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Methionine-90-Spin-Labeled Bovine α-Lactalbumin: Electron Spin Resonance and NMR Distance Measurements†

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ABSTRACT: The unique methionine residue of bovine α-lactalbumin was modified by irreversible alkylation with the bromoacetamido nitroxide spin-label 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl. The line shape of the electron spin resonance (ESR) spectrum was indicative of a fairly mobile spin-label and was sensitive to the calcium-induced conformational change. Paramagnetic broadening of the spin-label ESR lines by a Gd(III) ion substituted at the high-affinity calcium site of the protein yielded a distance between the spin-label and the metal-binding site of 8.0 ± 1.0 Å. The extent of the paramagnetic line broadening by the covalently attached nitroxide spin-label on the proton resonances of several amino acid residues of the protein at 500 MHz allowed estimation of intramolecular distances between the methionine-90 residue and several resolvable protons.

α-Lactalbumin (α-LA)[1] is the regulatory subunit of the "lactose synthase" complex. Upon binding to the enzyme galactosyltransferase (EC 2.4.1.22), lactose is efficiently synthesized from UDP-galactose and glucose. A putative, energy-minimized, three-dimensional structure was proposed by Warme et al. (1974) which was based, in part, on the high degree of primary structure homology between α-LA and lysozyme (Brew et al., 1970; Shewale et al., 1985). More recently, Smith et al. (1987) have reported a low-resolution crystallographic analysis of baboon α-LA, which, while not at the precise atomic resolution of most protein structures, confirmed that α-LA probably evolved from a lysozyme precursor. In spite of their structural similarities, the latter two proteins are quite functionally and physicochemically different. In particular, α-lactalbumin is a metalloprotein, with high affinities for Ca(II) (Permyakov et al., 1981, 1985; Murakami et al., 1982) and Zn(II) (Murakami et al., 1982; Musci & Berliner, 1985a). Although some controversy existed over the classification of α-LA as a strong calcium binding protein, it has been clearly confirmed that the dissociation constant lies in the low nanomolar range (Permyakov et al., 1987; Berliner & Johnson, 1987). Several lanthanides bind specifically to the calcium site (site I), most with higher affinity than that for Ca(II) (Murakami et al., 1982; Berliner et al., 1986).

One of our goals has been to structurally map the α-LA molecule in solution by several spectroscopic methods. A previous report documents several intramolecular distances by fluorescence measurements (Musci & Berliner, 1986). The precise location of the calcium binding site has recently been found definitively by X-ray crystallography (Stuart et al., 1986) to encompass Asp residues 82, 87, and 88 and the carbonyl oxygens of residues 79 and 84. Bovine α-LA contains the same calcium binding loop sequence as corrected by Shewale et al. (1985). A single methionine residue at position

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[1] Abbreviations: α-LA, α-lactalbumin; bromoacetamido spin-label, 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl; 2D NMR, two-dimensional nuclear magnetic resonance; ESR, electron spin resonance; FID, free induction decay; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Met-90-spin-labeled α-LA, α-LA labeled at Met-90 with the bromoacetamido spin-label 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl; Tris, tris(hydroxymethyl)aminomethane.

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90 also exists in the calcium binding loop region of bovine α-LA.

NMR is a powerful tool for elucidating structural relationships between different residues of a protein molecule *in solution*. The applications of two-dimensional NMR techniques to proteins of this size has led to the prediction of the three-dimensional structure *in solution* of several proteins (Wuthrich, 1986). While 2D NMR NOE methods alone may elucidate the primary sequence, secondary structure, and a limited number of short-range tertiary structural distances, methods for obtaining long-range distances have been most successful by incorporating paramagnetic spin-labels at a specific site(s) in the protein of interest (Schmidt & Kuntz, 1984). Nitroxide spin-labels are particularly powerful as their ESR spectra yield information about local conformation and mobility in a macromolecule (Berliner, 1976). Thus, one can take advantage of dipolar interactions (i.e., paramagnetic relaxation) between paramagnetic labels (or a lanthanide cation) and many proton nuclei in order to collect distances to several loci on a protein. Recently, Schmidt and Kuntz (1984) applied this method to obtain a series of intramolecular distances in lysozyme between a covalently bound nitroxide spin-label at His-15 and several amino acid residues. Since the lysozyme X-ray structure was known, and most of the NMR spectrum had been previously assigned, their results confirmed the validity of the paramagnetic relaxation method. In this paper, we report ESR and NMR studies with bovine α-lactalbumin that has been specifically modified at the unique methionine residue 90 with 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl.

Experimental Procedures

Proteins. Bovine α-lactalbumin (lot 52F-8075-1), which typically contained 0.3–0.4 mol of Ca(II)/mol of protein, was from Sigma Chemical Co.

Chemicals. Ultrapure $GdCl_3$ (99.9%, lot 33102) and $CaCl_2$ (99.9%, lot 110482) were from Alfa Products, while lanthanum chloride heptahydrate (99.999%, lot 0797) and $ZnSO_4$ (99.999%, lot 0208) were purchased from Aldrich Chemical Co. Tris(carboxymethyl)ethylenediamine–agarose (lot 121487-87) was from Pierce Chemical Co.; perdeuteriated tris(hydroxymethyl)aminomethane (95% atom, lot 6037) and deuterium oxide (99.8% D_2O) were from Merck Isotopes.

Methods. Spin-labeled α-lactalbumin was prepared by incubating a 1:10 mixture of ca. 1 mM α-LA and 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl for 4–6 days at 4 °C in 0.2 M acetate buffer at pH 3.6, followed by exhaustive dialysis vs the same buffer and finally vs 10 mM Tris buffer, pH 7.4. Met-90-oxidized α-LA was prepared according to Schachter and Dixon (1964). Briefly, the protein was incubated at room temperature for 1 h with 0.38 M H_2O_2 in 0.2 M acetate (pH 3.6), and the reaction was stopped by addition of a small amount of catalase. Gd(III)-α-LA was prepared as previously reported (Musci et al., 1986).

Protein concentration was estimated by optical absorption with an $\epsilon_{280} = 2.01$ mg^{-1} mL. ESR spectra were measured in quartz capillaries (Berliner, 1978) on a Varian E-4 spectrometer that was interfaced to a Varian E-935 Data System. The labeling stoichiometry was estimated by double integration of the spin-label ESR spectrum. Nitroxide rotational correlation times were calculated for an isotropic rapidly tumbling system [see, for example, Berliner (1976, 1978)]. Lactose synthetase rates were measured spectrophotometrically as previously reported (Musci & Berliner, 1985b).

Proton NMR spectra were obtained at 500 MHz on a Bruker AM-500 spectrometer at 300 ± 1 K. The sample buffer was 50 mM perdeuteriated Tris buffer (in D_2O), pH (uncorrected) 7.3. The residual water resonance was suppressed by presaturation with a single radio frequency for 1 s. Typically, 2000 transients were accumulated by digitization of 16K data points followed by zero filling to 32 K before Fourier transformation. The sweep width was 7500 Hz, the pulse width was 2 μs, and the pulse repetition rate was 2.1 s. The extent of broadening of each NMR line due to the presence of the paramagnetic nitroxide, W_p, was calculated after the methods of Schmidt and Kuntz (1984), assuming Lorentzian line shapes, according to

$$h_s/h_o = 1 - W_a/(W_a + W_p) \quad (1)$$

where h_s is the peak height difference between unlabeled and labeled species, h_o and W_a are the peak height and line width, respectively, of the unlabeled species, and W_p is the line-broadening contribution due to the presence of the paramagnetic label. In those regions where spectral overlap was severe, W_a and h_o values were evaluated by the method described in the Appendix.

The distance between the nitroxide group and each proton was calculated with a modified form of the Solomon–Bloembergen equation (Anglister et al., 1984):

$$1/T_{2p} = 2\pi W_p = \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \quad (2)$$

where τ_c is the correlation time of the proton–nitroxide interaction, r is the intramolecular distance, and the other symbols are constants [see, for example, Dwek (1973)].

Results

Met-90 Modification and ESR Experiments. Bovine α-LA was specifically alkylated at the unique Met-90 residue of α-LA by reaction with the (bromoacetamido)piperidine spin-label at pH 3.6 at 4 °C, where 54% labeling was achieved after incubation for 6 days. The low pH was chosen to ensure that only methionine was reactive under these conditions. In order to confirm that only Met-90 was modified, the exposed Met was first oxidized with H_2O_2 before addition of spin-label. The modified (Met-90-oxidized) α-LA did not react (nor did any other residues), as evidenced by no detectable covalently bound nitroxide ESR spectrum after dialysis. Several attempts were made to separate the labeled from the unlabeled protein on DEAE-Trisacryl; however, only slight separation was ever observed. The Met-90-labeled (54%) α-LA retained its ability to participate as a modifier protein in lactose synthase, compared to an unmodified α-LA control; recent work on several other chemically modified Met-90 α-LA's all showed retention of lactose synthase activity, although K_M values were altered in some derivatives (L. J. Berliner, G. Musci, S. A. Sonder, and M. P. Thompson, unpublished results).

The X-band ESR spectrum of spin-labeled apo-α-LA is shown in Figure 1a. The line shape is indicative of a weakly immobilized nitroxide moiety with a rotational correlation time, τ_c, of 1.2 ns. Upon addition of a stoichiometric concentration of Ca(II), a distinct increase in the mobility of the piperidine-*N*-oxyl group was observed ($\tau_c = 0.9$ ns), as evidenced by a narrower line width and increased peak height (Figure 1b). Double integration of the apo- and Ca(II)-α-LA ESR spectra (Figure 1a,b) yielded identical spin concentrations in both samples. This also appeared to rule out the possibility of aggregation of the apo conformer, which could result in possible dipolar interactions between spin-labels on adjacent molecules as a cause for the slightly broader line shape (the

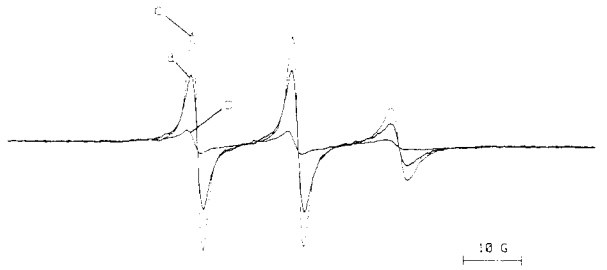

FIGURE 1: X-band ESR spectrum of Met-90-spin-labeled bovine α-LA, 10 mM Tris buffer, pH 7.4. The concentration of spin-labeled α-LA and metal ion was 0.67 mM throughout. (a) Apo-α-LA; (b) Ca(II)-α-LA; (c) Gd(III)-α-LA. Experimental conditions were as follows: microwave power, 20 mW; modulation amplitude, 0.2 G; field set, 3395 G; scan range, 100 G.

line shape did not change over 20 μM to 2 mM α-LA concentrations). The ESR spectrum was insensitive to further addition of Ca(II) (up to 10 mM). On the other hand, when a paramagnetic lanthanide [Gd(III)] was substituted for Ca(II) at *site I*, a substantial decrease (88%) in spectral intensity was observed, concomitant with some line broadening as well (Figure 1c). The effect was due specifically to paramagnetic broadening, since a control spectrum with diamagnetic La(III)-α-LA was identical with that for Ca(II)-α-LA (Figure 1b). The spectral reduction found in Figure 1c is expected when dipolar coupling between two spins occurs; the extent of the (apparent) decrease in signal intensity may be used to calculate a distance between the two paramagnetic species (Leigh, 1970). From the observed 88% signal decrease, we estimated a dipolar coupling constant value, C, of 100 G. The longitudinal relaxation time of Gd(III) was estimated as $(1.5–5.3) \times 10^{-10}$ s, analogous to the case of glyceraldehyde-3-phosphate dehydrogenase (Dwek et al., 1975). The distance, r, between the nitroxide group and Gd(III) was estimated as 8.0 ± 1.0 Å. While the theory of Leigh (1970) assumes that the spins are relatively rigid, the spin-label here was rotating rapidly. This, in effect, tends to reduce the mean dipolar interaction; hence, the distance calculated above is an *upper* limit. It is important to note that, while only 54% of the α-LA sample was modified, the ESR results reflect, of course, only the *labeled* fraction.

Addition of 5 mM Ca(II) to 0.67 mM Gd(III)-α-LA only partially restored the signal's original intensity, confirming previous reports that the lanthanides bind more strongly to α-LA than calcium (Murakami et al., 1982; Musci et al., 1986; Berliner et al., 1987).

NMR Studies. Figure 2 depicts the 500-MHz proton NMR spectrum of Met-90-spin-labeled Ca(II)-α-LA (Figure 2A) and its ascorbate-reduced diamagnetic derivative (Figure 2B). Spectra A and B of Figure 3 depict the same spectra after resolution enhancement by convolution difference. Met-90-spin-labeled α-LA (Figures 2A and 3A) displayed distinct paramagnetic broadening for most lines. Upon addition of 3 mM sodium ascorbate (which reduces the nitroxide to the diamagnetic hydroxylamine), the spectra (Figures 2B and 3B) were almost identical with that of the native protein (not shown). The covalently attached nitroxide moiety did not appear to perturb the overall Ca(II)-α-LA conformation. In order to quantitatively evaluate the paramagnetic relaxation contribution at each resonance, we calculated difference spectra for spin-labeled Ca(II)-α-LA before and after sodium ascorbate addition, as shown in Figure 3C. Note, for example, in the aromatic region, the substantially broadened lines in Figure 3A at 8.08, 7.64, 7.06, and 6.49 ppm (arrows) compared with those of the ascorbate-reduced derivative (Figure

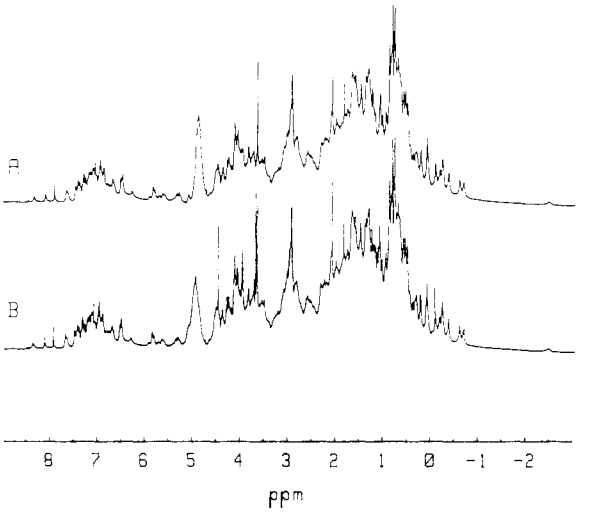

FIGURE 2: The 500-MHz ^{1}H NMR spectra of 1.34 mM Met-90-spin-labeled Ca(II)-α-LA, 50 mM Tris-d_{11}, pH 7.3. (A) Spin-labeled Ca(II)-α-LA. Water presaturation 1 s; pulse repetition time 2.1 s; 2000 scans; 0.5-Hz line broadening. (B) Reduced spin-labeled Ca(II)-α-LA (3 mM sodium ascorbate was added to sample A).

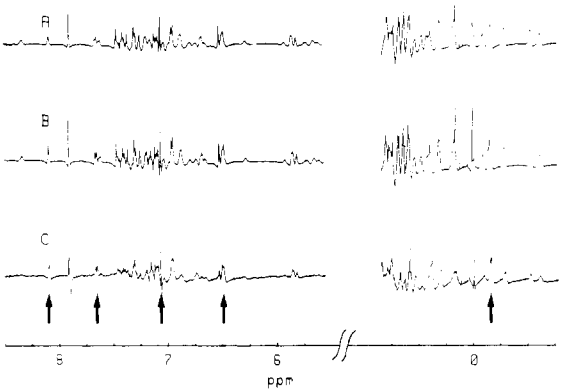

FIGURE 3: The 500-MHz ^{1}H NMR of 1.34 mM spin-labeled Ca(II)-α-LA. (A) Convolution difference spectra of spin-labeled Ca(II)-α-LA (line broadening of 0.5 Hz minus 5 Hz). (B) Convolution difference spectra of reduced spin-labeled Ca(II)-α-LA (line broadening of 0.5 Hz minus 5 Hz). (C) Difference spectra between spectrum B [reduced spin-labeled Ca(II)-α-LA] minus spectrum A [spin-labeled Ca(II)-α-LA]. Line broadening was 0.5 Hz. All other conditions were identical with those in Figure 2.

3B) where the paramagnetic broadening has been eliminated. Some distinct lines in the aliphatic region (e.g., −0.15 ppm) are also notable by their substantial broadening (spectrum A vs spectrum B of Figure 3). The resonance at −0.62 ppm has been previously assigned to the δ-CH_3 of Ile-95 (Koga & Berliner, 1985).

The peak height of each line in the difference spectrum is directly related to the extent of paramagnetic broadening from the spin-label, which is related to W_p (eq 1 under Experimental Procedures). The line-shape parameters, h_o and W_a, were more difficult to estimate where lines severely overlapped in the untreated (non-resolution-enhanced) spectra (Figure 2). In order to circumvent this problem, we developed a procedure to measure these parameters more precisely. Two sets of convolution difference spectra were calculated, from which quite accurate values of h_o and W_a were obtained by an iterative procedure described in the Appendix. Table I lists calculated W_a values, as well as the paramagnetic line-broadening contribution to several distinguishable resonance lines in the spin-labeled protein, several of which have been previously assigned. We have assumed $\tau_c = \tau_r$, the rotational

Table I: Distance Values from Protons to the Bromoacetamido Spin-Label on Methionine-90 of Bovine α-LA

ppm	residue	W_a (Hz)	W_p (Hz)	r(calcd) (Å)
−0.62 (t)	Ile-95 (CH_3^{δ})	10.7	0.74	22 ± 3
−0.54 (d)	NA[a]	10.6	0.64	22 ± 3
−0.03 (d)	NA	11.7	0.8	22 ± 3
−0.15 (d)	Ile-95 ($CH_3^{\gamma 2}$)	9.4	1.8	19 ± 3
5.85	Phe[b]	11.0	2.05	18 ± 3
6.49 (d)	Trp	12.8	3.9	16.5 ± 3
6.53 (s)	NA	5.1	1.55	19 ± 3
6.67	NA	13.4	2.2	18 ± 3
6.70	NA	9.2	1.0	21 ± 3
6.75	NA	4.05	11.0	14 ± 3
6.80	NA	5.2	0.8	21.5 ± 3
6.89 (d)	NA	13.5	1.05	20.5 ± 3
6.98 (d)	Trp-104[c]	8.4	3.6	17 ± 3
7.06	His-68 (C4)	1.2	10.0	14 ± 3
7.17 (t)	Phe[b]	3.3	9.1	14 ± 3
7.20 (d)	NA	7.6	2.8	17 ± 3
7.26 (s)	NA	4.4	0.9	21 ± 3
7.30 (d)	Trp (?)	4.5	3.8	17 ± 3
7.37 (s)	Trp	3.4	0.8	21 ± 3
7.41 (t)	NA	5.3	0.8	21.5 ± 3
7.46 (d)	NA	7.1	1.7	19 ± 3
7.48 (s)	Trp (?)	4.8	0.7	22 ± 3
7.62	His-32 (C2)	10.7	1.3	20 ± 3
7.64 (d)	Trp	5.3	4.5	16 ± 3
7.81	His-68 (C2)	2.4	1.7	19 ± 3
8.08	His-107 (C2)	4.9	11.4	14 ± 3
8.36	Trp-104[c]	12.4	1.6	19 ± 3

[a] Not assigned. [b] See footnote 3 in the text. [c] Recent evidence from temperature-dependent line-width studies of this pair of resonances in the bovine species (Kuwajima et al., 1986) strongly suggests that they arise from a Trp residue rather than initially assigned Tyr-103 (Berliner & Kaptein, 1981). This is confirmed as Trp-104 from 2D NMR studies on both the bovine and guinea pig species (C. M. Dobson, personal communication). Our data are, in fact, consistent with Trp-104, which is in very close proximity to Tyr-103 (Koga & Berliner, 1985); however, this necessitated some changes of previous assignments, which are corrected in Table I. Of course, further 2D NMR data analysis will be needed to fully assign the entire aromatic region.

correlation time of the proton–nitroxide interaction, as this has been shown to be quite valid for the case of a covalently attached nitroxide label. We assumed $\tau_c = 10^{-8}$ s from the overall rotational correlation time after the work on lysozyme (Bauer et al., 1975; Dill & Allerhand, 1979; Schmidt & Kuntz, 1984). The distances calculated from eq 2 are also listed in Table I; the calculations take into consideration the fact that only 54% of the α-LA sample was labeled. The accuracies of the calculated r values contain estimated uncertainties of up to 0.1 in h_s/h_o and up to 25% in line widths in the worst case.

Discussion

The thioether side chain of the unique Met-90 residue of bovine α-LA may be selectively modified with a paramagnetic spin-labeled alkylating agent. This site serves both as an excellent monitor of cation binding to the calcium site and as a "triangulation" or "survey" point for determining long-range intramolecular distances in solution. The modification reported here with the bromoacetamido spin-label 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl yielded a spin-labeled α-LA derivative that retained essentially all of the properties of the native protein (e.g., lactose synthase activity and calcium binding).

Upon binding a stoichiometric concentration of Ca(II) to Met-90-spin-labeled apo-α-LA (Figure 1a), a slight increase in segmental motion near the calcium binding loop was measured, as monitored by the mobility of the nitroxide ring covalently attached to the thioether side chain of this residue. This result is fascinating in the *sensitivity* of the spin-label to cation binding at a site which, while relatively near to Met-90, is nonetheless *several* bonds removed, of which ca. five to six are undergoing relatively free rotation. Yet a distinct, reproducible ESR spectral change was observed upon cation binding to the calcium site, an apparent *increase*, reflecting segmental mobility in the calcium-binding loop region during the apo- to Ca(II)-α-LA transition. This result was surprising in light of multiple evidence that apo-α-LA is partially (thermally) unfolded at 26 °C. Recent NMR evidence demonstrates that the interconversion between the unfolded and folded states is in slow exchange on the NMR time scale, whereas H–D exchange studies showed that the apo-α-LA structure was significantly more flexible than the Ca(II) conformer (Berliner et al., 1987; K. Koga and L. J. Berliner, unpublished results). Evidently, the calcium-binding loop *segmental* motion was slightly faster for Ca(II)-α-LA than for apo-α-LA as distinguished by a 1.2-ns vs 0.9-ns rotational correlation time for the nitroxide moiety. While an isotropic tumbling model was utilized for the τ_c calculations, it is unlikely that the anisotropic tumbling would contribute to the line shape when a model of the labeled protein was being examined [based on the putative structure of Warme et al. (1974)].[2] An alternate model would require that the apo conformer present additional steric hindrance or stronger hydrophobic van der Waals interactions with the nitroxide ring vs the Ca(II) conformer. This latter model also seems unlikely from consideration of the rate of nitroxide rotation and from examination of the protein model, both of which support an essentially freely rotating ring out in the solution environment, analogous to the case of His-15-labeled hen lysozyme (Schmidt & Kuntz, 1984).

The electron–electron dipole–dipole interaction between the paramagnetic cation Gd(III) at *site I* in the calcium-binding loop and the nitroxide moiety at Met-90 yielded a Gd(III)–electron distance of 8.0 ± 1.0 Å. This distance agrees excellently with the model of the labeled protein,[2] in agreement with the calcium-binding sequence location found crystallographically (Stuart et al., 1986).

The calculated distances between the nitroxide moiety and several proton resonances are shown in Table I. While the entire proton spectrum is not yet fully assigned, these calculated long-range distances will complement the complete 2D NMR structure. Of those resonances that have been previously assigned, the agreement with the bovine α-LA model was reasonable considering that the only X-ray structure available is baboon α-LA at 4.5-Å resolution (unknown precise sequence), where side-chain positions have not been satisfactorily resolved (Smith et al., 1987). We have modeled our results on a putative energy-minimized bovine α-LA structure

[2] We assumed that since the observed increase in line width depends on the sixth root of the distance, only the shortest possible distance between each proton and the nitroxyl oxygen should be considered. A three-dimensional model of α-LA was built from the coordinates calculated by Warme et al. (1974). The acetamido nitroxide moiety was then incorporated into the model, and then the widest angle of rotation (cone of tumbling) was estimated visually, taking into account the high mobility of the spin-label and any steric contacts between the label and the protein surface. We then obtained a conic distribution of the probability of finding the unpaired electron in a volume, whose apex is the γ-carbon of Met-90. The spin-label was found to extend 9 Å from the protein surface and to freely rotate in a 55° cone. A minimum distance between the nitroxide group and each proton was obtained, by an iterative analysis where two conditions were satisfied: (i) the nitroxide group was 9 Å from the γ-carbon atom on Met-90, and (ii) the vector traced from this point to the γ-carbon on Met-90 was 55° with respect to the cone axis. It is important to note that this calculated distance was a *forced* minimum from a putative model.

(Warme et al., 1974) which was calculated well before the cation-binding properties of the protein were known. Furthermore, a relatively unrestrained nitroxide rotation model was used in order to obtain only a very *approximate* estimate of proton distances (ca. up to 25%), since we solved for proton positions from carbon atom positions.[2] While some of the aromatic protons have been assigned to specific residues, not all have been unequivocally assigned to a specific ring position. This may account for some discrepancies in distances measured from the model compared to the experimentally measured distances. Nonetheless, the agreement was good for most of the resonances assigned to Tyr-103, Trp-104, and Trp-60.[3] While the distance measured to one proton on Ile-95 (−0.62 ppm) was consistently longer than that for the closest of approach to the nitroxide moiety in the free rotation simulation in the putative model, the overall agreement between model and experiment for the residues that have been assigned was quite satisfactory.

CONCLUSIONS

The unique Met-90 of bovine α-LA serves as a useful labeling point for paramagnetic dipolar relaxation measurements to various points on the protein structure. Experimentally measured distances by both ESR and NMR were in reasonable agreement with a putative bovine α-LA structure. Furthermore, the ESR spectra reported subtle features relating to local segmental motion in the apo- and Ca(II)-α-LA forms. The proton distances should provide valuable long-range distances for the eventual full calculated structure in solution.

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APPENDIX

Estimation of W_a and h_o Values for Lines in Crowded Spectral Regions. Two different sets of convolution difference spectra were obtained by applying exponential multiplication broadening factors of 0.5 Hz − 5 Hz and 0.5 Hz − 30 Hz to the original FID. The following relationships were applied to the peak heights in the convoluted spectra:

$$h_5/h_o = 1 - W_a/(W_a + 5) \quad (3)$$

$$h_{30}/h_o = 1 - W_a/(W_a + 30) \quad (4)$$

where W_a is the intrinsic line width (in Hertz), h_o is the peak height in the original non-resolution-enhanced spectrum, and h_5 and h_{30} are the peak heights in the 0.5 − 5 Hz and 0.5 − 30 Hz convolution difference spectra, respectively. Combining eq 3 and 4 above and solving for the values of W_a and h_o, one calculates

$$W_a = 30(h_{30} - h_5)/(6h_5 - h_{30}) \quad (5)$$

$$h_o = 5h_5[h_{30}/(6h_5 - h_{30})] \quad (6)$$

[3] The resonance at 5.85 ppm was assigned from 200-MHz NOE measurements by Koga and Berliner (1985), but at 500 MHz a group of three resonances (singlet, 5.829 ppm; doublet, 5.855 ppm; triplet, 5.935 ppm) exists. Although the 5.855 ppm proton resonance agreed satisfactorily with the model for Trp-60, this assignment remained somewhat tentative pending the full 2D NMR spin-system assignments which indicate that this pair of lines arises from a Phe side chain (C. M. Dobson, personal communication).

Similar calculations were made for doublets and triplets by taking the measured spin–spin coupling constants into account, although the discrimination between doublets and triplets was not critical to the final distance calculated. Several controls were made on isolated lines of precisely known line widths in the non-resolution-enhanced spectrum in order to experimentally verify the validity of this approach.

Registry No. Met, 63-68-3; Ile, 73-32-5; Trp, 73-22-3; Tyr, 60-18-4; His, 71-00-1; Ca, 7440-70-2.

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NMR Studies of Complex Formation between the Modified Oligonucleotide d(T*TCTGT) Covalently Linked to an Acridine Derivative and Its Complementary Sequence d(GCACAGAA)

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ABSTRACT: The oligodeoxynucleotide d(TTCTGT) was covalently attached to the 9-amino group of 2-methoxy-6-chloro-9-aminoacridine (Acr) through its 3′-phosphate via a pentamethylene linker (m_5). In order to avoid its hydrolysis by nucleases inside the cell, one of its phosphates (TpT) was substituted with a neopentyl group. Complex formation between each of the two purified isomers and the complementary strand d(GCACAGAA) was investigated by nuclear magnetic resonance. The COSY and NOESY connectivities allowed us to assign all the proton resonances of the bases, the sugars (except the overlapping 5′–5″ resonances), the acridine, and the pentamethylene chain. Structural information derived from the relative intensity of COSY and NOESY maps revealed that the duplex d(T*TCTGT)·d(GCACAGAA) adopts a B-type conformation and that the deoxyriboses preferentially adopt a 2′-endo conformation. The NOE connectivities observed between the protons of the bases or the sugars and the protons of the dye show the intercalation of the acridine between the base pairs. NOE connectivities as well as imino proton resonances show that, at room temperature, the C_7 base and the G_8 base belonging to two different duplexes are paired. The pseudoaxial and pseudoequatorial isomers were assigned, and the differences in stability of their complex with the complementary strand are discussed.

The regulation of gene expression in both procaryotes and eucaryotes requires molecules with a high specificity and strong affinity for a nucleic acid base sequence. These biological processes are usually controlled by specific proteins which are able to recognize a base sequence or a nucleic acid local structure (Hélène & Lancelot, 1982).

We have recently synthesized a new family of molecules which are aimed at selectively recognizing nucleic acid base sequences (Asseline et al., 1984a,b). These molecules involve an oligodeoxynucleotide covalently linked to an intercalating dye. The binding of the oligonucleotide to its complementary sequence is a highly specific process governed by stacking interactions between base pairs and by hydrogen-bond formation between complementary bases. The intercalating agent should provide an additional binding energy, through stacking interactions with the base pairs, which is expected to stabilize the specific complex. The intercalating dye was chosen in such a way as to be nonspecific with respect to base or base pair interaction.

Hence, a derivative of 9-aminoacridine (2-methoxy-6-chloro-9-aminoacridine) was covalently attached to the 3′-terminal residue of a deoxyoligonucleotide via a pentamethylene bridge: d(TTCTGT)m_5Acr.

Absorption studies showed that the presence of the dye strongly stabilized the complexes formed by the oligonucleotides with their target sequence (Asseline et al., 1984a,b).

We undertook a nuclear magnetic resonance investigation of the interaction of d(T*TCTGT)m_5Acr[1] with its complementary sequence. The conformation of the duplex is clearly shown. We have already demonstrated (Lancelot et al., 1985, 1986) that oligonucleotides linked to an acridine derivative self-associated at low temperature. In order to avoid aggregate formation, we have synthesized a complementary sequence, d(GCACAGAA), with two more bases on its 5′-terminal side than the oligonucleotide linked to the acridine derivative.

Preliminary investigations on the penetration of oligonucleotides linked to an acridine derivative across the cell membrane have shown that these oligonucleotides were rapidly hydrolyzed by nucleases inside the cell. In order to avoid such a degradation, the synthesized oligonucleotides linked to an acridine residue were substituted on one of their phosphates (TpT) by a neopentyl group (Np). The two isomers were

[1] Abbreviations: NMR, nuclear magnetic resonance; 1D and 2D, one and two dimensional; COSY DQF, double quantum filter 2D correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; $1\bar{3}3\bar{1}$, composite pulse of excitation with alternated phase; d(T*TCTGT)m_5Acr, deoxyribohexanucleotide d(TTCTGT) linked on its 3′-terminal phosphate to the 9-amino group of 2-methoxy-6-chloro-9-aminoacridine via a pentamethylene linker and substituted on its TpT phosphate with a neopentyl group; Acr, 2-methoxy-6-chloro-9-aminoacridine; m_5, pentamethylene chain, $-(CH_2)_5-$; Np, neopentyl group; Aro, H_6 or H_8 proton of base A, T, G, or C; $H_{1'}(5')$ and $H_{2'}(5')$, $H_{1'}$ and $H_{2'}$ proton of the 5′ neighboring nucleotide.

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