STRUCTURAL STUDIES OF CALCIUM-BINDING PROTEINS USING NUCLEAR MAGNETIC RESONANCE

LANA LEE,* DAVID C. CORSON,† AND BRIAN D. SYKES†
*Wayne State University, School of Medicine, Department of Biochemistry, Detroit, Michigan 48201; and †Department of Biochemistry and Medical Research Council Group on Protein Structure and Function, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

ABSTRACT Lanthanide-shifted ^1H nuclear magnetic resonance (NMR) spectroscopy has been used to compare the structure in solution of the EF-hand calcium-binding domains of four parvalbumins (isoelectric pH[pI] 3.95, 4.25, and 4.37 from carp, and pI from buffalo fish). These four parvalbumins are shown by NMR to have very similar structures at the level of resolution typical of x-ray structures. At the higher resolution possible by the lanthanide NMR technique, specific differences are noted between the pI 3.95 isoprotein from carp and the other two carp isoproteins, and the buffalo fish parvalbumin is shown to be different from all three carp isoproteins. The differences are estimated to correspond to changes of the order of 0.2 Å in the positions of some of the nuclei surrounding the EF calcium site.

INTRODUCTION

The structure of the calcium-binding protein parvalbumin, with an isoelectric pH(pI) of 4.25, from carp muscle has been determined by x-ray diffraction techniques (1–4). The crystallographic structure of parvalbumin indicates that each of its two calcium-binding domains, called the CD and EF hands, contains a contiguous polypeptide sequence consisting of 2–3 turns of α helix, a 12-residue loop around the metal ion, and a second 2–3 turns of α helix (1). The two aspartic and glutamic–acid-rich metal-binding loops contain regularly spaced carboxyl, carbonyl, and hydroxyl ligands. Homologous sequences to the CD and EF hands have been found in other parvalbumins (5), in other calcium-binding proteins such as troponin C, the myosin light chains, and calmodulin (6), and in the porcine and bovine intestinal calcium-binding proteins (7, 8). The x-ray structure of the bovine intestinal calcium-binding protein shows that one of its calcium-binding sites is a normal EF hand, whereas the second bonding site is modified because of the insertion of amino acids in the binding loop (9).

Carp muscle contains a group of parvalbumins that differ in their isoelectric points (10); four isoproteins with isoelectric points of 4.47, 4.37, 4.25, and 3.95 are found in carp muscle (10). The amino-acid sequences of three of the four parvalbumins (pI 4.47, 4.25, and 3.95) indicate a high homology between these proteins (5, 11). Moreover, the ligands of both calcium-binding sites are conserved in all three isoproteins. A large number of parvalbumins from other species such as pike, hake, coelacanth, thornback ray, whiting, cod, frog, rabbit, and rat have also been isolated and sequenced (12).

We would like to be able to compare the structure of the EF-hand calcium-binding domains of various parvalbumins in order to understand the influence of specific amino-acid substitutions on the equilibrium and kinetic properties of metal ion binding to these sites. It is difficult to independently determine the x-ray structure of each new parvalbumin because they do not crystallize in the same space group (13). We have presented elsewhere the details of an NMR methodology for the determination of the structure of calcium-binding sites of calcium-binding proteins in solution (14–20). Here we show that these methods can be used to compare the structure of the EF hand of a parvalbumin whose x-ray structure is unknown with the structure of the EF hand of a parvalbumin (carp pI 4.25) of known structure.

The nuclear magnetic resonance (NMR) method involves the study of shifted resonances when lanthanides such as Yb^{3+} are substituted for Ca^{2+} in the protein. For the carp muscle pI 4.25 isoprotein, we compared the Yb^{3+}-shifted ^1H, ^{13}C, and ^{113}Cd NMR spectra with those calculated on the basis of the x-ray structure (20). Our conclusion was that the solution structure as determined by NMR and the x-ray structure were very similar, with the caveat that the NMR observables were extremely sensitive to small structural differences below the level of the resolution of the x-ray data. This drawback becomes an advantage, however, when the goal is the comparison of small structural differences between different parvalbumins.

Here we present the ^1H HMR spectra of three carp parvalbumin isoproteins (pI 3.95, 4.25, 4.37), each of which contains the lanthanide Yb^{3+} in its EF calcium-

Address all correspondence to Dr. B. Sykes.

binding site. The lanthanide-shifted ^{1}H NMR resonances indicate that the structures of the calcium-binding domains of the three isoproteins are very similar; the differences that are noted occur mostly between the pI 3.95 isoprotein and the other two. However, the NMR spectrum of the Yb^{3+} substituted buffalo fish parvalbumin, which has an identical amino-acid composition as the pI 4.25 isoprotein of carp parvalbumin (Williams, T. C., and D. C. Corson, unpublished results), demonstrates more differences between its structure and that of the carp parvalbumins.

MATERIALS AND METHODS

Carp parvalbumins (*Cyprinus carpio*) with isoelectric points of 4.37, 4.25, and 3.95 were prepared by the method described in reference 10, buffalo fish (*Ictiobus*) parvalbumin (pI 4.30) was prepared by the method described in reference 21. Stock solutions of the lanthanide ytterbium were prepared and calibrated as described in reference 22.

The NMR samples were prepared by adding dithizoned buffer (15 mM PIPES, 0.15M KCl, 0.5 mM sodium 2, 2-dimethyl-2-silapentane-5-sulfonate [DSS] in D_2O, pH 6.7) to lyophilized protein. Microliter aliquots of ytterbium were added to each sample to attain total [Yb^{3+}] to total [protein] ratios indicated in the legend to Fig. 1. The pH of the resulting solution was remeasured to insure the pH had not changed upon metal addition. pH measurements were made on a pH meter (26; Radiometer America Inc., Westlake, OH) with a microelectrode (6030-04; Ingold Electrodes, Andover, MA); the values quoted are the readings directly observed and have not been corrected for the deuterium-isotope effect.

The ^{1}H NMR spectra were obtained on a spectrometer (HXS-270; Bruker Spectrospin [Canada] Ltd., Milton, Ontario) operating in the Fourier transform mode and equipped with quadrature detection (Nicolet Instrument Corp., Madison, WI). Typical instrumental settings for the spectra were an acquisition time of 0.2 s, a sweep width of ±10 kHz, a spectrum size of 8,192 data points, and 10-Hz line broadening. The sample temperature was 303°K. The chemical shifts were measured relative to the principal resonance of DSS as an internal standard. Bessel filters (model 4302; Ithaco, Inc., Ithaca, NY) set to twice the spectral width were used to minimize baseline distortion.

RESULTS

The 270 MHz ^{1}H NMR spectrum of carp parvalbumin (pI 4.25) in the presence of ytterbium at a total [Yb^{3+}] to total [protein] ratio of 0.69 is shown in Fig. 1 *B*. The downfield shifted ^{1}H NMR resonances range from 31.1 to 11.6 ppm. The corresponding spectra of other carp parvalbumin isoproteins (pI 4.37 and 3.95) are presented in Fig. 1 *C* and *A*, respectively. The spectrum of carp parvalbumin 4.25 (Fig. 1 *B*) is comparable to the spectra of these other carp parvalbumin isoproteins (Fig. 1 *A* and *C*) in terms of the range of chemical shifts observed (~31 to 11 ppm), number of shifted resonances, and in general appearance. Many of the resonances are, in fact, in identical positions. However, there are notable differences between the spectra of parvalbumins 4.25 and 3.95. The single proton resonance at 20.9 ppm in the spectrum of the 4.25 isoprotein (Fig. 1 *B*) is shifted to 19.5 ppm in the spectrum of the pI 3.95 isoprotein (Fig. 1 *A*), and the methyl resonance at 17.3 ppm is shifted to 18.1 ppm. In contrast, the spectrum of buffalo fish parvalbumin (Fig. 1 *D*) differs significantly

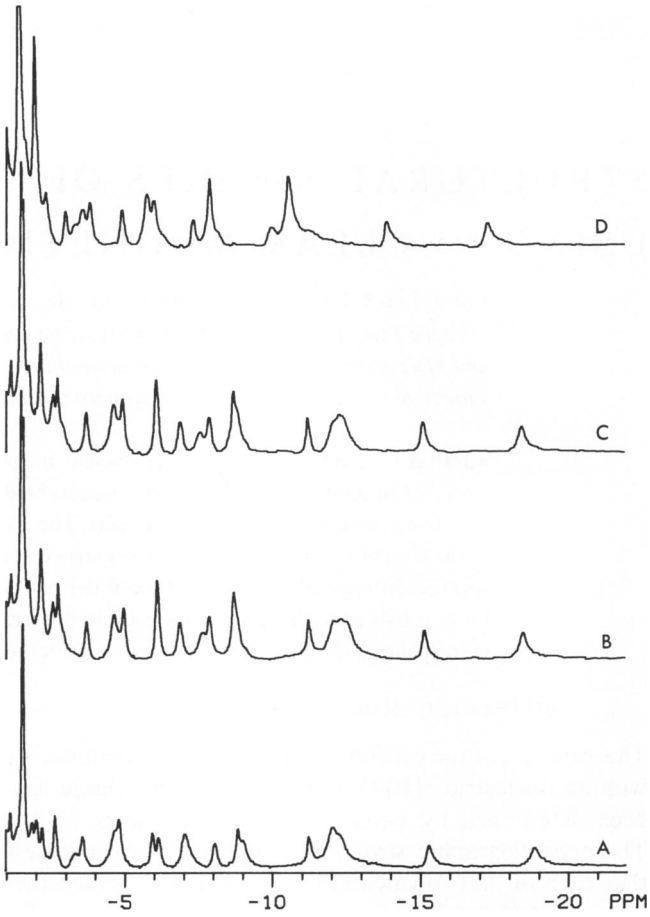

FIGURE 1 A comparison of the downfield region of the 270-MHz ^{1}H NMR spectra of (*A*) 1.0 mM carp parvalbumin (pI 3.95) at a total (Yb^{3+})/(protein) ratio of 0.53; (*B*) 0.83 mM carp parvalbumin (pI 4.25) at a total (Yb^{3+})/protein) ratio of 0.69; (*C*) 0.98 mM carp parvalbumin (pI 4.37) at a total (Yb^{3+})/(protein) ration of 0.58; (*D*) 0.88 mM buffalo fish parvalbumin at a total (Yb^{3+})/(protein) ration of 0.61.

from all the other spectra of carp parvalbumins. In particular, the range of observed chemical shifts is smaller, ranging from 28.7 to 10.7 ppm. Similar results are seen in the upfield region of the Yb^{3+}-shifted ^{1}H NMR spectra of these parvalbumins as seen in Fig. 2. Clearly, parvalbumins 4.25 and 4.37 are very similar, whereas some differences are seen between these two and the 3.95 isoprotein, and the buffalo fish parvalbumin spectrum is quite different. Two of the notable differences in the spectrum of the carp 3.95 parvalbumin are the collapse of the two methyl resonances at −1.5 ppm, and the splitting of the resonance near −6.0 ppm.

DISCUSSION

When parvalbumin has the diamagnetic metal ion Ca^{2+} present in both metal-binding sites, its ^{1}H NMR spectrum consists of resonances with chemical shifts in the range of ~10 to −0.5 ppm (15, 23). However, when the lanthanide ion Yb^{3+} is substituted for one or both of these Ca^{2+} ions in parvalbumin, the resulting ^{1}H NMR spectrum exhibits a

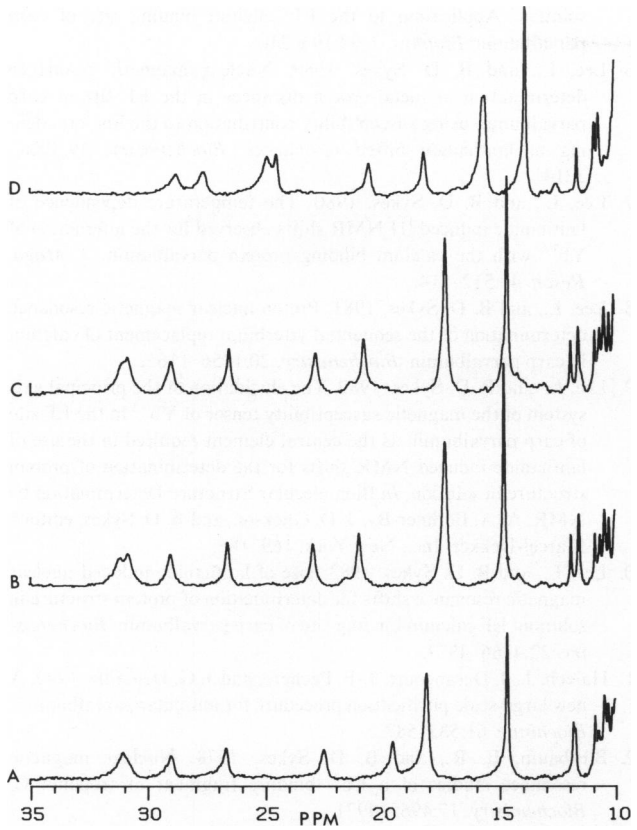

FIGURE 2 A comparison of the upfield region of the 270-MHz ^1H NMR spectra of (*A*) 1.0 mM carp parvalbumin (pI 3.95) at a total $(Yb^{3+})/(protein)$ ration of 0.53; (*B*) 0.83 mM carp parvalbumin (pI 4.25) at a total $(Yb^{3+})/(protein)$ ratio of 0.69; (*C*) 0.98 mM carp parvalbumin (pI 4.37) at a total $(Yb^{3+})/(protein)$ ratio of 0.58; (*D*) 0.88 mM buffalo fish parvalbumin at a total $(Yb^{3+})/(protein)$ ratio of 0.61.

series of new resonances that are shifted far outside the range of the Ca^{2+} form of the protein (~32 to −19 ppm) (14, 15). These shifted resonances result from the influence of the paramagnetic lanthanide Yb^{3+} on neighboring nuclei.

Elsewhere (15, 18, 24), we have presented details of the stoichiometry of Yb^{3+} binding to the two metal-binding sites of parvalbumin (pI 4.25) by an analysis of the areas of these lanthanide–induced-shifted resonances. Up to a total $[Yb^{3+}]$ to total [protein] ratio of 1, the series of resonances that appear correspond to nuclei near the first site filled, which is the EF site (18, 25). At total $[Yb^{3+}]$ to total [protein] ratios ranging from 1:1 to 2:1, a new and different set of resonances appears, corresponding to YB^{3+} filling the remaining metal-binding site, the CD site. This sequential filling of the two sites by Yb^{3+} has been confirmed by optical stopped-flow techniques (24, 26). Moreover, these studies explain and clarify the previous observation that other lanthanides, particularly those in the middle of the lanthanide series, appear to have equal affinities for the CD and EF sites of parvalbumin (27, 28). The same resonances, identified by their characteristic line widths and intensities, appear in the spectrum of all four isoproteins at the ratio of Yb^{3+} to protein used, with no evidence for the appearance of resonances corresponding to the filling of the CD site (18).

The spectra presented here are all of parvalbumins substituted with Yb^{3+} at a total $[Yb^{3+}]$ to total [protein] ratio of <1; thus, the series of shifted resonances shown in Fig. 1 result from nuclei near of the EF site of parvalbumin. We have shown that these shifts are dipolar in origin (14, 15, 20, 29) and that the positions of the shifted resonances are extremely sensitive to the orientation of the corresponding nuclei relative to the principal axis system (PAS) of the magnetic susceptibility tensor ($\underset{\sim}{\chi}$) of the Yb^{3+} bound to parvalbumin (20). For example, for nuclei such as the βCH_2 protons of Asp 92, which are amongst the most downfield-shifted resonances, a change in position relative to the metal by 1 Å could result in a change in chemical shift by as much as 20 ppm based upon the $1/r^3$ distance dependence of the lanthanide-induced chemical shift. These protons are ~4.5–4.7 Å from the metal. For the δCH_3 protons of Ile 97 at 14.8 ppm, a change in position relative to the metal by 1 Å could result in a change in chemical shift of roughly 7 ppm. These protons are ~7.2 Å from the metal.

Consequently, the very great similarity between spectra *A–C* in Fig. 1 leads to the unequivocal conclusions that (*a*) there has been no change in the orientation of the PAS of the magnetic susceptibility tensors for the Yb^{3+}'s bound to the EF sites of the three carp parvalbumins, and (*b*) there have been only very minor changes in position of some of the nuclei near the EF site. For example, the δCH_3 of Leu 86 at 17.3 ppm in Fig. 1 *B* and *C* has moved to 0.8 ppm upfield to 18.1 ppm in Fig. 1 *A* and the CH resonance at 20.9 ppm in Fig. 1 *B* has shifted upfield in Fig. 1 *C* and *A* by 0.2 ppm and 1.4 ppm, respectively. The movement of the δCH_3 of Leu 86 in the pI 3.95 isoprotein relative to the 4.25 and 4.37 isoproteins may reflect the substitution of ALA for THR at position 84. Similarly, the perturbation of the two methyl resonances at −1.5 ppm may reflect the substitution of ALA (pI 3.95) for THR (pI 4.25) at position 103. These are the only two changes near the EF site between these two isoproteins. Nonetheless, these three proteins have the same structure near the EF site; at least to a level of resolution that exceeds that possible by x-ray diffraction methods.

The situation is somewhat different for the buffalo fish protein. For the three carp isoproteins, even though some of the resonances in the Yb^{3+}-shifted ^1H NMR spectra were perturbed, most were unchanged between the three isoproteins. This indicates that there is no difference among these proteins that would affect all of the resonances, such as a slight movement in the position of the Yb^{3+} or change in the PAS of the $\underset{\sim}{\chi}$ for the Yb^{3+}. In the spectrum of the Buffalo fish protein the same resonances are present but all have been slightly shifted. This indicates either that the protein has a slightly different structure, or that something else has changed slightly like the position of the metal ion

or the PAS of the χ for the Yb^{3+}. The changes, however, can be estimated as $\tilde{0}.2$ Å for the most downfield resonance and therefore are minor.

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