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Thermodynamics of the Quenching of Tyrosyl Fluorescence by Dithiothreitol†

Joel K. Swadesh,‡ Philip W. Mui,§ and Harold A. Scheraga*
Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301
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ABSTRACT: Tyrosyl fluorescence quenching by oxidized dithiothreitol (DTT_o) in *N*-acetyl-L-tyrosine *N'*-methylamide, and native bovine pancreatic ribonuclease A and its reduced, S-methylated form, in aqueous solution is studied at pH 3.0. From the temperature dependence of the fluorescence quenching, it is demonstrated that the mechanism of the quenching process is probably static (formation of a complex), and not dynamic (collisional), in origin. Although other quenching mechanisms cannot be ruled out, our proposition that the quenching of tyrosyl fluorescence in these molecules is due to the formation of a complex between the tyrosyl moieties and DTT_o is consistent with previously reported evidence indicating a strong tendency for aromatics to complex with various disulfide-containing compounds. The strength of binding is approximately the same for these three tyrosine-containing compounds, indicating that the microenvironments of their tyrosyl residues may be similar. With 1 M as the reference standard state, the following average thermodynamic parameters are established for the complexation (at 298 K): $\Delta G° = -3.32$ kcal/mol, $\Delta H° = -1.1$ kcal/mol, and $\Delta S° = 7.4$ eu. The large positive value of $\Delta S°$ suggests that hydrophobic interactions may play an important role in the stabilization of such tyrosyl–disulfide complexes; the negative value of $\Delta H°$ suggests that polar interactions may also contribute to the formation of these complexes. Some possible implications with regard to protein-folding studies are discussed.

In a study of the fluorescence of polypeptides (Cowgill, 1967), it was demonstrated that intramolecular disulfides quench tyrosyl fluorescence. The details of the quenching mechanism were not known, but it was determined (Cowgill, 1967) that the quenching was neither collisional nor a direct through-bond effect. More recently, Bodner et al. (1980) noted that, in CCl_4 solution, dimethyl disulfide exhibits a chemical shift change in the proton nuclear magnetic resonance (NMR) spectrum on addition of 1-methylnaphthalene or other aromatic compounds. These authors attributed this behavior to the formation of a complex and estimated the enthalpy of complexation to be about −1 kcal/mol. It was further noted (Morgan et al., 1978; Morgan & McAdon, 1980) that residues containing aromatic rings and sulfur atoms tend to lie near each other in the three-dimensional structures of many globular proteins, suggesting that aromatic–disulfide interactions may stabilize the native conformation. A theoretical study (Némethy & Scheraga, 1981) indicated that the lowest energy complex of dimethyl disulfide and benzene is about 0.8 kcal/mol more stable than the corresponding complex with cyclohexane.

Disulfide-containing compounds have been used extensively in oxidative regeneration of proteins from their reduced forms, e.g., oxidized glutathione (Saxena & Wetlaufer, 1970; Hantgan et al., 1974; Ahmed et al., 1975; Konishi & Scheraga, 1980a,b; Konishi et al., 1981, 1982; Bouet et al., 1982), 2-mercaptoethanol (Creighton, 1979), and DTT_o[1] (Orsini et al., 1975; Creighton, 1977). In these and many other extensive

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[1] Abbreviations: DTT_o and DTT_r, oxidized and reduced forms, respectively, of dithiothreitol; Cys–SS–Cys, cystine; Cys–SH, cysteine; Ox–SS–Ox and Ox–SH, oxidized and reduced forms of a disulfide-containing redox couple; Tris, tris(hydroxymethyl)aminomethane; Gly, glycine; TFA, trifluoroacetic acid; Gdn·HCl, guanidine hydrochloride; RNase A, bovine pancreatic ribonuclease A; EDTA, ethylenediaminetetraacetic acid; 8SMe, disulfide-reduced and S-methylated RNase A; AcTyrNHMe, *N*-acetyl-L-tyrosine *N'*-methylamide.

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studies of oxidative refolding, it has been assumed implicitly that the oxidizing agent, in either its reduced or its oxidized form, interacted *only* with cystine or cysteine, respectively:

$$\text{Cys–SH} + \text{Ox–SS–OX} \rightleftharpoons \text{Cys–SS–Ox} + \text{Ox–SH}$$

$$\text{Cys–SS–Ox} + \text{Cys–SH} \rightleftharpoons \text{Cys–SS–Cys} + \text{Ox–SH}$$

Interaction of the oxidizing agent with residues other than cystine or cysteine was assumed implicitly to be weak or absent. In light of the cited evidence that aromatic compounds and disulfides can complex with each other, this assumption may be incorrect; i.e., such noncovalent interactions could affect the thermodynamics and kinetics of protein folding.

In this paper, we study the quenching of tyrosyl fluorescence by DTT_o and the associated thermodynamics of the quenching process. As fluorescent compounds, we use AcTyrNHMe, RNase A, and 8SMe. The first of these is a model compound, terminally blocked with groups that resemble the peptide bond. The second of these is a compact globular protein, containing six tyrosyl residues, of which three are fully exposed (Scheraga, 1967; Wyckoff et al., 1967). The final compound, 8SMe, is the reduced and S-methylated form of RNase A and should be a good model for a fully denatured protein molecule with little or no residual ordered structures, i.e., in a statistically coiled conformation. By studying the temperature dependence of the fluorescence quenching, we are able to show that the mechanism of the quenching of the tyrosyl fluorescence by DTT_o in these various compounds is probably static (complexation), rather than dynamic (collisional), in origin, in agreement with the evidence cited above, hence indicating a strong noncovalent interaction between disulfide-containing compounds and aromatics.

Experimental Procedures

Materials. Tris, DTT_r, DTT_o, Gly, and type II-A RNase A were purchased from Sigma Chemical Co. A mixed-bed ion-exchange resin (AG 501-X8) and a size-exclusion gel (P-6DG) were purchased from Bio-Rad Laboratories. Methyl 4-nitrobenzenesulfonate (99%) and TFA were obtained from Aldrich Chemical Co. The TFA was distilled from chromium trioxide before use (Pearson et al., 1981; McWherter et al., 1984). (Carboxymethyl)cellulose (CM-52) and Partisil 10 ODS-3 were purchased from Whatman, Inc. Ultrapure Gdn·HCl was obtained from Schwarz/Mann. Constant-boiling hydrochloric acid, used for amino acid analysis, was from Pierce Chemical Co. HPLC-grade acetonitrile was from J. T. Baker Chemical Co.

Purification of RNase A was carried out on a 2.5 × 40 cm column of (carboxymethyl)cellulose, under a linear gradient of 0–110 mM NaCl buffered at pH 8.0 with 10 mM Tris-HCl (Taborsky, 1959). The solution was then desalted by passage through the mixed-bed resin. Acetic acid was added to the desalted solution to a final concentration of 0.1 M, and the frozen solution was then lyophilized.

Preparation of S-Methylated RNase A. To reduce and methylate RNase A, we followed the procedure of Lee and Atassi (1973). A solution of 1.0 M Tris containing 70 mM EDTA was prepared, and the pH was adjusted to 8.5 with HCl. About 3.5 mL of this buffer was added to 7.5 g of Gdn·HCl to make up a solution of total volume 10.0 mL. This solution was purged with nitrogen gas for 10 min, and then 200 mg of RNase A and 180 mg of DTT_r were added. The solution was sealed under nitrogen and stirred for 2 h at ambient temperature. The temperature was raised to 40 °C, and 1.4 g of the methylating reagent, methyl 4-nitrobenzenesulfonate, in 5 mL of dioxane was added. The mixture was stirred for 30 min. Two milliliters of acetic acid and 10 mL of dioxane were added, and the cooled reaction mixture was desalted on a 5.6 × 43 cm P-6DG column at a flow rate of 3.9 mL/min, with 0.5 M acetic acid as the eluent. Following lyophilization, the 8SMe was subjected to reverse-phase HPLC on an SP-8000 liquid chromatograph (Spectra Physics) equipped with a variable-wavelength detector. The sample was loaded onto a 0.46 × 30 cm stainless steel column packed with Partisil 10 ODS-3. A 30-min linear gradient from 98% H_2O/0.09% TFA/2% CH_3CN to 50% H_2O/0.09% TFA/50% CH_3CN was used to elute the sample, which emerged as a single peak as monitored at either 280 or 220 nm. The sample was diluted to about 0.2 mg/mL, acetic acid was added to make the solution 0.5 M in acetic acid, and the frozen solution was lyophilized. The molecular weight of 8SMe was measured by equilibrium ultracentrifugation in a 0.1 M acetic acid solution containing 0.1 M NaCl and was found to be 13 100 [using a value of $\bar{v}$ = 0.703 (Richards & Wyckoff, 1971)], indicating that 8SMe is monomeric. Nitrogen analysis, using triplicate blanks and seven replicate samples, was used to obtain an extinction coefficient, which is reported below.

Table I: Amino Acid Analysis of S-Methylated RNase A (8SMe)

residue[a]	no. of residues	residue[a]	no. of residues
Asx	15.2 (15)	Leu	2.1 (2)
Thr	10.1 (10)	Tyr	5.5 (6)
Ser	13.9 (15)	Phe	2.9 (3)
Glx	12.7 (12)	His	4.0 (4)
Gly	3.0 (3)	Lys	9.8 (10)
Ala	13.1 (12)	Arg	4.2 (4)
Val	9.3 (9)	Cys	0.4 (0)
Met	3.8 (4)	*S*-methyl-Cys[b]	6.7 (8)
Ile	1.9 (3)	Pro[c]	NA

[a] Values of the number of each kind of residue are obtained from a linear least-squares fit (with a correlation coefficient of 0.99) of the normalized areas of the residue peaks to their respective theoretical numbers of residues. Theoretical values are listed in parentheses. Reported values are not corrected for losses due to hydrolysis. [b] Obtained by assuming that it has the same color value as glycine. [c] Not available because of interference from *S*-methyl-Cys, which exhibits a strong absorbance at 440 nm.

Amino acid analyses were carried out by hydrolyzing about 0.1 mg of the protein or peptide at 110 °C in vacuo in about 20 μL of constant-boiling HCl that had been treated with Na_2SO_3 (Swadesh et al., 1984) and then analyzing the hydrolysate on a Technicon TSM autoanalyzer. The amino acid analysis of 8SMe is reported in Table I. From the results of the amino acid analysis, it can be seen that the experimental value (6.7) obtained for *S*-methyl-Cys is lower than the theoretical value of 8.0. Such a discrepancy may reflect, in part, the relative insensitivity of the amino acid analysis as an analytical method for the assessment of the purity of a protein derivative such as 8SMe. Alternatively, the low value obtained for *S*-methyl-Cys can also be attributed to the incomplete methylation of all the eight cysteine residues in the preparation of 8SMe. However, it is reasonable to expect that, despite the incomplete methylation of all its cysteine residues, the fully reduced S-methylated RNase A prepared here will still be denatured, and its overall conformation should be close to that of a statistical coil.

Synthesis of AcTyrNHMe. For the synthesis of AcTyrNHMe, the procedure of Applewhite and Niemann (1959) was followed. The purity of the product was checked by HPLC and by thin-layer chromatography. No impurity was revealed.

Fluorescence Measurements. For fluorescence measurements, a solution of 25 mM Gly and 0.5 mM EDTA was adjusted to pH 3.0 and was filtered through 0.4-μm Millipore

membranes that had been prewashed to desorb fluorescent impurities (Lehrer & Leavis, 1978). Solutions of RNase A, 8SMe, DTT_o, and AcTyrNHMe were prepared from the Gly solution, and the pH of each solution was remeasured and found to be unchanged. Because of the higher solubility of 8SMe at lower pH, all fluorescence measurements were made at pH 3.0 (where RNase still has its native conformation), rather than under more physiologically relevant conditions. The ultraviolet absorption spectrum of each solution was recorded on either a modified Cary Model 14 (Denton et al., 1982) or a Hewlett-Packard Model 8450 spectrophotometer. The concentrations of the absorptive species were determined with $\epsilon_{275} = 9300\ M^{-1}\ cm^{-1}$ for RNase A (Sela & Anfinsen, 1957), $\epsilon_{285} = 273\ M^{-1}\ cm^{-1}$ for DTT_o (Cleland, 1964; Iyer & Klee, 1973), and $\epsilon_{276} = 9600\ M^{-1}\ cm^{-1}$ for 8SMe (determined here). For AcTyrNHMe, $\epsilon_{275} = 1420\ M^{-1}\ cm^{-1}$ (Bailey, 1966; *Handbook of Biochemistry*, 1970) was used. For DTT_o, absorption coefficients at 268 ($\epsilon_{268} = 210.5\ M^{-1}\ cm^{-1}$) and at 303 nm ($\epsilon_{303} = 170.8\ M^{-1}\ cm^{-1}$) were estimated from the absorption spectrum in order to correct the fluorescence measurements for self-absorption. To correct for self-absorption of the fluorophores, the absorption coefficients at 268 nm of 1200, 8100, and 7350 $M^{-1}\ cm^{-1}$ were estimated with a precision of ±10% for AcTyrNHMe, 8SMe, and RNase A, respectively. The concentrations of the solutions were determined to be 90.0 μM (AcTyrNHMe), 12.2 μM (8SMe), 1.91 μM (RNase A), and 24.9 mM (DTT_o); i.e., the solutions were sufficiently dilute to reduce the inner filter effect (which was corrected for nevertheless, see below) and to prevent aggregation.

Fluorescence measurements were carried out (in the presence and absence of DTT_o) with a Perkin-Elmer spectrofluorometer (Model MPF-44B) equipped with a jacketed cell holder connected to a constant-temperature bath. The slit width in the fluorescence measurements was 5 nm, and the excitation and emission wavelengths were 268 and 303 nm, respectively. An automated titrating system was constructed, with an Acculab multichannel pump to deliver the fluorescence quenching solution to a 1.0 cm × 1.0 cm cuvette in which was placed a precisely determined volume of the fluorophore-containing solution. A stirring bar inside the cuvette, rotated by an external magnet affixed to an overhead stirrer, afforded excellent mixing of the solution. The slight turbulence caused by the stirring did not disturb the measurement of the fluorescence. The pump and pipets used in this work were calibrated daily to a precision of no less than 1%. All solutions were purged with argon immediately prior to performing the fluorescence measurements, and the fluorescence compartment and DTT_o solution were kept under an atmosphere of nitrogen to prevent any possible fluorescence quenching by O_2 (Lakowicz & Weber, 1973a,b). Measurement of the UV absorbance of the DTT_o solution at various intervals revealed that possible losses due to evaporation did not measurably affect the DTT_o concentration. In a typical experiment, 2 mL of the fluorophore-containing solution was placed in the cuvette, and the DTT_o solution was added at about 40 μL/min. The fluorescence was measured as a function of time on the strip chart recorder with which the spectrofluorometer was equipped. No fluorescence impurities could be detected in the Gly solution used in this study, nor could any such impurities be detected in the DTT_o solution used for the fluorescence quenching experiments.

Correction for Effect of Dilution and Inner Filter Effect. During the fluorescence titration process, the effective concentration of the fluorophore decreases because of dilution (from the addition of the DTT_o solution) and because of the inner filter effect (Marsh et al., 1982; Lakowicz, 1983).

In our fluorescence titration experiments, DTT_o absorbs at both excitation and emission wavelengths, whereas the fluorophore (tyrosine residue) itself shows absorption only at the excitation wavelength. Thus, taking into account both the effect of dilution and the inner filter effects due to both the fluorophore and DTT_o, the following expression for the final (overall) corrected fluorescence intensity, F_{cor}, was obtained:

$$\log F_{cor} = \log F_a(V_T/V_0) + \log [C_0\epsilon^F_{268}V_0/2V_T + Q_0(\epsilon^{DTT_o}_{268} + \epsilon^{DTT_o}_{303})(V_T - V_0)/2V_T] \quad (1)$$

where F_a is the observed fluorescence intensity, V_0 and V_T are the volumes of the solution before and after the addition of DTT_o, C_0 and Q_0 are the initial concentrations of the fluorophore and DTT_o stock solution, respectively, and ϵ^F and ϵ^{DTT_o} denote the extinction coefficients of the fluorophore and DTT_o, respectively, at the wavelengths specified in the subscripts.

Results

Treatment of Fluorescence Data according to the Stern–Volmer Equation [See Also Moon et al. (1965) and Kunimitsu et al. (1968)]. The physical origin of fluorescence quenching arising from the addition of a quenching agent (quencher) to the fluorophore can be divided into two distinct mechanistic classes. In dynamic (collisional) quenching, the decrease in fluorescence is due to collision between the fluorophore and the quencher. In contrast, static quenching can arise from the formation of a dark complex between the fluorophore and the quenching agent. As will be shown later, the quenching of fluorescence observed in this study probably arises from static quenching and is presumably due to the formation of a nonfluorescent complex between the tyrosyl residue of the fluorophore and DTT_o.

For the association of a fluorophore (F) and a quencher (Q)

$$F + Q \rightleftharpoons F\cdots Q \quad (2)$$

the following relationship between the intensities of fluorescence in the absence (F_0) and presence (F) of the quenching agent can be obtained:

$$F_0/F = 1 + K_{as}[Q] \quad (3)$$

where K_{as} is the corresponding equilibrium (association) constant for the complexation reaction (eq 2). This equation is generally known as the Stern–Volmer relation (Stern & Volmer, 1919; Weber, 1948; Vaughan & Weber, 1970; Lehrer, 1971).

In the case of dynamic (collisional) quenching, the corresponding Stern–Volmer equation is given by (Stern & Volmer, 1919; Weber, 1948; Vaughan & Weber, 1970; Lehrer, 1971)

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_q[Q] \quad (4a)$$

$$F_0 = k_f\tau_0 \quad (4b)$$

where k_q is the bimolecular collisional quenching constant, k_f is the rate constant of the fluorescence process, and τ_0 denotes the excited-state lifetime of the fluorophore in the absence of the quencher. K_q is generally referred to as the Stern–Volmer collisional quenching constant. It should be noted that the Stern–Volmer equation has the same *mathematical* form for both types (static and dynamic) of quenching mechanism. In fact, on comparing eq 3 and 4a, it can readily be seen that the only difference between the two different forms of the Stern–Volmer equation lies in the *physical nature* of the quenching constants.

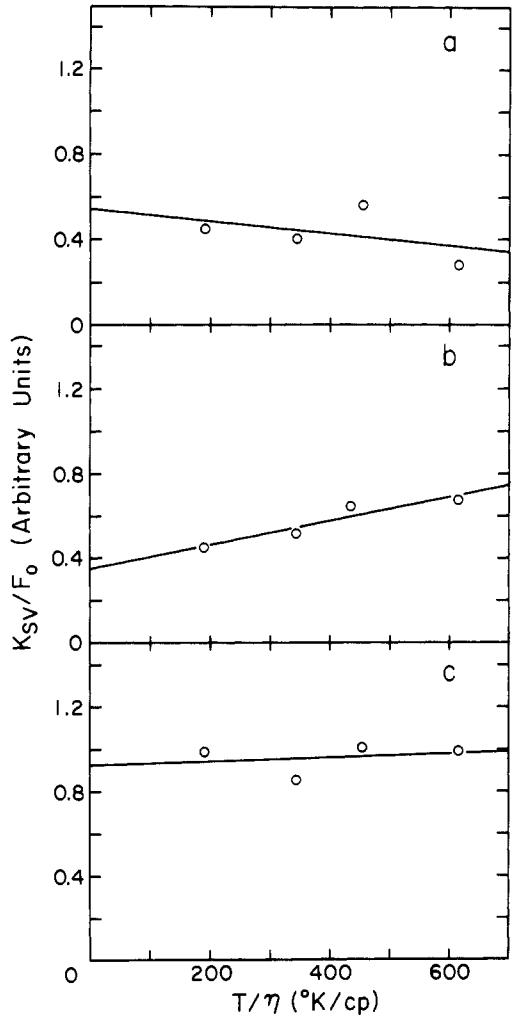

FIGURE 1: Dependence of the quantity K_{SV}/F_0 on T/η for (a) RNase A, (b) 8SMe, and (c) AcTyrNHMe. The viscosity of the solution, η, is expressed in centipoise and is taken to be that of water, at each temperature.

Distinction between the Two Types of Quenching Mechanism (Static vs. Dynamic). Fluorescence titration curves of the three tyrosyl-containing fluorophores (AcTyrNHMe, RNase A, and 8SMe) with the quenching agent were obtained in the concentration range of 0–3.3 mM *added* DTT_o [i.e., uncorrected for complexation (see later section)]. At concentrations of DTT_o higher than the above range, the uncertainty limits of the fluorescence measurements become very large because of the loss of precision in low values of the fluorescence intensity. The results of the fluorescence titrations were plotted according to the Stern–Volmer equation. In the above range of concentrations of *added* DTT_o, *linear* Stern–Volmer plots were obtained for all three fluorophores. In this representation, regardless of the specific type of quenching mechanism, the intercept is identically 1.0, and the slope of the plot corresponds to the association constant for the formation of the complex or the collisional quenching constant, depending on the particular mechanism of the quenching process.

As mentioned previously, the specific type of the quenching mechanism cannot be distinguished by such an approach, because of the fact that the Stern–Volmer equation is of the same mathematical form for both static and dynamic fluorescence quenching. However, for dynamic quenching, fluorescence is quenched through collisional encounters between the fluorophore and the quencher molecule, and the Stern–Volmer quenching constant for such a mechanism is proportional to the bimolecular collisional rate constant, k_q. In the diffusion-controlled limit, the bimolecular rate constant can be expressed by the Smoluchowski (1917) equation:

$$k_0 = \frac{4\pi N_0 R}{1000}(D_f + D_q) \tag{5}$$

where N_0 is Avogadro's number, R is the distance of closest approach between the fluorophore and the quencher (i.e., the sum of the molecular radii of the fluorophore and the quencher), and D_f and D_q are the diffusion coefficients of the fluorophore and the quencher, respectively. Here, k_0 is the diffusion-controlled bimolecular rate constant and is related to k_q by the quenching efficiency κ:

$$k_q = \kappa k_0 \tag{6}$$

For dilute solutions, the diffusion coefficient of a molecule can be approximated by the Stokes–Einstein equation, i.e.

$$D = \frac{kT}{6\pi\eta r} \tag{7}$$

where η is the viscosity of the solution and r is the radius of the molecule in question. From eq 4–7, it can be seen that, in the case of a dynamic (collisional) fluorescence quenching mechanism, the Stern–Volmer quenching constant is linearly proportional to the quantity T/η, viz.

$$K_q/F_0 \propto T/\eta \tag{8}$$

Moreover, in the limit of $T/\eta \rightarrow 0$, the value of K_q/F_0 should also approach zero. In contrast, for a static quenching mechanism, the Stern–Volmer quenching constant corresponds to the equilibrium constant for formation of a complex between the fluorophore and the quencher, and (according to the van't Hoff equation) its logarithm should show a temperature dependence proportional to $1/T$. Hence, the two different types of quenching mechanism (static vs. dynamic) can be distinguished by considering the dependence of the Stern–Volmer quenching constant on temperature and on the viscosity of the solution (Wawilow, 1929; Vaughan & Weber, 1970).

To differentiate between collisional and complexational quenching as a possible mechanism for the fluorescence quenching observed in the tyrosyl-containing fluorophores by DTT_o investigated in this study, K_{SV} is plotted against T/η, where K_{SV} is the observed Stern–Volmer quenching constant, T is the Kelvin temperature, and η is the temperature-dependent viscosity of the solution in centipoise, taken here to be that of water [Hardy & Cottington (1949); listed in the *Handbook of Chemistry and Physics* (1977)]. As can be seen in Figure 1, for all three tyrosyl-containing fluorophores studied here, there is little or no dependence of K_{SV}/F_0 on the quantity T/η. In the case of RNase A, the slope of the linear best fit of the plot is actually negative in sign, which is physically impossible for collisional quenching. More important, however, is the fact that the intercepts of the linear plots are nonzero for all three cases, indicating that the fluorescence quenching mechanism cannot be collisional in nature (Wawilow, 1929; Vaughan & Weber, 1970; Lehrer, 1971).

As a test for the possibility that the mechanism of fluorescence quenching is static, we again examine the temperature dependence of the Stern–Volmer quenching constants. For Figure 2, it can be seen that, within experimental errors, the logarithm of the Stern–Volmer quenching constants for all three systems does show a positive, linear dependence on $1/T$. Such a temperature dependence is, of course, consistent with an exothermic molecular association process.[2] Hence,

Table II: Stern–Volmer Parameters for Quenching of Tyrosyl Fluorescence

	AcTyrNHMe		RNase A		8SMe	
T (K)	slope (M^{-1})[a]	intercept[b]	slope (M^{-1})[a]	intercept[b]	slope (M^{-1})[a]	intercept[b]
279.1	393.1	0.95	302.9	0.99	277.7	1.00
298.3	267.5	1.02	179.9	1.00	230.2	0.96
299.7	275.8	1.06	248.8	0.97	359.4	1.04
310.6	302.1	1.05	311.3	0.98	241.3	1.09
325.1	250.1	0.99	209.7	1.01	219.4	0.98

[a] Depending on the type of quenching mechanism, the slope corresponds either to K_{as} (static quenching) or to K_q (dynamic quenching). See text for a detailed discussion. [b] Ideally, the intercept (a dimensionless quantity) should be identically 1.0.

the Stern–Volmer slopes can be interpreted as association constants of the complexation in all three systems. With the measurements made in the present investigation, we cannot exclude alternative models of fluorescence quenching that have been proposed (Förster, 1959; Eftink & Ghiron, 1981).

Thermodynamic Parameters of Complexation Processes. As mentioned in the previous section, our results indicate that DTT_o quenches tyrosyl fluorescence probably by complexation. However, in order to obtain the true value of the association constant of the complex, K_{as}, it is necessary to correct the initial concentrations of DTT_o and fluorophore for the amounts due to the formation of the complex. To correct for this effect, we use the method of successive approximations, as follows.

The association (equilibrium) constant of the complex is given by

$$K_{as} = \frac{[F\cdots DTT_o]}{[DTT_o][F]} \tag{9}$$

where [F] and $[F\cdots DTT_o]$ are the equilibrium concentrations of the uncomplexed and complexed fluorophore, respectively. The equilibrium concentrations of complexed and uncomplexed fluorophore and DTT_o are related by

$$[F\cdots DTT_o] = [DTT_o]_0 - [DTT_o] \tag{10}$$

$$[F] = [F]_0 - [F\cdots DTT_o] \tag{11}$$

where $[DTT_o]_0$ and $[F]_0$ are the total concentrations (in both complexed and uncomplexed forms) of DTT_o and of the fluorophore, respectively. Substituting eq 10 and 11 into eq 9, the concentration of free (uncomplexed) DTT_o can be expressed in terms of $[F]_0$ and $[DTT_o]_0$:

$$K_{as} = \frac{[DTT_o]_0 - [DTT_o]}{[DTT_o]([F]_0 - [DTT_o]_0 + [DTT_o])} \tag{12}$$

For AcTyrNHMe, $[F]_0$ was obtained directly from UV absorbance measurements. For RNase A, it is known that three of its tyrosine residues are solvent-exposed (Scheraga, 1967; Wyckoff et al., 1967), and $[F]_0$ was taken to be 3 times the concentration of the protein. In the case of 8SMe, $[F]_0$ was estimated from the ratio of the molar fluorescence intensity of 8SMe relative to that of AcTyrNHMe. According to such an estimation, 4.2 tyrosine residues of 8SMe are solvent-exposed. With the above values of $[F]_0$, and the values of K_{as} from the initial Stern–Volmer plots, the concentration of *uncomplexed* DTT_o, $[DTT_o]$, was recalculated from eq 12 for each initial (total) concentration of DTT_o. These newly

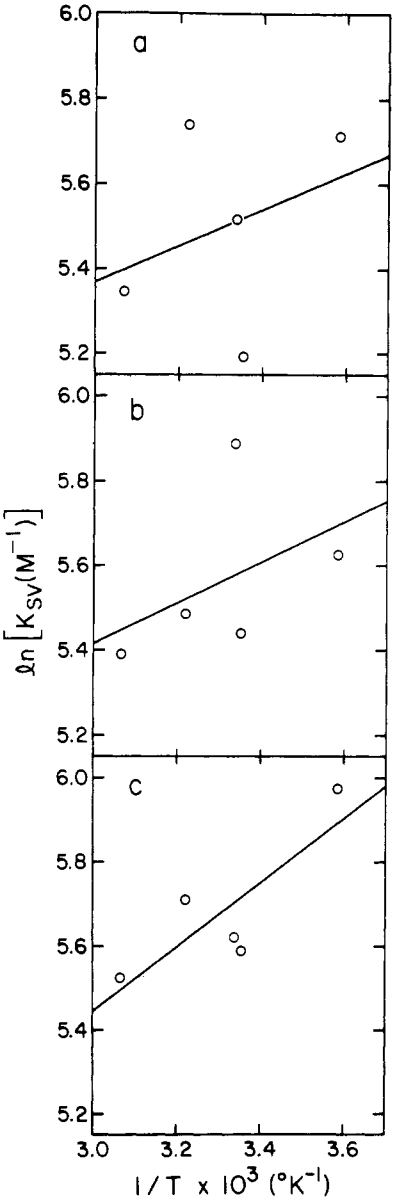

FIGURE 2: Dependence of ln K_{SV} on $1/T$ for (a) RNase A, (b) 8SMe, and (c) AcTyrNHMe.

[2] It should be pointed out that the thermodynamic parameters, $\Delta G°$, $\Delta H°$, and $\Delta S°$, pertaining to the formation of a hydrophobic bond have been shown to be temperature dependent (Némethy & Scheraga, 1962). For a rigorous thermodynamic analysis, it is necessary to obtain the values of $\Delta H°$ and $\Delta S°$ by a parametric fitting of the values of $\Delta G°$ determined at different temperatures to

$$\Delta G° = a + bT + cT^2$$

$$\Delta H° = a - cT^2$$

$$\Delta S° = -b - 2cT$$

However, the large uncertainties inherent in the experimental fluorescence measurements in this study preclude the adoption of such a rigorous approach. Instead, we have obtained the values of $\Delta H°$ and $\Delta S°$ (presented in Table III) from a linear van't Hoff plot, thereby in essence treating these two thermodynamic parameters as constants in our experimental temperature range. Accordingly, the values presented in Table III represent, as a first approximation, the average values of $\Delta H°$ and $\Delta S°$ for the complexation reactions in the temperature range of 279.1–325.1 K.

Table III: Thermodynamic Parameters for Complexation Reactions of AcTyrNHMe, RNase A, and 8SMe with DTT_o at 298 K[a]

	Ac-TyrNHMe	RNase A	8SMe
$\Delta G°$ (1 M)	−3.38 (0.03)	−3.26 (0.07)	−3.31 (0.06)
$\Delta G°$ (unit mole fraction) (kcal/mol)	−5.79 (0.03)	−5.67 (0.07)	−5.71 (0.06)
$\Delta H°$ (kcal/mol)	−1.53 (0.6)	−0.85 (1.3)	−0.96 (1.1)
$\Delta S°$ (1 M)	6.2 (2.0)	8.1 (4.4)	7.9 (3.5)
$\Delta S_u°$ (unit mole fraction) (cal mol^{-1} K^{-1})	14.2 (2.0)	16.1 (4.4)	15.9 (3.5)

[a] Values for the standard enthalpy and standard entropy are obtained as the slopes and the negative of the intercepts of plots of $-R \ln K_{as}$ vs. $1/T$. Values of the standard free energy (at 298 K) are calculated from the relation $\Delta G° = \Delta H° - T\Delta S°$ (see also footnote 2). The values of K_{as} are obtained from Table II. Standard errors are presented in parentheses.

generated values of $[DTT_o]$ were then used to obtain another value of K_{as} from an updated Stern–Volmer plot. The whole procedure was repeated until the value of K_{as} converged to within 0.1%. By the technique of successive approximations, the values of K_{as} typically increased by about 6%, but never by more than 11% of the original value. The corrected values for the Stern–Volmer slopes and intercepts are presented in Table II. It is possible that DTT_o binds to sites other than tyrosyl moieties in RNase A and 8SMe, but the similarity in the values of K_{as} for all three fluorogenic substrates (including that for AcTyrNHMe) indicates that alternative site binding is probably not significant.

Thermodynamic parameters of the complexation reactions for all three fluorophores of AcTyrNHMe, RNase A, and 8SMe, pertaining to both standard states of 1 M and unit mole fraction, are presented along with their associated standard errors in Table III. It is worthwhile to point out one important aspect about such reactions, namely, that these complexation (binding) reactions occur intermolecularly, giving rise to a single product species. As is well-known, the value of the equilibrium constant of such a $2 \rightarrow 1$ reaction depends on the choice of the standard state (Mui et al., 1986). Consequently, the associated change in standard free energy will also be dependent on the reference standard state. The corresponding value of $\Delta S°$ (designated in Table III as $\Delta S_u°$), which pertains to the standard state of unit mole fraction (i.e., pure substance), is generally referred to as the unitary entropy change of the reaction (Gurney, 1953; Kauzmann, 1959). Moreover, it should be further noted that the standard enthalpy change of the reaction is *independent* of the choice of the standard state. By statistical methods,[3] it was shown that the behavior of the three fluorogenic substrates is essentially equivalent.

Discussion

In the foregoing sections, we have presented results demonstrating that the addition of DTT_o can lead to the quenching of tyrosyl fluorescence from all three fluorophores, AcTyrNHMe, RNase A, and 8SMe. With regard to the mechanism of quenching, it is imperative to distinguish between the two major possible types of quenching: (i) dynamic quenching, which is due to collisional encounters between the fluorophore and the quencher, and (ii) static quenching, which arises from the formation of a dark complex between the fluorophore and the quenching agent. The linearity of the Stern–Volmer plots indicates that a single mechanism probably dominates in the quenching process. On the basis of the dependence of K_{SV}/F_0 on the quantity T/η, it is shown that dynamic quenching does *not* play a significant role in the quenching of tyrosyl fluorescence by DTT_o. This conclusion is supported by the fact that the magnitudes of the values of the Stern–Volmer quenching constants presented in Table II are much larger than those typically observed for collisional quenching of tyrosine (Altekar, 1977). The Stern–Volmer constants for the quenching of tyrosyl fluorescence by molecular oxygen, which is a very efficient collisional quencher, are only about 20% in magnitude as compared to the values of K_{as} presented in Table II (Lakowicz & Weber, 1973a,b). Moreover, it is also determined that the magnitude (efficiency) of the quenching observed in the present study decreases with temperature, a characteristic that is inconsistent with a collisional type of quenching mechanism.

The characteristics mentioned above are not inconsistent with a static type of quenching mechanism, however. As shown in the previous section, the logarithm of the Stern–Volmer quenching constants obtained in our present study does display a positive linear dependence on $1/T$, a characteristic consistent with complexation, where such a positive dependence implies a negative value of the enthalpy which, in turn, implies molecular association.[2] The observed Stern–Volmer constants, much larger than those reported for very efficient collisional quenching (Lakowicz & Weber, 1973a,b), may merely reflect a strong molecular association between the fluorophore and the quencher. However, it must be emphasized that there are other types of quenching mechanisms that we have not considered here [e.g., long-range energy transfer (Förster, 1959)]. In order to rule out these alternative mechanisms completely, a more extensive study involving excited-state lifetime measurements would have to be carried out.

There is some corroborative evidence in the current literature that suggests that disulfide-containing compounds form complexes with aromatic compounds. It has been established that the presence of disulfide bonds can lead to a reduction in the fluorescence output of tyrosine and tryptophan residues in proteins (Steiner, 1964; Churchich, 1966; Cowgill, 1966). More important, from a fluorescence quenching study of model compounds and disulfide-containing peptides, it has also been shown that the quenching of tyrosyl fluorescence is very short range and the adjacency (probably within van der Waals distance to each other) of the cystine group to the fluorophore itself is of critical importance (Cowgill, 1967). The requirement of such intimate contact between the cystine and the tyrosine groups is entirely consistent with the above assertion that static quenching plays a significant role in the quenching of tyrosyl fluorescence by DTT_o.

Using statistical methods, Morgan et al. (1978) noted that there are regions in the three-dimensional structures of several different globular proteins in which residues with sulfur-containing side chains (Cys and Met) and aromatic groups (His, Phe, Trp, and Tyr) lie near each other. These authors suggested that the existence of some unusually strong noncovalent interactions between the sulfur atoms and the π-electronic systems of the aromatics might be the underlying physical reason for the presence of such regions (Morgan et al., 1978; Morgan & McAdon, 1980). The existence of a 1:1 complex between various divalent sulfur and aromatic compounds in CCl_4 solution was subsequently demonstrated by NMR spectroscopy (Bodner et al., 1980), and the enthalpy of formation of such complexes was estimated to be about −1

[3] Analysis of the covariance (Sokal & Rohlf, 1969) in the plots of $-R \ln K$ vs. $1/T$ gave a mean sum of squares among the slopes (two degrees of freedom) of 0.02 and a mean sum of squares within the regression (nine degrees of freedom) of 0.16. The value of the F test (i.e., the ratio of the two mean sums of squares) indicated that there is only a 10–25% possibility that the slopes differ from one another.

kcal/mol. In a theoretical study, the interaction energy of dimethyl disulfide with benzene was calculated to be about 0.8 kcal/mol stronger than that with cyclohexane,[4] thus confirming the presence of a strong noncovalent interaction between disulfide-containing and aromatic compounds (Némethy & Scheraga, 1981).

The thermodynamic parameters presented in Table III pertain to the formation of complexes between tyrosyl residues and DTT_o. For all three cases of AcTyrNHMe, RNase A, and 8SMe, our results indicate that formation of a complex between the tyrosyl residue and DTT_o is exothermic, with a value of about −1 kcal/mol for the associated $\Delta H°$. This value of $\Delta H°$ compares well with the range of −0.79 to −0.96 kcal/mol established for the enthalpy of formation of a 1:1 complex between various divalent sulfur containing and aromatic compounds in CCl_4 solution (Bodner et al., 1980), although it must be pointed out that the solution conditions (aqueous vs. CCl_4) are different in these two cases and a direct comparison of the energetics corresponding to the two complexation processes may not be appropriate. However, the fact that both complexation processes are exothermic suggests that the formation of such a complex between aromatics and disulfide-containing compounds may be a general phenomenon.

From the values of the thermodynamic parameters of the complexation reactions presented in Table III, it is obvious that the major contributing factor in the stabilization of the complex between the tyrosyl moieties and DTT_o is entropic, rather than enthalpic, in origin. As a consequence of the complexation process, the solvent molecules surrounding the solvent-exposed tyrosyl residue (Scheraga, 1967; Wyckoff et al., 1967) will have to be displaced and, subsequently, replaced by the complexing molecule, the quencher. Qualitatively, because of the partially nonpolar character of the DTT_o molecule, the net result of the complexation would correspond to a process in which the aromatic side chain of the tyrosyl residue is transferred from a polar (aqueous) to a nonpolar (hydrophobic) environment (Némethy & Scheraga, 1962).

The values of the *unitary* entropy of the complexation reactions investigated in this study range from 14.2 eu in the case of AcTyrNHMe to 16.1 eu for RNase A (Table III). We emphasize that the difference in the values of the unitary entropy for the three systems is not statistically significant.[3] Unfortunately, there are currently no experimental data pertaining to the *entropy* of transfer of the side chain of the amino acid tyrosine. As a comparison, the unitary entropy of formation of a pairwise hydrophobic bond (of maximum strength) between the side chains of two residues, one containing a polar end group (e.g., tyrosine) and the other having a nonpolar side chain, is estimated to be 4–8 eu at 25 °C (Némethy & Scheraga, 1962), whereas the values of the entropies of transfer of aliphatics from a polar to a nonpolar environment are typically in the range of 18–23 eu (Kauzmann, 1959). However, entropic parameters for the formation of hydrophobic bonds in protein molecules, as modeled by the Kauzmann approach, can be treated only as upper bounds to such values (due to the neglect of the presence of the polypeptide backbone in the Kauzmann approach). Interestingly, the values of the entropic parameters obtained in this study for the complexation reactions between DTT_o and a protein fluorophore (viz., RNase A and 8SMe) lie nicely intermediate between the range specified by the two theoretical approaches (Kauzmann vs. Némethy–Scheraga).

It is also important to point out that the reaction pertaining to the formation of a pairwise hydrophobic bond is typically endothermic (i.e., $\Delta H° > 0$), whereas the values of the enthalpy change for the complexation reactions investigated in this study are exothermic ($\Delta H° \simeq -1$ kcal/mol). However, it must also be noted that there are two –OH groups present in the DTT_o molecule. It is possible that a hydrogen bond(s) between the two complexing molecules, or between DTT_o and other neighboring regions of the protein molecule, may be formed as a result of the complexation reaction, thus rendering the energetics of the overall process to be slightly exothermic, rather than endothermic (as would be expected from the standpoint of hydrophobic bond formation *alone*).

On the basis of the results presented in this work, and also those from other studies (Morgan et al., 1978; Bodner et al., 1980; Morgan & McAdon, 1980; Némethy & Scheraga, 1981), it seems that there is a strong tendency for aromatics to be associated (complexed) with disulfide-containing compounds. In the particular case of tyrosyl residues, our results indicate that hydrophobic interactions may play an important role in the stabilization of such complexes. It has been proposed that such hydrophobic interactions may be the dominant physical force behind the formation of certain "nucleation" (or chain folding initiation) structures (Matheson & Scheraga, 1978), which may initiate and direct subsequent conformational folding events (Scheraga, 1973; Wetlaufer, 1973; Tanaka & Scheraga, 1977; Matheson & Scheraga, 1978; Némethy & Scheraga, 1979; Wetlaufer & Rose, 1981; Montelione et al., 1984). Our present study suggests that the formation of a complex between tyrosyl and cystine residues may play a role in the stabilization of certain nucleation structures in the refolding process of RNase A. In fact, tyrosines and disulfides exist in four of the six nucleation sites (viz., B, D, E, and F) proposed by Némethy and Scheraga (1979) for the initial stages of folding of ribonuclease.

It should be pointed out that, in the regeneration of RNase A from the *reduced* protein, the reoxidation of the disulfide bonds is usually accomplished by the addition of an external disulfide reagent, such as oxidized glutathione or DTT_o (Hantgan et al., 1974; Creighton, 1977, 1979, 1980; Konishi & Scheraga, 1980a,b; Konishi et al., 1981, 1982). If, at any stage of the regeneration process, a large excess of such external disulfide-containing reagents is present, the tendency to form *intra*molecular tyrosyl–disulfide complexes (within the protein molecule) may be lowered due to the external competition from the formation of *inter*molecular complexes with the disulfide-containing reagent. However, it must be stressed that, while the formation of an *intra*molecular tyrosyl–disulfide complex corresponds to a unimolecular reaction, an *inter*molecular complex is formed by a bimolecular reaction. In general, a unimolecular reaction is energetically favored over its bimolecular counterpart (Mui et al., 1986; Menger, 1985), although the *magnitude* of such an energetic advantage depends critically on the nature of the reaction itself. Hence, depending on the actual magnitude of the energetic advantage of the formation of *intra*molecular as compared to *inter*molecular tyrosyl–disulfide complexes, the regeneration pathway of the protein molecule (RNase A) from its reduced form may, conceivably, be altered, because of the presence of the large excess of external disulfide-containing reagents in the regeneration process. Interestingly, the pathway for the refolding of RNase A has been shown to depend on the concentrations of oxidized and reduced glutathiones (Konishi et al., 1982).

[4] In the theoretical calculations of Némethy and Scheraga (1981), there was no need to include special π-electron interactions explicitly, since the parameterization of aromatic carbons in the ECEPP (Empirical Conformational Energy Program for Peptides) algorithm (Momany et al., 1975) includes this effect implicitly.

Further work is needed to determine how important disulfide–aromatic interactions are in the folding of proteins accompanying the oxidative formation of disulfide bonds.

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Registry No. DTT_o, 24891-61-0; AcTyrNHMe, 6367-14-2; RNase, 9001-99-4; Tyr, 60-18-4.

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High-Resolution Proton and Laser Photochemically Induced Dynamic Nuclear Polarization NMR Studies of Cation Binding to Bovine α-Lactalbumin†

Lawrence J. Berliner,* Keiko Koga,‡ Hiroyasu Nishikawa,§ and Julie E. Scheffler‖
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210
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ABSTRACT: α-Lactalbumin (α-LA) is a calcium binding protein that also binds Mn(II), lanthanide ions, Al(III), Zn(II), Co(II). The structural implications of cation binding were studied by high-resolution proton (200 MHz) NMR and photochemically induced dynamic nuclear polarization (CIDNP) spectroscopy. Marked changes were observed in the NMR spectra of the apoprotein upon addition of a stoichiometric amount of calcium to yield Ca(II)-α-LA, manifested particularly in ring current shifted aliphatic peaks and in several shifts in the aromatic region, all of which were under slow exchange conditions. The CIDNP results showed that two surface-accessible tyrosine residues, assigned as Tyr-18 and -36, became inaccessible to the solvent upon addition of 1:1 Ca(II) to apo-α-lactalbumin, while Tyr-103 and Trp-104 remained completely accessible in both conformers. The proton NMR spectra of apo-α-LA and Al(III)-α-LA were extremely similar, which was also consistent with intrinsic fluorescence results [Murakami, K., & Berliner, L. J. (1983) *Biochemistry 22*, 3370–3374]. The paramagnetic cation Mn(II) bound to the strong calcium binding site on apo-α-LA but also to the weak secondary Ca(II) binding site(s) on Ca(II)-α-LA. It was also found that Co(II) bound to some secondary sites on Ca(II)-α-LA that overlapped the weak calcium site. All of the lanthanide shift reagents [Pr(III), Eu(III), Tb(III), Dy(III), Tm(III), and Yb(III)] bound under slow exchange conditions; their relative affinities for apo-α-lactalbumin from competitive binding experiments were Dy(III), Tb(III), and Pr(III) > Ca(II) > Yb(III).

α-Lactalbumin (α-LA)[1] is the noncatalytic protein subunit of the lactose synthase complex that functions by modifying the acceptor specificity of galactosyltransferase (UDP-galactose:D-glucose 4-galactosyltransferase, EC 2.4.1.22) from GlcNAc to glucose. It is also known that α-LA is highly homologous to lysozyme in primary structure (Brew et al., 1970) from which three-dimensional modeling studies were reported (Browne et al., 1969; Warme et al., 1974). Koga and Berliner (1985) found a strong structural identity between the two proteins *in solution* in the form of a hydrophobic box comprised of Trp-60, Trp-104, Tyr-103, and Ile-95. In spite of the similarities in structures, these two proteins have dramatically different biological functions. Furthermore, another significant difference between them is their respective metal ion affinities. Recently, α-LA was found to be a metalloprotein that binds Ca(II) very strongly with a dissociation constant K_d = 0.2–3.0 nM (Berliner et al., 1978; Permyakov et al., 1981a, 1987; Murakami et al., 1982; Bryant & Andrews, 1984; Van Ceunebroeck et al., 1985). On the other hand, the K_d values for lysozyme and Ca(II)-like cations are millimolar or higher (Secemski & Lienhard, 1974; Kurachi et al., 1975; Ostroy et al., 1978). We have described the ligand properties of the calcium site by NMR (^{113}Cd) and ESR [Mn(II) and Gd(III)] as essentially identical with those of parvalbumin, troponin C, and calmodulin (Berliner et al., 1983; Musci et al., 1986).

Since this specific multiple cation site binding had been well characterized by other spectroscopic methods, we were interested in attempting to characterize this cation binding from a more detailed structural perspective. The work presented here encompasses high-resolution Fourier transform and CIDNP ^{1}H NMR studies of the effects of diamagnetic and paramagnetic cation binding to bovine α-LA.

Experimental Procedures

Materials. Bovine apo-α-LA (lots 75C8110, 86C8020, 52F80751, and 50F8105) and bovine Ca(II)-α-LA (lot 33F8145) were from Sigma Chemical Co. Metal-free apo-α-LA was prepared by chromatography on tris(carboxymethyl)ethylenediamine–agarose (Pierce Chemical Co., lot 121483-87) at pH 8.0 (Koga & Berliner, 1985). Residual

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*Author to whom correspondence should be addressed.
‡Present address: Tokushima Research Institute, Otsuka Pharmaceutical Co., Tokushima 771-01, Japan.
§On leave from the Department of Physiology, Kyoto Prefectural University of Medicine, Kyoto 602, Japan.
‖Present address: Schering-Plough Corp., Bloomfield, NJ 07003.

[1] Abbreviations: α-LA, α-lactalbumin; GlcNAc, *N*-acetylglucosamine; NOE, nuclear Overhauser effect; CIDNP, chemically induced dynamic nuclear polarization; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; Tris-d_{11}, tris(hydroxymethyl)aminomethane-d_{11}; FT, Fourier transform; FID, free induction decay; bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate].

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