stable or catalytically more active per se than the complex with ADP. But in contrast to ADP and ATP, AMP does not form a catalytically active complex. A preliminary examination of the ability of the ADP–Fe(II) complex to remain active in catalyzing OH formation from H_2O_2 has shown that it is nearly completely inactive after 10 min at room temperature. In view of the substantially reduced activity of the Fe(III)–nucleotide complexes and the results demonstrating the enhanced reduction in activity due to dissolved O_2, we speculate that the nucleotides hold Fe(II) in a configuration such that their oxidation to Fe(III) is less rapid and also prevent Fe(II), as well as Fe(III), from becoming ligated with buffer anions which are inactive in the catalysis of OH from H_2O_2.

The results with ascorbate emphasize the fact that Fe(III)–nucleotide complex can be reduced to the ferrous complex by a biological reductant which is then effective in catalyzing OH formation from H_2O_2. This work also strengthens the interpretation that it is the ferrous complex which is the catalytically active unit. This appears to be of high biological significance in oxidative damage due to oxygen free radicals.

Acknowledgments

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Registry No. ADP, 58-64-0; ATP, 56-65-5; AMP, 61-19-8; Fe^{2+}, 15438-31-0; Fe^{3+}, 20074-52-6; hydroxyl radical, 3352-57-6; H_2O_2, 7722-84-1; O_2, 7782-44-7; N_2, 7727-37-9.

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Proton Nuclear Magnetic Resonance Studies of Porcine Intestinal Calcium Binding Protein†

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ABSTRACT: ^{1}H nuclear magnetic resonance has been employed to study the environment of several proton nuclei (primarily those arising from aromatic residues) of the porcine intestinal calcium binding protein. An assignment for the single tyrosine (Tyr-16) residue has been made on the basis of laser photochemically induced dynamic nuclear polarization (CIDNP) and homonuclear decoupling experiments. pH titration studies have shown that the tyrosine pK_a is unusually high in the apoprotein and increases even further upon the addition of calcium. However, the observation of a CIDNP effect with this tyrosine in both the presence and absence of calcium indicates that it is solvent accessible and therefore exposed on the surface of the molecule. Under the conditions of these experiments, the protein was observed to bind calcium with a 2:1 stoichiometry, at a rate of exchange slow enough that the NMR spectra are in the slow-exchange limit. The presence of upfield shifted phenylalanine and methyl resonances in the apoprotein indicates that there is a well-defined tertiary structure in the absence of calcium.

High-affinity calcium-binding proteins (CaBP)[1] with molecular weights near 10 000 have been found in the small intestines of several mammalian species (Kallfelz et al., 1967; Drescher & DeLuca, 1971; Hitchman & Harrison, 1972). Porcine intestinal calcium binding protein is a compact, globular protein (M_r 8799), whose amino acid sequence of 78 residues has recently been determined (Hofmann et al., 1979). An examination of the evolutionary relationships among

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[1] Abbreviations: ICaBP, intestinal calcium binding protein; CaBP, calcium binding protein; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; ppm, parts per million; δ, chemical shift; CIDNP, chemically induced dynamic nuclear polarization; FMN, flavin mononucleotide; TnC, troponin C; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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various CaBP's shows that the porcine intestinal CaBP is distantly related to parvalbumin and troponin C (Barker et al., 1977). The amino acid composition is typical of CaBP's, and it contains a sequence (residues 55–69) which is very similar to the EF calcium binding site of carp muscle parvalbumin. A second portion of the sequence (residues 15–30) shows a limited homology to the CD site of carp parvalbumin which includes an insertion of Pro at residue 23 (Hofmann et al., 1979). Both the tyrosine and one or more of the phenylalanine residues have been shown to be perturbed when ICaBP binds metals (Dorrington et al., 1978). This paper describes the assignment of resonances in the ^{1}H NMR spectrum of the protein and the use of ^{1}H NMR to study the environment of aromatic residues within the protein, conformational changes in the protein caused by calcium binding, and the calcium binding stoichiometry.

Materials and Methods

Protein Samples. Porcine intestinal CaBP was purified from the mucosa of the duodenum and the jejunum as described previously (Hitchman et al., 1973). It was of high purity as judged by the absence of histidine, half-cystine, methionine, and tryptophan upon amino acid analysis. The protein, as isolated, was judged to be calcium saturated by comparison of its ^{1}H NMR spectrum with that of the desalted protein after addition of excess calcium and especially by the presence of nonexchangeable NH resonances even after the protein was dissolved in D_2O for several days. These resonances disappear when the the apoprotein is dissolved in D_2O (see below). The protein was desalted by using the following procedure. A 4-mL solution of 2 mg/mL ICaBP was made up in 10 mM Tris, 150 mM NaCl, and 10 mM EDTA, pH 8.5, and let sit a minimum of 1 h at 4 °C. It was then passed down a Sephadex G-25 medium column (34 × 2.2 cm; Pharmacia) over Chelex (2 × 2.2 cm; Bio-Rad, 100–200 mesh) in the same buffer without EDTA (O'Neil et al., 1982); the flow rate was ca. 0.7 mL/min. The eluted protein was then freeze-dried and made up to 4 mL with double-distilled, demineralized water. This sample was immediately passed down a G-25 medium (16 × 1.5 cm) over G-25 course (69 × 1.5 cm) over Chelex (7 × 1.5 cm) column in the same water; the flow rate was ca. 0.2–0.6 m/min. The eluted protein (referred to as the apoprotein hereafter) was then freeze-dried in aliquots which corresponded to the approximate sample size and lyophilized once from dithizoned D_2O.

After lyophilization, each sample was made up to 1 mL in dithizoned D_2O, and the protein concentration was determined by UV spectrophotometry by using the following extinction coefficients: $\epsilon_{280}^{1.0\%} = 0.170$ mL mg^{-1} cm^{-1} $\epsilon_{276.9}^{1.0\%} = 0.190$ mL mg^{-1} cm^{-1}. The concentration of the sample used in the calcium titration was further confirmed by amino acid analysis. The protein samples were made up to volume in 30 mM imidazole-d_4 (Merck Sharp & Dohme) and 20 mM KCl, pH 6.5. The only exception was the CIDNP experiments for which the buffer was 10 mM Tris and 40 mM KCl, pH 7.5. The typical sample size was 350 μL (1.2 mL in the CIDNP experiments). Both NMR buffers were pretreated with dithizone to remove contaminating metal ions; the Tris buffer used in the G-25 over Chelex column (see above) was pretreated by running it down a Chelex-100 column (25 × 4.5 cm; 100–200 mesh). All columns were run at 4 °C.

The pH measurements were made with an Ingold microelectrode (Model 6030-04) attached to a Beckman Expandomatic SS-2 or a Radiometer 24 pH meter; the pH values quoted are those observed and are not corrected for the deuterium isotope effect on the glass electrode. Values above pH 11 were not corrected for Na^+ interference and thus cannot be considered more accurate than ±0.2 pH unit (Snyder et al., 1975). Electrode standardization was achieved prior to each measurement with Fischer standard buffers. pH adjustments were made by the addition of small volumes of dithizoned DCl and/or chelexed NaOD (0.5 M). The sample pH was measured immediately prior to the accumulation of spectra. The total volume change during the pH titrations was 12%.

Stock Metal Solutions. Stock calcium solutions were prepared from $CaCl_2$ (anhydrous) by weight, and the final concentrations were determined by titration with EDTA in 0.1 M NaOH, with murexide as the indicator, and by atomic absorption spectrophotometry. The atomic absorption experiments were run at the Alberta Agriculture Toxicology Laboratory, and the resulting calcium determinations agreed with those determined by EDTA titration within an experimental error of ±6%. The total volume change over the calcium NMR titration was 2%.

NMR Spectra. The NMR spectra were obtained on a Bruker HXS-270 NMR spectrometer operating in the Fourier transform mode and equipped with quadrature detection. The ambient temperature for the samples was 299 K (except the decoupling experiment for the calcium-saturated protein, which was carried out at 333 K), and all samples were equilibrated 10–15 min prior to acquisition. The parameters used for the spectra were typically 4K data points, sweep width = ±2300 Hz, filter width = 2500 Hz (Butterworth) or 5000 Hz (Bessel), and 8 μs pulse (~80°). The HDO resonance was suppressed with homonuclear decoupling. Chemical shift values are relative to the major resonance of DSS, which was measured separately under identical conditions (30 mM imidazole-d_4 and 20 mM KCl, pH 6.5). For the spectra which are resolution enhanced, the FID was apodized by double-exponential multiplication (DM = 4.0, Nicolet 1180 software). Laser photo-CIDNP experiments were performed by utilizing a Spectra Physics Model 164 argon ion laser operating at 3.2 W in the multiline mode; 10-mm o.d. flat-bottomed NMR tubes were used. The sample was irradiated for 1 s, the laser beam being directed into the bottom of the sample via a computer-controlled shutter and mirror (Hincke et al., 1981b). The initial concentration of FMN for the apoprotein was 0.21 mM, and it was 0.42 mM for the calcium-saturated protein experiment.

CD Spectra. The CD spectra were measured at room temperature on a J-500 spectropolarimeter (Japan Spectroscopic Co.) equipped with a built-in data processor. The spectra were signal averaged 8 times to increase the signal to noise ratio in the spectra. The results are expressed as molar ellipticity ([θ] in degrees-centimeters squared per decimole) calculated by using the equation $[\theta]\lambda = 100\theta/(cl)$ where c is the molar protein concentration, l is the path length in centimeters, and θ is the observed ellipticity in degrees. The samples were 0.14 mM ICaBP, 30 mM imidazole-H_4, and 20 mM KCl, pH 8.00, and the path length was 1 cm. The apoprotein spectrum sample size was 500 μL, and the EDTA/ICaBP ratio was 5/1. The calcium-saturated protein spectrum sample size was 100 μL, and the Ca^{2+}/ICaBP ratio was 69/1. The respective molar ellipticity values at 275 nm were −1747 and +2887, respectively.

Results

The complete ^{1}H NMR spectrum of the apoprotein is shown in Figure 1, along with an expanded view of the aromatic region. Of particular interest in the aliphatic region of the spectrum are the upfield shifted methyl groups at 0.49 and

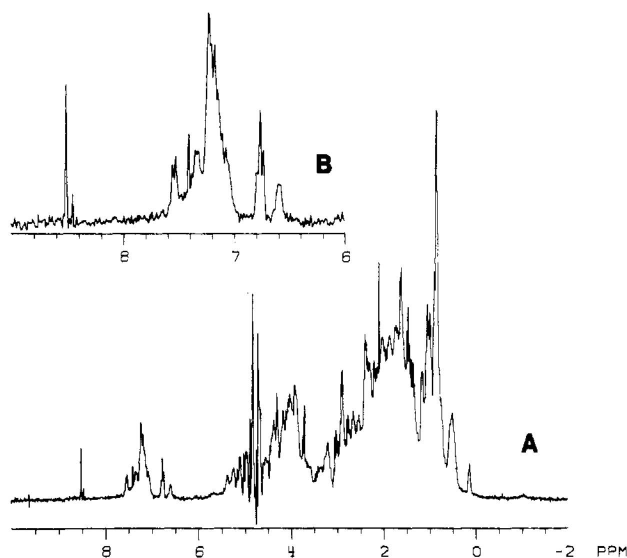

FIGURE 1: (A) Complete 270-MHz ^{1}H NMR spectrum of 0.47 mM apo-ICaBP (see Materials and Methods). The sample was preexchanged in the presence of 30 mM imidazole-d_4 and 20 mM KCl, pH 6.5, buffer in D_2O for several hours prior to acquisition. The inset (B) shows the expanded spectrum of the aromatic region. The spectra are resolution enhanced with a Lorentzian to Gaussian conversion and represent 2000 acquisitions.

0.12 ppm. The unique resonance frequency of such methyl groups has been shown (Sternlicht & Wilson, 1967) to arise when these protons are in close proximity to one or more aromatic groups. The resonances in the expanded aromatic region are derived from several sources. No backbone amide (NH) proton resonances are apparent. The narrow resonances at 8.50 and 7.40 ppm are from the imidazole of the buffer, which is not completely deuterated. These peaks were useful as an internal pH indicator; in the pH range used in these experiments their chemical shift is very sensitive to pH. The small resonance at 8.44 ppm is from formate ion, and the remainder of the resonances are derived from the one tyrosine and five phenylalanines. The spectrum in the region 7.0–6.4 ppm is typical of and similar to that of other CaBP's such as skeletal TnC (Seamon et al., 1977; Levine et al., 1977) and cardiac TnC (Hincke et al., 1981a) in that it contains a number of upfield shifted phenylalanine resonances at ca. 6.77 ppm and at 6.58 ppm, in addition to tyrosine (6.74) resonances. The spectrum is also sensitive to the addition of Ca^{2+} as will be discussed.

The laser photochemically induced dynamic nuclear polarization experimental results are shown in Figure 2 for apo-ICaBP and Ca^{2+}-saturated ICaBP. The only significant peaks in the difference spectra, which occur at positions where there is a resonance in the corresponding dark spectrum, are at 6.73 ppm for the apoprotein and 6.76 ppm for the calcium-saturated protein. This allows the assignment of these doublet resonances to the 3,5-protons of Tyr-16 (Kaptein et al., 1978); it is apparent that the chemical shift of these protons are essentially unaffected by the addition of calcium. The fact that one observes a CIDNP effect in both the presence and absence of calcium indicates that the tyrosine is on the surface of the molecule as it must be accessible to the flavin dye in the solvent (Kaptein et al., 1978; Hincke et al., 1981b). This is in good agreement with earlier solvent perturbation studies (Dorrington et al., 1978) and with the recently published crystal structure of the highly homologous bovine ICaBP (Szebenyi et al., 1981).

The homonuclear decoupling experiments, shown in Figure 3 for apo-ICaBP and calcium-saturated ICaBP, allow for the

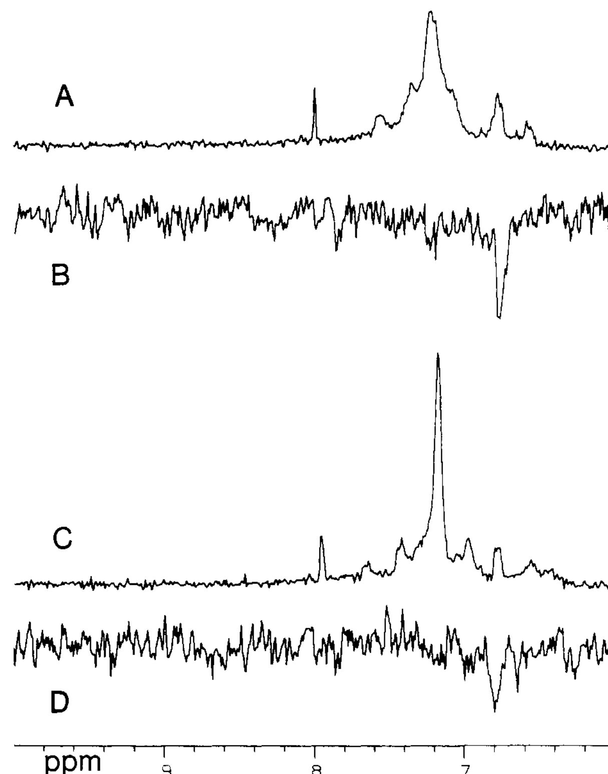

FIGURE 2: ^{1}H NMR laser photo-CIDNP experiments: (A) A "dark" spectrum of 0.57 mM apo-ICaBP at pH 7.5. The spectrum is resolution enhanced with a Lorentzian to Gaussian conversion and represents 2048 acquisitions. (B) The difference spectrum for apo-ICaBP which is a dark spectrum subtracted from a light spectrum. The spectrum has a line broadening of 2.5 Hz and represents 16 acquisitions. (C) A dark spectrum of the laser CIDNP sample shown in (A) after the addition of excess calcium to a $[Ca^{2+}]_0/[ICaBP]_0$ ratio of 4.9. The spectrum is resolution enhanced with a Lorentzian to Gaussian conversion and represents 2048 acquisitions. (D) The difference spectrum (light–dark) for the calcium-saturated sample shown in (C). The spectrum has a line broadening of 2.5 Hz and represents 16 acquisitions.

assignment of the 2,6-protons of tyrosine-16 in both forms of the protein. Irradiation at 7.53 ppm in the apoprotein (Figure 3B) and a 7.39 ppm in the calcium-saturated protein (Figure 3D) produced a collapse in the multiplicity of the 3,5-proton resonances at 6.73 and 6.76 ppm, respectively, indicating that these respective pairs were the spin-coupled protons of the tyrosine in the two states of the protein. The environment of the 2,6-protons, therefore, is slightly perturbed by the addition of calcium. The decoupling experiment in the presence of calcium (Figure 3C,D) was done at a higher temperature (333 K), but there was no change in the spectrum of the calcium-saturated protein as a function of temperature beyond the narrowing of resonances and the loss of some of the intensity of the slowly exchangeable NH resonances (these are the additional resonances observed in the range of ca. 7.4–9.0 ppm). Even at these high temperatures and also at high salt (0.5 M KCl) concentrations, intensity in the NH resonances remains for days. These resonances exchange readily (within hours) for the apoprotein.

The pH titration of the apoprotein is shown in Figure 4. One can see that the tyrosine protons (7.58 and 6.75 ppm) shift upfield as the pH increases, but the shifts are small compared with those observed for other CaBP's (Hincke et al., 1981a) and are not apparent until one is above pH 10.3. Thus the tyrosine has a very high pK_a, even though it is on the surface of the protein. The titration was not taken above pH 12 as this protein has been observed to irreversibly denature under

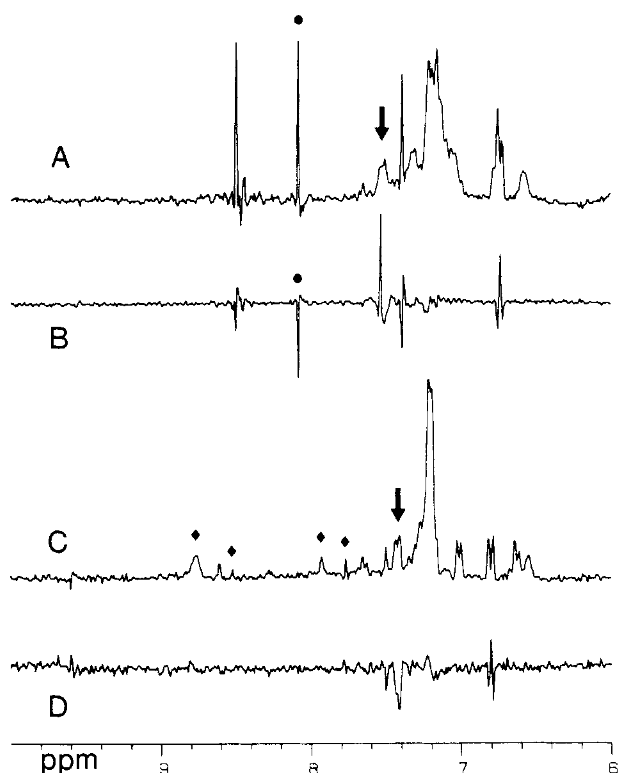

FIGURE 3: ^{1}H NMR homonuclear decoupling experiments. The spectra are resolution enhanced with a Lorentzian to Gaussian conversion. (A) The undecoupled spectrum of 0.50 mM apo-ICaBP at 299 K and pH 6.5. (B) A difference spectrum for the apo-ICaBP with the decoupler set at 7.53 ppm. The frequency of the decoupling γ-H_2 radio-frequency field is indicated by the arrow. The resonance marked by a (●) is the frequency of the decoupling (γ-H_2) radio-frequency field in the blank. The spectra represent 2000 acquisitions. (C) The undecoupled spectrum of 0.86 mM calcium-saturated ICaBP at 333 K. (D) The difference spectrum for the calcium-saturated protein with the decoupler set at 7.39 ppm. The frequency of the decoupling γ-H_2 radio-frequency field is indicated by the arrow. The NH backbone resonances are marked by a (♦). The spectra represent 4000 acquisitions.

such conditions (O'Neil et al., 1982). For this reason one cannot obtain a complete titration curve, and it is thus difficult to determine the pK_a; it is sufficient to indicate, however, that it is unusually high. As the pH is raised there are significant shifts in two phenylalanine resonances: One shifts downfield from 6.78 ppm; it comes out from underneath the 3,5-protons of the tyrosine, the latter of which can then clearly be seen as a well-resolved doublet. The other, at 7.34 ppm, shifts upfield.

In the presence of calcium (results not shown), there is no significant effect on the aromatic region of the spectrum throughout the titration; the tyrosine pK_a therefore is further increased. These titration results are in excellent agreement with spectroscopic studies (O'Neil et al., 1982), where a pK_a for tyrosine in the calcium-saturated protein was determined to be 12.9. Readjustment of the pH 12 spectrum for both protein states to pH 6.5 resulted in the original spectrum; thus no denaturation had occurred. The backbone NH resonance at 8.86 ppm is resistant to exchange even at pH 12, which further demonstrates structural integrity of the protein in the presence of Ca^{2+}. There were no significant spectral changes in the aliphatic region throughout either of these titrations.

Figure 5 shows the results of titration of the apo-ICaBP with calcium. It is apparent in Figure 5 that the binding of calcium causes perturbations in the aromatic spectrum, most strikingly in the broad envelope of phenylalanine resonances from 7.0 to 7.4 ppm; this envelope sharpens up and increases in intensity

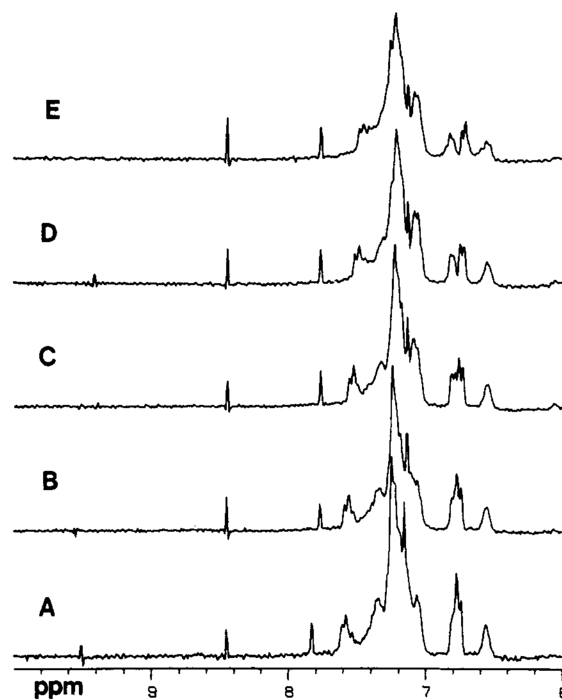

FIGURE 4: pH titration of 0.90 mM ICaBP in the presence of excess EDTA. The pH values are (A) 8.48, (B) 10.29, (C) 10.98, (D) 11.52, and (E) 11.92. The EDTA/ICaBP ratio was 3.25 except for (A), where it was 0.81. The spectra represent 4000 acquisitions and are resolution enhanced with a Lorentzian to Gaussian conversion.

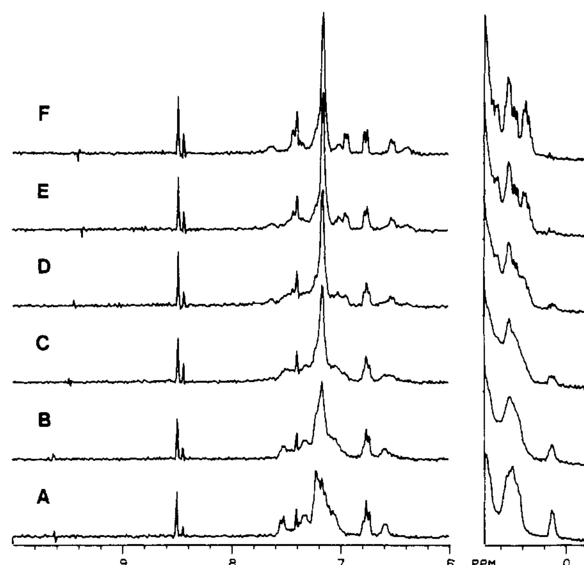

FIGURE 5: Ca^{2+} titration of 0.47 mM apo-ICaBP at pH 6.5. Both the low- and high-field portions of the ^{1}H NMR spectrum are shown. The $[Ca^{2+}]_0/[ICaBP]_0$ ratios are (A) 0.28, (B) 0.74, (C) 1.16, (D) 1.59, (E) 2.04, and (F) 3.33. The spectra are resolution enhanced with a Lorentzian to Gaussian conversion and represent 2000 acquisitions.

as the calcium/ICaBP ratio increases. The intensity of the apo-ICaBP tyrosine 2,6-proton resonance at 7.56 ppm decreases, and the intensity of the ICaBP calcium-saturated tyrosine 2,6-protons at 7.39 ppm increases, indicating that calcium binding is in the NMR slow-exchange limit. The tyrosine 3,5-protons appear to become resolved into a clear doublet at 6.76 ppm as calcium is added because the overlapping phenylalanine resonance in the free form decreases in intensity; the other upfield-shifted phenylalanines (6.4–6.7 ppm) undergo a significant change as well. The upfield-shifted methyl groups are also significantly perturbed, as shown in Figure 5. When the intensity change in the broad phenyl-

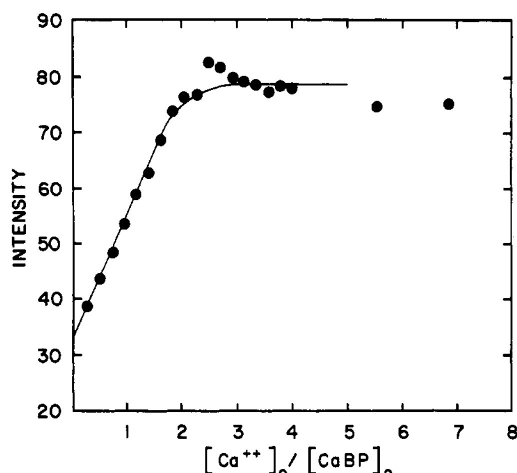

FIGURE 6: Ca^{2+} titration data. The resonance intensity (in arbitrary units) at 7.16 ppm in Figure 5 is plotted vs. the ratio of total Ca^{2+} concentration to total ICaBP concentration. The protein was found to initially be 14% saturated with calcium after desalting (see the text).

alanine envelope is taken and plotted as a function of $[Ca^{2+}]_0/[ICaBP]_0$, the results show a 2:1 stoichiometry (Figure 6). In this plot we have taken into account the fact that the "apo" protein is not initially completely calcium free. Comparison of the circular dichroism of a sample of protein identical with that used in the calcium NMR titration, with the CD of the same sample after addition of excess EDTA and then excess calcium (Dorrington et al., 1978), indicated that the protein was initially 14% calcium saturated. The CD measurements were done at pH 8.0 where the molar ellipticity change measured at 275 nm between the Ca^{2+}-free and Ca^{2+}-saturated forms is large (O'Neil et al., 1982). Assuming there are 2 mol of calcium bound per mol of ICaBP, the initial point in the titration thus corresponds to a $[Ca^{2+}]_0/[ICaBP]_0$ ratio of 0.28.

Discussion

In this discussion we concentrate primarily on the assigned Tyr-16 aromatic ring resonances and their use as probes of the effects of Ca^{2+} binding to porcine ICaBP. If the aromatic ring of Tyr-16 is free to undergo at least a 180° flipping motion, then one would expect to observe doublets for both the 2,6 and 3,5 pairs of protons, and they should titrate upfield with the loss of the Tyr phenolic proton (Snyder et al., 1975, 1976). The CIDNP and homonuclear decoupling experiments provide a definite assignment for the 2,6 and 3,5 resonances of tyrosine-16 of the porcine ICaBP in both the presence and absence of calcium.

The 3,5-protons of Tyr-16 in the apoprotein appear initially as a triplet as they are superimposed on phenylalanine resonance and are resolved into a clear doublet only by the addition of calcium or an increase in pH. Note that the difference ^{1}H decoupling pattern is appropriate for a doublet. In the apoprotein above pH 10 this doublet resonance titrates upfield with a $pK_a \geq 11-12$. The CIDNP experiments indicate that the tyrosine is exposed to the solvent in both the apo and calcium-saturated form. This agrees well with solvent perturbation studies (Dorrington et al., 1978). A model for the existence of such a high pK_a for a surface tyrosine has been proposed in which the hydroxyl group of tyrosine-16 is hydrogen bonded to glutamic acid residue 38 (O'Neil et al., 1982). This model is consistent with the X-ray crystal structure of bovine ICaBP (Szebenyi et al., 1981).

In the presence of calcium no resonances shift with pH. This implies that the tyrosine has a very high pK_a when calcium is bound. This is in agreement with a UV spectrophotometric titration that shows a tyrosine $pK_a = 12.9$ (O'Neil et al., 1982). The lack of observation of any significant changes in the aliphatic region of the protein from pH 6.5 to 12, in either the absence or presence of calcium (not shown), indicates that there is no large change in protein conformation over this pH range. The chemical shift of 6.76 ppm for the tyrosine 3,5-protons is the same as for the apoprotein and is similar to the values observed for skeletal and cardiac TnC and calmodulin (Seamon et al., 1977; Seamon, 1980; Hincke et al., 1981a). The addition of calcium to the apoprotein causes changes in the resonance attributed to the 2,6-protons of Tyr and changes in several resonances associated with Phe residues. This is consistent with the spectral work of Dorrington et al. (1978).

Under the conditions of these experiments the stoichiometry of calcium binding appears to be 2:1.[2] The stoichiometry of 2:1 is in disagreement with earlier published results which indicate a stoichiometry of 2:1 at lower protein concentrations (~0.1 mM) but a stoichiometry of 1:1 at higher (0.5 mM) protein concentrations (Dorrington et al., 1978). It agrees well, however, with results for the highly homologous bovine ICaBP (Fullmer et al., 1977; Szebenyi et al., 1981).

That the rate of calcium binding is slow enough to result in NMR spectra in the slow-exchange limit is in agreement with the results for the high-affinity sites of TnC and calmodulin (Seamon et al., 1977; Seamon, 1980; Hincke et al., 1981a).

The presence of upfield-shifted phenylalanine resonances in the aromatic region and upfield shifted methyl groups in the aliphatic region (Figure 1) in the apoprotein indicates that a well-defined tertiary structure exists in the protein even in the absence of calcium, as was observed for apo-cTnC (Hincke et al., 1981a; Seamon et al., 1977). The insensitivity of the calcium-saturated protein to high temperature, high pH, and high salt concentration demonstrates the enhanced structural stability of the protein in the presence of calcium.

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Registry No. Ca, 7440-70-2; tyrosine, 60-18-4; phenylalanine, 63-91-2.

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[2] All titrations seem to show some decrease in intensity beyond ≈2:1, which may indicate weak third site binding and/or nonspecific binding of excess Ca^{2+}.

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Conformational Stability of Mixed Disulfide Derivatives of β-Lactoglobulin B[†]

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ABSTRACT: To probe the relationship between chemical structure and conformational stability, the urea denaturation of bovine β-lactoglobulin B and four mixed disulfide derivatives of this protein was investigated. The following groups were attached to the single sulfhydryl group of β-lactoglobulin B through a disulfide bond: propyl, aminoethyl, carboxyethyl, and hydroxyethyl. The optical rotatory properties in the near- and far-UV wavelength range suggest that β-lactoglobulin B and the propyl, carboxyethyl, and hydroxyethyl derivatives have similar conformations but that the conformation of the aminoethyl derivative differs substantially from that of the unmodified protein. In all cases, denaturation was shown to be reversible, and the derivatives were less stable than unmodified β-lactoglobulin B. The midpoints of the isothermal urea denaturation curves at pH 2.83 and 25 °C occur at 4.97 M urea for β-lactoglobulin B and at 4.46, 4.23, 4.19, and 1.68 M urea for the carboxyethyl, propyl, hydroxyethyl, and aminoethyl derivatives, respectively. An analysis of these data shows that the corresponding decreases in the conformational stability are approximately 1.1, 1.6, 1.7, and 7.3 kcal/mol for the modified proteins. The conformational stability of the aminoethyl derivative is decreased to such an extent that the protein is partially unfolded even in the absence of a denaturant. The urea denaturation curves were less steep for the derivatives than for the unmodified protein. The dependence of the free energy of denaturation on urea concentration, $d(\Delta G_D)/[d(urea)]$, was 2.23 kcal/mol per M urea for β-lactoglobulin B and 1.46, 1.26, 0.92, and 0.43 kcal/mol per M urea for the carboxyethyl, hydroxyethyl, propyl, and aminoethyl derivatives, respectively. This suggests a greater deviation from a two-state mechanism for the unfolding of the derivatives.

Estimates of the conformational stability of about 25 proteins are now available (Pace, 1975; Privalov, 1979; Pfeil, 1981). In general, the globular conformation is from 2 to 15 kcal/mol more stable than unfolded conformations. There is considerable interest in determining how small changes in the chemical structure of a protein can alter the conformational stability. One approach is to compare genetic variants of a protein which differ only slightly in amino acid sequence (Knapp & Pace, 1974; Yutani et al., 1980; Schellman et al., 1981; Matthews et al., 1980). Another approach is to compare proteins whose structure has been changed slightly by chemical modification (Imoto & Rupley, 1973; Jacobson & Braun, 1977; Stoesz & Lumry, 1979; Hollecker & Creighton, 1982). These experimental studies of the relationship between structure and conformational stability are essential to test predictions based on theory and model compound data and the increasingly well-defined and well-understood three-dimensional structures of globular proteins determined by using X-ray diffraction (Richardson, 1981). The successful development of a useful approach for analyzing electrostatic interactions in globular proteins by Gurd's laboratory provides a good example of the progress which can be made when reliable experimental results are available for refining a theoretical approach (Friend & Gurd, 1979; Matthew & Richards, 1982).

We report studies of the effect of chemical modification of β-lactoglobulin B on the conformational stability. β-Lactoglobulin is well suited for this purpose since large amounts of the pure protein are readily available and the denaturation of the protein has been investigated in detail (Pace & Tanford, 1968; Alexander & Pace, 1971; Creighton, 1980). In addition, β-lactoglobulin contains a single sulfhydryl group (cysteine residue 121) which can be chemically modified (Townend et al., 1969; Ralston, 1972). We have prepared the propyl (P),[1]

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