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Local phenomena and distribution of molecular species during the unfolding of heme-free myoglobin in the presence of GdnHCl and urea as seen by time-resolved fluorescence spectroscopy

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We have used time-resolved fluorescence spectroscopy for following the unfolding of apomyoglobin in urea and guanidine hydrochloride (GdnHCl). The data have been compared with those obtained using classical techniques such as CD and steady-state emission spectroscopy. Both the average intensity of the lifetimes and the size of the librational cone of the fluorophores, as measured by time-resolved fluorescence, increased with denaturant concentration and their changes largely preceded the modifications detectable with CD and the shift of the maximum of emission spectra. The data indicate that the changes in the local environments of the tryptophans were completed when the global modification monitored by CD and the emission spectra was still minimal. This suggests that an initial event in the denaturation of apomyoglobin is localized at the tryptophan residues. The correlation times of native apomyoglobin showed the rotational diffusion characteristics of a rigid rotor. In 3.6 M GdnHCl and 7.5 M urea, where the secondary structure is practically absent, the correlation times of the two systems became very short, as expected from the motion of a flexible polymer. In GdnHCl, under conditions of partial unfolding, it was not possible to detect the presence of native totally folded molecular species.

1. Introduction

Protein denaturation is a complex phenomenon, which has elicited a great deal of investigation in order to elucidate the nature and thermodynamic characteristics of the intermediate steps [1–4].

Time-resolved spectrofluorimetry opens a novel approach to the understanding of unfolding phenomena in proteins. In fact, it can detect modifications both of the environments around the fluorophores (tryptophans), and of the shape and flexibility of the entire protein.

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We have explored the lifetimes and correlation times associated with heme-free sperm whale myoglobin (apomyoglobin) in the presence of increasing concentrations of either urea or GdnHCl. These data were compared with those obtained with CD and steady-state emission measurements. Apomyoglobin is a globular monomeric protein (molecular mass 16 kDa), with two tryptophans at positions 7 and 14, and has a high degree of helical content which gives a strong signal in CD spectroscopy. Also, it is devoid of sulfhydryl groups, which may alter denaturation processes by forming inter- and intramolecular disulfide bridges during the unfolding.

The results showed that time-resolved fluorescence spectroscopy is a powerful tool for observing local phenomena and distribution of molecu-

lar species during denaturation. They suggest that in apomyoglobin the unfolding process begins with a local modification of the environment of the tryptophan residues. Also, they indicate that even at denaturant concentrations, where global unfolding is limited, native, totally folded molecules are not detectable.

2. Materials and methods

Sperm whale myoglobin was purchased from Sigma. The apo derivative was prepared by extraction with methyl ethyl ketone as described by Teale [5]. Before use, the protein was extensively centrifuged and forced through a 0.2 μm filter.

Time-resolved fluorescence lifetimes and correlation times were measured with an Edinburgh 119 nanosecond pulse fluorometer. Excitation was at 296 nm and a cut-off filter ($T = 50\%$ at 340 nm) was used in emission. The data were deconvolved and analyzed using nonlinear least-squares procedures based on the Marquardt algorithm [6], and classical equations, as previously described [7]. In all cases, it was assumed that the decays of either emission intensity or fluorescence anisotropy were described by linear sums of exponentials. For the correlation times the anisotropy at zero time was assumed to be that of the frozen fluorophore. This was determined to be 0.27 for *N*-acetyltryptophanamide (NATA) in 100% glycerol at $-5\,^{\circ}$C. All correlation times were normalized to water at $25\,^{\circ}$C.

Emission spectra and steady-state anisotropy were measured upon excitation at 296 nm with an SLM 3000 fluorometer.

CD was recorded on a JASCO-20 spectropolarimeter.

The relative viscosities of 6 M GdnHCl and 7 M urea were ascertained using calibrated Hubbelohde viscometers, following classical procedures [8]. All of the correlation times were normalized to water at $25\,^{\circ}$C.

The measurements were conducted in 0.05 M phosphate buffer (pH 6.5) at $4\,^{\circ}$C. The protein was equilibrated with the desired concentration of denaturant for 24 h in the cold.

3. Treatment of data

3.1. Estimation of correlation time

The correlation time of native apomyoglobin was estimated using the equation

$$r_e = \frac{M(1-\bar{v}\rho_0)}{6N\pi\eta s} \tag{1}$$

which is obtained by combining the equation of Stokes' law with those describing the sedimentation velocity. In eq. 1, N represents Avogadro's number, r_e the effective radius (in cm), ρ_0 the density of the solvent and s the sedimentation velocity (in s) of the system. Also, $\bar{v}$ and η denote the partial specific volume of the protein and the viscosity of the solvent, respectively. From this, the effective volume of the molecule was estimated, and hence its correlation time, σ, from

$$\sigma = \frac{V\eta}{kT} \tag{2}$$

where V (in cm^3) is the volume of the molecule, η the viscosity of the solvent (in P), k (in erg degree^{-1} mol^{-1}) Boltzmann's constant, and T the absolute temperature (in K). The practical value of this treatment is discussed elsewhere [9], using a molecular mass of 16.8 kDa, $\bar{v} = 0.74$ cm^3 g^{-1}, $s_{20,w} = 2.04$ s^{-13}, a value of 8.0 ns was obtained for the expected correlation time of native apomyoglobin.

3.2. Estimation of the size of the librational cones

When the local librational modes of the fluorescent probes can be resolved from the global rotational modes of the protein to which they belong, it is possible to compute the semiapex angle of the cones which define the local librations of the probes [9]. If the assumptions are made that the probes have cylindrical symmetry and that either the emission or the absorption transition moments are parallel to the main axis of symmetry, two models are possible.

If the axis of the probe is considered as wobbling within the cone, the 'soft' angle δ' is given by

$$f = \cos^2\delta'(1 + \cos^2\delta')^2/4 \quad (3)$$

where f is the fraction of the initial anisotropy depolarized by the local motion.

An alternative model is that for which the probe rotates about a fixed axis, to which the emission transition moment makes a constant, 'rigid', angle δ. In this case

$$f_i = \{(3\cos^2\delta - 1)/2\}^2 \quad (4)$$

These equations were used for computing the possible amplitudes of the angles of the cone which enclosed the local librational motions of tryptophans under the various experimental conditions.

In the Edinburgh 119 time-correlated photon-counting fluorometer, the bandwidth of the light pulses was between 1.5 and 2 ns. This implies that the time-resolving power was not sufficient for assigning precise values to decay times below 1 ns. The fractional depolarization produced by the local motions of tryptophan was therefore computed from the difference between the anisotropy of the frozen probe, measured as reported in section 2, and the initial anisotropy detected for the longer correlation times, which represented the global motion of the protein.

Another characteristic of the equipment was the presence, on certain days, of a very small radiofrequency signal in the baseline. The signal did not produce distortion of the data. Its only effect was to lower the value of the χ^2 of the computer fits well below the unit value, when the noise of the data was not enough in order to mask the systematic instrumental error.

3.3. Measurements of emission intensity

Accurate measurements of emission intensities in the presence of spectral shift require time-consuming and often inaccurate integrations across all of the emission. Time resolution of the emission decay offers a very precise means for estimating changes in the quantum yield of fluorophores by using the weighted average of the lifetimes, τ_{av}. This quantity is independent of the shape and position of the emission spectra. It was computed from

$$\tau_{av} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \quad (5)$$

4. Results

4.1. Denaturation in urea

4.1.1. CD in urea

Fig. 1 reports the far-ultraviolet CD spectra of apomyoglobin in the presence of various concentrations of urea. Increasing concentrations of urea produced a decrease in the proportion of secondary structure. The spectra remained constant between 6.5 and 8 M urea, suggesting the complete elimination of secondary structure. The fractional changes in ellipticity at 222 nm are

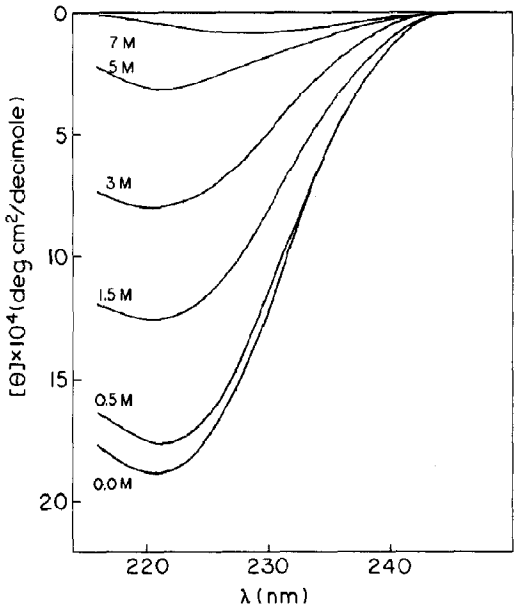

Fig. 1. CD spectra of apomyoglobin in the presence of increasing concentrations of urea, in 0.05 M phosphate buffer at pH 6.5 and 4° C. Protein concentration near 0.1 mg/ml; 1 mm path length cuvette. Urea concentrations given by each trace.

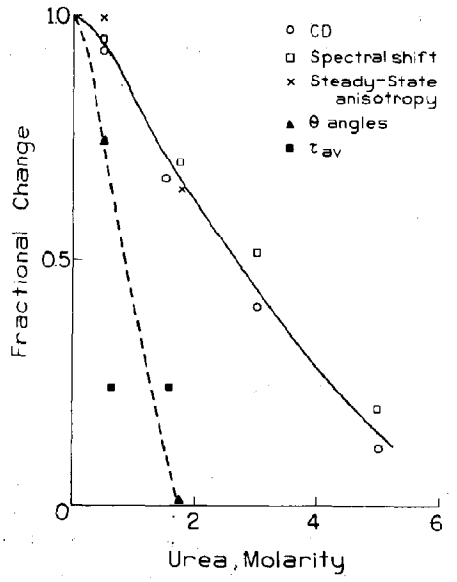

Fig. 2. Variation with urea concentration of: (□) the peak of the emission spectrum of apomyoglobin; (○) ellipticity at 222 nm; (×) steady-state anisotropy; (▲) size of the local librational cones; (■) average time of intensity decay. In 0.05 M phosphate buffer at pH 6.5 and 4° C.

reported in fig. 2, together with those of the other parameters used for following denaturation.

4.1.2. Emission spectra in urea

Denaturation of sperm whale apomyoglobin in urea produced a red shift of the emission spectra of tryptophan and an increase in intensity of emission. Fig. 2 shows the fractional shift of the wavelength of maximum intensity between 0 and 7.5 M urea.

4.1.3. Steady-state anisotropy in urea

Fig. 2 also shows changes of the steady-state anisotropy of the system at different urea concentrations. Static anisotropy, CD spectroscopy and the shift of the maximum of the emission spectra appear to follow the same denaturation curve.

4.1.4. Lifetimes in urea

As shown in table 1 the lifetimes of the system were dependent on the concentration of urea. In the absence of denaturant, apomyoglobin showed

Table 1

Lifetimes (τ) and amplitudes (α) of sperm whale apomyoglobin (0.3 mg/ml) upon denaturation with urea in 0.05 M phosphate buffer at pH 6.5 and 4° C (excitation, 296 nm; cut-off filter at 340 nm in emission)

[Urea] (M)	τ_i (S.D.) (ns)	α_i	χ^2	τ_{av} (ns)
Native	0.45 (0.13)	0.33		
	1.68 (0.28)	0.45		
	4.65 (0.38)	0.22	2.7	3.1
0.58	1.90 (0.08)	0.28		
	5.30 (0.29)	0.72	2.34	4.8
1.76	1.85 (0.05)	0.54		
	5.93 (0.13)	0.46	0.95	4.8
7.50	2.43 (0.08)	0.41		
	6.21 (0.10)	0.59	1.75	5.4

three lifetime components, however, only two were detectable in the presence of the denaturant. The presence of three lifetime components in native apomyoglobin has also been reported by Janes et al. [10]. The average decay of emission intensity,

Table 2

Correlation times ϕ of sperm whale apomyoglobin (0.3 mg/ml) in the presence of urea in 0.05 M phosphate buffer at pH 6.5 and 4° C

The reported values are adjusted to water at 25° C. Excitation at 296 nm; cut-off in emission at 340 nm. The anisotropy of the frozen probe was measured to be 0.27. If the detectable initial anisotropy was less than 0.27 the balance was included in the fraction of the shorter correlation time. The angles δ and δ' correspond to the rigid and soft semiapex angles of the cone of librations of the local motions of tryptophans (eqs. 3 and 4).

[Urea] (M)	ϕ_i (S.D.) (ns)	f_i	χ^2	δ (°)	δ' (°)
Native	1.0 (0.4)	0.25		17	22
	7.5 (1.6)	0.75	0.90		
0.58	1.0 (0.7)	0.39		22	30
	6.4 (1.3)	0.61	0.81		
1.76	0.7 (0.2)	0.80		37	54
	7.3 (0.8)	0.20	0.61		
7.50	0.3 (0.1)	0.71		n/a	
	1.4 (0.2)	0.29	2.10		

τ_{av} computed from eq. 5, increased with concentration of denaturant. Fig. 2 shows that the fractional change of τ_{av} preceded the unfolding of the protein monitored by either CD or the shift of the emission maximum.

4.1.5. Correlation times in urea

Under all conditions two correlation times satisfied the analyses of the data. The use of a third component did not improve in a significant way the statistics of the simulations.

As shown in table 2, one of the correlation times was in any case near 1 ns. It reflected the local librational motions of the tryptophan residues, while the longer correlation time reflected the global motion of the protein. In the absence of denaturant, the long correlation time was that expected from the rotational diffusion of monomeric apomyoglobin as a rigid rotor. Intermediate concentrations of urea did not modify appreciably the detectable correlation times. Unfolding of the system was revealed by modifications of the relative amplitudes of the short and long correlation times. The relative amplitude of the short correlation time rose from 25% in the native protein to about 80% in the presence of 1.76 M urea. This was consistent with an increase in the semiapex angles of the cone of librational motions of the tryptophans from about 20° in the native protein to more than 45° in the partially unfolded system.

At high denaturant concentrations (7.5 M urea) the longer correlation time decreased to a value near 2 ns, consistent with the presence of a flexible polymer.

Fig. 2 shows that the fractional modifications of the librational cones followed very closely the modifications of τ_{av} of the system. They both largely preceded the unfolding detected by CD, steady-state emission spectra and steady-state anisotropy.

4.2. Denaturation in GdnHCl

4.2.1. CD in GdnHCl

Fig. 3 shows the CD spectra obtained in the presence of increasing amounts of GdnHCl. The decrease of the signal originating from the secondary structure is maximal at 3.6 M GdnHCl.

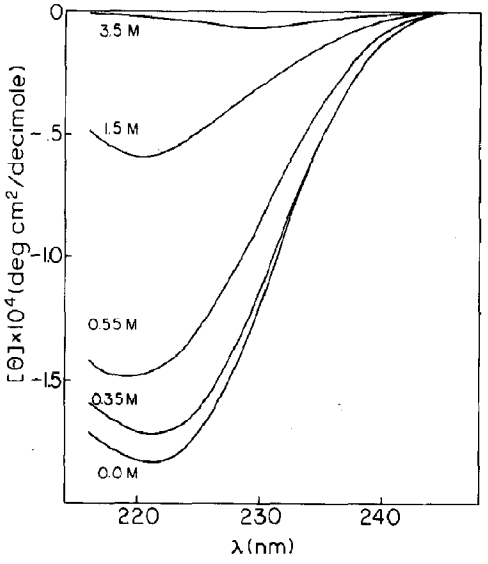

Fig. 3. CD spectra of apomyoglobin in the presence of increasing concentrations of GdnHCl, in 0.05 M phosphate buffer at pH 6.5 and 4° C. Protein concentration near 0.1 mg/ml; 1 mm path length cuvette. GdnHCl concentrations given by each trace.

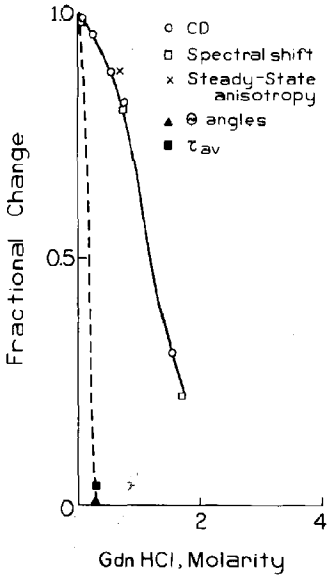

Fig. 4. Variation with GdnHCl concentration of: (□) the peak of the emission spectrum of apomyoglobin; (○) ellipticity at 222 nm; (×) steady-state anisotropy; (▲) size of the local librational cones; (■) average time of intensity decay. In 0.05 M phosphate buffer at pH 6.5 and 4° C.

Fig. 4 shows the fractional changes in ellipticity at 222 nm, together with those of the other parameters.

4.2.2. Emission spectra in GdnHCl

Also in this case, with increasing concentrations of denaturant there occurred an increase in intensity and a progressive shift of the emission maximum toward the red. Fig. 4 shows the fractional shift of the emission maximum with increasing concentrations of GdnHCl.

4.2.3. Steady-state anisotropy in GdnHCl

Also in this case the fractional modification of anisotropy followed the denaturation curves described by the ellipticity at 222 nm and by the shift of the maximum of the emission intensity.

4.2.4. Lifetimes in GdnHCl

Table 3 shows that, as already noted for urea, unfolding of the protein reduced the number of lifetime components of the system to two. In this case as well, the lifetimes became longer, with the amplitude favoring the longer components, and therefore the τ_{av} value increased with denaturant concentration. As discussed below, low concentrations of denaturant induced the formation of aggregates, which are known to increase the intensity of emission. This was probably the origin of the scattering of the intensity data (τ_{av}) in table 3. Nevertheless, they are consistent with the analogous data obtained in urea in the sense that they show an increase in τ_{av} which largely preceded the unfolding of the secondary structure monitored by CD and the shift of the emission maximum of the system (fig. 4).

Table 3

Lifetimes (τ) and amplitudes (α) of sperm whale apomyoglobin (0.3 mg/ml) upon denaturation with GdnHCl in 0.05 M phosphate buffer at pH 6.5 and 4°C (excitation at 296 nm; cut-off filter at 340 nm in emission)

[GdnHCl] (M)	τ_i (S.D.) (ns)	α_i	χ^2	τ_{av} (ns)
Native	0.45 (0.13)	0.33		
	1.68 (0.28)	0.45		
	4.65 (0.38)	0.22	2.7	3.1
0.38	1.56 (0.10)	0.53		
	4.95 (0.20)	0.47	3.8	4.1
0.75	1.98 (0.09)	0.41		
	6.33 (0.01)	0.59	0.8	5.5
3.60	1.45 (0.39)	0.49		
	4.90 (0.26)	0.51	4.1	4.3

Table 4

Correlation times ϕ of sperm whale apomyoglobin (0.3 mg/ml) in the presence of GdnHCl in 0.05 M phosphate buffer at pH 6.5 and 4°C

The reported values are adjusted to water at 25°C. Excitation at 296 nm; cut-off in emission at 340 nm. The anisotropy of the frozen probe was measured to be 0.27. If the detectable initial anisotropy was less than 0.27 the balance was included in the fraction of the shorter correlation time. The angles δ and δ' correspond to the rigid and soft semiapex angles of the cone of librations of the local motions of tryptophans (eqs. 3 and 4).

[GdnHCl] (M)	ϕ_i (S.D.) (ns)	f_i	χ^2	δ (°)	δ' (°)
Native	1.0 (0.7)	0.25		17	22
	7.5 (2.6)	0.75	0.9		
0.38	1.4 (0.4)	0.51		26	37
	20.9 (1.9)	0.49	1.1		
0.75	0.9 (0.2)	0.49		26	37
	20.4 (3.4)	0.51	0.5		
1.50	0.1 (0.2)	0.56		26	37
	6.3 (0.4)	0.44	2.5		
3.60	0.5 (0.1)	0.70		n/a	
	1.8 (0.8)	0.30	1.8		

4.2.5. Correlation times in GdnHCl

As shown in table 4, here also the time-dependent anisotropy was always satisfied by two components. In analogy with the data reported in table 2, the correlation times near 1 ns reflected the local librational motions of tryptophans, the longer values reflecting the global motions of the protein.

In the presence of low concentrations of denaturant the long correlation time increased and was concentration dependent, indicating the formation of aggregates. At higher concentrations of GdnHCl (1.5 M) it decreased again, suggesting inhibition of aggregation.

The fractional amplitudes of the two correlation times changed with GdnHCl. The amplitude of the short correlation time rose from 25% in the native protein to a maximum of 40% in 1.5 M GdnHCl. This indicated an increase in the semi-apex angle of the cone of the librational motion of the tryptophans from near 20° in the native protein to more than 30° in the presence of the denaturant.

Fig. 4 shows that also in GdnHCl the increasing freedom of the local motions of the tryptophans largely preceded the unfolding of the secondary structure detected by CD spectra, static anisotropy and the shift of the emission maximum.

5. Discussion

5.1. Local vs. global phenomena

The data reported in figs. 2 and 4 and tables 2 and 4 show the presence of two distinct processes. The modifications of the parameters obtained from time-resolved fluorescence spectroscopy, namely, modifications of the angles, δ, of the librational cones of the tryptophans and of the average lifetimes, τ_{av}, largely preceded those of all of the other classic parameters, namely, CD spectra, steady-state anisotropy and position of the emission maximum. The two sets of parameters followed, respectively, local and global structural changes of the protein.

The increase in τ_{av} was a local phenomenon due to modifications of the collisional quenching of the tryptophan residues with nearby residues in the polypeptide chain [11]. This hypothesis is supported by the observation that in the presence of denaturant the lifetime below 1 ns detectable in native apomyoglobin disappeared, as if this component was the one originating from the quenching. It should be stressed that quenching may also be produced by energy transfer between the tryptophans (in positions 7 and 14, respectively). This can be inhibited by local phenomena which modify the distance and angular relationships of the two residues.

The increase in size of the librational cones of the tryptophans also monitors local phenomena. In fact, in the absence of global modifications detectable in CD spectroscopy, it can be explained only by a modification of the structural environment around the tryptophans, which allowed a larger degree of librational freedom of these residues. This hypothesis is consistent with the decrease in quenching phenomena, as discussed above.

Thus, it appears that while the τ_{av} and δ values monitored the local interaction of tryptophans with the denaturants, CD, steady-state anisotropy and the shift of the emission maximum followed global unfolding phenomena. The detection of local initial events during the denaturation of apomyoglobin is probably the novel contribution brought by time-resolved spectroscopy to the study of protein unfolding.

5.2. Denaturation steps

Tanford [12] originally proposed that denaturation in urea and GdnHCl is controlled by the free energy of transfer of the peptide groups and of the amino acid side chains from the hydrophobic interior of proteins to the surrounding denaturant-solvent. According to the free energies of transfer estimated by Nozaki and Tanford [13,14] tryptophans are the residues which most readily undergo solubilization in the denaturants. This is very consistent with our observation that the interaction of the tryptophans with denaturants is the first event detectable in the unfolding process.

Our data are in agreement with the findings of the group in Naples [15–17] who followed the disappearance of the fluorescence of 8-anilino-1-naphthalenesulfonic acid embedded in the heme pocket of apomyoglobin at low concentrations of GdnHCl. This phenomenon preceded the global unfolding of the molecule detected by CD spectroscopy and emission maximum. They also proposed local distortions of the structure to be responsible for the phenomenon. Our data suggest that the distortion was produced by solubilization of the two tryptophans which are both located in the A helix of the protein.

The relevance of the initial portion of the polypeptide chains to the structure of the heme pocket in hemoproteins is also supported by the findings of Franchi et al. in our laboratory [18]. We observed the presence in the isolated β-subunits of a heme-linked domain which disappears at very low concentrations of GdnHCl. This domain was assigned to the first 50 amino acids of the polypeptide chain, which include the two tryptophans of the molecule in positions 14 and 37.

The question may be posed as to whether there is proof of both tryptophans undergoing denaturation simultaneously. However, there is no conclusive evidence in favor of this process in any of our experiments, or of the data reported by the various authors quoted herein, multimodal behavior being noted during the denaturation process, when monitored by the emission of tryptophans.

5.3. The correlation times of native and fully denatured apomyoglobin

The comparison of the estimated (from eqs. 1 and 2) values and those found for the correlation times of apomyoglobin indicated that the native protein is essentially a rigid rotor, except for the local librational activity of the tryptophan residues. In 7 M urea and 3.6 M GdnHCl the polypeptide chain showed only very short correlation times, as expected from a flexible polymer.

5.4. The distribution of molecular species during unfolding

As expected, the correlation times distinguished very well between native and totally unfolded molecules, which behaved like flexible polymers.

An important piece of information was obtained from the data in GdnHCl. In 0.38 M GdnHCl, where aggregates were formed, the shift of the emission maximum, the ellipticity at 222 nm and the steady-state anisotropy indicated the extent of denaturation to be only 10% or less. In spite of this low degree of global unfolding, it was not possible to detect in the system rotational correlation times corresponding to those of the native protein. At the same concentration of denaturant the local phenomena detected by the size of the librational cone and by the average lifetimes were practically completed.

Thus, it appears that the global unfolding detected by CD and the other steady-state techniques must be interpreted as the average unfolding of each molecule of protein rather than the equilibrium between native and denatured forms.

5.5. Analyses of lifetime distributions

Recently, Bismuto et al. [19] have published data on the effect of GdnHCl on the Lorentzian distribution of the lifetimes of tryptophan emissions in tuna apomyoglobin (*Thunnus tinnus*).

From their data, it appears that the bandwidths of the Lorentzians used to fit the intensity decay of the system detected global unfolding phenomena. In this respect, such an analysis appears to be less useful than those performed in more conventional ways, as presented in this paper.

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