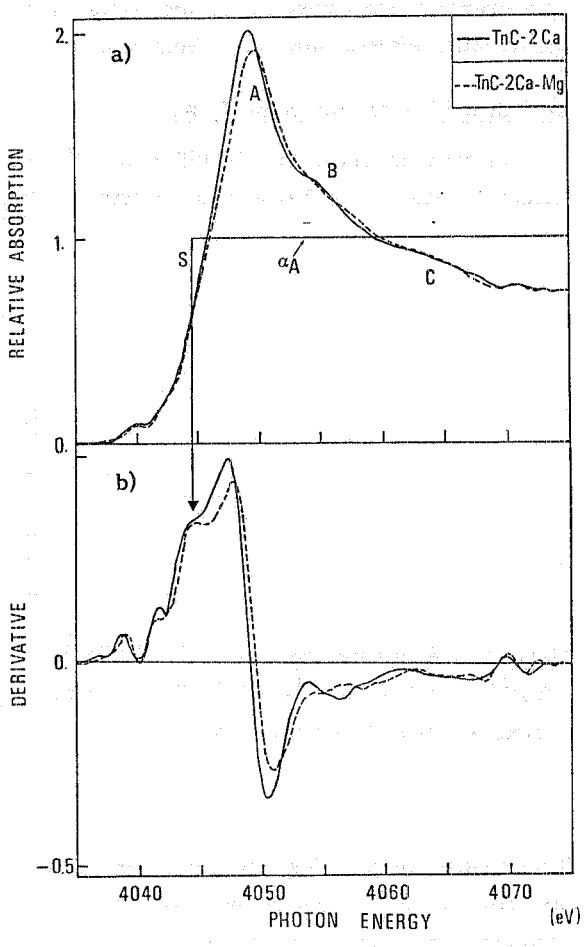

FIG. 4 - Effect of Mg^{2+} on XANES of troponin C (TnC-2Ca^{2+} and TnC-4Ca^{2+}). a) XANES of TnC-4Ca^{2+} with and without added Mg^{2+}. b) Derivative spectra of the curves in the upper panel.

d) XANES of carp parvalbumin

Fig. 5 shows the XANES spectrum of carp parvalbumin compared with that of TnC. The two spectra are clearly different in the XANES region while only subtle differences are observed in the edge region. In parvalbumin, peak A as well as peak C and B, are shifted to higher energy by 0.8 eV and are broader compared with TnC. The energy splitting between peaks A and B is 7 ± 0.2 eV. We know from crystallographic data that parvalbumin binds two Ca^{2+} at sites with different coordination (Moews & Kretsinger, 1975). Therefore, the measured XANES spectrum of parvalbumin is the sum of two different spectra characteristic of each site: the "CD hand", site, where Ca^{2+} is coordinated by 6 oxygen atoms and the "EF hand" site where Ca^{2+} is eigth fold coordinated.

To obtain the XANES of the two parvalbumin sites separately, we have assumed the metal binding sites in TnC as a model for the "CD hand" site of parvalbumin whose sequence is very similar to that of sites II, III and IV of TnC (Kretsinger & Barry, 1975) (see Table I). We have then subtracted the contribution of this site to the measured spectrum of parvalbumin in order to obtain the spectrum of the "EF hand" site. The result is plotted in the panel b of Fig. 5. The obtained spectra of the "CD and EF hand" sites in the "edge region" (from E_1 to S) show a different change of the E_1 and E_2 peaks. Clear differences appear on the multiple scattering resonances of the XANES: peak A of the obtained "EF hand" site is as narrow as peak A of TnC, and it is shifted toward higher

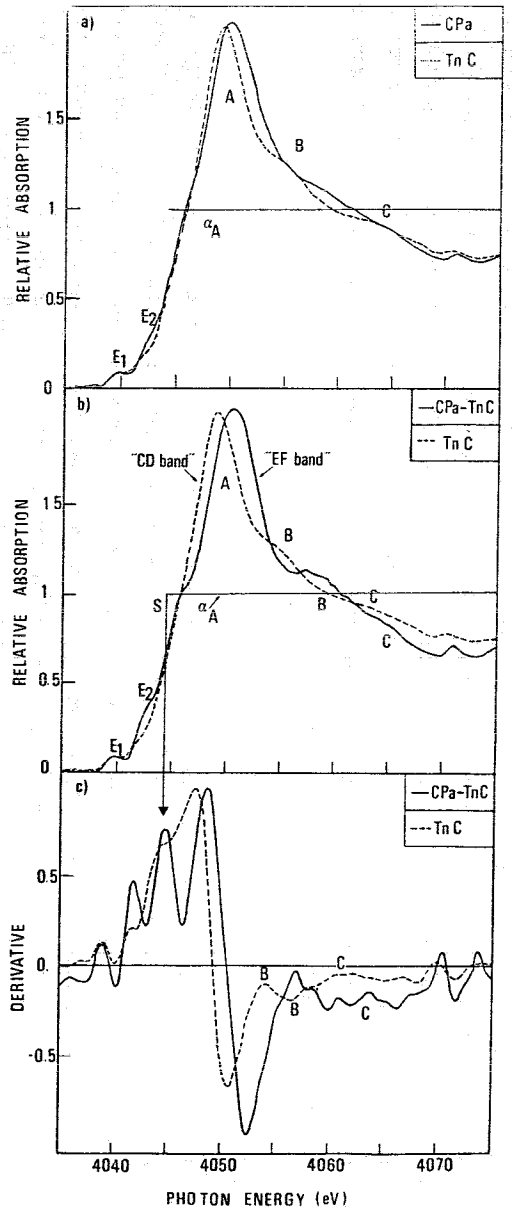

FIG. 5 - XANES spectrum of carp parvalbumin (CPa). a) XANES of CPa-2Ca^{2+} compared with TnC-2Ca^{2+}, b) XANES of the calcium site in the "EF hand" region (solid line) obtained by subtracting the XANES of TnC-2Ca^{2+} from the XANES of parvalbumin CPa-2Ca^{2+} (CPa-TnC). This calculation implies that the "CD hand" calcium site of CPa (dashed line) has the same feature of the Ca^{2+}-binding sites of TnC. c) Derivative spectra in panel b.

TABLE I - Amino acids bound by Ca^{2+} in troponin (TnC) and parvalbumin (Parv) sites from Kretsinger (1980).

	X	Y	Z	-Y	-X	-Z
Parv CD	Asp	Asp	Ser	C=0	Glu	Glu
EF	Asp	Asp	Asp	C=0	H_2O	Glu
TnC I	Asp	Asp	H_2O?	C=0	Ser	Glu
II	Asp	Asp	Ser	C=0	Asp	Glu
III	Asp	Asn	Asp	C=0	Asp	Glu
IV	Asp	Asn	Asp	C=0	Asp	Glu

energy of 1.2 eV. This shift was expected from analysis of model compounds when the C.N. increases from 6 to 8.The changes in intensity and the larger shift of peak B are also in agreement with this interpretation.

We have measured the effect of Mg^{2+} on the XANES of parvalbumin to test possible analogies with TnC. No effect of Mg^{2+} on the structure of Ca^{2+} sites in parvalbumin has been found.

DISCUSSION

a) Affinity and specificity

One of the primary aims of this investigation was to explore whether specific configurations of the Ca^{2+}-binding sites could be correlated with properties such as affinity (or stability of the complex) and selectivity for Ca^{2+} relative to that of other cations. From the study of Ca^{2+} bound to TnC and parvalbium these correlations have not been found.

In the proteins studied here Ca^{2+} is expected to be bound in the helix-loop-helix domain. The loop is characterized by a sequence of 12 residues. The amino acid sequences of the four loops of TnC show some differences concerning the order of the amino acids and the positions of COO^- groups. Generally Ca^{2+} is coordinated in an ideal octahedron by 4 COO^- from aspartic and glutaminc residues and by a carbonyl peptide (Kretsinger, 1980) at it is shown in Table I. In this paper we report that all the sites of TnC have similar structure in spite of the sequence differences between sites. From EXAFS and XANES spectra we estimate that both the average distance Ca-O and the arrangement of oxygens and Ca-O-C bonding angles are similar in all four Ca^{2+} sites. Therefore our data confirm the prediction of Kretsinger & Barry (1975) that TnC sites III, IV and II are like the Ca^{2+} sites of "CD hand" but no evidence for a different structure predicted for site I (see Table I) has been found.

The absence of a characteristic local structure for Ca^{2+} bonding in the sites I and II, which are the calcium specific sites responsible for muscle contraction, demonstrates that the answer to the question raised by Kretsinger (1980): "why are TnC-I and TnC-II the only loops (between calcium modulated proteins) that do not bind Mg^{2+} with $pK_d(Mg^{2+}) > 3$?" cannot be find in the local chemical bonding of calcium.

The different affinities for Ca^{2+} exhibited by TnC sites seem not to be related to significant differencies in near neighbour atomic arrangement therefore the energy necessary to form the complex should depend on several factors such as total amino acid sequence, fold energy, inter-domain interactions. All these factors may contribute to affinity and selectivity for Ca^{2+} of the binding sites. Differences between binding sites are expected when Ca^{2+} is not bound. According to circular dichroism measurements, in fact, the binding of Ca^{2+} to the high affinity sites occurs to domains of the protein which do not contain a preformed α-helical region in the Ca^{2+}-free state. Similar measurements in the region of the low affinity sites indicate that α-helices are present. The binding of Ca^{2+} to the low affinity sites propably requires only a movement of the two "helical fingers" of the helix-loop-helix "CD hand" domains relative to each other.

b) Effect of Mg^{2+}

Binding of Mg^{2+} to TnC appears to modify the structure of the Ca^{2+} binding sites. The same changes are observed both in TnC-2Ca^{2+} and TnC-4Ca^{2+}. So far the observed reduced affinity for Ca^{2+} of the Ca^{2+}-Mg^{2+} sites in the presence of millimolar concentrations of Mg^{2+} has been interpreted simply on competition grounds. However a conformational change of those sites induced by Mg^{2+} bound to the so-called Mg^{2+}-specific sites cannot be excluded. Even if the experimental conditions for the preparation of the samples (Ca^{2+}/Mg^{2+} concentration ratio= 0.1-0.2; $K_{Ca}/K_{Mg} \geq 10^3$) would predict a full occupancy of the high affinity sites by Ca^{2+} in both TnC-2Ca^{2+} and TnC-4Ca^{2+}, Mg^{2+} binding confined to the Mg^{2+} sites, we cannot exclude a partial occupancy of the Ca^{2+}-Mg^{2+} sites by some Mg^{2+}. If the former prediction is correct, the effect of Mg^{2+} would

be exerted on all the four sites. At this stage of our study it is premature to speculate on the biological relevance of this finding and clearly further experiments are needed. At any rate the Mg^{2+}-induced coordination geometry of Ca^{2+} in all TnC binding sites represent a third type of Ca^{2+} coordination detected by XANES spectra, different from that observed in parvalbumin and Mg^{2+}-free TnC. The specificity of the effect of Mg^{2+} on TnC Ca^{2+}-binding sites is supported by the fact that excess of monovalent cations is unable to produce similar changes and by the non-specific effect of Mg^{2+} on Ca^{2+} binding sites of parvalbumin.

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REFERENCES

- M. Belli, A. Scafati, A. Bianconi, S. Mobilio, L. Palladino, A. Reale, and E. Burattini, Sol. St. Commun. 35, 355 (1980).
- A. Bianconi, S. Doniach and D. Lublin, Chem. Phys. Lett. 59, 121 (1978).
- A. Bianconi, B. Oesh, S. Alemà, L. Castellani, I. Davoli, P. Fasella and S. Mobilio, "Calcium-Binding Proteins: Structure and Function" Ed. by F.L. Siegel, E. Carafoli, R.N. Kretsinger D.H. Mac Lennan and R.H. Wasserman (Elsevier North Holland, Amsterdam 1980), p. 297.
- A. Bianconi, "EXAFS for Inorganic Systems": Ed. by S.S. Hasnain and C.D. Garner Daresbury Report DL/SCI/R17 E (1981) p. 13.
- A. Bianconi, M. Dell'Ariccia, P. Durham and J.B. Pendry (1982) Phys. Rev. B to be published.
- N. Burger, H. Fuess and S.A. Mason, Acta Crys. B33, 1968 (1977).
- L. Castellani, E.P. Morrison and E.J. O'Brien, Biochem. Biophys. Res. Commun. 96, 558 (1980).
- S. Cummings, J.E. Enderby and R.A. Howe, J. Phys. C13, 1 (1980).
- S. Doniach, P. Eisenberger and K.O. Hodgson, "Synchrotron Radiation Research" Ed. by H. Winick and S. Doniach, Plenum Press, New York, p. 425 (1980).
- P.J. Durham, J.B. Pendry and C.H. Hodges, Sol. St. Commun. 38, 159 (1981).
- H. Einsphar and C.E. Bugg, Acta Cryst. B30, 1037 (1974).
- H. Einspar and C.E. Bugg, in "Calcium Binding Proteins and Calcium Function" Ed. by R.H. Wasserman, R.A. Corradino, E. Carafoli, R.H. Kretsinger, D.H. Mac Lennan and F.L. Siegel North Holland (1977), pp. 13-20.
- P. Eisenberger, R.G. Shulman, B.M. Kincaid, G.S. Brown and S. Ogawa, Nature 274, 30 (1978).
- P. Eisenberger and B. Lengeler, Phys. Rev. B22, 3551 (1980).
- R.H. Kretsinger and C.E. Nockolds, J. Biol. Chem. 248, 3313 (1973).
- R.H Kretsinger and C.D. Barry, Biochim. Biophys. Acta 405, 40 (1975).
- R.H. Kretsinger, Crit. Rev. Biochem. 8, 119 (1980).
- F.W. Kutzler, C.R. Natoli, D.K. Misemer, S. Doniach and K.O. Hodgson, J. Chem. Phys. 73, 3274 (1980).
- P.C. Levis, B. Nagy, S.S. Lehrer, H. Biolokowska and I. Gergeley, Arc. Biochem. and Biophys. 200, 17 (1980).
- G. Licheri, G. Piccalunga and G. Pinna, J. Chem. Phys. 64, 2437 (1976).
- M.G. Lynch, D. Dill, J. Siegel and J.L. Dehmer, J. Chem. Phys. 71, 4249 (1979).
- U.K. Laemmli, Nature, 227, 680 (1970).
- R.M. Miller, D.W.L. Hukins, S.S. Hasnain and P. Lagarde, Daresbury, Lab. Report DL/SCI/P250E, to be published.
- S. Mobilio, F. Comin and L. Incoccia, Frascati report LNF-82/19 unpublished.
- P.C. Moews and R.H. Kretsinger, J. Mol. Biol. 91, 201 (1975).
- J.F. Pechére, J. Demaille and J.P. Capony Biochim. Biophys. Acta 236, 391 (1971).
- S.V. Perry and H.A. Cole, Biochemistry J. 141, 733 (1974).
- J.D. Potter and J. Gergeley, J. Biol. Chemistry 250, 4628 (1975).
- L. Powers, P. Eisenberger and J. Stamatoff, Ann. New York Acad. Sciences 307, 113 (1978).
- L. Powers, B. Chance, Y. Ching and P. Angiolillo, Biophys. J. 34, 465 (1981).
- G.S. Smith and J.L. Hoard, J. Chem. Soc. Am. 81, 556 (1959).
- D.M.E. Szebenyi, S.K. Obendorf and K. Moffat, Nature 294, 327 (1981).
- B.K. Teo, J. Am. Chem. Soc. 103, 3990 (1981).
- H.A. Weakliem and J.L. Hoard, J. Chem. Soc. Am. 81, 549 (1959).
- R.J.P. Williams, in "Calcium Binding Proteins" Ed. by R.H. Wasserman, R.A. Corradino, E. Carafoli, R.H. Kretsinger, D.H. Mac Lennan and F.L. Sigel, North Holland (1977), p. 3.