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Intramolecular Distance Measurements in α-Lactalbumin†

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Received January 6, 1986; Revised Manuscript Received March 18, 1986

ABSTRACT: The distance between the calcium site (*site I*) and the zinc site (*site II*) in α-lactalbumin was estimated from Forster energy-transfer measurements between donor Eu(III) [or Tb(III)] at *site I* and acceptor Co(II) at *site II* to be 11.5 ± 1.5 Å. Intersite distances were also measured between the bis-ANS [4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]] binding locus and cobalt at *site II* (13.6 ± 1.0 Å), between bis-ANS and a fluorescein moiety covalently bound to Met-90 (33.5 ± 3.0 Å), and between Met-90 (fluorescein) and cobalt at *site II* (16.7 ± 1.0 Å). The apparent K_d for cobalt binding to *site II* agreed well with the value measured previously by intrinsic fluorescence [Murakami, K., & Berliner, L. J. (1983) *Biochemistry 22*, 3370–3374]. A Zn(II) titration of Eu(III)–α-lactalbumin reconfirmed that both *sites I* and *II* can be occupied simultaneously [Musci, G., & Berliner, L. J. (1985) *Biochemistry 24*, 3852–3856], since the lanthanide fluorescence was unaffected.

α-Lactalbumin (α-LA)[1] is a low molecular weight protein found in mammalian milk that plays a crucial role in the biosynthesis of lactose. Furthermore, it has been shown that α-LA is a calcium-binding protein with K_d = 0.2–3 nM (Permyakov et al., 1981, 1985; Murakami et al., 1982). A second cation site, *site II*, is specific for Zn(II), Co(II), Cu(II), and Al(III) (Murakami & Berliner, 1983; Musci & Berliner, 1985a). Specific cation binding to α-LA is important in modulating protein conformation and hence its activity in the lactose synthase complex (Murakami & Berliner, 1983; Musci & Berliner, 1985a,b). A hydrophobic site was found from fluorescence studies with the apolar dye bis-ANS by Musci and Berliner (1985a). The affinity of α-LA for the fluorophore was dependent on the state of metal-ion binding. The precise location of this hydrophobic binding region is important to our understanding of the α-LA–galactosyltransferase interaction, as suggested from kinetic result studies (Berliner et al., 1984) and by the inhibition of lactose synthesis by bis-ANS (Musci

† This work was supported in part by a grant from the U.S. Public Health Service (HD 17270).
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[1] Abbreviations: α-LA, α-lactalbumin; bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]; EDTA, ethylenediaminetetraacetic acid; EF, enhancement factor for bis-ANS binding to α-LA (i.e., the intensity ratio of the binary complex to that of the free dye); ESR, electron spin resonance; GT, galactosyltransferase; IAF, (iodoacetamido)fluorescein; NMR, nuclear magnetic resonance; UDP-Gal, uridine 5′-diphosphate galactose; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

0006-2960/86/0425-4887$01.50/0

& Berliner, 1985b). Although a putative three-dimensional structure of α-LA was proposed from its high sequence homologies with lysozyme (Browne et al., 1969; Warme et al., 1974), no X-ray structure has been reported to date.

Structural studies in solution, such as NMR (Koga & Berliner, 1985) or spectroscopic measurements of specific intersite distances, are quite valuable in determining spatial relationships *in solution*. Lanthanides are well-known for their ability to substitute for calcium ion in proteins; their spectroscopic features make them powerful tools for biochemical investigations at the molecular level (Matthews & Weaver, 1974; Moews & Kretsinger, 1975).

In this paper, we report intramolecular distance measurements in the α-LA molecule between the two principal metal-binding sites, the hydrophobic bis-ANS binding site, and fluorescein-labeled Met-90.

Materials and Methods

Proteins. Bovine α-LA, from Sigma Chemical Co. (lot 52F-8075-1), typically contained 0.34 mol of Ca(II)/mol of protein (untreated "Sigma α-LA").

Chemicals. Tris(carboxymethyl)ethylenediamine was from Pierce Chemical Co. DEAE-Sephacryl was purchased from LKB. Bis-ANS and IAF were from Molecular Probes, Junction City, OR. Ultrapure manganese chloride (99.999%, lot 0518), zinc chloride (99.999%, lot 0208), and cobalt chloride (99.999%, lot 0918) were from Aldrich Chemical Co. Ultrapure europium chloride (99.9%, lot 010578), terbium chloride (99.9%, lot 030878), and EDTA (99.9+%, lot 011581) were from Alpha Products. All other reagents were analytical grade and were used without further purification.

Methods. Apo-α-LA was prepared by repeatedly passing the protein down a column of tris(carboxymethyl)ethylenediamine. It contained less than 2% bound calcium, as estimated from atomic absorption and NMR measurements (Koga & Berliner, 1985). Eu(III)-α-LA and Tb(III)-α-LA were prepared by addition of 1.2–1.4 equiv of the lanthanide to untreated "Sigma α-LA" or apo-α-LA, followed by exhaustive dialysis vs. Chelex 100 treated 10 mM Tris buffer, pH 7.4.

Fluoresceinyl-α-LA was prepared by incubation of a 1:20 mixture of α-LA and IAF for 3–5 days at 4 °C in 0.2 M acetate buffer, pH 3.6, followed by exhaustive dialysis vs. the same buffer and finally vs. 10 mM Tris buffer, pH 7.4. Separation of labeled IAF–α-LA from unlabeled protein was achieved by chromatography by stepwise elution on DEAE-Sephacryl with increasing concentrations of Tris buffer, pH 7.4. A stoichiometrically pure IAF–α-LA sample was obtained after two passes down the column. The labeling stoichiometry was estimated spectrophotometrically by assuming E_{495} = 42 600 M^{-1} cm^{-1}, as used previously for the fluorescein isothiocyanate conjugate of conalbumin (Tenderly & Chang, 1966). Met-90-oxidized α-LA was prepared according to the method of Schachter & Dixon (1964). The protein was incubated at room temperature for 1 h with 0.38 M H_2O_2 (0.2 M acetate buffer, pH 3.5) and then stopped by addition of a small amount of catalase. N-Terminal dansylated α-LA was prepared according to the method of O'Keeffe et al. (1980). Lifetime measurements were also consistent with a single site.

Fluorescence measurements were carried out on an SLM Model 4800S spectrofluorometer at 25 °C without spectral correction. Equilibrium binding data were fit by nonlinear regression analysis (Murakami et al., 1982). Protein concentration was estimated either chemically (Bio-Rad protein assay) for IAF–α-LA, since the IAF moiety also absorbs at 280 nm, or spectrophotometrically on a Perkin-Elmer Lambda 5 instrument, with E_{280} = 2.01 mg^{-1} mL. Bis-ANS concentration was estimated spectrophotometrically with E_{385} = 16 790 M^{-1} cm^{-1} (Farris et al., 1978). Atomic absorption measurements were carried out on a Perkin-Elmer Model 360.

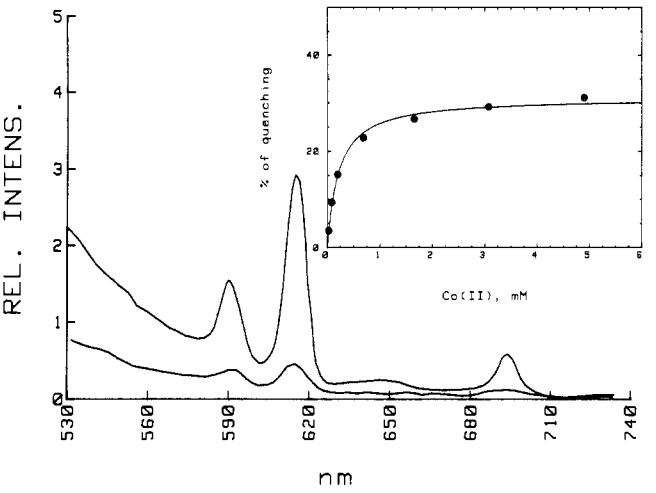

FIGURE 1: Fluorescence emission spectra of 30.3 μM $EuCl_3$ in the absence (lower spectrum) or presence (upper spectrum) of an equimolar concentration of α-LA. Experimental conditions were 10 mM Tris buffer, pH 7.4, 25 °C, and λ_{ex} = 395 nm. (Inset) Co(II) titration of 30.3 μM Eu(III)–α-LA; λ_{em} = 590 nm.

The distance r between a fluorescent donor and an absorbing acceptor can be estimated from the efficiency of energy transfer (Lakowicz, 1983). The "Forster distance" R_0 is defined as the distance, in angstroms, at which 50% energy transfer occurs:

$$R_0 = (9.79 \times 10^{-3})(JK^2\phi\eta^{-4})^{1/6} \quad (1)$$

J, the overlap integral of the donor emission and the acceptor absorption spectra, is described by the expression

$$J = \int_0^\infty F_d(\lambda)\epsilon_a(\lambda)\lambda^4 \, d\lambda \quad (2)$$

where $F_d(\lambda)$ and $\epsilon_a(\lambda)$ refer respectively to the corrected, normalized emission intensity of the donor and the absorption extinction coefficient of the acceptor at wavelength λ. K^2 is a factor describing the relative orientation of the transition dipoles of the donor and the acceptor, ϕ is the quantum yield of the donor, and η is the refractive index of the medium. While K^2 theoretically ranges from 0 to 4, an average value of $K^2 = {}^2/_3$ accounts for random orientations between the emission and absorption dipoles. The refractive index, η, is usually equal to 1.33 for protein media.

The actual distance, r, is then calculated as

$$r = [R_0^6(1 - E)/E]^{1/6}$$

where $E = (1 - I/I_0)$ is the efficiency of energy transfer and I and I_0 are the fluorescence intensities in the presence and absence of the acceptor, respectively.

Results and Discussion

Calcium–Zinc Site Distance. Figure 1 depicts the fluorescence spectrum of a fixed amount of $EuCl_3$ in the absence and presence of a stoichiometric amount of α-LA (10 mM Tris buffer, pH 7.4). Several bands (593, 617, 652, and 698 nm) characterize the Eu(III) fluorescence spectrum. Eu(III)–α-LA complex formation resulted in a 10-fold increase in the Eu(III) emission intensity as shown (upper spectrum). The inset (Figure 1) depicts a cobalt titration of the same 1:1 Eu(III)–α-LA complex, where a net 31% quenching was found

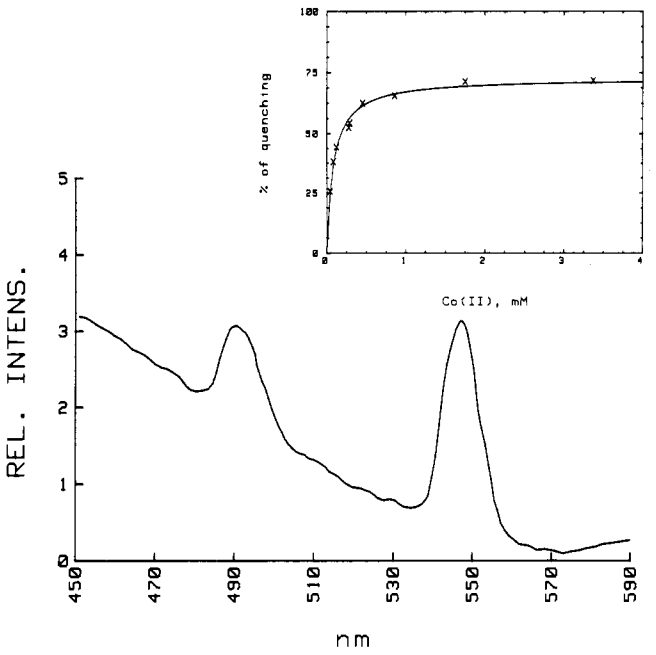

FIGURE 2: Fluorescence emission spectrum of 78.1 μM Tb(III)–α-LA. Experimental conditions were 10 mM Tris buffer, pH 7.4, 25 °C, and λ_{ex} = 374 nm. (Inset) Co(II) titration of 78.1 μM Tb(III)–α-LA; λ_{em} = 545 nm.

for the fully saturated complex. We also examined Tb(III)–α-LA, which displayed two principal emission bands at 490 and 547 nm, respectively (Figure 2). The corresponding cobalt titration is plotted in the inset where the maximum efficiency of quenching was 73%. The data from Figures 1 and 2 were used to calculate a distance between the lanthanide at *site I* and cobalt ion at *site II*. The overlap integral, J, was computed graphically from the emission spectrum of each donor [$J = 2.0 \times 10^{-17}$ M^{-1} cm^3 for Eu(III)–α-LA or 4.5×10^{-17} M^{-1} cm^3 for Tb(III)–α-LA] and the absorption spectrum of Co(II)–α-LA [ϵ_a(515 nm) = 19.0 M^{-1} cm^{-1}, about 4-times higher than for the aquo ion]. We approximated the orientation factor $K^2 = {}^2/_3$, which is a valid assumption when both donor and acceptor are metal ions. The least certain parameter in these calculations was ϕ, the fluorescence quantum yield of the bound lanthanide donors, since their extremely low extinction coefficients were impossible to measure. On the other hand, values of ϕ = 0.2–0.6 have been reported for Tb(III) (Holmquist, 1980) from fluorescence lifetime measurements of inorganic model compounds that contain oxygen donors. We used these values for both Tb(III) and Eu(III), since the dependence of r on the sixth root of the quantum yield leads to a very small uncertainty in the computed distance. We calculated r = 9.7–11.4 Å for Tb(III)–α-LA and r = 10.8–13.0 Å for Eu(III)–α-LA. Since both cations bind to the same site (Murakami et al., 1982), we assigned a mean *site I–site II* distance, 11.5 ± 1.5 Å, with an estimated uncertainty that spans the ranges for r calculated above for each lanthanide. These data were also consistent with ESR measurements of Gd(III)–Cu(II)–α-LA, which predicted that the *site I–site II* distance must be greater than 10 Å (Musci et al., 1985).

The assumption that the observed cobalt energy-transfer quenching was strictly from binding only to *site II* was supported by several pieces of evidence. The binding data fit a simple, single hyperbola, consistent with a single class of binding site. The quenching of lanthanide fluorescence was reversible by competitively displacing the Co(II) with other *site II* specific cations, such as Zn(II) or Al(III) (data not shown). Furthermore, neither Zn(II) nor Al(III) affected Eu(III)–α-LA fluorescence in the absence of Co(II), which also reconfirmed that both *site I* and *site II* could be occupied simultaneously (Musci & Berliner, 1985a).

Bis-ANS–Zinc Site Distance. A cobalt titration of 1 μM bis-ANS/59.4 μM apo-α-LA in 10 mM Tris buffer, pH 7.4, λ_{ex} = 385 nm, was carried out (data not shown). Since K_d = 6.2 μM for the bis-ANS–apo-α-LA complex (Musci & Berliner, 1985a), more than 90% of the fluorophore was complexed under these conditions; furthermore, the large difference in quantum yield between the free and bound forms of the dye precluded the necessity to correct the observed fluorescence intensity. The maximum efficiency of Forster energy-transfer quenching of the 485-nm emission band of bis-ANS by Co(II) was 49%. Almost identical results were obtained for the Ca(II)–α-LA conformer (data not shown). Furthermore, the K_d(app) for Co(II) binding to bis-ANS–α-LA was identical in both cases (0.15 ± 0.01 mM and 0.16 ± 0.01 mM for apo-α-LA and Ca(II)–α-LA, respectively), which agreed well with previously reported values (Murakami & Berliner, 1983). It is important to point out that here the observed titration curves were not simple hyperbolic, since each α-LA conformer [apo-Co(II)–, Ca(II)–, and Ca(II)–Co(II)–α-LA] has different K_d values and enhancement factors (EF) with bis-ANS (Musci & Berliner, 1985a). Therefore, the energy-transfer efficiency calculated for the Co(II)-saturated protein was relative to the Zn(II)-saturated protein as a control, which does not contain a fluorescence acceptor. The details of these calculations are presented in the Appendix. This yielded a corrected energy-transfer efficiency from bis-ANS to cobalt in apo-α-LA and Ca(II)–α-LA of 0.59 and 0.57, respectively, confirming again that the intramolecular distance was identical for apo-α-LA and Ca(II)–α-LA. On the basis of a quantum yield for the bound fluorophore of 0.54 (relative to quinine sulfate in 0.1 N H_2SO_4 as 0.7), an estimated J of 7.3×10^{-17} M^{-1} cm^3, and an assumed $K^2 = {}^2/_3$, a distance of 13.6 ± 1.0 Å was calculated between the bis-ANS and Co(II) sites.

Distances from Met-90. Treatment of α-LA with excess IAF at acid pH under conditions where only the selective alkylation of methionine residues occurs resulted in a stoichiometric incorporation of the label. In order to confirm that only Met-90 was modified under these conditions, we first selectively oxidized Met-90 by hydrogen peroxide according to the method of Schachter and Dixon (1964) and subsequently labeled it with excess IAF, as above, where less than 2% of labeling was observed within experimental error. The excitation band of the bound fluorescein at 495 nm overlapped significantly with the emission band of bis-ANS at 485 nm. In Figure 3 the upper emission spectrum depicts the bis-ANS–α-LA complex, while the lower emission spectrum is the bis-ANS–IAF–α-LA complex at the same concentrations. From the difference in emission intensities at 485 nm, we estimated an energy-transfer efficiency of 75% between the two fluorophores.[2] Where neither the donor nor the acceptor is a metal ion, uncertainties in the value of the orientation factor, K^2, become more significant. We adopted the treatment of Steinberg (1971), who assumed a range of static donor–acceptor orientations that do not change during the lifetime of the excited state, yielding a K^2 = 0.476. For fluorophores bound to macromolecules, segmental motions at the donor or

[2] This is based on the percent quenching of bound bis-ANS at λ_{ex}^{max} = 485 nm by the acceptor IAF, which absorbs at ca. 490 nm. The contribution to the overall fluorescence emission intensity by free bis-ANS is so small that it can be neglected (Musci & Berliner, 1985a).

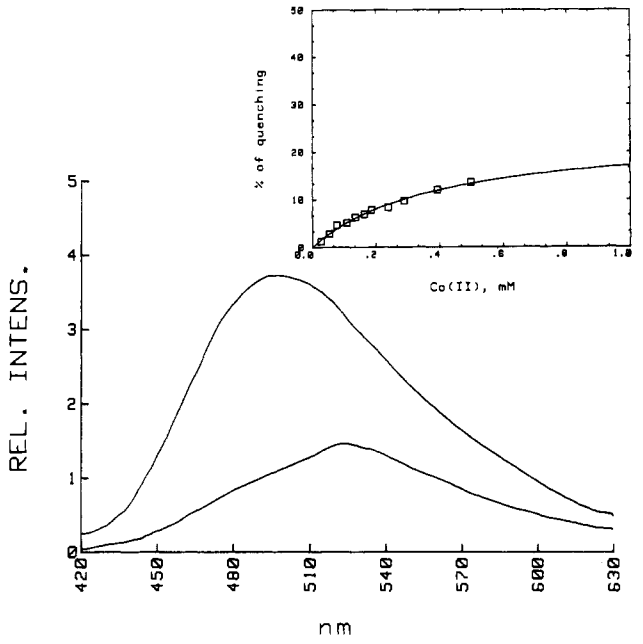

FIGURE 3: Fluorescence emission spectra of bis-ANS complexes with α-LA (upper spectrum) and IAF–α-LA (lower spectrum). Bis-ANS concentration was 7.2 μM and α-LA or IAF–α-LA was 0.38 μM. Experimental conditions were 10 mM Tris buffer, pH 7.4, 25 °C, and λ_{ex} = 385 nm. (Inset) Co(II) titration of 5.7 μM IAF–α-LA in 10 mM Tris buffer, pH 7.4; λ_{ex} = 490 nm, λ_{em} = 515 nm.

the acceptor site could randomize the orientation. However, if the polarization of the donor or the acceptor is less than 0.3, errors in calculated distances are likely to be less than 10% (Haas et al., 1978). We measured polarization values here of 0.26 at 385 nm for bis-ANS–α-LA and 0.34 at 490 nm for IAF–α-LA, which can be assumed to approximate a random orientation model. A fluorescein (Met-90) to bis-ANS distance of 33.5 ± 3.0 Å was calculated from the measured quantum yield for the bound fluorescein of 0.67 and the overlap integral $J = 5.9 \times 10^{-14}$ M^{-1} cm^3.

The inset to Figure 3 depicts a Co(II) titration of 5.7 μM IAF–α-LA (10 mM Tris buffer, pH 7.4), where a maximum energy-transfer efficiency of 0.23 was calculated. With the quantum yield above, an assumed $K^2 = {}^2/_3$, and a measured $J = 5.2 \times 10^{-17}$ M^{-1} cm^3, the distance between the fluorescein moiety on Met-90 and Co(II) at *site II* was calculated as 16.7 ± 1.0 Å.

Dansyl-α-LA. The amino-terminal Glu residue of α-LA was covalently modified with the fluorescent dye dansyl chloride by O'Keeffe et al. (1980), who reported Forster energy-transfer measurements to a cobalt ion bound to galactosyltransferase in a cross-linked GT–α-LA complex. They reported a distance of ca. 32 Å between Co(II) at *site I* of GT and the α-amino group of α-LA. Since it was not known at that time that Co(II) could bind to *both* subunits of that complex (albeit with higher affinity for GT than for α-LA), we investigated Co(II) binding to dansyl-α-LA in the *absence* of GT. Figure 4 shows a Co(II) titration of amino-terminal dansylated α-LA (10 mM Tris, pH 7.4), where 60% quenching was observed at saturating cobalt concentrations. A nonlinear regression best fit of the data, however, more closely approximated two classes, rather than one class, of Co(II) sites in this case (K_1 = 0.22 mM, EF_{max1} = 39.4%; K_2 = 7.8 mM, EF_{max2} = 38.7%). Moreover, titrations with several other nonacceptor cations revealed that essentially every metal ion affected the dansyl quantum yield. A rough estimate for the distance between the dansyl group and the *closest* Co(II) ion was ca. 10 Å,[3] based on a donor quantum yield of 0.05 (relative

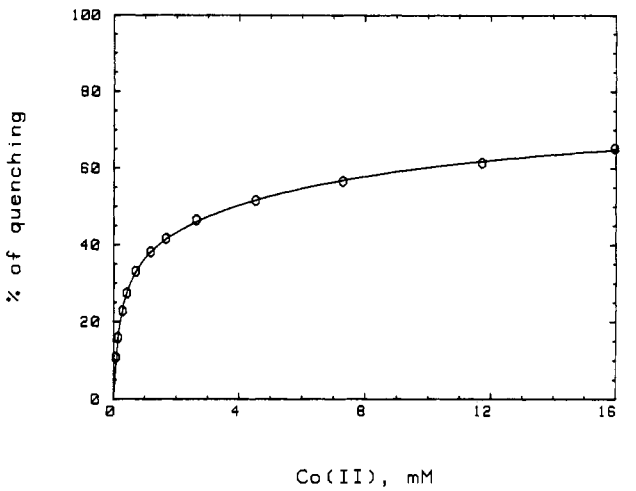

FIGURE 4: Co(II) titration of 29.8 μM dansyl-apo-α-LA. Experimental conditions were 10 mM Tris buffer, pH 7.4, 25 °C, λ_{ex} = 360 nm, and λ_{em} = 515 nm.

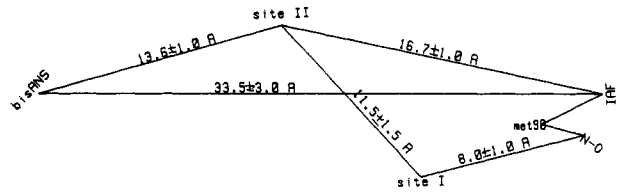

FIGURE 5: A distance map of α-lactalbumin derived from the fluorescence data in this work. The *site I* to N–O (Met-90) distance of 8.0 ± 1.0 Å is derived from an ESR measurement between Gd(III) and a covalently attached piperidinylacetamide spin-label on the Met-90 side chain (L. J. Berliner, K. Koga, and G. Musci, unpublished results).

to a quinine sulfate standard) and $J = 7.2 \times 10^{-17}$ M^{-1} cm^3.

Lastly, it is worth noting that the Co(II) concentration range necessary to significantly quench the dansyl fluorescence (i.e., up to 15 mM) was much higher than that used by O'Keeffe et al. (1980) in the dansyl-α-LA–GT study (up to 80 μM). Therefore, we may conclude that contributions from Co(II) binding to α-LA were not significant in the latter experiments and probably had little effect on the calculated measurement in the GT–α-LA complex.

CONCLUSIONS

The topographical relationships between critical binding loci on the α-LA molecule are important for a detailed understanding of the conformational role of α-LA in lactose synthesis. Fluorescence spectroscopy is a highly sensitive technique that can yield valuable distance information between *specifically* placed donors and acceptors. The experiments outlined above allowed the measurement of several intramolecular distances on the α-LA molecule, which are summarized pictorially in Figure 5. These results, in conjunction with data from other spectroscopic techniques, such as ESR (Musci et al., 1985) and NMR (Koga & Berliner, 1985), should contribute significantly to a structural description of α-LA *in solution.*

ACKNOWLEDGMENTS

We are extremely grateful to Drs. F. G. Prendergast (Mayo Foundation) and R. D. Fugate (SLM-Aminco) for several helpful discussions.

[3] It is worthwhile noting that even if both sites were exactly 10 Å away from the dansyl moiety, the (underestimated) calculated distance would be off by only 10%.

APPENDIX

Calculation of Energy-Transfer Efficiencies for Various Co(II)–α-LA–Bis-ANS Complexes. In the cobalt titration of apo-α-LA the protein goes from an "apo" to a "zinc" conformation, as defined by Musci and Berliner (1985a). There is a difference between the two forms in the EF but not in K_d values. The corrected value for the energy-transfer efficiency (E_{cor}) is therefore described by

$$E_{cor} = 1 - [(1 - E_{obsd})(EF_{apo}/EF_{Zn})] \quad (3)$$

where E_{obsd} is the experimentally observed value ($E_{obsd} = 0.49$) and EF_{apo} and EF_{Zn} are from Musci and Berliner (1985a). We calculated $E_{cor} = 0.59$.

In the case of Co(II) titration of Ca(II)–α-LA, there is a transition from the "calcium" to the "apo-like" conformation as defined by Musci and Berliner (1985a,b). We must take into account a difference both in enhancement factors and in K_d values between the two conformers. Thus here

$$E_{cor} = 1 - (1 - E_{obsd})\left[\frac{(\%\text{ bound bis-ANS})(EF)_{\text{apo-like}}}{(\%\text{ bound bis-ANS})(EF)_{\text{calcium}}}\right]$$

where the percent of bound bis-ANS was calculated from the K_d values reported by Musci and Berliner (1985a). Here, from a $E_{obsd} = 0.50$, we obtained $E_{cor} = 0.57$.

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