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Authors

Marzola, Pasquina
Gratton, Enrico

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Hydration and Protein Dynamics: Frequency Domain Fluorescence Spectroscopy on Proteins in Reverse Micelles

Pasquina Marzola[†] and Enrico Gratton*[‡]

Dipartimento di Chimica, Università degli Studi, Via Risorgimento 35, 56126 Pisa, Italy, and Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois at Urbana-Champaign, 1110 West Green Street, Urbana, Illinois 61801 (Received: October 29, 1990; In Final Form: June 18, 1991)

Lysozyme, human serum albumin (HSA), and liver alcohol dehydrogenase (LADH) have been studied in reverse micelles by frequency domain fluorescence spectroscopy. The emission of the tryptophanyl residues of the proteins was monitored. Fluorescence and anisotropy decays were measured from 2 to 350 MHz for each protein in reverse micelles and in aqueous solutions. The wide range of modulation frequencies available allowed direct monitoring of the internal motions of tryptophan residues, occurring in the subnanosecond time range, together with the whole protein rotational dynamics in the micelles. The results indicate that the rotational correlation times for the internal motions and the overall protein rotation in reverse micelles decrease with increasing water concentration. Lysozymes showed peculiar rotational dynamics which reflect denaturation occurring as the protein increases its water content in the reverse micelle. This effect was not observed for the other proteins. Dynamic measurements appear useful in understanding structural changes arising from the interactions between proteins and micellar systems.

Introduction

The activation of dynamics in proteins is of major importance for their functionality. Despite the amount of experimental evidence on protein dynamics^{1,2} and the advances in theoretical methods,^{3,4} there are still some basic aspects that need to be understood. Among these aspects is the role of hydration.¹⁻⁵ The effect of water is minimal on the protein structure but it is paramount on its dynamics.¹⁻⁵ The effect of hydration on protein dynamics has been addressed very little in the literature, and its physical origin is virtually unknown. A proposed way to elucidate the role of water on protein dynamics is to study the process of adding water to dry proteins up to the solution state. This kind of study has provided insight on the relationships between protein-water interactions and particular protein functions.^{5,6} At room temperature, in the absence of hydration, proteins are essentially in a frozen state;^{1,5} when the water is gradually added to the proteins, a sudden increase in their dynamics occurs.^{1,5} A recent study on lysozyme hydration indicates that the internal protein dynamics are completely activated (i.e., the characteristic parameters are equal to the solution values) at $h = 0.15$, (where h is defined as $h = \text{grams of } H_2O/\text{gram of protein}$) and that the internal motions are relatively uncoupled from the surface water motions.⁵

The major problem in carrying out these studies is the physical state of the samples; these are dry samples, powders or films, on which the application of standard spectroscopic techniques is difficult.⁷

An alternative way to overcome this problem could be the use of reverse micelles which can host proteins in a small water pool.^{8,9} Reverse micelles are spherical aggregates formed by dissolving amphiphiles in organic solvents.^{10,11} The polar head of the amphiphilic molecule is directed toward the interior of the aggregate, and the hydrophobic tail is in the organic phase. Large biomolecules can be solubilized in the polar core of the inverted micelle with the aid of small quantities of water. Being optically transparent, the micellar suspensions can be studied by optical spectroscopic techniques, and, in addition, the total amount of water in the pool can be easily controlled. Besides the potential in studying protein hydration, and more generally proteins in water-restricted environments, reverse micellar systems have also a practical and technological interest. In fact, it has been shown that some enzymes, when hosted in reverse micelles, display activity which can be larger than in aqueous solution.¹¹ In addition, their use is particularly important in carrying out enzymatic

reactions involving water-insoluble substrates or reaction products.¹² In this frame, the spectroscopic investigation of proteins in reverse micelles could also have a technological interest to elucidate protein location and dynamics and evidence possible correlations among dynamics, location, and activity.

With the purpose of studying protein dynamics in the micellar water pool, we carried out experiments on different proteins in reverse micelles constituted by AOT (sodium bis(2-ethylhexyl) sulfosuccinate) in isooctane, using frequency domain fluorimetry.¹³ This technique allows the measurement of the time dependence of fluorescence decay and anisotropy decay (see Theoretical Equations and Data Analysis). Two experimental approaches to measure the time course of the fluorescence decay are currently used: time and frequency domain fluorescence spectroscopies. In time domain spectroscopy, the sample is excited by a short pulse of light and the emission is detected as a function of the time. The time course of fluorescence intensity is directly measured. In frequency domain spectroscopy, the sample is excited by light with a sinusoidally modulated intensity at a given angular frequency ω ; the emitted light intensity is sinusoidally modulated at the same frequency but delayed in phase and demodulation with respect to the excitation.¹³ The frequency dependence of the phase and modulation of the emitted light, with respect to the incident light, contains all the information needed to determine the time dependence of fluorescence intensity. The usefulness of frequency domain fluorimetry in studying rotational and internal dynamics in proteins has been demonstrated.¹⁴ The possibility to carry out frequency domain fluorescence studies up to 350 MHz allows measurement of the correlation time for motions in the subna-

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* Address correspondence to this author.

[†] Università degli Studi.

[‡] University of Illinois.

nosecond time range.^{14,15} In the present work we focused our attention on three proteins: lysozyme, human serum albumin (HSA), and liver alcohol dehydrogenase (LADH); their fluorescence properties have been characterized in aqueous solution,¹⁶ and two of them (lysozyme and LADH) have been extensively studied in reverse micellar systems.^{11,17,18}

Fluorescence lifetimes and time-resolved differential polarization were measured for the proteins in the micellar environment at varying water contents and compared with the results obtained in aqueous solution. In aqueous solution the tryptophanyl residues of the investigated proteins showed internal dynamics with correlation times in the subnanosecond range, and the overall rotational correlation times were in good agreement with the Stokes-Einstein relationship. The correlation times for the overall rotation and for internal motions changed by insertion in reverse micelles and by varying the water pool size. The protein internal dynamics were activated by increasing the hydration. The comparison of both fluorescence lifetimes and anisotropy decays obtained in reverse micelles with those obtained in aqueous solution showed that structural-dynamical modifications occur in proteins hosted in reverse micelles as detected by fluorescence.

Materials and Methods

Chemicals. Lysozyme (3 times crystallized and lyophilized) and human serum albumin (essentially fatty acid free) were purchased from Sigma and dissolved in buffer Tris-HCl (20 mM, pH 7). The protein solutions were chromatographed on a FPLC apparatus (Pharmacia) to remove possible dimers. A Superose 12 column (Pharmacia), equilibrated with the same buffer, was used. The chromatogram of HSA showed three well-resolved peaks; only the last protein-containing fraction (monomer) was collected.

Liver alcohol dehydrogenase was purchased from Bohering as a crystalline suspension in 20 mM potassium phosphate (pH 7) containing 10% ethanol. To solubilize the protein, the phosphate concentration of the solution was increased up to about 200 mM by adding concentrated phosphate buffer at pH 7; the insoluble fraction was removed by centrifugation. The solution was then applied to the Superose 12 column equilibrated with glycine-NaOH buffer (100 mM, pH 8.8); a single elution peak was obtained, and only its central part was collected. After the purification procedure, the active-sites concentration of LADH was measured by the method of ref 19 and found to be about 96%.

AOT (sodium bis(2-ethylhexyl) sulfosuccinate) was obtained from Sigma and purified according to the method given in ref 20. Relatively high purity was obtained according to the procedure of ref 11. However, even after the purification, the AOT suspension showed a small fluorescence background upon excitation at 300 nm. In all experiments the signal from AOT was less than 5% of the total signal and, in addition, the background fluorescence of the micellar suspension was always subtracted.²¹ High purity isooctane was purchased from American Burdick & Jackson (Muskegon, MI) and used without further purification.

Reverse micellar suspensions were prepared by dissolving AOT in isooctane at a final concentration of 0.11 M. Concentrated stock solutions of proteins in buffer (20 mM Tris-HCl (pH 7) for HSA and lysozyme and 100 mM glycine-NaOH (pH 8.8) for LADH) were *directly* added to the micellar suspension in the fluorescence cuvette by using a Hamilton microsyringe and gently shaken by hand until completely clarified. Varying amounts of buffer solution were added to change the water content, which is expressed as $w_0 = [\text{H}_2\text{O}]/[\text{AOT}]$. Fresh samples were prepared at each w_0 to avoid a long exposure to laser light that could damage

the protein chromophores. For spectroscopic measurements, the protein concentration in aqueous solution was adjusted to give an absorbance of 0.1 at 295 nm. The absorbance in reverse micelles was approximately 0.1 for lysozyme, 0.07 for HSA, and 0.04 for LADH.

Experimental Apparatus. Fluorescence decays and time-resolved anisotropies were measured by using the multifrequency phase modulation fluorometer previously described.¹³ Excitation was provided by a mode-locked Nd-YAG laser (Coherent Antares Model 76S-SGH). The light from the mode-locked laser was used to pump a Rhodamine 6G dye laser (Coherent Model 701-3) which was cavity dumped and externally frequency doubled, providing light at 300 nm. Fluorescence emission was observed after a WG-335 filter. Temperature was kept at 25 °C by use of a thermostated sample holder. For lifetime measurements, color errors were minimized by using a reference solution of *p*-terphenyl in ethanol (lifetime of 1.06 ns). Phase and modulation data were acquired at 10–12 frequencies in the range of 2–350 MHz, with uncertainties of ± 0.2 and ± 0.004 for phase and modulation, respectively.

The subtraction of the signal due to the micellar suspension was carried out according to the method given in ref 21. Briefly, the signals from the blank (in the present case a micellar suspension containing only buffer) and from the sample (micellar suspension containing protein at the same w_0 of the blank) were separately acquired at all frequencies. These signals are essentially sinusoidal oscillating at the frequency of modulation¹³ and can be completely defined by a phase and an amplitude. They can be regarded, on the complex plane, as rotating vectors, and they can be subtracted according to vector subtraction rules. In this way it is possible to obtain the actual signal for the sample, under the hypothesis of a linear system (i.e., no interactions between the fluorophore under investigation and the impurities contained in the blank). All data in reverse micelles, both for the lifetime determination and for the measurement of the decay of the emission anisotropy, were acquired with blank subtraction.

Theoretical Equations and Data Analysis. In frequency domain fluorescence spectroscopy, the samples are excited by using a light sinusoidally modulated in the radio-frequency range. The phase and the modulation of the emitted light, relative to the incident light, measured as a function of the modulation frequency, contain all information to determine the time dependence of fluorescence decay.¹³ Analogously, to determine the time dependence of fluorescence anisotropy, the samples are excited by using linearly polarized, modulated light. The phase difference as well as the modulation ratio between the perpendicular and parallel components of the emission are measured. These terms, which will be referred to as differential phase and modulation ratio, contain all information to determine the time-resolved fluorescence anisotropy.¹⁵

The theory of frequency domain fluorimetry and the relationships between experimental observable quantities in frequency and time domain experiments have been described.^{13,15} The theoretical equations giving the decay of fluorescence intensity and anisotropy are more readily understandable if written in the time domain. In general, the fluorescence decay intensity, $I(t)$, has the expression

$$I(t) = \sum (f_i/\tau_i) e^{-t/\tau_i} \quad i = 1, \dots, n \quad (1)$$

where f_i is the fraction of light contributing to the total fluorescence by the i th component and it is called fractional intensity. Sometimes the pre-exponential factor, α_i , proportional to the product $f_i\tau_i^{-1}$, is used in describing the fluorescence decay. If the decay is described by a distribution of lifetimes,²² the expression for $I(t)$ is

$$I(t) = \int_0^\infty f_i(\tau) \tau^{-1} e^{-t/\tau} d\tau \quad (2)$$

where $f_i(\tau)$ is the assumed distribution function for fluorescence

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TABLE I: Proteins in Aqueous Solutions

Fluorescence Decay Multiexponential Fit Analysis							
	f_1	τ_1/ns	f_2	τ_2/ns	τ_3/ns	$\langle\tau\rangle/\text{ns}$	χ^2
lysozyme	0.82 ± 0.01	1.98 ± 0.03		0.51 ± 0.02		1.7	2.4
HSA	0.67 ± 0.01	7.4 ± 0.2	0.29 ± 0.01	2.8 ± 0.1	0.21 ± 0.02	5.8	3.3
LADH	0.63 ± 0.01	6.2 ± 0.1	0.36 ± 0.01	2.9 ± 0.1	0.10 ± 0.01	4.9	1.0
Gaussian Distributional Fit Analysis							
	c/ns	σ/ns	χ^2				
lysozyme	1.74 ± 0.02	0.81 ± 0.03	2.9				
HSA	5.99 ± 0.07	3.8 ± 0.2	4.7				
LADH	4.94 ± 0.05	1.5 ± 0.1	2.8				
Anisotropy Decay							
	ϕ_p/ns	ϕ_f/ns	f_L	r_0^a	χ^2		
lysozyme	4.8 ± 0.4	0.21 ± 0.05	0.77 ± 0.02	0.31	1.8		
HSA	34 ± 3	0.14 ± 0.05	0.76 ± 0.02	0.31	2.4		
LADH	36 ± 4	0.30 ± 0.06	0.84 ± 0.05	0.31	1.5		

^a r_0 was fixed to 0.31.

lifetimes, generally a Gaussian or a Lorentzian function, completely defined by the center and the width.²² In the present paper, the width of the Gaussian distribution functions is expressed by the variance, σ . Both eq 1 and 2 were used in the analysis of phase and modulation data. The software developed in our laboratory (Globals Unlimited) allows for the simultaneous analysis of multiple data sets²³ (in the present case the varying parameter is the water content). Different types of analysis were carried out on the data and will be reported in the following section. In lifetime measurements, the error in the parameters is calculated by using the covariance matrix as discussed in ref 24.

The time-resolved anisotropy for a protein-bound fluorophore contains contributions from the rotational diffusion of the protein as well as from segmental motions of the fluorophore with respect to the protein itself. Assuming that the segmental motions occur independently from protein rotation, the anisotropy decay $r(t)$ is the product of these separate processes²⁵

$$r(t) = r_0 e^{-t/\phi_p} [\beta e^{-t/\phi_f} + (1 - \beta)] \quad (3)$$

where ϕ_p is the correlation time for the overall rotation of the protein, ϕ_f the correlation time for the segmental motions, and β the fraction of the overall polarization which is lost by the faster process. The constant r_0 is the time-zero anisotropy and has a value of 0.31 for the indole at 300-nm excitation wavelength.²⁶ Equation 3 can be written in the form of a double-exponential decay

$$r(t) = r_0 [f_S e^{-t/\phi_S} + f_L e^{-t/\phi_L}] \quad (4)$$

where $1/\phi_S = 1/\phi_f + 1/\phi_p \approx 1/\phi_f$, since $\phi_p \gg \phi_f$.

The factor f_L can be used to calculate the angular displacement, θ , responsible for the faster motion:²⁵

$$\cos^2 \theta = (2f_L + 1)/3 \quad (5)$$

For anisotropy decay measurements, the error in the parameters is calculated by using the rigorous error analysis provided by the Globals Unlimited software.²⁷

Results

In the present investigation three proteins have been studied; the results obtained are reported separately for the different proteins. For each protein we report the results of lifetimes and fluorescence anisotropy measurements in aqueous solutions and in reverse micelles at varying w_0 .

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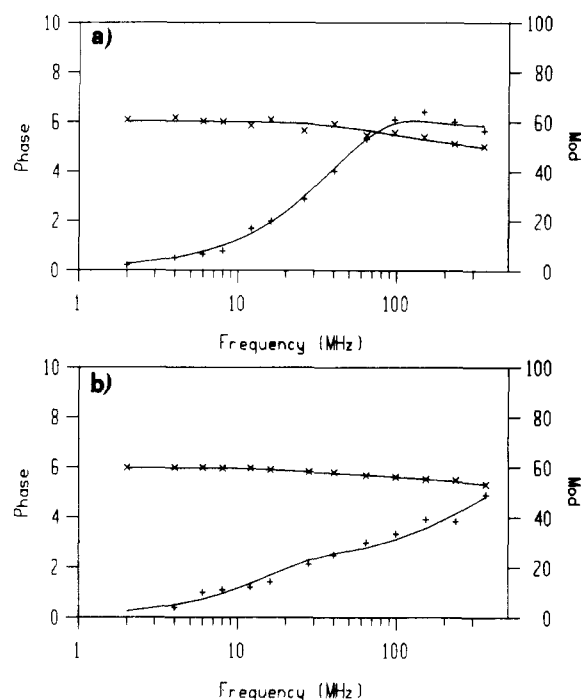


Figure 1. (a) Differential phase and modulation ratio for lysozyme in aqueous solution at pH 7. (b) Differential phase and modulation ratio for lysozyme in reverse micelles at $w_0 = 25.2$.

1. Lysozyme. a. Aqueous Solutions. The frequency domain phase and modulation data for lysozyme in aqueous buffered solution at pH 7 are analyzed in terms of a multiexponential decay and continuous unimodal Gaussian distribution of lifetimes. The results from the analysis are presented in Table I. The best fit is obtained by using a double-exponential decay. The lifetimes measured for lysozyme in aqueous solution (1.98 and 0.51 ns) are in good agreement with previously reported data.²⁸ Lysozyme contains six tryptophan residues, but more than 80% of the fluorescence arises from Trp-62 and/or Trp-108.²⁹ On the basis of experiments with modified derivatives, the long component was assigned to Trp-62 and the short component to Trp-108 and other emitting residues.²⁸ The analysis in terms of a continuous unimodal Gaussian distribution results in a distribution centered at 1.74 ns, close to the average lifetime, calculated as $\tau = \sum f_i \tau_i$.

Figure 1a shows the differential phases and the modulation ratios as a function of the modulation frequency for lysozyme in

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TABLE II: Lysozyme in Reverse Micelles

Fluorescence Decay Multiexponential Fit Analysis ^a $\chi^2_{\text{GLO}} = 1.7$						
w_0	f_1	τ_1/ns	f_2	τ_2/ns	τ_3/ns	$\langle\tau\rangle/\text{ns}$
2.7	0.51 ± 0.01	4.99 ± 0.06	0.40 ± 0.01	1.72 ± 0.02	0.37 ± 0.01	3.3
4.2	0.46 ± 0.01		0.44 ± 0.01			3.1
8.4	0.41 ± 0.01		0.47 ± 0.01			2.9
12.6	0.40 ± 0.01		0.48 ± 0.01			2.9
16.8	0.40 ± 0.01		0.47 ± 0.01			2.8
19.3	0.44 ± 0.01		0.45 ± 0.01			3.0
25.2	0.42 ± 0.01		0.47 ± 0.01			2.9
33.6	0.41 ± 0.01		0.47 ± 0.01			2.9
42.0	0.40 ± 0.01		0.49 ± 0.01			2.9
50.4	0.39 ± 0.01		0.49 ± 0.01			2.8

Gaussian Distributional Fit Analysis $\chi^2_{\text{GLO}} = 3.3$		
w_0	c/ns	σ/ns
2.7	2.77 ± 0.08	2.2 ± 0.1
4.2	2.57 ± 0.08	2.1 ± 0.1
8.4	2.33 ± 0.08	2.1 ± 0.1
12.6	2.20 ± 0.08	2.1 ± 0.1
16.8	2.19 ± 0.07	2.1 ± 0.1
19.3	2.20 ± 0.08	2.1 ± 0.1
25.2	2.19 ± 0.07	2.1 ± 0.1
33.6	2.20 ± 0.07	2.1 ± 0.1
42.0	2.25 ± 0.07	2.0 ± 0.1
50.4	2.28 ± 0.07	2.0 ± 0.1

^aThe analysis was carried out by linking the values of lifetimes throughout the varying water contents.

buffer. The solid line represents the best fit of the experimental data obtained by using eq 4. The fit parameters are shown in Table I; r_0 was fixed to the theoretical value of 0.31. (Note: The anisotropy decay data for proteins in aqueous solution were also analyzed by allowing the parameter r_0 to vary. In this way we obtained the following results: (1) lysozyme, $\phi_p = 5.1$, $\phi_r = 0.52$, $f_L = 0.80$, $r_0 = 0.295$; (2) HSA, $\phi_p = 33$, $\phi_r = 0.090$, $f_L = 0.70$, $r_0 = 0.337$; (3) LADH, $\phi_p = 34$, $\phi_r = 0.10$, $f_L = 0.69$, $r_0 = 0.366$. It can be observed that while ϕ_p does not change, with respect to the data reported in Table I, ϕ_r is affected by the value of r_0 .) The two rotational correlation times, 0.21 and 4.8 ns, can be attributed to rapid internal motions of tryptophanyl residues and to the overall rotation of the protein, as previously suggested.¹⁴ The longer value is in agreement with the value calculated by the Stokes-Einstein equation for a molecule of the size of lysozyme tumbling in water. The coefficient f_L allows estimation of the average angular displacement relative to the fast motion, which is about 23° according to eq 5. The lysozyme dynamics probed by time-resolved fluorescence anisotropy of tryptophan residues was previously reported.^{14,16} In ref 16, the anisotropy decay of lysozyme at 20 °C was analyzed by using a single exponential with a rotational correlation time of 3.8 ns. This low value of ϕ_p was explained by assuming that internal motion of the tryptophan residues may influence the observed anisotropy decay. The measurements shown in the present work allow us to distinguish between rotational and internal dynamics.

b. Reverse Micelles. The steady-state fluorescence (spectra not shown) of lysozyme in reverse micelles shows a significant blue shift (about 10 nm) in the emission maximum at low w_0 compared with the spectrum in buffer. This shift decreases by increasing the water content; at high w_0 ($w_0 \geq 10$) the maximum of emission spectrum approaches the value of buffer. This finding is consistent with the data reported for lysozyme in reverse micelles constituted by AOT-heptane.⁹

For time-resolved experiments w_0 values from 2.7 to 50.4 were explored. The frequency domain phase and modulation data for lysozyme in reverse micelles are more complex than in aqueous solution. A good fit of the experimental data in reverse micelles is obtained by using a triple-exponential decay, composed by a relatively long lifetime (between 4.9 and 6 ns), accounting for more than 40% of the total fluorescence, and two shorter lifetime components of about 2 and 0.4 ns. This finding holds for all w_0 values. A good fit is also obtained by linking the three lifetime values throughout the w_0 range and allowing the fractional in-

tensties to vary. The results from this global analysis are presented in Table II. From Table II it can be observed that two of the three lysozyme lifetimes in reverse micelles are close to the lifetimes in aqueous solution. In fact, reliable fits are also obtained when two of the lifetimes are fixed at the values found in aqueous solution (1.98 and 0.51 ns) while the third lifetime is allowed to vary. It could be tempting to conclude that the fluorescence from Trp-108 and Trp-62 does not change when lysozyme is incorporated in reverse micelles and that the long lifetime corresponds to an enhanced fluorescence from other residues, owing to conformational alterations. However, since the ratios of the values of $\alpha_i = f_i/\tau_i$ change significantly with respect to the values found in solution, we believe that a better explanation is that the fluorescence of the emitting lysozyme residues is changed by incorporation in reverse micelles, due to conformational changes or to solvent effect. Lysozyme is positively charged at pH 7 (its isoelectric point is between 10.5 and 11.0³⁰); however, the dramatic conformational changes observed in lysozyme hosted in reverse micelles seem to not depend on the pH¹⁸ and are likely due to denaturation. The fluorescence decay data for lysozyme in reverse micelles are also analyzed by assuming a Gaussian distribution of lifetimes. The fit parameters are reported in Table II. The center of the distribution decreases from 2.77 (at $w_0 = 2.7$) to 2.28 ns (at $w_0 = 50.4$). The distribution width in reverse micelles is higher than the width obtained by the distribution fit in solution, being 2.2 ns at $w_0 = 2.7$. This value slightly decreases at high w_0 . This finding is in agreement with data previously reported for time-resolved fluorescence decay of lysozyme in reverse micelles.⁹ The larger lifetime distribution width observed in reverse micelles is probably correlated with a higher heterogeneity of conformations and environments experienced by the protein.²²

In Figure 1b, a representative curve of the differential phase and the modulation ratio data for lysozyme in reverse micelles ($w_0 = 25.2$) is shown. In the w_0 range investigated, these curves are found to be significantly different from those obtained in aqueous solution (Figure 1a). At each w_0 a good fit of the data can be obtained by using two rotational correlation times according to eq 4. In all cases, the longer correlation time is around 20 ns, and the shorter is always less than 0.5 ns. The best fit of the experimental data is obtained by linking the shorter rotational correlation time and the r_0 value throughout the different water contents. Table III shows the fitting parameters obtained when

TABLE III: Lysozyme in Reverse Micelles

w_0	Anisotropy Decay ^a		f_L	r_0
	ϕ_p /ns	ϕ_t /ns		
2.7	23 ± 2	0.21 ± 0.02	0.72 ± 0.01	0.291 ± 0.003
4.2	21 ± 2		0.71 ± 0.01	
8.4	21 ± 2		0.68 ± 0.01	
12.6	25 ± 3		0.64 ± 0.01	
16.8	18 ± 2		0.67 ± 0.01	
19.3	23 ± 2		0.66 ± 0.01	
25.2	20 ± 2		0.68 ± 0.01	
33.6	20 ± 2		0.66 ± 0.01	
42.0	21 ± 2		0.64 ± 0.01	
50.4	20 ± 2		0.55 ± 0.01	

^aThe analysis was carried out by linking the values of ϕ_t and r_0 throughout the varying water contents.

this linking scheme is used. The value of ϕ_p is not significantly dependent on w_0 and is always higher than the rotational correlation time of the protein in aqueous solution. This long value may indicate conformational alterations and interactions between lysozyme and AOT molecules. The internal dynamics of lysozyme is dependent on the water content: the f_L value decreases from 0.72 ($w_0 = 2.7$) to 0.55 ($w_0 = 50.4$), indicating a parallel increase of the angular displacement responsible for the faster motion. The value found for f_L at $w_0 = 2.7$ (0.72) indicates that local motions occur with an angle of about 25° even at low w_0 . Since the diffusion coefficient (which is expressed by $R = (1/6\phi_p)[4\theta^2/\pi^2]$, for a motion occurring within a cone of semiangle θ)³¹ is higher than that in water, we could conclude that higher amplitude internal protein motions are allowed in reverse micelles than in solutions.

2. Human Serum Albumin. a. Aqueous Solutions. The results from the analysis of phase and modulation data of HSA in aqueous solution at pH 7 are presented in Table I. Although a single tryptophanyl residue is present in HSA, the protein shows a quite complicated fluorescence decay, even in aqueous solution. The time dependence of fluorescence intensity is analyzed by using a triple-exponential decay in agreement with previous results.¹⁶ Table I also shows the results obtained by analyzing the HSA decay in terms of a unimodal Gaussian distribution of lifetimes; the distribution width appears to be considerably wide. In a protein containing a single tryptophanyl residue, this finding indicates a wide heterogeneity of conformational substates.²²

Figure 2a shows the differential phase and the modulation ratio for HSA in buffer at pH 7. The continuous line represents the best fit of the experimental data obtained with the parameters reported in Table I; the limiting anisotropy, r_0 , is fixed to the theoretical value of 0.31. (See Note under section 1a.) The anisotropy decay of HSA in buffer is analyzed by using two rotational correlation times: 34 and 0.14 ns. The longer value, which represents the rotational correlation time of HSA, is slightly higher than the value calculated by using the Stokes-Einstein relationship, but it could reflect the nonspherical shape of this protein.³² The second component is attributed to internal motion of the single tryptophan residue of HSA; from the value of f_L we estimate that this internal motion corresponds to a rotation of about 23°. Studies of HSA dynamics in aqueous solution by time-resolved fluorescence spectroscopy were previously reported.^{16,31} In ref 31, the correlation times for the overall protein rotation, as well as for the tryptophanyl internal motion, were measured: the results reported are consistent with the values measured in the present investigation.

b. Reverse Micelles. The HSA emission spectra in reverse micelles (spectra not shown) are significantly blue shifted (about 10 nm) when compared to the spectrum in buffered aqueous solution. This shift is not dependent on the w_0 value, at least in the investigated range ($w_0 = 4.1$ –50.4). The isoelectric point of HSA is 5.85,³³ and at pH 7, the protein is negatively charged.

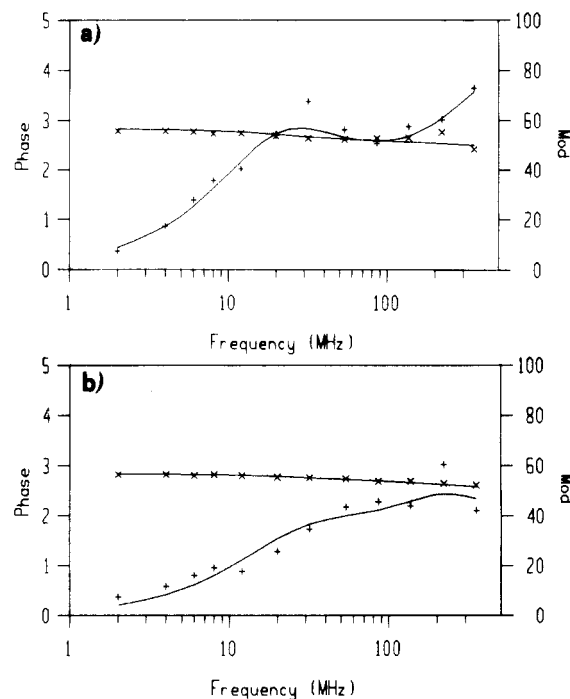


Figure 2. (a) Differential phase and modulation ratio for HSA in aqueous solution at pH 7. (b) Differential phase and modulation ratio for HSA in reverse micelles at $w_0 = 33.5$.

The phase and modulation data are analyzed by using a three-component exponential decay or a Gaussian distribution of lifetimes. A good fit of the experimental data in reverse micelles is obtained by using a triple-exponential decay composed by a long lifetime component (between 6 and 7 ns) accounting for about 30% of the fluorescence, a component with lifetime around 2.5 ns, accounting for about 60%, and a 0.5-ns component. This finding holds for all investigated w_0 . A good fit is obtained also by linking the three lifetime values throughout the w_0 range and allowing the fractional intensities to vary. The results from this global analysis are shown in Table IV. The data were not analyzable by fixing the lifetime values to the values found in water. The average lifetimes, reported in the seventh column of Table IV, are significantly shorter than the average lifetime of HSA in aqueous solution. The results from the distribution analysis are also shown in Table IV. The center and the width of the Gaussian distribution decreases by increasing the water concentration. The width of the Gaussian distribution is lower with respect to that found in aqueous solution, in contrast to lysozyme.

A representative curve of the differential phase angles and the modulation ratios for HSA in reverse micelles, obtained at $w_0 = 33.5$, is shown in Figure 2b. The continuous line represents the best fit of the experimental data. This fit is obtained by using eq 4 and linking r_0 throughout the varying water contents. The fitting parameters are reported in Table V. The longer component

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TABLE IV: HSA in Reverse Micelles

Fluorescence Decay Multiexponential Fit Analysis ^a $\chi^2_{\text{GLO}} = 3.7$						
w_0	f_1	τ_1/ns	f_2/ns	τ_2/ns	τ_3/ns	$\langle\tau\rangle/\text{ns}$
4.1	0.42 ± 0.01	6.1 ± 0.2	0.51 ± 0.01	2.35 ± 0.05	0.48 ± 0.02	3.8
8.3	0.35 ± 0.01		0.58 ± 0.01			3.5
25.2	0.32 ± 0.01		0.58 ± 0.01			3.3
33.5	0.31 ± 0.01		0.59 ± 0.01			3.3
50.4	0.31 ± 0.01		0.58 ± 0.01			3.3
Gaussian Distributional Fit Analysis $\chi^2_{\text{GLO}} = 5.5$						
w_0	c/ns		σ/ns			
4.1	3.48 ± 0.08		2.0 ± 0.2			
8.3	3.23 ± 0.07		1.8 ± 0.1			
25.2	3.03 ± 0.07		1.9 ± 0.1			
33.5	2.96 ± 0.07		1.8 ± 0.1			
50.4	2.95 ± 0.07		1.8 ± 0.1			

^aThe analysis was carried out by linking the values of lifetimes throughout the varying water contents.

TABLE V: HSA in Reverse Micelles

Anisotropy Decay ^a $\chi^2_{\text{GLO}} = 4.5$				
w_0	ϕ_p/ns	ϕ_r/ns	f_L	r_0
4.1	68 ± 6	1.1 ± 0.3	0.92 ± 0.06	0.250 ± 0.005
8.3	59 ± 5	0.9 ± 0.3	0.87 ± 0.06	
25.2	48 ± 3	0.8 ± 0.2	0.84 ± 0.06	
33.5	41 ± 3	0.5 ± 0.1	0.86 ± 0.05	
50.4	32 ± 3	0.2 ± 0.05	0.87 ± 0.06	

^aThe analysis was carried out by linking the value of r_0 throughout the varying water contents.

corresponding to the whole molecule rotation decreases from 68 ns at $w_0 = 4.1$ to 32 ns at $w_0 = 50.4$. At high w_0 this longer component is equal to the rotational correlation time of HSA in pure water. This result is in agreement with an investigation of HSA dynamics in reverse micelles carried out by electron paramagnetic resonance spin labeling technique.³¹ The decreasing trend of ϕ_r indicates that the internal motions become faster with increasing micellar water pool size; the water content seems to significantly influence the structural flexibility of the protein. The trend observed for the HSA rotational correlation time is expected for the rotation of a protein located in the micellar water pool, which rotational freedom increases when the water pool size is increased.

3. Liver Alcohol Dehydrogenase. a. Aqueous Solution. The fit parameters obtained by analyzing the phase and modulation data for LADH in aqueous solution (buffer pH 8.8) are shown in Table I. The data are analyzed by a sum of three exponential decays: the longer lifetimes (6.2 and 2.9 ns) account for 99% of the total fluorescence intensity, while the remaining 1% is due to a 0.1-ns component. The average lifetime is 4.9 ns. This finding is quite surprising since previous studies report a double-exponential decay for LADH.³⁵ LADH is a dimer composed of two identical subunits. Each subunit contains two tryptophanyl residues; one of these residues (Trp-15) is located near the surface of the protein, while the other (Trp-314) is buried inside the protein. The two lifetimes generally found for LADH are believed to be independent; the short one is attributed to Trp-314 and the long one to Trp-15 emission.³⁵ Since our data can be analyzed also by fixing the short lifetime to a very short value, 0.001 ns (the χ^2 goes up to 1.05, the values of the two long lifetimes are substantially not changed), we interpret this short component as due to scattered light.

By analyzing the data with an unimodal Gaussian distribution of lifetimes and taking into account 1% of scattered light, we obtain a distribution centered at 4.94 and having a width of 1.5 ns.

The differential phase angles and the modulation ratios for LADH at pH 8.8 and 25 °C are shown in Figure 3a. The best

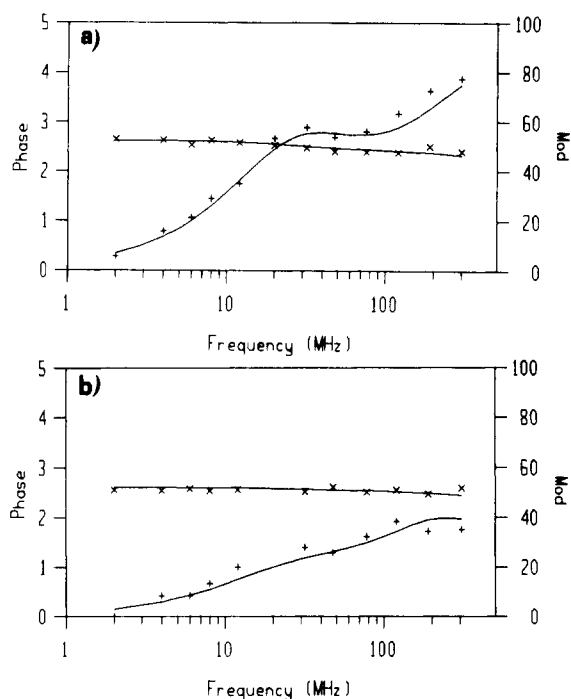


Figure 3. (a) Differential phase and modulation ratio for LADH in aqueous solution at pH 8.8. (b) Differential phase and modulation ratio for LADH in reverse micelles at $w_0 = 25.2$.

fit of the curves is obtained by using two rotational correlation times of 36 and 0.30 ns. The r_0 value is fixed at 0.31. (See Note under section 1a.) The fit parameters are shown in Table I. The longer value represents the whole molecule rotation. The 0.30-ns rotational correlation time is due to internal dynamics of tryptophan residues of LADH. The LADH dynamics have been previously studied by fluorescence anisotropy; the overall rotational correlation time³⁵ is in good agreement with the present result. The existence of internal motion for tryptophan residues of LADH was not reported.³⁵ However, a rapid internal motion has been detected by measuring the steady-state anisotropy of Trp-15.³⁶

b. Reverse Micelles. The emission spectra (not shown) of LADH in reverse micelles of AOT-isooctane present a small blue shift of the emission maximum (about 2–3 nm), with respect to the aqueous solution. Values of w_0 from 7.4 to 50.5 are investigated in time-resolved fluorescence experiments. We were not able to carry out measurements at w_0 values smaller than 7.4 because of the reduced solubility of LADH. Buffer solution at pH 8.8 is utilized since this pH is close to the isoelectric point of LADH, thus allowing us to minimize the electrostatic interactions with the micellar wall.³⁵ The phase and modulation data

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TABLE VI: LADH in Reverse Micelles

Fluorescence Decay Multiexponential Fit Analysis ^a $\chi^2_{\text{GLO}} = 4.3$						
w_0	f_1	τ_1/ns	f_2	τ_2/ns	τ_3/ns	$\langle\tau\rangle/\text{ns}$
7.4	0.72 ± 0.01	4.74 ± 0.08	0.26 ± 0.02	1.78 ± 0.04	0.67 ± 0.02	3.9
12.6	0.74 ± 0.02		0.24 ± 0.02			3.9
25.2	0.86 ± 0.02		0.12 ± 0.01			4.3
37.8	0.79 ± 0.02		0.16 ± 0.01			4.1
50.5	0.85 ± 0.02		0.13 ± 0.01			4.3
Gaussian Distributional Fit Analysis $\chi^2_{\text{GLO}} = 6.0$						
w_0	c/ns			σ/ns		
7.4	3.91 ± 0.05			1.5 ± 0.1		
12.6	3.93 ± 0.05			1.5 ± 0.1		
25.2	4.41 ± 0.06			1.6 ± 0.1		
37.8	4.11 ± 0.06			1.7 ± 0.1		
50.5	4.39 ± 0.08			1.6 ± 0.1		

^aThe analysis was carried out by linking the values of lifetimes throughout the varying water contents.

TABLE VII: LADH in Reverse Micelles

Anisotropy Decay ^a $\chi^2_{\text{GLO}} = 4.5$				
w_0	ϕ_p/ns	ϕ_r/ns	f_L	r_0
7.4	128 ± 15	0.9 ± 0.1	0.90 ± 0.06	0.270 ± 0.005
12.6	89 ± 8	0.9 ± 0.1	0.91 ± 0.06	
25.2	95 ± 9	0.7 ± 0.1	0.89 ± 0.05	
37.8	90 ± 8	0.6 ± 0.1	0.89 ± 0.05	
50.4	83 ± 6	0.6 ± 0.1	0.87 ± 0.05	

^aThe analysis was carried out by linking the value of r_0 throughout the varying water contents.

in reverse micelles are more complex than in aqueous solution. They are analyzed by using a triple-exponential decay. The best fit is obtained by linking the values of lifetimes throughout varying water contents. The results are presented in Table VI. More than 70% of the fluorescence is due to a 4.74-ns component, a small percentage (2–5%) to a 0.67-ns component, and the remainder to a 1.78-ns component. The data were not analyzable by fixing the third lifetime to a lower value, thus excluding that it can be attributed to scattered light. The average lifetime is shorter than the average lifetime of LADH in water. The fluorescence decays of LADH in reverse micelles are analyzed by a Gaussian distribution of lifetimes as well (see Table VI). The center of the distribution slightly changes from 3.91 (at $w_0 = 7.1$) to 4.39 ns (at $w_0 = 50.5$). The distribution width is almost the same at all w_0 investigated and is slightly larger than in aqueous solution. Analogously to the average lifetimes, the distribution centers are close to the center obtained in aqueous solution. A time-resolved fluorescence spectroscopic investigation of LADH in AOT–isooctane reverse micelles has been previously reported, where the LADH fluorescence decay in reverse micelles has been analyzed by using a sum of three exponential decays with an average lifetime of about 2.5 ns.³⁵ At present, we are not able to justify the difference between our and literature data.

In Figure 3b, a representative curve of differential phases and modulation ratios for LADH at $w_0 = 25.2$ is shown. The best fit of the experimental data is obtained by using a double-exponential and linking the r_0 value throughout the varying water content. The fit parameters are presented in Table VII. The longer rotational correlation time is due to the overall protein rotation; it decreases with increasing water content but remains longer than the rotational correlation time of the protein in water even at high w_0 . The rotational correlation time for the fast component, present at each investigated w_0 , decreases from 0.9 ($w_0 = 7.1$) to 0.6 ns ($w_0 = 50.5$). For LADH, also, the internal motions, i.e., the flexibility, are affected by the increasing micellar water pool size.

Discussion

In the present work we have investigated the dynamics of lysozyme, HSA, and LADH in reverse micelles by using frequency domain fluorescence spectroscopy. These proteins were chosen

because their fluorescence is well characterized in aqueous solutions and two of them, lysozyme and LADH, were extensively studied in reverse micelles.^{11,17,18} The results obtained are discussed in connection with previously reported data on L-Trp,⁹ *N*-acetyl-L-tryptophanamide (NATA),^{9,37} and some small Trp-containing peptides³⁷ (adrenocorticotropin (1–24) [ACTH(1–24)], its fragment ACTH(5–10), and glucagon).

Lifetimes. In all proteins studied, a blue shift in the fluorescence emission occurred. The blue shift was limited to 1–2 nm for LADH, while it was large (about 10 nm) for lysozyme at low w_0 and for HSA. This finding is not surprising since a significant blue shift is generally found in the emission spectra of Trp-containing molecules;^{9,37} this effect is attributed to a decreased environmental polarity in reverse micelles.⁹ Lysozyme is known to undergo a major conformation change in AOT–isooctane reverse micelles;¹¹ from CD spectra it was inferred that it is probably denatured.¹⁸ The presence of lysozyme substrate does not prevent these alterations but only delays them.¹⁸ In the presence of a substrate, the structural alterations, as probed by CD, occur in times on the order of 10 min.¹⁸ For this reason, the experiments reported in this study were performed in the absence of substrate. In this case, the relevant blue shift can be reasonably attributed to the severe structural modifications in the protein which can make the tryptophanyl residues more exposed to the solvent. The average lifetime of lysozyme in reverse micelles is longer than in solution. This finding is consistent with a lower polarity of the environment experienced by the protein tryptophanyl residues. From the distributional analysis, it can be inferred that lysozyme experiences a higher heterogeneity of conformations and environments. The decrease in both the center and the width of the lifetime distribution observed when the water content is increased probably indicates a faster interconversion rate between substates.⁹

HSA has not been extensively studied in reverse micelles. The blue shift observed in the emission spectrum does not necessarily indicate that the protein is denatured. To assess this point, we studied CD spectra of HSA in reverse micelles.³⁴ A decrease of about 20% in the ellipticity in the far-UV region was found in reverse micelles,³⁴ indicating a loss of approximately 15% of the α -helical content (Dr. M. Waks, personal communication). Minimal alterations were found in the near-UV region.³⁴ A certain degree of conformational changes was found by use of ESR spin labeling,³⁴ but the spectra in reverse micelles were drastically different from a spectrum of denatured protein. A blue shift in the emission spectrum of HSA could be related to binding surfactant molecules.³⁸ In fact, HSA shows a blue shift of about 10 nm in aqueous solution in the presence of AOT (at [AOT]/[HSA] > 2).³⁹ In the present investigation, a considerable decrease of the average lifetime is observed by inserting HSA in

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reverse micelles. Since the emission spectrum of HSA is blue shifted with respect to the spectrum in aqueous solution, this observation cannot be attributed to a different polarity of the tryptophan environment. A decrease in the average lifetimes in reverse micelles, with respect to the aqueous solution, was found in all model compounds investigated in ref 37 (at $w_0 > 11$). This behavior was explained in part by a modification of the hydrogen bonds between the indole derivatives and the structured water as compared with the bulk water.³⁷ Although this mechanism can also be true for the Trp in HSA, and partially account for the decrease in $\langle \tau \rangle$, we believe that the alterations occurring in the protein structure in reverse micelles can also contribute.

LADH in reverse micelles of AOT-isooctane does not undergo any relevant conformational changes, as probed by CD and UV absorption techniques;^{17,35} in addition, its activity is retained in reverse micelles.^{17,42} The fluorescence decay of LADH tryptophanyl residues seems to be affected by the micellar environment. In particular, a third lifetime appears in reverse micelles. Subtle conformational changes, not detected by UV and CD, could be responsible for alterations in the chemico-physical environment of tryptophanyl residues. Nevertheless, the average lifetime is close to the value found in solution, indicating that the possible conformational changes are not very important as compared to the other proteins.

Anisotropy Decay Results. Two rotational correlation times were necessary to analyze the data in reverse micelles and in water. The longer rotational correlation time was attributed to the whole molecule tumbling and the shorter time to internal motions of the Trp residues with respect to the protein structure. In all model compounds investigated in ref 37, a double exponential was necessary to analyze the anisotropy decays in reverse micelles and in water (with the exception of NATA in water), indicating that the existence of internal motions in reverse micelles is not just a property of proteins.

The correlation time for the overall protein rotation is expected to be influenced by the pool size of the protein-micelle system. In HSA and LADH, the rotational correlation times depend on the value of w_0 and, at high w_0 , tend to be higher than the rotational correlation times for the proteins in water. This finding indicates that at low w_0 the protein rotation is hindered by the interaction with the micelle and by the high viscosity of the micellar water, while at high w_0 the rotation becomes faster and the physical environment becomes closer to the bulk water. Lysozyme shows a particular behavior, since, at each w_0 , its rotational correlation time remains approximately constant and significantly higher than in aqueous solution. This should be related with the dramatic alterations in lysozyme structure.

It is important to note that the measured rotational correlation time, ϕ_p , is determined by the rotation time of the protein in the

micelle, ϕ_{int} , and by the rotation of the micelle in the organic solvent ϕ_{mic} .

$$1/\phi_p = 1/\phi_{int} + 1/\phi_{mic}$$

The rotation of the micelles that contain proteins is not easily determined, since the micellar parameters change by insertion of the macromolecules.⁴⁰ However, by use of the simple model of ref 41, ϕ_{mic} can be estimated. According to ref 41, we calculated that at high w_0 $\phi_{mic} \gg \phi_{int}$, so $\phi_p \approx \phi_{int}$. At low w_0 , ϕ_{mic} can contribute to the measured value of ϕ_p . The possible effect is that the rotational correlation time of the protein in the micelle, ϕ_{int} , is longer with respect to the measured value, ϕ_p . The possible contribution from the micellar rotation to the measured protein rotational correlation time does not affect our considerations on overall protein rotational dynamics in reverse micelles.

A large decrease of the rotational dynamics, influenced by the rotation of the micelles, was also found in the Trp-containing peptides in transferring from water to reverse micelles.³⁷

The protein internal dynamics is obviously not affected by the rotation of the micelle. In all cases investigated, the internal dynamics of tryptophanyl residues, as measured by the correlation time (or by the diffusion coefficient), increases with water content. This is a direct observation of the effect of hydration on the protein flexibility. A similar behavior was observed in ACTH(5-10) and glucagon.³⁷

Conclusion

The main goal of the present work was to investigate the usefulness of micellar water pool in studying the protein hydration, i.e., in reproducing the physical state of a dehydrated protein at low w_0 which is progressively hydrated with increasing water content. While the results from anisotropy decay in reverse micelles are readily understandable and reflect, to a certain extent, the effect of the presence of water, the fluorescence lifetime results are less clear. The relatively simple behavior previously reported for fluorescence lifetime distribution of lysozyme and azurin⁹ is not found for each protein investigated in the present work. Fluorescence lifetimes are very sensitive to any modification of the protein tertiary structure as well as to the microphysical environment. Structural modifications can occur in proteins hosted in reverse micelles which can obscure the effect of the interaction with water. In some instances, probably, the effects observed on fluorescence lifetimes are related to structural modifications of the protein or to interactions with the micellar environment and not only to hydration effects.

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Registry No. LADH, 9031-72-5; lipozyme, 9001-63-2; tryptophanyl, 73-22-3.

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