Steady state and time resolved effects of guanidine hydrochloride on the structure of *Humicola lanuginosa* lipase revealed by fluorescence spectroscopy

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Abstract

Effects of guanidine hydrochloride (GdnHCl) on the structure and dynamics of wild-type *Humicola lanuginosa* lipase (HLL) and its two mutants were studied. The latter were S146A (with the active site Ser replaced by Ala) and the single Trp mutant W89m, with substitutions W117F, W221H, and W260H. Steady-state, stopped-flow, and time-resolved laser-induced fluorescence spectroscopy were carried out as a function of [GdnHCl]. The maximum emission wavelength and fluorescence lifetimes revealed the microenvironment of the tryptophan(s) in these lipases to become more polar upon increasing [GdnHCl]. However, significant extent of tertiary structure in GdnHCl is suggested by the observation that both wild-type HLL and W89m remain catalytically active at rather high GdnHCl concentrations of >6 and 4.0 M, respectively. Changes in steady-state emission anisotropy, as well as variation in rotational correlation times and residual anisotropy values, demonstrate that upon increasing [GdnHCl] the structure of the lipases became more loose, with increasing amplitude of structural fluctuations. Finally, intermediate states in the course of exposure of the proteins to GdnHCl were revealed by stopped-flow fluorescence measurements.

Keywords: fluorescence spectroscopy; *Humicola lanuginosa* lipase; unfolding

The structure of the *Humicola lanuginosa* lipase (HLL, triacylglycerol hydrolase, E.C. 3.1.1.3) has been solved at 3.25 Å (Lawson et al., 1994b) and more recently for another crystal form at 1.8 Å resolution (Derewenda et al., 1994a). HLL consists of a single, roughly spherical domain containing a central eight-stranded, predominately parallel β -pleated sheet and five interconnecting α -helices, compacted to a volume of $\sim 35 \times 45 \times 50$ Å³. The active site of HLL is comprised of a Ser-Asp-His catalytic triad that is closely reminiscent to that in serine proteases (Brady et al., 1990). In addition to its catalytic role, the active site Ser seems to be also structurally important. Accordingly, modification of the active site Ser to yield the inactive mutant S146A results in conformational alterations and different lipid binding affinities (Peters et al., 1998).

Lipases exhibit a characteristic property called “interfacial activation,” which describes their augmented catalytic activity when

the substrate is presented in an aggregated state such as micelles or membranes (Hermoso et al., 1997; Berg et al., 1998). A dynamic structural feature of lipases involved in interfacial activation is opening of the “lid” (Martinelle et al., 1995; Juszczak et al., 1997). In HLL, the lid is constituted by an α -helical surface loop of amino acids 86–93, lying directly over the active site (Brady et al., 1990; Berg et al., 1998). Accordingly, two conformations have been suggested for HLL, with the lid “closed” and “open” (Svendsen et al., 1997). In an aqueous medium, the conformation is closed and access to the catalytic triad is blocked by the lid. Instead, in complexes with substrate analogues or proper inhibitors the lid helix is displaced and the active site becomes exposed (Brzozowski et al., 1991; Derewenda et al., 1992; Lawson et al., 1994a). The lid is highly mobile also in HLL crystals (Derewenda et al., 1994b).

The 269 residue sequence of HLL includes four tryptophans (Martinelle et al., 1995) out of which the lid contains one at position 89. Chemical modification studies have shown some Trp residues to be involved in catalysis (Liu et al., 1977). Accordingly, site-directed mutagenesis revealed (Holmquist et al., 1994, 1995) Trp89 to be important for efficient hydrolysis of tributyrin and it was concluded to play a role in the catalytic steps after the absorption of the lipase to the substrate interface. In brief, Trp89 seems to influence the binding of substrates into the active site (Holmquist et al., 1995).

To gain more insight into the structural characteristics of HLL and the lid in particular, we compared the effects of a widely used

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Abbreviations: carboxy-DCFDA, carboxy-2',7'-dichlorofluorescein diacetate; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; G_u , free energy of unfolding; GdnHCl, guanidine hydrochloride; Hepes, N-(2-hydroxyethyl) piperazine-N'-2-ethanesulphonic acid; HLL, *Humicola lanuginosa* lipase; RFI, relative fluorescence intensity; W89m, single Trp HLL mutant with substitutions W117F, W221H, and W260H; τ , fluorescence lifetime; ϕ , rotational correlation time; r_{∞} , residual anisotropy.

chaotrope, guanidine hydrochloride (GdnHCl) on the *H. lanuginosa* wild-type lipase, the inactive mutant S146A, and the single Trp-mutant W89m (W117F, W221H, W260H). More specifically, we monitored changes in the environment and dynamics of Trps by steady-state, time-resolved, and stopped-flow fluorescence measurements as a function of increasing concentrations of the chaotrope. The functional integrity of the active site was assessed by measuring the catalytic activity towards a fluorogenic water soluble substrate.

Results

Steady-state fluorescence measurements

The wavelength of the emission maximum λ_{max} for Trp depends on its microenvironment. More specifically, a low polarity, hydrophobic microenvironment is characterized by $\lambda_{max} \approx 331$ nm, while for Trp in an aqueous phase λ_{max} is 350–353 nm (Brustein et al., 1973). A convenient way to measure λ_{max} is to record synchronous fluorescence spectra (Miller, 1979). At $\Delta\lambda = 60$ nm only Trp fluorescence is observed, whereas emission from Tyr is excluded. Such spectra for the wild-type HLL, S146A, and W89m mutants are depicted in Figure 1. For all these enzymes, λ_{max} is at ≈ 340 nm, suggesting their Trps to reside in environments of intermediate polarity. As there are four Trps in the wild-type and S146A, only average microenvironments of Trp in these proteins can be assessed. Notably, the fluorescence intensity of W89m is almost half of that for the wild-type, thus revealing the quantum yield for Trp89 to exceed the average values for quantum yields of the three other Trps in the wild-type HLL and S146A.

For all three lipases Trp maximum emission wavelengths increase slightly upon increasing concentrations of GdnHCl (data not shown), whereas the emission intensities decrease (Fig. 2). The equilibrium parameters for GdnHCl-induced structural alterations

Fig. 1. Synchronous fluorescence spectra for the wild-type (●), S146A (Δ), and W89m (*) lipases. Difference between the excitation and emission wavelengths $\Delta\lambda = 60$ nm. Concentration of the lipases is $1 \mu\text{M}$ in 20 mM Hepes, 0.1 mM EDTA, pH 7.0. The temperature was 25°C .

Fig. 2. The effect of [GdnHCl] on the fluorescence intensity for wild-type (●), W89m (*), and S146A (Δ). Concentration of the lipases is $1 \mu\text{M}$ in 20 mM Hepes, 0.1 mM EDTA, pH 7.0. Excitation and emission wavelengths were 295 and 342 nm, respectively. The temperature was maintained at 25°C .

of lipases were calculated from these data according to Equation 1 and are compiled in Table 1. The values of [GdnHCl] corresponding to the midpoints of the transition curves C_m were 3.1, 2.3, and 1.75 M for the wild-type, S146A, and W89m, respectively, reflecting the relative stabilities of these lipases. These values for C_m reveal S146 to be important in the stabilization of HLL, in keeping with our previous results (Peters et al., 1998). Likewise, the unfolding of W89m reported by its single Trp in the lid occurs at a lower [GdnHCl] compared to the wild-type. The estimated free energy of unfolding of wild-type HLL, 9.92 kcal/mol is higher than the value for S146A, 8.74 kcal/mol. Lowest free energy changes was calculated for W89m, 6.86 kcal/mol. Denaturant m_{eq} values, correlating with the amount of protein surface exposed to solvent upon unfolding (Myers et al., 1995) were 3.2, 3.8, and 3.9 for the wild-type, S146A, and the single Trp mutant W89m, respectively.

Rotational diffusion of fluorophores is the dominant cause of fluorescence depolarization. In proteins, the mobility of fluorescent amino acid residues bears a close relationship with the overall state of protein and any factor that affects its size, shape, or seg-

Table 1. Thermodynamic parameters for the unfolding of lipases derived from equilibrium measurements at 25°C

	m_{eq} (kcal M^{-1} mol $^{-1}$)	C_m (M)	ΔG_u^0 (kcal mol $^{-1}$)
Wild-type	3.20 ± 0.0028	3.10 ± 0.066	9.92 ± 0.030
S146A	3.80 ± 0.0025	2.30 ± 0.006	8.74 ± 0.028
W89m	3.92 ± 0.49	1.75 ± 0.007	6.86 ± 0.89

mental flexibility will also affect the observed steady state anisotropies (Lakowicz, 1983). Changes in fluorescence anisotropy in the course of unfolding by increasing [GdnHCl] are shown in Figure 3. At GdnHCl concentrations up to 2.5 and 1.5 M value for anisotropy for wild-type and S146A HLL, respectively, first decrease slowly, whereafter exceeding these chaotrope concentrations significant and rather abrupt further conformational changes occur. Judged by the observed changes in anisotropy, effects of GdnHCl on these proteins seem to be complete at 3.5 and 2.5 M chaotrope. For W89m there are essentially no changes in anisotropy up to 1 M GdnHCl, whereafter a steep decrease is evident, reaching saturation at 2.5 M.

To this end, when urea was used instead of GdnHCl, the maximum fluorescence emission wavelengths and steady state anisotropies of wild-type HLL did not change, even up to 8.0 M concentration of this chaotrope (data not shown). This is somewhat unexpected as it suggests HLL cannot be denatured by urea. Accordingly, in experiments described here, only GdnHCl was used.

Time-resolved fluorescence measurements

In addition to its spectral features, fluorescence lifetimes of Trp also depend on its microenvironment thus allowing to obtain further insight into conformational changes of proteins. The mean fluorescence lifetimes for the three proteins studied here decrease with increasing concentrations of GdnHCl (Fig. 4). Notably, mean fluorescence lifetimes decrease stepwise, in distinction from the changes in steady state anisotropies. Except for the native state of W89m, which revealed a single-exponential decay with $\tau \approx 5.4$ ns, biexponential fluorescence decays were measured for the lipases both in their native and denatured states. The respective lifetimes, τ_1 in the range of 0.2–1.5 ns and τ_2 of ~ 3 –5.5 ns, are depicted in

Fig. 3. Steady state fluorescence anisotropy for wild-type (●), W89m (*), and S146A (△) as a function of [GdnHCl]. The excitation and emission wavelengths were 295 and 345–355 nm. Both excitation and emission bandpasses were 10 nm. Conditions were as described in the caption for Figure 1.

Fig. 4. The average fluorescence lifetime τ for wild-type (●), W89m (*), and S146A (△) as a function of [GdnHCl]. See caption for Figure 1 for the conditions used.

Figure 5. Values of τ_1 for wild-type and S146A reveal insignificant changes with [GdnHCl], varying between ≈ 1.0 and 1.2 ns. Different behavior of τ_1 was evident for W89m and at [GdnHCl] = 0.5 M, the value of τ_1 is ≈ 0.3 ns, whereas at [GdnHCl] ≥ 1.0 M it prolongs to ≈ 0.8 ns. The longer lifetime component τ_2 for the wild-type, S146A, and W89m first remains constant at ≈ 4.4 , 5.6, and 5.4 ns, respectively, for [GdnHCl] up to 4.5 M, whereafter the corresponding values decrease to 3.5, 3.8, and 4.1 ns. The fractional intensities of τ_2 decrease with increasing [GdnHCl] (Fig. 6). The above changes indicate that Trp(s) become exposed to more polar microenvironments with increasing concentrations of GdnHCl (Kungl et al., 1998). Notably, τ_2 of the different lipases begins to decrease at different concentrations of GdnHCl, reflecting different stabilities of the three enzymes against loosening of their structure by the chaotrope.

Time-dependent fluorescence anisotropy decays provide information on the diffusive motions of the fluorophore during its excited lifetime. More specifically, these data can reveal if a fluorophore is free to rotate over all angles, or if the environment surrounding the fluorophore restricts its angular displacement. Moreover, these measurements can reveal if the anisotropy decays as a result of a single rate of rotation, or if several rotational processes are present (Lakowicz, 1983). We measured fluorescence anisotropy decays at different [GdnHCl] and calculated the rotational correlation times (ϕ) and residual anisotropy (r_∞) from these data. Two correlation times (ϕ_1 and ϕ_2) were obtained. For the wild-type HLL and S146A, values for the shorter correlation time ϕ_1 changed insignificantly with the increase in [GdnHCl], the average values for these proteins being 1.45 and 0.79 ns, respectively (data not shown). Instead, the shorter correlation time of W89m was dramatically affected by the chaotrope and upon increasing GdnHCl to 2.0 M, its value increased 4.4-fold, from 0.24 to 1.29 ns, no further significant changes were evident at [GdnHCl] ≥ 2.0 M, and the minor decrement by ≈ 0.15 ns at [GdnHCl] between 5–5.5 M should not

Fig. 5. Variation of the (A) shorter and (B) longer lifetime components τ_1 and τ_2 , respectively, of wild-type (●), W89m (*), and S146A HLL (Δ) as a function of [GdnHCl]. Conditions as in Figure 1.

be paid too much attention (Fig. 7A). The latter data suggest the frequency of segmental motions of the Trp in the lid to decrease upon unfolding. In the native state, the values of the longer correlation time ϕ_2 for the wild-type and W89m are identical (≈ 20 ns) whereas for S146A $\phi_2 \approx 11.8$ ns. For all three lipases, ϕ_2 increases with [GdnHCl], yet for S146A the value for ϕ_2 is augmented more than for the wild-type and W89m HLL (Fig. 7B). Upon [GdnHCl] exceeding the value of 3.5, 2.5, and 3.5 M for wild-type, S146A, and W89m HLL, respectively, the values for ϕ_2 became too long to be measured accurately.

The residual fluorescence anisotropy (r_∞) values approach zero with increasing [GdnHCl]. However, different concentrations were required for the three enzymes (Fig. 8), viz. 4.0, 3.5, and 2.5 M for

Fig. 6. The fractional intensities of the shorter lifetime component τ_1 for the wild-type (●), W89m (*), and S146A HLL (Δ) as a function of [GdnHCl].

the wild-type HLL, S146A, and W89m, respectively. Also the residual anisotropies for S146A, W89m, and the wild-type were different as a function of [GdnHCl]. More specifically, r_∞ for the wild-type decreases progressively with increasing [GdnHCl]. For S146A two apparent threshold concentrations of ~ 1.0 and 2.5 M GdnHCl are observed, whereas for W89m there is a single threshold at 1.5 M chaotrope. There is a lot of scatter in the data, particularly for wt HLL. Accordingly, the absolute values of r_∞ should perhaps not be considered too much in detail.

Stopped-flow fluorescence measurements

The time courses of unfolding of lipases in the millisecond to second timescale at different [GdnHCl] were followed by recording the intrinsic fluorescence emission as a function of time up to 48 s after rapid mixing. Kinetic traces measured in the 0 to 40 ms domain after exposure of wild-type and W89m HLL to GdnHCl are illustrated in Figures 9A and 9B. The behavior of S146A was indistinguishable from the wild-type (data not shown). Exposure of lipases to [GdnHCl] ≥ 2.0 M causes first a rapid (within ~ 2 – 3 ms) transient decrease in Trp fluorescence. This decrease is followed by a slow increase in fluorescence. At lower concentration of GdnHCl, only the slow enhancement of Trp emission is seen. We did not study these fast processes in more detail at this stage and their origin thus remains uncertain. Representative traces of fluorescence changes in the time regime up to 48 s are shown in Figure 10. Accordingly, the above changes in fluorescence occurring in the 0 to ~ 20 ms time range were followed by much more slower (up to ≈ 50 s) attenuation of fluorescence emission. Rate constants and the lag times were obtained as described in Materials and methods and are compiled in Tables 2 and 3, respectively.

The above kinetic data provide evidence for four distinct intermediates. Immediately after mixing with the chaotrope, there is a

Fig. 7. Correlation times ϕ_1 for W89m (A) and ϕ_2 for the wild-type (●), W89m (*), and S146A HLL (Δ) (B), as a function of [GdnHCl]. The values of ϕ_1 for wild-type and S146A HLL varied insignificantly as a function of [GdnHCl] and, accordingly, for the sake of clarity these data are not included in figure. Excitation was at 298 nm and emission wavelength range 345–350 nm.

transient change in the conformation of the three lipases produced by [GdnHCl] > 1.0 M. In the 0 to 48 s time domain, only one rate constant is seen for all three lipases at 1.0 M [GdnHCl], whereas two rate constants are evident at 3.0 M chaotrope. At 4.0 M [GdnHCl], however, there are three rate constants for W89m and two rate constants for the wild-type and S146A HLL. Accordingly, the unfolding processes to vary depending on the concentrations of the denaturant, the rate constants increasing with [GdnHCl]. Lag time for W89m was always shorter than for the wild-type and S146A HLL, which were approximately equal. In general, the lag times decreased with increase in [GdnHCl].

Fig. 8. Dependency of residual anisotropy r_∞ for W89m (*), S146A (Δ), and wild-type HLL (●) on the concentration of the chaotrope.

Activity measurements

The relative catalytic activities of wild-type and W89m toward a water soluble nonspecific esterase substrate carboxy-DCFDA as function of [GdnHCl] are shown in Figure 11. Both enzymes reveal the same tendency, their activities first increasing at low [GdnHCl] and then decreasing above 1.5 and 1.0 M chaotrope for wild-type and W89m, respectively. Wild-type HLL retains ~69% catalytic activity at 6.0 M GdnHCl, whereas W89m became inactive already at 4.0 M GdnHCl.

Discussion

Protein unfolding can be induced by factors such as pressure (Valente-Mesquita et al., 1998), temperature (Scalley & Baker, 1997), pH (Jamin & Baldwin, 1998), lipids (Pinheiro et al., 1997), and chaotropes (Pace, 1986), and these studies have provided information about the interactions governing protein structure as well as on the involvement of intermediates and transition states (Privalov, 1996; Steer & Merrill, 1997). Because of its inherent dynamics, fluorescence spectroscopy is an excellent tool for studies on protein structure. More specifically, maximum emission wavelengths, intensities, and fluorescence lifetimes of the intrinsic fluorophores, tryptophan in particular, are sensitive to their environment. Moreover, emission anisotropy, residual anisotropy, and rotational correlation times report on the conformational dynamics of the protein. Combined with stopped-flow, fluorescence measurements can be used to monitor the kinetics of protein unfolding (Otlewski et al., 1996; Malecki & Wasylewski, 1997; Steer & Merrill, 1997). Fluorescence data also allow to derive thermodynamic parameters attributed to protein stability (Otlewski et al., 1996; Park et al., 1997). Most proteins contain several tyrosine and tryptophan residues. Accordingly, contribution of an individual fluorophore to overall changes in fluorescence is difficult to ascertain as in this case changes in fluorescence may reflect global

Fig. 9. Unfolding of the (A) W89m and (B) wild-type HLL by GdnHCl monitored by intrinsic fluorescence intensity. Fluorescence was recorded at 350 nm with 295 nm excitation. Temperature was maintained at 25 °C. The concentration of GdnHCl was 1.0 M (Δ), 3.0 M ($\circ$), and 4.0 M ($\square$), in 20 mM Hepes, 0.1 mM EDTA, pH 7.0. The temperature was 25 °C. All the kinetic traces were normalized to the same start point and the dead times were subtracted.

changes in protein structure. The use of mutant proteins possessing single tryptophan residue avoids this problem and allows for probing site-specific alterations (Steer & Merrill, 1995).

The free energy at zero concentration of denaturant ΔG_u^0 can be used to estimate the conformational stability of the lipases. We used a two-state model (native and unfolded) to calculate the ΔG_u^0 and m_{eq} -value. Soulages (1998) pointed out most mutant proteins to be characterized by a lower m_{eq} value and diminished stability compared to their wild-types. Accordingly, the

Fig. 10. Unfolding of the (A) W89m and (B) wild-type HLL monitored by Trp fluorescence at GdnHCl concentrations of (a) 1.0 M, (b) 3.0 M, and (c) 4.0 M. Fluorescence was recorded at 350 nm with excitation at 295 nm. Data were collected as described in Materials and methods. Temperature was 25 °C.

estimated values could be at error due to the two-state assumption and should be taken as tentative only. Yet, our results indicate that compared to the wild-type HLL, both S146A and W89m have a reduced ΔG_u^0 and an increased m_{eq} value. The latter suggest more apolar residues in the course of unfolding to become solvent exposed in these mutants (Pace et al., 1989). This is in keeping with the view that in addition to its central role in catalysis (Brady et al., 1990) S146 is also important to the stability of the lipase and, accordingly, changing it to Ala decreases the stability of this protein (Peters et al., 1998). Alanine lacks the hydroxyl moiety and it can thus be expected that the replace-

Table 2. Rate constants for the unfolding of the three lipases induced by GdnHCl, as derived from stopped-flow measurements^a

[GdnHCl] (M)	Wild-type		S146A		W89m		
	k_1 (s ⁻¹)	k_2 (s ⁻¹)	k_1 (s ⁻¹)	k_2 (s ⁻¹)	k_1 (s ⁻¹)	k_2 (s ⁻¹)	k_3 (s ⁻¹)
1.0	0.064 ± 0.011		0.023 ± 0.003		0.035 ± 0.008		
3.0	0.022 ± 0.007	0.017 ± 0.003	0.011 ± 0.006	0.026 ± 0.004	0.0094 ± 0.003	0.01 ± 0.002	
4.0	0.03 ± 0.003	0.18 ± 0.02	0.03 ± 0.002	0.11 ± 0.02	0.093 ± 0.02	0.057 ± 0.003	0.14 ± 0.01

^aRates were measured by stopped-flow fluorescence emission measurements at 25 °C, and the data were fitted with either single or double exponential functions as outlined under Materials and methods. The rate constants k_1 , k_2 , and k_3 correspond to three distinct phases in the course of unfolding.

ment of serine abrogates hydrogen bonding of the -OH to -NH₂ and -OH moieties of vicinal amino acids.

Our data reported here thus comply with the general notion that a single amino acid substitution can have a significant impact on the conformational stability of a protein (Dill & Shortle, 1991). Even more important to the stability of HLL are the three Trps, W117, W221, and W260. In HLL, W89 and W117 are located in different α -helices and W221 and W260 in different β -pleated sheets. The single Trp of W89m resides in the α -helix constituting the lid. Tryptophan residues 117, 221, and 260 may contribute to the structural stability of HLL not only by hydrophobicity but also π -cation interaction (Dougherty, 1996) could be involved. In this respect, it is possible that the replacement of Trp221 and Trp260 with His abrogates π -cation interactions between Trp and the vicinal basic amino acids, viz., Lys223, Arg195, and His145. To this end, π -cation interactions would also explain the low quantum yields of W117, W221, and W260.

GdnHCl has been suggested to perturb water structure so as to cause it to become a better solvent for nonpolar amino acids (Lee & Timasheff, 1974), thus causing weakened hydrophobic interactions. As the latter have been concluded to be of primary importance in maintaining protein folding (Dill & Shortle, 1991), addition of the chaotrope will result in loosening of protein structure. Accordingly, the protein may stretch out and expose amino acids initially residing in its interior. On the other hand, weakening binding of GdnHCl to proteins has been proposed (Timasheff, 1993). Interestingly, HLL was not denatured by urea. To this end,

in contrast to urea GdnHCl is a salt. This difference may thus indicate that HLL is significantly stabilized by electrostatic interactions, which are disrupted by GdnHCl but not by urea. In this respect, it may be relevant to note that HLL acts at the interface between water and the hydrophobic substrate. Accordingly, ionic interactions would seem to be well suited for this function, so as to eliminate denaturation due to surface tension at the water/substrate interface.

In the experiments described here, conformational changes due to unfolding were revealed by a number of observations. The maximum emission wavelength for Trp shifted to longer wavelength (data not shown), thus indicating Trp(s) to move into a more polar environment (Brustein et al., 1973). The steady-state anisotropy decreased with increasing of [GdnHCl], also reflecting increased mobility of the fluorophores (Fig. 3) (Lakowicz, 1983). It can be anticipated that the Trp(s) rotate more freely when the protein chain stretches and interactions of Trp(s) with vicinal amino acids become weaker.

Table 3. The lag times for the unfolding of lipases induced by different concentrations of GdnHCl^a

[GdnHCl] (M)	Wild-type		S146A		W89m		
	t_1 (s)	t_2 (s)	t_1 (s)	t_2 (s)	t_1 (s)	t_2 (s)	t_3 (s)
1.0	38.0		36.4		31.2		
3.0	15.5	30.7	16.8	30.7	14.4	25.2	
4.0	14.3	29.5	13.6	30.7	2.5	12.7	28.3

^aKinetic traces were determined by stopped-flow fluorescence measurements at 25 °C as described under Materials and methods. Values for t_1 , t_2 , and t_3 represent the lag times for the distinct subsequent unfolding processes.

Fig. 11. The relative catalytic activities toward 0.1 mM carboxy-DCFDA of W89m (*) and wild-type HLL (●) as a function of [GdnHCl]. The concentration of the lipases was 1.0 μ M in 20 mM Hepes, 0.1 mM EDTA, pH 7.0. Temperature was 25 °C.

Also fluorescence lifetime of Trp is related to its microenvironment and reduced solvent exposure is associated with longer lifetimes (Kungl et al., 1998). The average fluorescence lifetimes for the three lipases decreased with increasing [GdnHCl] in keeping with Trp(s) becoming exposed to more hydrophilic microenvironments. Except for W89m at [GdnHCl] = 0, two fluorescence lifetimes were measured for the lipases (Fig. 5). Multiple fluorescence lifetimes of Trp(s) in proteins are generally attributed to different Trp rotamer conformations (Chen et al., 1991). The longer fluorescence lifetime τ_2 decreased with increase in [GdnHCl], reaching a minimum of 3.5 ns at 4.5 M chaotrope. This value still exceeds the fluorescence lifetime of free Trp in water (2.7 ns) and suggests that the environment of the Trp(s) is not equivalent to a bulk aqueous phase. Recent theories and experimental data suggest that even denatured proteins may often remain relatively compact, with persistent hydrophobic clustering and considerable residual secondary structure. Accordingly, even in 6 M GdnHCl proteins can still retain significant amount of internal structure (Dill & Shortle, 1991). Our results are in keeping with this and suggest that HLL possesses significant extent of secondary and tertiary structure even at high concentrations of chaotrope.

This view is further supported by the measurement on the catalytic activity of both the wild-type as well as the W89m as a function of [GdnHCl]. Both enzymes are first activated by GdnHCl (up to 4.5 and 1.5 M, respectively). This suggests that a general "loosening" of the tertiary structure could result in increased turnover number for these enzymes. Yet, also weakened hydrophobic interactions in GdnHCl may allow for an easier access of the water soluble substrate into the hydrophobic cleft accommodating the catalytic site. In this connection, it is of interest to note that Holmquist et al. (1993) reported an ~12% inhibition of HLL activity at 0.1 M GdnHCl. This observation was suggested to be due to destabilization by GdnHCl of the salt bridge involving Arg86 in the open conformation of the lipase. Yet these authors used as a substrate tributuroylglycerol emulsified by sonication in the presence of gum arabic. Accordingly, direct comparison with the present results is not unambiguous. Increasing [GdnHCl] to 4 M results in complete loss of activity of the W89m while even at 6 M chaotrope ~69% of the activity is retained by the wild-type. Accordingly, although changes in Trp emission are quite large, the catalytic triad Ser146–Asp201–His258 remains by and large functional. The latter suggests the unfolding of the wild-type HLL to be only partial at 6 M GdnHCl. Taking into account structural dynamics, a more accurate description is that the amplitude for conformational fluctuation of HLL increases with increasing [GdnHCl]. Accordingly, the time-averaged fraction of the enzyme molecules in a conformation with catalytically functional active site decreases when [GdnHCl] exceeds 5.0 M. To this end, it is of interest that the acyl chain specificity of HLL is altered by substitution of Trp89 (Martinelle et al., 1996). Accordingly, it would be of interest to study if also GdnHCl would affect their catalytic specificity. However, it should be stressed that GdnHCl affects water structure and, accordingly, also the hydrophobicity driven aggregation of these substrates and the properties of the oil/water interfaces. Due to the above, interpretation of the data on the effects of GdnHCl would be complicated. This was also the reason why we choose to use the water soluble carboxy-DFDCA.

Fluorescence anisotropy decays are the result of the fluorophore dynamics and the overall rotation of the molecule (Steiner, 1991), thus yielding rotational correlation times for the fluorophore. Two correlation times were observed for each of the enzymes. It is

commonly assumed that the fluorescence from proteins becomes depolarized due to (1) rapid local motions of the tryptophan side chains and (2) by the overall rotation of the entire macromolecule (Steiner, 1991). The shorter correlation time ϕ_1 is thus likely to reflect the side-chain rotations, whereas the longer correlation time ϕ_2 is due to the rotation of the entire molecule. The latter relates to the hydrodynamic volume by the Einstein–Stoke's equation $\phi_2 = \eta(v + h)MW/RT$, where η is the viscosity of the medium, MW is the molecular mass of protein, v is the specific volume of protein, h is the hydration factor, and T and R have their usual meanings. Unfortunately, changes in hydration upon the interaction of GdnHCl with the lipase cannot be assessed by our techniques. Accordingly, calculation of the values for the hydrodynamic volume is ambiguous. Yet, increase in ϕ_2 upon increasing [GdnHCl] indicates the volume of the protein to increase upon denaturation. It has been reported that proteins can aggregate upon unfolding (Swaminathan et al., 1996). However, no fluorescence energy transfer between fluorescein and rhodamine B labeled wild-type HLL was observed when [GdnHCl] was increased (data not shown), thus making the latter possibility unlikely.

Residual anisotropy r_∞ reports on the amplitude of the fluorophore motions (Lakowicz, 1983). In case the angular range of rotational motion of the fluorophore is restricted, the anisotropy does not decay to zero. The values for r_∞ decreased upon unfolding of the lipases, indicating diminished constraints for the motion of Trp(s) (Fig. 8). For wild-type and S146A r_∞ became zero at 3.5 and 4.0 M [GdnHCl], respectively. The ratio of values of ϕ_2 for wild-type and S146A in buffer is 1.7. This suggests that the average volume of the wild-type HLL to be larger than that of S146A and again reveals the replacement of S146 with alanine to result in a conformational changes. Yet, S146A should still possess a relatively compact structure. In the light of ϕ_2 , it appears that the volume change for S146 upon unfolding process is more dramatic than that of the wild-type HLL. This view is also supported by the m_{eq} value, which indicates in the course of GdnHCl induced unfolding more apolar residues of S146A to become solvent exposed than for the wild-type.

The shorter correlation time ϕ_1 depends on the frequency of the rapid local motions of the Trp side chains. The increase in ϕ_1 of W89m can thus be assigned to changes in the packing of Trp of the "lid" and reveals the segmental motions to become slower. At low [GdnHCl] the movement of the α -helix constituting the lid should thus be retarded due to partial unfolding, whereas increasing [GdnHCl] further ≥ 2 M, the mobility of the "lid" remains unaffected. There are four Trps in the wild-type and S146A. As the correlation times for the rapid local motions of can become shorter upon denaturation (Swaminathan et al., 1996), a possible explanation for the insignificant change in ϕ_1 for the wild-type HLL and S146A is that increased mobility of the other three Trps could offset the change resulting from the reduced mobility of the Trp in the lid.

Intermediate states during unfolding and refolding of proteins have recently gained considerable interest (Privalov, 1996; Steer & Merrill, 1997). The involvement of intermediates is readily indicated by our steady state measurements on lipases. Stopped-flow spectroscopic techniques can be used to investigate unfolding in the millisecond time regime, revealing the involvement of intermediate states in unfolding as well as structural rearrangement not observed in equilibrium studies. Fluorescence changes in the initial stages of the denaturation process observed in stopped-flow measurements are depicted in Figure 9. The rapid decrease in fluorescence intensity indicates that Trp(s) reside transiently in a more

polar microenvironment, whereafter they become again accommodated in a less polar microenvironment (She et al., 1998). While this phenomenon is seen already at 1 M GdnHCl, it became more pronounced at higher concentrations of GdnHCl. This suggests that different unfolding mechanism may result depending on the concentration of the chaotrope.

The calculated rate constants obtained from the kinetic curves are compiled in Table 2 and correspond to the distinct unfolding processes during denaturation by GdnHCl. For the wild-type and S146A, there are two possibilities to explain the fluorescence changes, as follows. First alternative is the involvement of intermediate states in the course of unfolding with different rate constants describing the changes in protein conformation from one state to another. The other possibility is that the four Trps present in these proteins located in different region of the protein unfold at different rates. Assuming the Trps to become exposed to solvent one by one, fluorescence intensities should decrease stepwise. However, the observations on the single Trp mutant W89m rules out the second possibility. Accordingly, the data can be rather interpreted in terms of intermediates.

Unfolding of HLLs is readily evident at different concentrations of GdnHCl (Fig. 10). In the time range in which the fluorescence of lipases was monitored, the number of intermediate states and the rate constants of unfolding did depend on [GdnHCl] (see Table 2). In general, the observations listed above would imply the possibility that the unfolding of lipases proceeds rapidly at a high concentration of GdnHCl, so that the intermediate state appears early or their population is more pronounced. Wild-type HLL and W89m reveal different behavior, especially during the first 15 s after mixing at [GdnHCl] = 4.0 M. The reason for this difference could be the three Trps absent in W89m, and the insensitivity of their emission to the unfolding by GdnHCl.

As the decrease in fluorescence intensity reveals Trp(s) to become exposed to an environment of higher dielectric constant, their quantum yield thus reflects the rate of unfolding. Our results suggest that in 4.0 M GdnHCl the conformational changes are induced in W89m within 40 s, whereas for wild-type HLL and S146A slightly longer times is required, ~50 s. The conformational changes involve intermediates, transition from one state to another taking ≤ 20 s. The transition times of wild-type and S146A are almost equal. This implies that while the replacement of serine by alanine affects the overall stability of the lipase, it has little effect on the rate of unfolding.

The steady state data depicted in Figure 2 indicate unfolding of lipase induced by GdnHCl to proceed via a simple two-state mechanism (native and unfolded) with no evidence for stable intermediate states, contrary to what has been reported (Palleros et al., 1993; Tsaprailis et al., 1998). However, stepwise changes in mean fluorescence lifetimes suggest intermediate states in the unfolding process. There are several possible explanation to this discrepancy (Swaminathan et al., 1996). As the transition between the intermediate states is fast, the population of each intermediate state could be too small to be evident in steady state fluorescence spectroscopy. Accordingly, stopped-flow fluorescence spectroscopy of the dynamics of protein unfolding provides more sensitive means than steady state measurements to study this process in detail.

Conclusions

Effects of GdnHCl on the conformational dynamics of wild-type lipase from *H. lanuginosa* (HLL) and its mutants, W89m (with

single Trp at position 89), and S146A (with substitution in the active site), were compared using Trp fluorescence spectroscopy. The decrease in [GdnHCl], corresponding to the midpoints of the transition curves (C_m), and the diminished free energies of unfolding in the absence of GdnHCl (ΔG_u^0) indicate the replacement of S146 with alanine as well the replacement W117, W221H, and W260 in W89m to decrease the stability of HLL. Compared to their native states lower steady state anisotropies (r), increased longer correlation times (ϕ_2), and decreased residual anisotropies (r_∞) reveal the structure of HLL and its mutants to become less compact in the presence of the chaotrope. The red shift of maximum emission wavelength and decrease in average fluorescence lifetimes with increase in [GdnHCl] report the Trps to become exposed to more hydrophilic microenvironments.

Changes in fluorescence intensity and shorter correlation times (ϕ_1) for W89m coincide and are saturated at ≈ 2.0 M GdnHCl whereas for the wild-type HLL the corresponding changes require a two times higher chaotrope concentration. Significant amounts of tertiary structure even at high GdnHCl concentrations are indicated by the activity measurements. Changes in the average fluorescence lifetimes suggest unfolding by GdnHCl to proceed via intermediate states depending on chaotrope concentrations. This could be confirmed in stopped-flow fluorescence measurements. The duration and number of these intermediates depend on [GdnHCl]. In brief, with an increase in [GdnHCl] the number of these intermediate states increases and they become more transient.

Materials and methods

Materials

Wild-type, inactive mutant (S146A), and W89m *H. lanuginosa* lipases (HLL) were kindly provided by Drs. Allan Svendsen and Shamkant Anant Patkar (Novo Nordisk, Bagsvaerd, Denmark). The concentrations of lipases were determined as described previously (Bradford, 1976). The specific activities of wild-type HLL and W89m mutant were 2,158 and 1,130 $\mu\text{mol}/\text{mg}/\text{min}$, respectively (Svendsen et al., 1997). The above lipases were subjected to the given concentrations of guanidine hydrochloride (GdnHCl, Fluka AG, Buchs SG, Switzerland) or urea (Merck, Darmstadt, Germany) for 24 h at room temperature prior to fluorescence measurements. GdnHCl (8.0 M) and urea (10.0 M) stock solutions were made in 20 mM Hepes, 0.1 mM EDTA, pH 7.0 and then diluted to the desired concentrations. Water used was deionized in a Milli RO/Milli Q (Millipore, Bedford, Massachusetts) filtering system.

Steady-state fluorescence measurements

Steady-state fluorescence measurements were carried out with a Perkin Elmer LS 50B spectrofluorometer equipped with a magnetically-stirred, thermostated cuvette compartment. The excitation wavelength was 295 nm (Das & Mazumdar, 1995; Barry & Matthews, 1997). Both excitation and emission bandpasses were 5 nm. When recording synchronous fluorescence spectra, the difference $\Delta\lambda$ between emission and excitation wavelength was set at 60 nm. In steady-state anisotropy measurements, excitation and emission bandpasses were 10 nm, the emission wavelength centered at 350 nm. Fluorescence intensity values were corrected for dilution.

Analysis of GdnHCl denaturation curves

Steady-state fluorescence emission intensity at 342 nm was recorded at equilibrium as a function of [GdnHCl], so as to provide a measure of the conformational changes in the lipases. These data were analyzed assuming the free energy of unfolding, ΔG_u to be linearly dependent on [GdnHCl] denoted here by C essentially, as described in detail previously (Pace, 1975):

$$\Delta G_u = \Delta G_u^0 - m_{eq}C = m_{eq}(C_m - C) \quad (1)$$

in which ΔG_u^0 and ΔG_u represent the free energy of unfolding in the absence and presence of GdnHCl, respectively, C_m is the mid-point concentration of GdnHCl required for unfolding, and m_{eq} stands for the slope of the unfolding curve at C_m . The RFI vs. [GdnHCl] curves were fitted and values for m_{eq} and C_m calculated using a program (Park et al., 1997) kindly provided by Dr. Mike Sauder (Fox Chase Cancer Center, Institute for Cancer Research, Philadelphia, Pennsylvania).

Time-resolved fluorescence measurements

Commercial laserspectrometer (Photon Technology International, Ontario, Canada) was used to measure fluorescence lifetimes, rotational correlation times, and residual anisotropies. A train of 500 ps excitation pulses at 337 nm at a repetition rate of 10 Hz was produced by a nitrogen laser, pumping a dye (rhodamine 6G, Merck, Darmstadt, Germany, 5 mM solution in methanol) laser. Pulses from the dye laser were frequency doubled. Excitation wavelength was 298 nm, and emission wavelengths for lipases were from 345 to 355 nm.

For the measurement of fluorescence lifetimes, the average of five emission decay curves was analyzed using software provided by the instrument manufacturer. The validity of the fit of a particular model was judged by the value of the reduced chi-square, χ^2 (Gratton et al., 1984; Jameson & Hazlett, 1991).

Fractional intensities $I(t)$ were calculated according to the equation:

$$I(t) = \alpha_i \tau_i / \sum_{i=1}^N \alpha_i \tau_i \quad (2)$$

where α_i is amplitude and τ_i is lifetime.

For polarized light, the decay of fluorescence intensity $F(t)$ can be calculated from the raw data:

$$F(t) = I_{||}(t) + 2 \times G \times I_{\perp}(t) \quad (3)$$

where $I_{||}(t)$ is the intensity of light detected with both the excitation and emission polarizer being vertical (i.e., parallel polarizers) and $I_{\perp}(t)$ is the intensity of light detected with vertical excitation polarizer and horizontal emission polarizer (i.e., perpendicular polarizers), G is the correction term for the relative throughput of each polarization through the emission optics.

Anisotropy $r(t)$ is defined as

$$r(t) = \frac{I_{||}(t) - G \times I_{\perp}(t)}{I_{||}(t) + 2 \times G \times I_{\perp}(t)} \quad (4)$$

where G , $I_{||}$, and $I_{\perp}$ are as defined above.

Anisotropy can also be expressed as

$$r(t) = r_0 e^{-t/\phi} \quad (5)$$

When the angular range of the rotational motion of fluorophore is limited,

$$r(t) = (r_0 - r_{\infty})e^{-t/\phi} + r_{\infty} \quad (6)$$

where r_0 is the anisotropy in the absence of rotational diffusion.

Rotational correlation times (ϕ) and residual anisotropies (r_{∞}) can be derived from the fitted curves. It should be recalled that being constructed from fitted curves $r(t)$ contains no experimental noise and therefore criteria such as the χ^2 value do not apply. For the determination of rotational correlation times and residual anisotropies, each emission decay was measured ten times.

Stopped-flow fluorescence measurements

Fluorescence changes in the millisecond to second time range were monitored using a stopped-flow spectrofluorometer (Olis RSF 1000f, On-line Instrument System Inc., Bogart, Georgia) equipped with a quartz glass fluorescence observation chamber (20 mm pathlength, 1.5 mm diameter) and a rapid scanning emission monochromator. The dead time of the instrument is 2 ms. When unfolding of lipases by GdnHCl was observed, one of the syringes was filled with 2 μ M lipase solution and the other syringe with a solution of GdnHCl. The concentrations of the lipase and GdnHCl in the syringes were twice the final concentrations in the observation chamber. Excitation wavelength of 295 nm was selected with a monochromator and fluorescence emission spectra were recorded in the range 285 to 425 nm. All experiments were carried out at 25 °C. Temperature in the reaction chamber and in the syringes was regulated by a computer controlled circulating waterbath (Neslab Instrument, Portsmouth, New Hampshire). Fluorescence intensities were detected using rates of 1,000 and 21 scans/s, with the respective data collection times of 0.04 and 48 s. Due to photobleaching, longer collection times could not be used. To determine the extent of photobleaching, buffer was used instead of the chaotrope solution and these traces were subtracted from those recorded in the presence of GdnHCl.

Each experiment was repeated at least three times and averaged values were used for data analysis. For the 0.04 s collection time, the data could not be properly fitted. When the collection time was 48 s, the fluorescence changes were corrected for the decrease in emission intensity due to photobleaching. The fluorescence processes could not be fitted as a single exponential decay process. Accordingly, parts of the curves were fitted separately using either single- or two-exponential equations:

$$Y = Ae^{-kt} + C \quad (7)$$

$$Y = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + C. \quad (8)$$

These were solved with nonlinear least-squares fitting procedures both Levenberg–Marquardt algorithm and successive integration method with the routines of the software provided by the instrument manufacturer. Parameters A_1 and A_2 represent the amplitudes of the processes, and k_1 and k_2 are the respective rate constants. C is a constant accounting for the baseline of the background at

infinite time. All collected decays were characterized by a delay after the mixing of the protein and chaotrope solution. Lag time for the first decay process was determined as the intersection of the horizontal baseline and the slope of the decay of fluorescence intensity. Lag times for the initiation of the subsequent unfolding processes were defined as the discontinuities evident in the fluorescence decays.

Assay for catalytic activity

HLL activity measurements were carried out with a spectrofluorometer equipped with a magnetically-stirred, thermostated (25 °C) cuvette compartment. The excitation and emission wavelengths were 511 and 526 nm, respectively, both excitation and emission bandpasses set at 5 nm. Carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA, Molecular Probe, Inc., Oregon) was dissolved in dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) to 10 mM concentration and stored at -70 °C. For assay, the concentration of HLL was 1.0 µM in 20 mM Hepes, 0.1 mM EDTA, pH 7.0 incubated in the indicated concentration of GdnHCl for 24 h. The reactions were started by the addition carboxy-DCFDA to an initial concentration of 0.1 mM. Upon hydrolysis of carboxy-DCFDA, a more intensively fluorescing product is formed (Thomas et al., 1979). The increase in fluorescence intensity with time was recorded and the slopes of the kinetic curves were determined so as to obtain changes in relative activity. The data were corrected for the decrease in the quantum yield of the fluorescent product caused by GdnHCl in a concentration dependent manner.

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References

- Barry JK, Matthews KS. 1997. Ligand-induced conformational changes in lactose repressor: A fluorescence study of single tryptophan mutants. *Biochemistry* 36:15632-15642.
- Berg OG, Cajal Y, Butterfoss GL, Grey RL, Alsina MA, Yu BZ, Jain MK. 1998. Interfacial activation of triglyceride lipase from *Thermomyces (Humicola) lanuginosa*: Kinetic parameters and a basis for control of the lid. *Biochemistry* 37:6615-6627.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.
- Brady L, Brzozowski AM, Derewenda ZS, Dodson E, Dodson G, Tolley S, Turkenburg JP, Christiansen L, Høge-Jensen B, Nørskov L, et al. 1990. A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature* 343:767-770.
- Brustein EA, Vedenkina NS, Irkova MN. 1973. Fluorescence and the location of tryptophan residues in protein molecules. *Photochem Photobiol* 18:263-279.
- Brzozowski AM, Derewenda U, Derewenda ZS, Dodson GG, Lawson DM, Turkenburg JP, Björklund F, Høge-Jensen B, Patkar SA, Thim LA. 1991. Model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* 351:491-494.
- Chen R, Knutson J, Ziffer H, Porter D. 1991. Fluorescence of tryptophan dipeptides: Correlations with the rotamer model. *Biochemistry* 30:5184-5195.
- Das TK, Mazumdar S. 1995. pH-induced conformational perturbation in horseradish peroxidase. Picosecond tryptophan fluorescence studies on native and cyanide-modified enzymes. *Eur J Biochem* 227:823-828.
- Derewenda U, Swenson L, Green R, Wei Y, Dodson GG, Yamaguchi S, Haas MJ, Derewenda ZS. 1994a. Current progress in crystallographic studies of new lipases from filamentous fungi. *Protein Eng* 7:551-557.
- Derewenda U, Swenson L, Green R, Wei Y, Dodson GG, Yamaguchi S, Haas MJ, Derewenda ZS. 1994b. An unusual buried polar cluster in a family of fungal lipases. *Struct Biol* 1:36-47.
- Derewenda ZS, Derewenda U, Dodson GG. 1992. The crystal and molecular structure of the *Rhizomucor miehei* triacylglyceride lipase at 1.9 Å resolution. *J Mol Biol* 227:818-839.
- Dill KA, Shortle D. 1991. Denatured states of proteins. *Annu Rev Biochem* 60:795-825.
- Dougherty DA. 1996. Cation- π interaction in chemistry and biology: A new view of benzene, Phe, Tyr, and Trp. *Science* 271:163-168.
- Gratton E, Jameson DM, Hall RD. 1984. Multifrequency phase and modulation fluorometry. *Annu Rev Biophys Bioeng* 13:105-124.
- Hermoso J, Pignol D, Penel S, Roth M, Chapus G, Fontecilla-Camps JC. 1997. Neutron crystallographic evidence of lipase-colipase complex activation by a micelle. *EMBO J* 16:5531-5536.
- Holmquist M, Clausen IG, Patkar S, Svendsen A, Hult K. 1995. Probing a functional role of Glu87 and Trp89 in the lid of *Humicola lanuginosa* lipase through transesterification reaction in organic solvent. *J Protein Chem* 14:217-224.
- Holmquist M, Martinelle M, Clausen IG, Patkar S, Svendsen A, Hult K. 1994. Trp89 in the lid of *Humicola lanuginosa* lipase is important for efficient hydrolysis of tributyrin. *Lipid* 29:599-603.
- Holmquist M, Norin M, Hult K. 1993. The role of arginines in stabilizing the active open-lid conformation of *Rhizomucor miehei* lipase. *Lipid* 28:721-726.
- Jameson DM, Hazlett TL. 1991. Timersolved fluorescence in biology and biochemistry. In: Dewey G, ed. *Biophysical and biochemical aspect of fluorescence spectroscopy*. New York: Plenum Press. pp 105-133.
- Jamin M, Baldwin RL. 1998. Two forms of the pH 4 folding intermediate of apomyoglobin. *J Mol Biol* 276:491-504.
- Juszczak LJ, Zhang ZY, Wu L, Gottfried DS, Eads DD. 1997. Rapid loop dynamics of Yersinia protein tyrosine phosphatases. *Biochemistry* 36:2227-2236.
- Kungl AJ, Visser NV, van Hoek A, Visser AJWG, Billich A, Schilk A, Gstach H, Auer M. 1998. Time-resolved fluorescence anisotropy of HIV-1 protease inhibitor complexes correlates with inhibitory activity. *Biochemistry* 37:2778-2786.
- Lakowicz JR. 1983. *Principles of fluorescence spectroscopy*. New York: Plenum.
- Lawson DM, Brzozowski AM, Rety S, Verma C, Dodson GG. 1994a. Probing the nature of substrate binding in *Humicola lanuginosa* lipase through X-ray crystallography and intuitive modeling. *Protein Eng* 7:543-550.
- Lawson DM, Dodson GG, Hubbard RE, Høge-Jensen B, Boel E, Brzozowski AM, Derewenda ZS. 1994b. In: Woolley P, Peterson S, eds. *Lipase—Their biochemistry, structure and application*. United Kingdom: Cambridge University Press. pp 77-94.
- Lee JC, Timasheff SN. 1974. Partial specific volumes and interactions with solvent components of proteins in guanidine hydrochloride. *Biochemistry* 13:257-265.
- Liu WH, Beppu T, Arima K. 1977. Studies on the lipase of thermophilic fungus *Humicola lanuginosa*. Part VII. The chemical modification of the lipase of *Humicola lanuginosa* by N-bromosuccinimide in urea solution. *Agric Biol Chem* 41:131-135.
- Malecki J, Wasylewski Z. 1997. Stability and kinetics of unfolding and refolding of cAMP receptor protein from *Escherichia coli*. *Eur J Biochem* 243:660-669.
- Martinelle M, Holmquist M, Clausen IG, Patkar S, Svendsen A, Hult K. 1996. The role of Glu87 and Trp89 in the lid of *Humicola lanuginosa* lipase. *Protein Eng* 9:519-524.
- Martinelle M, Holmquist M, Hult K. 1995. On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim Biophys Acta* 1258:272-276.
- Miller JM. 1979. Recent advances in molecular luminescence analysis. *Anal Proc* 16:203-208.
- Myers JK, Pace CN, Scholtz JM. 1995. Denaturant *m* values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding. *Protein Sci* 4:2138-2148.
- Otlewski J, Sywula A, Kolasinski M, Krowarsch D. 1996. Unfolding kinetics of bovine trypsinogen. *Eur J Biochem* 242:601-607.
- Pace CN. 1975. The stability of globular proteins. *CRC Crit Rev Biochem* 2:1-43.
- Pace CN. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol* 131:266-280.
- Pace CN, Shirley BA, Thomson JA. 1989. Protein unfolding. In: Creighton TE, ed. *Protein structure and function: A practical approach*. Oxford, England: IRL Press. pp 311-330.
- Palleros DR, Shi L, Reid KL, Fink AL. 1993. Three-state denaturation of DnaK induced by guanidine hydrochloride. Evidence for an expandable intermediate. *Biochemistry* 32:4314-4321.

- Park SH, O'Neil KT, Roder H. 1997. An early intermediate in the folding reaction of the B1 domain of protein G contains a native-like core. *Biochemistry* 36:14277–14283.
- Peters GH, Svendsen A, Langberg H, Vind J, Patkar SA, Toxvaerd S, Kinnunen PKJ. 1998. Active serine involved in the stabilization of the active site loop in the *Humicola lanuginosa* lipase. *Biochemistry* 37:12375–12383.
- Pinheiro TJT, Elöve GA, Watts A, Roder H. 1997. Structural and kinetic description of cytochrome *c* unfolding induced by the interaction with lipid vesicles. *Biochemistry* 36:13122–13132.
- Privalov PL. 1996. Intermediate states in protein folding. *J Mol Biol* 258:707–725.
- Scalley ML, Baker D. 1997. Protein folding kinetics exhibit an Arrhenius temperature dependence when corrected for the temperature dependence of protein stability. *Proc Natl Acad Sci USA* 94:10636–10640.
- She M, Dong WJ, Umeda PK, Cheung HC. 1998. Tryptophan mutants of tropomyosin C from skeletal muscle—An optical probe of the regulatory domain. *Eur J Biochem* 252:600–607.
- Soulages JL. 1998. Chemical denaturation: Potential impact of undetected intermediates in the free energy of unfolding and *m*-values obtained from a two-state assumption. *Biophys J* 75:484–492.
- Steer BA, Merrill AR. 1995. Guanidine hydrochloride-induced denaturation of the colicin E1 channel peptide: Unfolding of local segments using genetically substituted tryptophan residues. *Biochemistry* 34:7225–7233.
- Steer BA, Merrill AR. 1997. Characterization of an unfolding intermediate and kinetic analysis of guanidine hydrochloride-induced denaturation of the colicin E1 channel peptide. *Biochemistry* 36:3037–3046.
- Steiner RF. 1991. Fluorescence anisotropy. In: Lakowicz JR, ed. *Topics in fluorescence spectroscopy*, vol 2. New York/London: Plenum Press.
- Svendsen A, Clausen IG, Patkar SA, Borch K, Thellersen M. 1997. Protein engineering of microbial lipase of industrial interest. *Methods Enzymol* 284:317–340.
- Swaminathan R, Nath U, Udgaonkar JB, Periasamy N, Krishnamoorthy G. 1996. Motional dynamics of a buried tryptophan reveals the presence of partially structured forms during denaturation of barstar. *Biochemistry* 35:9150–9157.
- Thomas JA, Buchsbaum RN, Zimniak A, Racker E. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* 18:2210–2218.
- Timasheff SN. 1993. The control of protein stability and association by weak interactions with water: How do solvents affect these processes? *Annu Rev Biophys Biomol Struct* 22:67–97.
- Tsapralis G, Chan DWS, English AM. 1998. Conformational states in denaturants of cytochrome *c* and horseradish peroxidases examined by fluorescence and circular dichroism. *Biochemistry* 37:2004–2016.
- Valente-Mesquita VL, Botelho MM, Ferreira ST. 1998. Pressure-induced subunit dissociation and unfolding of dimeric beta-lactoglobulin. *Biophys J* 75:471–476.