Deibel, M. R., & Coleman, M. S. (1980b) *J. Biol. Chem. 255*, 4206–4212.
Deibel, M. R., Riley, L. K., & Coleman, M. S. (1983) *Isozymes: Curr. Top. Biol. Med. Res. 7*, 297–315.
Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.
Eftink, M. R., Zajicek, J. L., & Ghiron, C. A. (1977) *Biochim. Biophys. Acta 491*, 473–481.
Greenwood, M. F., Coleman, M. S., Hutton, J. J., Lampkin, B., Krill, C., Bollum, F. J., & Holland, P. (1977) *J. Clin. Invest. 59*, 889–899.
Grinvald, A., & Steinberg, I. Z. (1974) *Anal. Biochem. 59*, 583–598.
Grinvald, A., & Steinberg, I. Z. (1976) *Biochim. Biophys. Acta 427*, 663–678.
Kato, K.-I., Goncalves, J. M., Houts, G. E., & Bollum, F. J. (1967) *J. Biol. Chem. 242*, 2780–2789.
Knutson, J. R., Walbridge, D. G., & Brand, L. (1982) *Biochemistry 21*, 4671–4679.
Knutson, J. R., Beechem, J. M., & Brand, L. (1983) *Chem. Phys. Lett. 102*, 501–507.
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, p 44, Plenum, New York.
Lakowicz, J. R., & Cherek, H. (1980) *J. Biol. Chem. 255*, 831–834.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
McCaffrey, R., Smoler, D. F., & Baltimore, D. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 521–525.
Peterson, R. C., Cheung, L. C., Mattaliano, R. J., Chang, L. M. S., & Bollum, F. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4363–4367.
Ross, J. B. A., Schmidt, C. J., & Brand, L. (1981) *Biochemistry 20*, 4369–4377.
Servillo, L., Colonna, G., Balestrieri, C., Ragone, R., & Irace, G. (1982) *Anal. Biochem. 126*, 251–257.
Teale, F. W. J., & Weber, G. (1957) *Biochem. J. 65*, 476–482.

Structural Elucidation of a Hydrophobic Box in Bovine α-Lactalbumin by NMR: Nuclear Overhauser Effects†

Keiko Koga and Lawrence J. Berliner*
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210
Received April 26, 1985

ABSTRACT: The proton nuclear Overhauser effects of bovine α-lactalbumin were studied at 200 MHz by irradiation of an upfield ring current shifted methylene at −2.45 ppm (assigned to Ile-95) and two aromatic protons, Tyr-103 (8.36 ppm) and Trp-60 (5.85 ppm). The experimental results were consistent with a putative three-dimensional α-lactalbumin model [Warme, P. K., Momany, F. A., Rumball, S. V., Tuttle, R. W., & Scheraga, H. A. (1974) *Biochemistry 13*, 768–782], which predicted the close proximity of Ile-95, Tyr-103, Trp-60, and Trp-104. Several of the assignments correlated with those previously made from chemically induced dynamic nuclear polarization experiments [Berliner, L. J., & Kaptein, R. (1981) *Biochemistry 20*, 799–807]. Subtle differences in the structure of this hydrophobic box region in α-lactalbumin were found between the Ca(II) and apo forms of the protein. The existence of this "hydrophobic box" in α-lactalbumin was strikingly similar to that in lysozyme, as verified *in solution*.

α-Lactalbumin (α-LA)[1] is a modifier protein in the lactose synthase complex whose function is to modify the acceptor specificity of galactosyl transferase from GlcNAc to glucose. The molecular details of the interaction between α-LA and galactosyl transferase still remain unclear, nor has an X-ray structure of α-LA been reported to date. However, an energy-minimized, three-dimensional structure was described by Warme et al. (1974) based on primary sequence homologies to hen egg white (HEW) lysozyme. It is desirable to test the validity of this putative model *in solution* by experiments that may elucidate detailed molecular structural features. The nuclear Overhauser effect (NOE) is an extremely powerful method for investigating the tertiary structure of proteins *in solution* [for example, see Keller & Wüthrich (1981)]. The NOE reflects specific dipolar interactions between nuclei, which are distance-dependent and thus provide information about the relative positions of an irradiated, saturated nucleus and those nuclei that experience a change in intensity as a result of this initial irradiation (Noggle & Schirmer, 1971). Recently, Poulsen et al. (1980) verified the existence of a "hydrophobic box" in HEW lysozyme by ^{1}H NMR NOE measurements, consistent with its X-ray structure.

It has recently been shown that α-LA is a calcium binding protein (Hiroaka et al., 1980) that undergoes a distinct conformational change upon cation binding (Permyakov et al., 1981a,b; Murakami et al, 1982). In this work, we show by NOE measurements the existence of a hydrophobic box structure in bovine α-LA that is remarkably similar to that of HEW lysozyme. Furthermore, we note some subtle dif-

† This work was supported in part by Grant HD 17270 from the USPHS. The 200-MHz NMR instrument used in these studies was funded by NIH Grant GM 27431 to L.J.B. (co-PI) for core research.

[1] Abbreviations: α-LA, α-lactalbumin; GlcNAc, *N*-acetylglucosamine; HEW, hen egg white; NOE, nuclear Overhauser effect; Tris-DCl, tris(hydroxymethyl)aminomethane-d_{11}; FT, Fourier transform.; FID, free induction decay; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; CIDNP, chemically induced dynamic nuclear polarization; 1D, one dimensional.

0006-2960/85/0424-7257$01.50/0

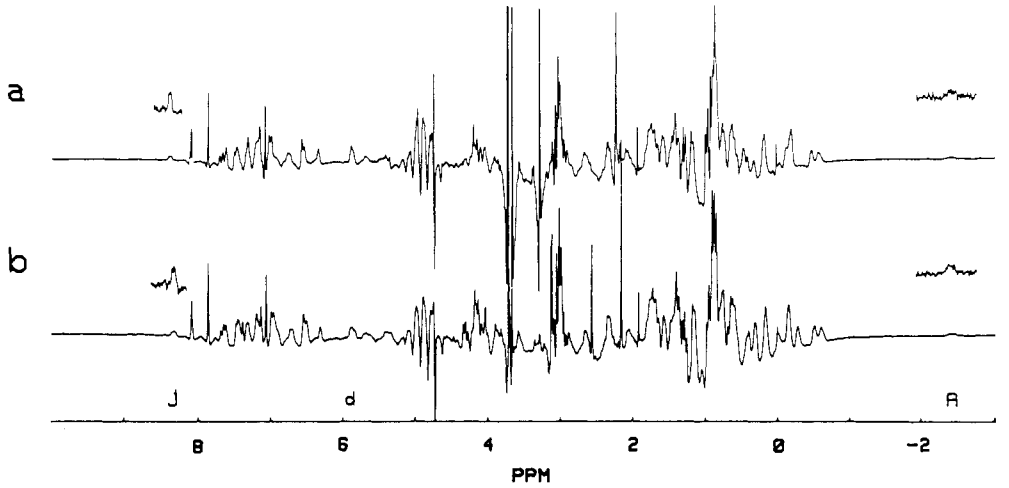

FIGURE 1: 200-MHz ^{1}H NMR spectra of bovine α-LA at pH 7.2 (uncorrected) in 50 mM Tris-DCl. Spectrometer parameters were as follows: 8K data points, 1000 scans, 3000-Hz sweep width, 3.7-μs pulse width (=90°), and 1.4-s acquisition time. Spectral resolution enhancement was by convolution difference (line broadening 0.5 Hz minus line broadening 5 Hz). (a) Apo-α-LA, 1.6 mM; (b) Ca(II)-α-LA, 1.9 mM.

ferences between the apo- and Ca(II)-α-LA conformers.

Experimental Procedures

Materials

Bovine α-LA (lot no. 52F-80751) was from Sigma Chemical Co. Metal-free apo-α-LA was prepared by chromatography on *N,N,N'*-tris(carboxymethyl)ethylenediamine–agarose (Pierce Chemical Co., lot no. 121483–87) at pH 8.0, 25 °C. After two passes, the residual calcium content was checked by fluorescence (Murakami et al., 1982), proton NMR, and atomic absorption and was found to be consistently less than 2%. Protein concentration was determined by using $E^{1\%}_{1cm}$ = 20.1 at 280 nm. Samples for NMR experiments were 3 mM α-LA in 50 mM tris(hydroxymethyl)aminomethane-d_{11} (95 atom %, lot no. 6037, Merck Stable Isotopes, Inc.), pH 7.2. Deuterium oxide (D_2O) was from Merck or Sigma Chemical Co. (99.8%). All pH values were uncorrected.

NMR Methods

Proton NMR spectra were measured on a Bruker WP-200 at 301 ± 1 K. For normal FT-NMR spectra, the residual water resonance was suppressed by presaturation with a single radiofrequency for 1 s, which was gated off during acquisition. An average of 1000 transients were accumulated by digitization of 8K data points. The sweep width was 3000 Hz; the pulse repetition time was 2.4 s. NOE difference spectra were obtained by alternately collecting 80 acquisitions with *on-resonance* irradiation and 80 acquisitions with *off-resonance* irradiation. The total number of accumulated FID's was typically 8000 each, which were then subtracted to yield the NOE difference spectrum. All chemical shifts were measured from internal DSS and are reported with an accuracy of ±0.01 ppm for normal FT spectra and ±0.02 ppm for NOE difference spectra.

Results and Discussion

The 200-MHz proton NMR spectra of apo- and Ca(II)-α-LA are shown in parts a and b, respectively, of Figure 1. In both cases, several ring current shifted resonances were found in the upfield region (0 to −2.5 ppm). Of particular note was a broad line (A) at −2.45 ppm (inset) that occurred in both apo-α-LA and Ca(II)-α-LA. This proton must be influenced by strong ring currents, by virtue of the unusually large upfield shifts, and must therefore be located very close to the ring plane of an aromatic residue. The remaining upfield ring current shifted peaks differed somewhat in chemical shift between apo- and Ca(II)-α-LA, evident of two different conformations (Murakami et al., 1982; Musci & Berliner, 1985a,b). Another unusual resonance was the broad downfield line j at 8.36 ppm (inset, Figure 1). This broad aromatic resonance (which was severely reduced after convolution difference) was not pH-dependent, which rules out its assignment as one of the three His residues (at positions 107, 68, and 32) in the protein (Bradbury & Norton, 1975). The other distinct resonance of interest was the unusual aromatic proton d at 5.85 ppm (Figure 1), assigned earlier by CIDNP methods as a buried, cross-polarized proton from the indole ring of Trp-60 (Berliner & Kaptein, 1981). These three resonances, j, d, and A, meet the requirements salient to a 1D NOE study, i.e., preferably *well-isolated* proton resonances. Also, since a protein of this size would be a good candidate for general spin diffusion phenomena upon irradiation at a specific frequency (Kalk & Berendsen, 1976), it was absolutely necessary to avoid further nonspecific irradiation during attempts to irradiate specifically in a crowded peak region. The contribution, in part, of general spin diffusion to NOE difference spectra cannot be totally ruled out for any protein at high magnetic fields (Kalk & Berendsen, 1976). For quantitative measurements of interproton distances in a protein one must examine the time dependence of these NOE's at short irradiation times (Wagner & Wüthrich, 1979). Poulsen et al. (1980) showed with lysozyme a simple qualitative correlation between observed NOE's and residue positions, even when measured under conditions approaching steady state. Thus, even if a small contribution from spin diffusion occurred, it would not significantly alter the overall qualitative structural analyses. In our experiments, however, only well-isolated peaks were irradiated with relatively short irradiation times (0.4 s). For example, Figure 2 contains quite specific NOE resonances; i.e., any general spin diffusion effects are minimal.

NOE Measurements of Ca(II)-α-LA. NOE experiments were carried out on the three well-isolated protons described above: protons d (5.85 ppm), j (8.36 ppm), and A (−2.45 ppm), respectively. Figure 2 shows the NOE difference spectrum of 3 mM Ca(II)-α-LA, pH 7.2, irradiated at proton d (5.85 ppm, Trp-60). A large negative NOE was observed at the aromatic resonance g (7.18 ppm). Other weaker NOE's in the aromatic region were resonances at 6.48 (e), 6.98 (f), 7.64 (i), and 5.40 ppm, respectively. Some NOE's were also observed in the aliphatic region at 0.43, 1.12 (b), 3.07 (F),

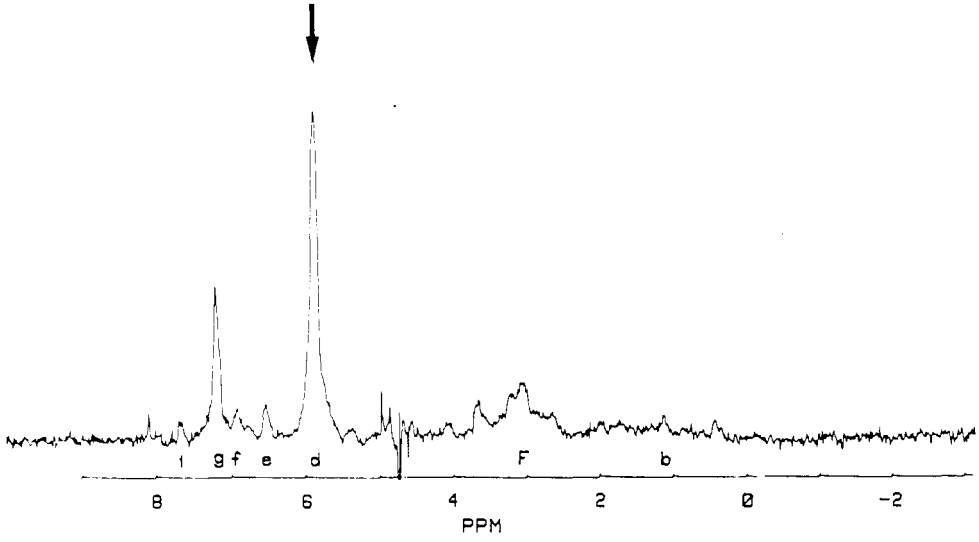

FIGURE 2: NOE difference spectrum of 3 mM Ca(II)-α-LA, produced by irradiation of peak d (arrow) at 5.85 ppm for 0.4 s (8000 scans). Exponential multiplication was 0.5 Hz. All other conditions were as in Figure 1.

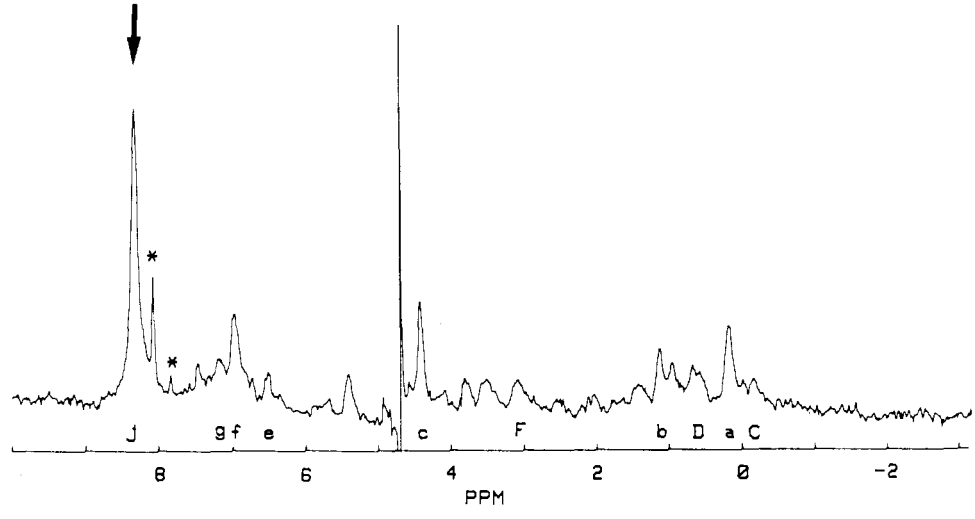

FIGURE 3: NOE difference spectrum of 3 mM Ca(II)-α-LA, produced by irradiation of peak j (arrow) at 8.36 ppm for 0.4 s (5600 scans). The asterisks (*) denote nearby lines partially irradiated by the 8.36 ppm pulse. All other conditions were as in Figure 1.

3.19, and 3.63 ppm. The observed percent NOE's for the aromatic peaks were ~30% (g, 7.18 ppm), ~6% (e, 6.48 ppm), ~3% (f, 6.98 ppm), and ~3% (i, 7.64 ppm). While we did not consider precise distance calculations from these NOE values because of potential spin diffusion contributions, it was clear that the large NOE effect at peak g (7.18 ppm, Figure 2) reflected a very short interatomic distance between this proton (g) and the irradiated proton (d) at 5.85 ppm; i.e., these two protons must be on the same residue (Trp-60). The protons e (6.48 ppm), f (6.98 ppm), and i (7.64 ppm) must also be located near proton d (Trp-60, 5.85 ppm). Fortunately, several of these resonances have been assigned earlier from CIDNP studies on α-LA by Berliner & Kaptein (1981). Resonance e (6.48 ppm) was assigned as a solvent-accessible (directly polarized) Trp-104 proton that cross-relaxed to protons d (5.85 ppm, Trp-60) and i (7.64 ppm). Thus the NOE from proton d (5.85 ppm) to protons e (6.48 ppm) and i (7.64 ppm) served as a reconfirmation of the cross-polarization observed from CIDNP experiments. Since resonance i (7.64 ppm) showed a weak NOE from Trp-60 (d, 5.85 ppm) and a strong cross-polarization from CIDNP when Trp-104 was directly polarized (Berliner & Kaptein, 1981) and it was clearly a doublet from both 200- and 500-MHz experiments (K. Koga and L. J. Berliner, unpublished experiments), we could assign resonance i to the C(7) H of Trp-104 since the only other Trp doublet, C(4) H, was *directly* polarized in CIDNP experiments. The third aromatic resonance f (6.98 ppm) was shown from CIDNP to belong to the ortho proton(s) of one of the three exposed Tyr residues which, according to the NOE results of Figure 2, should be situated close to Trp-60 and Trp-104 in the α-LA structure.

Figure 3 displays the NOE difference spectrum of Ca(II)-α-LA obtained by irradiation of resonance j at 8.36 ppm. Strong NOE difference peaks were observed at 0.18 (a, ~20%), 1.12 (b, ~10%), 4.42 (c, ~20%), and 6.98 ppm (f, ~20%), respectively. These large NOE values imply that these protons were most likely from the *same* residue. Since, as noted above, CIDNP studies showed that the 6.98 ppm resonance f belongs to a Tyr, we have assigned resonance j (8.36 ppm) to the *same* Tyr residue. Furthermore, the strong NOE aliphatic resonances a, b, and c most likely also originate from aliphatic protons on this same Tyr residue. Noting further that the weak NOE's in the aromatic region at 6.48 (e) and 7.18 ppm (g) have been assigned above as Trp-104 and Trp-60, respectively (Figure 2), we reconfirm here that a close structural relationship must exist between this specific Tyr [6.98 (f) and 8.36 (j) ppm] and Trp-60 and Trp-104. Additional NOE's in the aromatic region were at 5.40 and 7.46

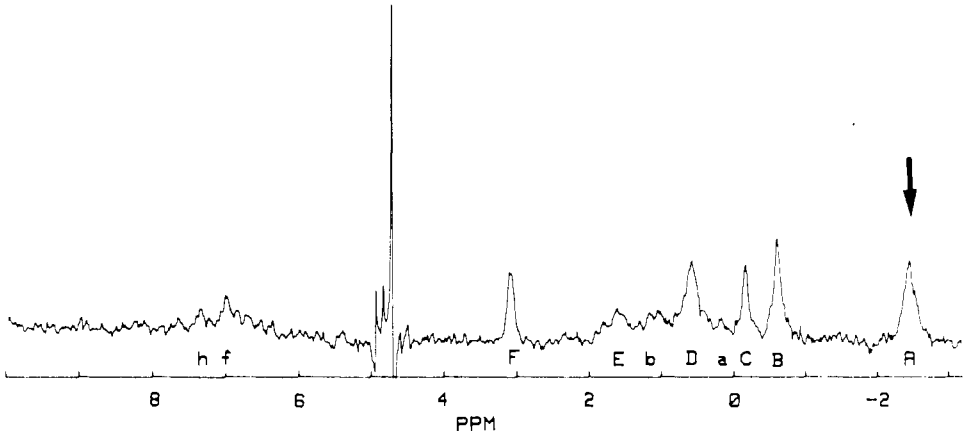

FIGURE 4: NOE difference spectrum of 3 mM Ca(II)-α-LA, produced by irradiation of peak A (arrow) at −2.45 ppm for 0.4 s (5600 scans). All other conditions were as in Figure 1.

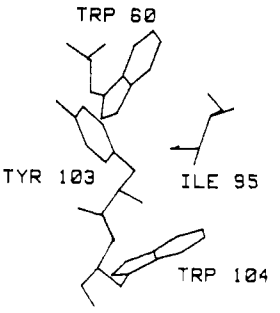

FIGURE 5: Two-dimensional projection of the hydrophobic box region of bovine α-LA based on the coordinates of Warme et al. (1974).

ppm, respectively. Other protons in the aliphatic region appeared at 0.56 (D), 0.67, 0.96, 1.41, 3.07 (F), 3.51, and 3.80 ppm, respectively, as well as at −0.17 ppm (C), an unusual upfield-shifted proton. These latter lines must arise from aliphatic residues situated close to the unique tyrosine [6.98 (f) and 8.36 (j) ppm].

Figure 4 shows the NOE difference spectrum obtained by irradiation of the −2.45 ppm line (A). Several strong peaks appeared in both the aliphatic and aromatic regions at −0.62 (B, ~80%), −0.17 (C, ~50%), 0.56 (D, ~50%), 1.59 (E, ~20%), and 3.07 ppm (F, ~45%), respectively. The large NOE's clearly indicated that proton A (−2.45 ppm) and these five peaks (B–F) are from the same aliphatic residue. Additional, weaker NOE's were observed at 0.18 (a) and 1.12 ppm (b) in the aliphatic region; aromatic NOE's appeared at 6.98 (f, ~10%) and 7.33 ppm (h, ~7%), respectively. It follows that the ring current effects on the −2.45 ppm proton (A) must arise from the aromatic protons (f, h) above. Since, from Figures 2 and 3 we assigned resonances a, b, and f to a Tyr that resides near Trp-60 and Trp-104, the aliphatic residue that corresponds to protons A–F (Figure 4) must also be closely situated to these aromatic residues.

In the absence of a high-resolution X-ray structure of α-LA we have examined the putative structure of Warme et al. (1974), which was already shown to correlate well with laser CIDNP results on this protein (Berliner & Kaptein, 1981). Of the four Tyr residues in bovine α-LA, the putative models of both Warme et al. (1974) and Browne et al. (1969) show clearly that only Tyr-103 is close enough to Trp-60 and Trp-104 to yield significant cross-polarization or NOE's. It follows that the unique Tyr, which was assigned as peaks f and j in Figure 3, is residue 103. Figure 5 depicts a two-dimensional projection of this region based on coordinates from Warme et al. (1974), provided by Professor H. A. Scheraga, which was also consistent with a low-resolution partial map

Table I: Comparisons of Ile-95 (α-LA) and Ile-98* (Lysozyme)

		chemical shift (ppm)		
		Ile-95		
peak	proton moiety	Ca(II)-α-LA[a]	apo-α-LA[a]	Ile-98*, lysozyme[b]
A	$H^{\gamma 12}$	−2.45	−2.45	−2.10
B	CH_3^{δ}	−0.62	−0.61	−0.01
C	$CH_3^{\gamma 2}$	−0.17	−0.16	−0.26
D	$H^{\gamma 11}$	0.56	0.59	0.63
E	H^{β}	1.59	1.52	1.56
F	H^{α}	3.07	3.05	2.88

[a] Conditions were as follows: pH 7.2, 50 mM Tris-DCl. All chemical shifts are ±0.02 ppm from DSS. [b] Assignments taken from Poulsen et al. (1980).

of baboon α-LA (D. Stuart, personal communication). As discussed earlier, several of the NOE peaks that appeared after irradiation of the 8.36 ppm line (j) of Tyr-103 (Figure 3) were protons from this same Tyr (Tyr-103); i.e., peaks a (0.18 ppm) and b (1.12 ppm) should correspond to H^{β}, peak c (4.42 ppm) to H^{α}, peak f (6.98 ppm) to the ring 3,5 ortho proton(s), and peak i (8.36 ppm) to the 2,6 meta proton(s). Resonances a (0.18 ppm) and b (1.12 ppm) have chemical shift values very different from those of a Tyr in a random coil state (Bundi & Wüthrich, 1979), since large ring current shifts would be expected from the nearby aromatic residues, Trp-104 and Trp-60. Resonance e (6.48 ppm), (which was assigned to Trp-104 from CIDNP experiments (Berliner & Kaptein, 1981), corresponds most closely to C(4) H, which is also very near to a Tyr-103 2,6 meta proton in the putative model.

The assignment of peak f (6.98 ppm) to Tyr-103 also enabled us to assign NOE difference peaks that resulted from the irradiation of resonance A (−2.45 ppm) in Figure 4. In the model (Figure 5), the aliphatic residue that is closest to Tyr-103 is Ile-95. This resonance (A) corresponds to Ile-98* in lysozyme,[2] which was assigned from homodecoupling experiments by Chapman et al. (1978) as $H^{\gamma 12}$. Upon irradiation of this resonance (−2.10 ppm in lysozyme) Poulsen et al. (1980) found several aliphatic and aromatic resonances in the NOE difference spectrum that were *very similar* to the α-LA NOE spectra of Figure 4. [For comparison see Figures 6a and 7a of Poulsen et al. (1980).] Therefore, we have assigned the −2.45 ppm peak (A) to $H^{\gamma 12}$, the −0.62 ppm peak (B, ~30%) to CH_3^{δ}, the −0.17 ppm peak (C, ~20%) to $CH_3^{\gamma 2}$, the 0.56 ppm peak (D, ~60%) to $H^{\gamma 11}$, the 1.59 ppm peak (E,

[2] Residues that correspond to the *same* position are nonetheless designated with the residue number of the respective numbering systems. The lysozyme numbering system is designated with an asterisk (*).

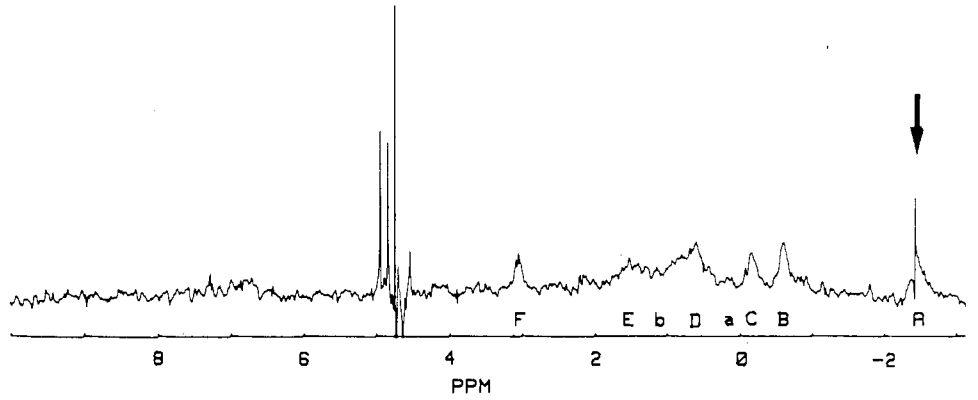

FIGURE 6: NOE difference spectrum of 3 mM apo-α-LA, produced by irradiation of peak A (arrow) at −2.45 ppm for 0.4 s (8000 scans). All other conditions were as in figure 1.

~20%) to H^{β}, and the 3.07 ppm peak (F, ~45%) to H^{α} of Ile-95 of Ca(II)-α-LA.[3] The analogous assignments in lysozyme are noted in Table I. The relative sizes of the NOE's for peaks D ($H^{\gamma 11}$), E (H^{β}), and F (H^{α}) are in good agreement with their relative distances from $H^{\gamma 12}$ (−2.45 ppm, A) on Ile-95 in the putative α-LA model (Warme et al., 1974). Overall, the chemical shift values of Ile-95 (α-LA) and Ile-98* (lysozyme) are very similar. However, comparison of the NOE's to $CH_3^{\gamma 2}$ and CH_3^{δ} suggests a subtle structural difference between the two proteins. The NOE of peak B (−0.62 ppm, ~80% per three protons) in Figure 4 is larger than that of peak C (−0.17 ppm, ~50% per three protons). Since the distance between CH_3^{δ} and $H^{\gamma 12}$ is *shorter* than that between $CH_3^{\gamma 2}$ and $H^{\gamma 12}$, we assigned peak B to CH_3^{δ} and peak C to $CH_3^{\gamma 2}$, which is *opposite* to that in lysozyme (Table I). In both proteins these chemical shifts are very different from those of an Ile in a random coil. Ile-98* of lysozyme is known from X-ray analysis to be in a hydrophobic box surrounded by Trp-108* and Trp-63* (Blake et al., 1965), which were also shown by difference NOE experiments to be in close proximity to Ile-98* (Poulsen et al., 1980). By analogy, we have verified the existence of a hydrophobic box in α-LA *in solution*, involving the *same* residues (Trp-104, Trp-60, and Ile-95). In addition, Figure 4 showed that the ortho proton of Tyr-103 (6.98 ppm, f) gave the largest interresidue NOE (~10%) to $H^{\gamma 12}$ of Ile-95. Peak h (7.33 ppm) was assigned to Trp-60 [C(2) H] as it was the nearest proton to Ile-95 $H^{\gamma 12}$ from the putative model (Warme et al., 1974). The proton–proton distance in these pairs was 3–4 Å, which is certainly close enough to show interresidue NOE's. A summary of the assignments from this study is compiled in Table II.

Other Assignments. We have attempted to tentatively assign other NOE peaks principally on the basis of the putative model. The resonance at 5.40 ppm (Figures 2 and 3) should be either from Trp-104 or from Trp-60. Since it was not observed in CIDNP experiments (Berliner & Kaptein, 1981), we tentatively assigned it to Trp-60. The 3.80 ppm peak in Figure 3 was assigned to C(β) H of Trp-104, which also gave a strong emission in CIDNP spectra (Berliner and Kaptein, unpublished results). Of the remaining unassigned aliphatic peaks, those in Figure 2 at 0.43, 3.19, and 3.63 ppm, respectively, most probably arose from Ile-59, which is quite close to Trp-60. In particular, the 0.43 ppm resonance must be strongly effected by ring currents. In Figure 3, the peaks at 0.67, 0.96, 1.41, and 3.51 ppm, respectively, are most consistent with Val-99, which is the other aliphatic residue nearest Tyr-103.

Table II: Nuclear Overhauser Effects and Assignments of Ca(II)-α-LA

peak[a]	chemical shift[b] (ppm)	NOE for irradiated peak 5.85 ppm	8.36 ppm	−2.45 ppm	assignments
A	−2.45			irrad[d]	Ile-95, $H^{\gamma 12}$
B	−0.62			++	Ile-95, CH_3^{δ}
C	−0.17		+	++	Ile-95, $CH_3^{\gamma 2}$
a	0.18		++	+	Tyr-103, H^{β}
D	0.56		+	++	Ile-95, $H^{\gamma 11}$
b	1.12	+	++	+	Tyr-103, H^{β}
E	1.59			+	Ile-95, H^{β}
F	3.07	+	+	++	Ile-95, H^{α}
c	4.42		++		Tyr-103, H^{α}
d	5.85	irrad			Trp-60[c]
e	6.48	+	+		Trp-104[c], C(4) H
f	6.98	+	++	+	Tyr-103, ortho H
g	7.18	++	+		Trp-60
h	7.33			+	Trp-60, C(2) H
i	7.64	+			Trp-104[c], C(7) H
j	8.36		irrad		Tyr-103, meta H

[a] Capital letters refer to Ile-95 proton resonances; lower case letters refer to aromatic amino acid proton resonances. [b] Conditions were as follows: pH 7.2, 50 mM Tris-DCl. All chemical shifts are ±0.02 ppm from DSS. [c] Assignments from Berliner & Kaptein (1981). [d] irrad = irradiated peak.

Comparisons between Ca(II)-α-LA and Apo-α-LA. Figure 6 shows the NOE difference spectrum of apo-α-LA by irradiation at −2.45 ppm for 0.4 s. While the spectrum was *generally* similar to that for Ca(II)-α-LA (Figure 4), there were some differences that distinguished the two conformers. Note that the broad NOE peak at 0.8 ppm appeared *only* in apo-α-LA. Although we have not yet assigned this peak, it is obviously one of the differences in an aliphatic proton in this region of apo-α-LA vs. Ca(II)-α-LA. Where the strongest aromatic NOE resonances (f, h) appeared at 6.98 and 7.33 ppm in Ca(II)-α-LA (Figure 4), these peaks were essentially absent in apo-α-LA (Figure 6). This suggests most likely that the distaince between the $H^{\gamma 12}$ proton of Ile-95 and the ortho proton of Tyr-103 increased in apo-α-LA vs. Ca(II)-α-LA. The chemical shift differences in NOE peak position between Ca(II)-α-LA and apo-α-LA are mostly identical within experimental error (Table I); however, preliminary measurements at 500 MHz indicate small but measurable differences (K. Koga and L. J. Berliner, unpublished results). Lastly, the NOE spectral comparisons show that the hydrophobic box region of lysozyme is more similar to Ca(II)-α-LA than apo-α-LA.

[3] The line shapes of Ile-95 H^{α} (F) and H^{β} (E) were consistent with the expected doublet and multiplet hyperfine structures, respectively. Futhermore, the observed percentage NOE's for these two protons directly reflect the differences in interproton distance from $H^{\gamma 12}$ (A) on the same residue.

Structure/Function Significance of This Region. The results with α-LA confirm that Ile-95, Tyr-103, Trp-104, and Trp-60 comprise part of a hydrophobic box region. The results were internally consistent from NOE irradiations of three different—but interrelated—resonances, which were also consistent with laser CIDNP results (Berliner & Kaptein, 1981) and the putative model of Warme et al. (1974). This region exists in both Ca(II)- and apo-α-LA *in solution* and is very similar to an analogous region in lysozyme. In the case of lysozyme this hydrophobic box comprises Tyr-20*, Tyr-23*, Trp-28*, Trp-108*, Trp-111*, Leu-17*, Ile-98*, and Met-105*, which border the substrate (saccharide) binding region (Blake et al., 1967). One of the major changes that occurred upon evolution of the α-LA structure from lysozyme was substitution of Ala-107* in the B saccharide binding subsite by a bulky Tyr-103, forming another corner of the hydrophobic box while blocking potential saccharide binding. Several recent experimental results have suggested that the interaction site between α-LA and galactosyl transferase is a very apolar patch (Lindahl & Vogel, 1984; Musci & Berliner, 1985a,b; Berliner et al., 1984). Should this involve the hydrophobic box region, the findings reported here may be useful in studying the interaction site in the lactose synthase complex. There are other differences between α-LA and lysozyme as well. Of the two key carboxylate moieties in lysozyme function, Asp-52* and Glu-35* are missing in α-LA. Also, as noted earlier, α-LA is a strong calcium binding protein whereas lysozyme binds calcium-like cations very weakly (Secemski & Lienhard, 1974; Kurachi et al., 1975; Ostroy et al., 1978). When the calculations of Warme et al. (1974) were completed, the calcium binding properties of α-LA were unknown. Thus, until a crystal structure of both forms is available, a detailed analysis of α-LA structure *in solution* is invaluable. More details of these spectra will be described in later work on the effects of cation binding (H. Nishikawa, K. Koga, J. E. Scheffler, and L. J. Berliner, unpublished results).

ACKNOWLEDGMENTS

We are indebted to Professor Harold A. Scheraga for providing a set of three-dimensional coordinates for the calculated conformations of bovine α-LA. We thank Dr. David Stuart, Oxford University, for sharing some preliminary details of the low-resolution structure of baboon α-LA. We also thank Drs. J. E. Scheffler and H. J. Vogel for helpful discussions.

Registry No. Lysozyme, 9001-63-2.

REFERENCES

Berliner, L. J., & Kaptein, R. (1981) *Biochemistry 20*, 799–807.

Berliner, L. J., Davis, E. D., Ebner, K. E., Beyer, T. A., & Bell, J. E. (1984) *Mol. Cell. Biochem. 62*, 37–42.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1965) *Nature (London) 206*, 757–761.

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967) *Proc. R. Soc. London, B 167*, 378–388.

Bradbury, J. H., & Norton, R. S. (1975) *Eur. J. Biochem. 53*, 387–396.

Browne, W. J., North, A. C. T., Phillips, D. C., Brew, K., Vanaman, T. C., & Hill, R. L. (1969) *J. Mol. Biol. 42*, 65–86.

Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 285–297.

Chapman, G. E., Abercrombie, B. D., Cary, P. D., & Bradbury, E. M. (1978) *J. Magn. Reson.* 31, 459–469.

Hiraoka, Y., Segawa, T., Kuwajima, K., Sugai, S., & Murai, N. (1980) *Biochem. Biophys. Res. Commun. 95*, 1098–1104.

Kalk, A., & Berendsen, H. J. C. (1976) *J. Magn. Reson. 24*, 343–366.

Keller, R. M., & Wüthrich, K. (1981) *Biol. Magn. Reson. 3*, 1–52.

Kurachi, K., Sieker, L. C., & Jensen, L. H. (1975) *J. Biol. Chem. 250*, 7663–7667.

Lindahl, L., & Vogel, H. J. (1984) *Anal. Biochem. 140*, 394–402.

Murakami, K., Andree, P. J., & Berliner, L. J. (1982) *Biochemistry 21*, 5488–5494.

Musci, G., & Berliner, L. J. (1985a) *Biochemistry 24*, 3852–3856.

Musci, G., & Berliner, L. J. (1985b) *Biochemistry 24*, 6945–6948.

Noggle, J. H., & Schirmer, R. E. (1971) *The Nuclear Overhauser Effect*, Academic Press, New York.

Ostroy, F., Gams, R. A., Glickson, J. D., & Lenkinski, R. E. (1978) *Biochim. Biophys. Acta 527*, 56–62.

Permyakov, E. A., Yarmolenko, V. V., Kalinichenko, L. P., Morozova, L. A., & Burstein, E. A. (1981a) *Biochem. Biophys. Res. Commun. 100*, 191–197.

Permyakov, E. A., Kalinichenko, L. P., Morozova, L. A., Yarmolenko, V. V., & Burstein, E. A. (1981b) *Biochem. Biophys. Res. Commun. 102*, 1–7.

Poulsen, F. M., Hock, J. C., & Dobson, C. M. (1980) *Biochemistry 19*, 2597–2607.

Secemski, I. I., & Lienhard, G. E. (1974) *J. Biol. Chem. 249*, 2932–2938.

Wagner, G., & Wüthrich, K. (1979) *J. Magn. Reson. 33*, 675–687.

Warme, P. K., Momany, F. A., Rumball, S. V., Tuttle, R. W., & Scheraga, H. A. (1974) *Biochemistry 13*, 768–782.