A DETERMINATION OF THE RELATIVE COMPACTNESS OF THE Ca^{2+}-BINDING SITES OF A Ca^{2+}-BINDING FRAGMENT OF TROPONIN-C AND PARVALBUMIN USING LANTHANIDE-INDUCED ^1H NMR SHIFTS

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Received 21 November 1978

1. Introduction

The calcium regulation of muscle contraction in vertebrate skeletal muscle results from the interaction of calcium with the protein complex troponin [1]. Troponin is composed of three subunits, of which only TnC appears to bind Ca^{2+} [2,3]. The amino acid sequence of TnC has been determined [4,5]. TnC has a strong sequence homology to carp parvalbumin [6,7]. Parvalbumin is roughly half of the size of TnC and binds 2 mol Ca^{2+} [8,9] compared to 4 mol Ca^{2+} in TnC [10]. The crystal structure of parvalbumin from carp has been determined [11,12]. The similarity between the primary sequences of TnC and parvalbumin suggests a possible location in the sequence of TnC for the 4 Ca^{2+}-binding sites [4,6].

Recently there has been considerable interest in studying the properties of fragments of TnC and parvalbumin containing single Ca^{2+}-binding sites. A cyanogen bromide fragment of TnC, labelled CB-9, that binds a single Ca^{2+} and mimics the conformational change of TnC upon Ca^{2+} binding has been reported [13–15]. This fragment contains 52 residues from LYS 84 through homoserine 135, corresponds to the third Ca^{2+}-binding site in the intact protein, and binds Ca^{2+} with an affinity about two orders of magnitude lower than the intact protein. Similarly, parvalbumin has been split into two fragments A and B corresponding to the amino acids 1→75 and 76→108 in the intact molecule [16,17]. These fragments bind Ca^{2+} with dissociation constants several orders of magnitude less strongly than intact parvalbumin [9].

In this paper we have used ^1H NMR methods to compare the Ca^{2+}-binding sites of CB-9, which is the best fragment prepared to date with respect to strength of Ca^{2+} binding, and parvalbumin, which is the simplest homologous intact protein. In particular we have estimated the relative compactness of the immediate ligands around the Ca^{2+}-binding site in the fragment versus the intact protein by analyzing the shifts induced in their ^1H NMR spectrum when the lanthanide ion praseodymium is used as an analog of Ca^{2+}. Several workers have proposed the substitution of the lanthanides for Ca^{2+} [18,19]. From the NMR standpoint this is particularly attractive because the lanthanides are paramagnetic and the shifts and broadenings induced in the spectrum of the protein can be used to extract the structure of the protein in solution [20–24].

2. Materials and methods

The CB-9 fragment from rabbit skeletal TnC was isolated as in [14]. Carp parvalbumin (pI 4.25) was isolated by method in [25]. Stock solutions of the lanthanide metal ions were prepared as in [14].

The CB-9 solution for the spectrum presented in fig.1D was prepared by adding lyophilized CB-9 to buffer (20 mM PIPES, 0.1 M KCl, 2 mM DSS in D_2O, pH 6.8) which had been pre-dithizoned. The concentration of CB-9 determined by amino acid analysis was 0.9 mM. To this Ca^{2+}-free solution was added a stock $PrCl_3$ solution to give a final total $[Pr^{3+}]$ of

1.5 mM. The parvalbumin solutions (1.2 mM) for the spectra shown in fig.1A, 1B,1C were made up by dissolving the lyophilized protein in 15 mM PIPES, 0.15 M KCl, 0.5 mM DSS in D_2O, pH 6.6, then adding small aliquots of a stock $PrCl_3$ solution. No attempt to prepare Ca^{2+}-free protein was made for this study since the lanthanide metals readily displaced the Ca^{2+} (see section 4). The spectrum of parvalbumin prepared in the above manner (fig.1A) is identical to the spectrum of parvalbumin from which the Ca^{2+} has been removed and then added back at saturating levels [26]. All pH values were measured on a Beckman Expandomatic SS-2 pH meter with an Ingold model 6030-04 microelectrode. pH values reported are meter readings and are not corrected for the deuterium isotope effect.

NMR spectra were obtained using a Bruker HXS-270 NMR spectrometer operating in the Fourier transform mode and equipped with a Nicolet quadrature accessory. The ambient temperature for all samples was 27°C. The CB-9 spectra were obtained with a pulse width of 9 μs (~90°), an acquisition time of 0.68 s, a sweep width of ±6024 Hz, and a filter width (Butterworth) of 6000 Hz. The pulse response of the Butterworth filters produced a rolling baseline which made the presentation of the lanthanide-shifted peaks difficult. Consequently, the spectrum presented in fig.1D is a convolution difference spectrum [20]. The parvalbumin spectra were taken with a pulse width of 8 μs (~80°), an acquisition time of 0.164 s, and a sweep width of ±12 500 Hz. For these spectra Bessel filters were used (Ithaco model 4302) with a filter width of 25 000 Hz and no baseline imperfections were observed.

3. Results

The 270 MHz ^1H NMR spectrum of parvalbumin, at pH 6.6 and saturated with Ca^{2+}, is shown in part in fig.1A. The non-exchangeable NH resonances appear in the region of +10.5 to +7.8 ppm downfield from DSS. The aromatic resonances (10 Phe, 0 Tyr, 0 Trp, 1 His) appear upfield of the NH resonances in the region beginning at +7.8 ppm. The only aliphatic resonance shown is the most upfield peak at −0.48 ppm from DSS which has been attributed to Val 106 [27]. The spectrum of parvalbumin in the presence of a 1.1:1 molar ratio of Pr^{3+} to parvalbumin is shown in fig.1B, and at a 2.1:1 molar ratio in fig.1C. In fig.1B a large number of new resonances appear both upfield and downfield of the main envelope of the protein spectrum. In going from spectrum 1B to 1C some of the resonances present in spectrum 1B remain, some

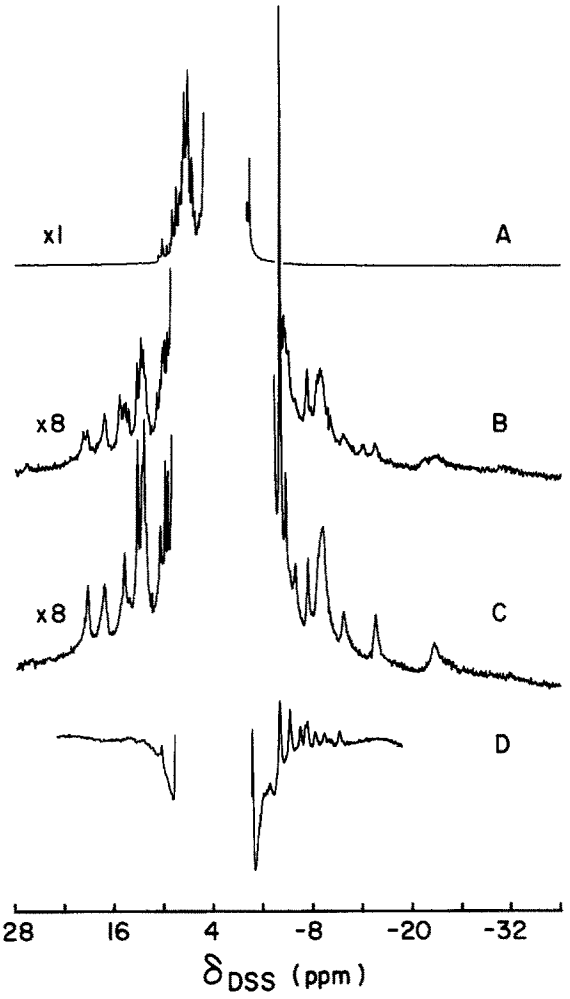

Fig.1. A comparison of the 270 MHz ^1H NMR spectra of: (A) 1.2 mM native parvalbumin in 15 mM PIPES, 0.15 M KCl, 0.5 mM DSS in D_2O, pH 6.6; (B) 1.08 mM parvalbumin at a $[Pr^{3+}]/[protein]$ ratio of 1.1, vertical scale × 8; (C) 0.98 mM parvalbumin at a $[Pr^{3+}]/[protein]$ ratio of 2.1, vertical scale × 8; (D) 0.9 mM CB-9 in 10 mM PIPES, 0.1 M KCL, 2 mM DSS, in D_2O (pH 6.8). $[Pr^{3+}]/[CB-9] = 1.7$. Spectrum is the result of deconvolution. See section 2 for details of sample preparation and spectral acquisition parameters.

disappear, and some new peaks appear, but the range of shifted peaks remains approximately constant (+20 to −23 ppm). The spectrum of CB-9 with Pr^{3+} added at a ~1.7:1 molar ratio is shown in fig.1D. Shifted resonances appear outside of the main protein envelope, but the maximum observed shifts are not as large as with parvalbumin (+10 to −12 ppm). The spectrum of CB-9 in the presence of La^{3+} extends from 8.3 to 0 ppm [14].

4. Discussion

The spectra of both parvalbumin and CB-9 in the presence of Pr^{3+} show a large number of new resonances which are shifted well outside of the main envelope of the ^1H NMR spectrum of either protein. These shifts are very much larger than the shifts seen with CB-9 when the diamagnetic metals Ca^{2+} or La^{3+} are added [14], or with parvalbumin when Ca^{2+} is added [27]. The large shifts in spectra 1B–1D must therefore result from the influence of the paramagnetism of the Pr^{3+} on the nuclei neighboring the metal ion binding site.

We have studied the stoichiometry of the appearance of these shifted resonances in detail for parvalbumin titrated with several of the lanthanides [26]. For the purposes of this discussion it is sufficient to note first that all of the resonances increase and/or decrease in intensity with no change in resonance position as the lanthanide ion concentration is varied and therefore are in the NMR slow exchange limit as expected from the strong metal ion binding constants and slow off rate constants [28]. Therefore these resonances represent the spectrum of the protein Pr^{3+} complex. The second feature, is that the spectra of parvalbumin with added lanthanides at a molar ratio of 2:1 are very similar regardless of whether one starts from parvalbumin free of Ca^{2+} or parvalbumin as isolated containing ~2 mol Ca^{2+}/mol parvalbumin. This indicates that the binding of the lanthanides to parvalbumin is stronger than the binding of Ca^{2+}. Similar displacement of Ca^{2+} from parvalbumin by lanthanides has been observed in Tb^{3+} fluorescence experiments [29] and in X-ray occupancy studies [29,30]. Similarly the displacement of Cd^{2+} from parvalbumin by Gd^{3+} has been observed [31]. Also the binding constants for lanthanides are several orders of magnitude stronger for other Ca^{2+} ligands such as EGTA [32]. It is also interesting to note here that the binding of Pr^{3+} to the CB-9 fragment is not greater than the binding of Ca^{2+} ($K_d^{Pr^{3+}}$ ~2.2×10^{-5} M, vs $K_d^{Ca^{2+}}$ ~3.7×10^{-5} M [14]). The third point is that only relatively small changes in the spectrum of a few of the bound peaks were observed with lanthanide ions in excess indicating that the effects of non-specific binding can be neglected for this discussion. Even if non-specific binding occurs, the weaker binding leads to spectra in the fast exchange limit and much smaller shifts [20–24].

The paramagnetic shifts caused by proximity to the lanthanide metal are a function of the distance and orientation of the nucleus relative to an axis system at the metal, defined by the magnetic susceptibility tensor of the metal in the binding site [33]:

$$\delta = A_1 \left(\frac{3\cos^2\theta - 1}{r^3}\right) + A_2 \left(\frac{\sin^2\theta\cos 2\phi}{r^3}\right)$$

where δ is the shift from the diamagnetic resonance position and contact shifts for lanthanides will be assumed negligible. If we, for the moment, assume that the $A_2 \sin^2\theta\cos 2\phi$ term is small relative to the A_1 $(3\cos^2\theta - 1)$ term, that $(3\cos^2\theta - 1)$ is of the order of unity, and that A_1 taken from model compounds is equal to ~1000 ppm Å^3 [23], we get r=3.5 Å for the maximum shift observed of ~23 ppm (the exact diamagnetic position of the most-shifted peak is not known). This is about as close as the β,β' protons of a ligand such as ASP 51 of parvalbumin could approach the metal ion. Most other nuclei will be more removed. For r=7 Å the shift drops to ~3 ppm neglecting angular factors. Therefore the shifted peaks observed represent the nuclei closely surrounding the metal ion; that is, within a ~7 Å radius. We therefore have a very sensitive probe of the details of the conformation of the ligands directly surrounding the metal.

We come now to the comparison of the spectrum of CB-9 + Pr^{3+} and parvalbumin + Pr^{3+}. The main difference is that the shifts are smaller for CB-9, the peaks shifting only as far upfield as −11.5 ppm and as far downfield as 10.1 ppm remembering that most of the resonances are shifted from the aliphatic region of

the spectrum at ~1–3 ppm since few aromatic amino acids are in the vicinity of metal ion site. The smaller range could be the result of the different amino acid composition surrounding the binding site [4,6], but it is unreasonable to expect that the different amino acids will all have nuclei farther away from the metal. In addition, the two sites on parvalbumin which are surrounded by different amino acids contributed shifts over the same range (see spectra 1B and 1C). A second possibility is that CB-9 is missing large regions of the liganding sphere and only has ligands in the angular orientations resulting in small shifts. This would, however, require large vacant regions around the metal ion and a model built of the CB-9 fragment shows that no portion of the amino acids surrounding site 3 in TnC is missing, assuming CB-9 folds into the same structure [14,15]. The final possibility is that the angular factors cover the same range but that on average the structure of the fragment is less compact than the intact protein resulting in the ligands being on average further from the metal ion. Taking the maximum shift in parvalbumin as ~23 ppm and CB-9 as ~12 ppm and neglecting the angular factors, this indicates that the nuclei on the ligands are ~25% further from the metal ions in CB-9 than in the parvalbumin. This moves the closest nuclei as estimated above from 3.5 Å to 4.4 Å on average. We suggest that the further stabilization of the binding of Ca^{2+} suggested for the intact molecule [15] is the result of the intact molecule providing a more compact integral binding site for the metal ion.

It is clear that the lanthanide-shifted resonances observed in this study provide a detailed fingerprint of structural information that is very useful in characterizing Ca^{2+}-binding sites in solution. The quantitative analysis of this information is underway at present.

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