Effects of Urea and Guanidine Hydrochloride on the Activity and Dynamical Structure of Equine Liver Alcohol Dehydrogenase

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ABSTRACT: The inactivation of equine liver alcohol dehydrogenase by guanidine hydrochloride and urea has been studied by monitoring the intrinsic tryptophan fluorescence and phosphorescence emission. The use of triplet-state lifetimes to probe the flexibility of protein structure at the site of tryptophan-314 reveals a distinct behavior between the two denaturants. At predenaturational concentrations, the loss of enzyme activity in guanidine hydrochloride is associated with a loosening of intramolecular interactions resulting in a greater fluidity of the interior region of the macromolecule. In contrast, the interaction with urea, even at high concentrations, does not alter the dynamics of the native conformation. Enzyme activity is irreversibly lost as a result of a drastic unfolding of the macromolecule which occurs in a highly cooperative two-stage process.

It has been recognized for a long time that biological macromolecules must possess a fluctuating structure in order to carry out the multitude of functions in which they are involved. Only in recent years, however, have serious efforts been made from both experiment and theory to try to establish whether mobility is correlated with function and whether such a correlation implies a direct role of mobility rather than being incidental to it (Richards, 1983; Käiväräinen, 1985).

The detection of long-lived tryptophan phosphorescence from solutions of horse liver alcohol dehydrogenase (LADH)[1] at room temperature (Saviotti & Galley, 1974; Kishner et al., 1979) has opened the possibility to investigate its conformational fluctuations in the slow end of the spectrum. Knowledge of the dependence of the triplet-state lifetime of the indole nucleus on the viscosity of its microenvironment (Strambini & Gonnelli, 1985) has further increased the potentiality of this approach. Through the intrinsic phosphorescence lifetime, one can probe the fluidity at a specific site of the macromolecule. As this parameter has been shown to be sensitive to even very large effective viscosities, it may be particularly suitable in monitoring the mobility of highly structured rigid regions which undergo slow, large-amplitude fluctuations. It is these motions which are believed to play a determining role in the functional mechanism (Janin & Wodak, 1983; Käiväräinen, 1985).

The present work deals with the dynamical changes brought about at the site of Trp-314 in LADH by interaction with two perturbants of protein structure, namely, Gdn·HCl and urea. The results clearly distinguish between the action of the two denaturants. Predenaturational concentrations of Gdn·HCl inhibit completely the catalytic activity of LADH. The loss of function is not tied to a specific inhibition by Gdn·HCl or to a visible unfolding of the macromolecule. Instead, what accompanies the decrease in enzyme activity is a greater flexibility of this internal region of the protein. Concentrated urea, in contrast, is unable to alter the mobility at the site of Trp-314, and the loss of enzyme activity in the predenaturational concentration range is the result of competitive inhibition. Drastic structural changes in concentrated urea proceed parallel to the irreversible loss of catalytic activity and are found to take place at a slow rate as a highly cooperative two-stage process.

[1] Abbreviations: LADH, horse liver alcohol dehydrogenase; Trp, tryptophan; Gdn·HCl (GuHCl in figures), guanidine hydrochloride; CD, circular dichroism.

Materials and Methods

The LADH used in this study was the crystalline suspension supplied by Boehringer (Mannheim). Highest purity urea and Gdn·HCl were from Carlo Erba (Milan) and Merck (Darmstadt), respectively. These compounds were twice recrystallized and fresh solutions prepared daily. The enzyme was dialyzed for at least 24 h against 0.03 M pyrophosphate buffer, pH 8.6. Any remaining insoluble precipitate was removed by centrifugation. Fresh preparations were made weekly, and no loss of activity was found during that time.

The activity of LADH preparations, as measured by the Dalziel method (Dalziel, 1957), ranged between 130% and 145%. Active-site concentrations were also determined by spectrophotometric titration of LADH coenzyme binding sites with NAD^+ in the presence of excess pyrazole (Theorell & Yonetani, 1963). On the basis of a molar extinction coefficient of $E_{280} = 3.53 \times 10^4\ M^{-1}\ cm^{-1}$, the coenzyme binding capacity was typically 95% or better.

Sample Preparation. In both absorption and emission studies, the final concentration of LADH in 0.03 M pyrophosphate buffer, pH 8.6, was typically $(1.5–2) \times 10^{-5}$ M. Urea and Gdn·HCl concentrations were determined by density measurements.

For satisfactory deoxygenation of the sample prior to phosphorescence measurements, the following procedure has been adopted. Enzyme and denaturant solutions undergo separately a first stage of degassing by repeated application of moderate vacuum followed by an inlet of very pure nitrogen. In a glovebag, these solutions are then transferred in measured amounts to a specially constructed cell of Spectrosil quartz tubing (4 mm i.d.) provided with a side arm and a vacuum-tight cap. The denaturant is placed in the side arm and is mixed with the protein solution only before a kinetic run is started. The cap allows connection of the cell to a vacuum line from which it can be detached following deoxygenation without any danger of air leakage (Swagelok pat D-316). To free the empty cell from the O_2 adsorbed on its walls, it is kept under vacuum at about 80 °C for a period of 10 min. The final deoxygenation step consists of bringing the cell with the sample to an all-steel vacuum line. Multiple exchanges of nitrogen (0.1 ppm in O_2 content; SIO, Florence, Italy) ac-

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companied by gentle agitation of the sample decreases the O_2 content of the solution to its lowest value in about 5 min. A check on the thoroughness of O_2 removal is obtained from the dependence of the phosphorescence lifetime on the amount of excitation absorbed by the sample (Strambini, 1983).

A lifetime of 0.40 ± 0.02 s obtained for LADH with the present deoxygenation method does not show signs of O_2 depletion and compares favorably with the value of 0.25 ± 0.05 s reported by Calhoum et al. (1983), who used an enzymatic system for the removal of O_2. Measurements of enzyme activity before and after the degassing process showed no deterioration of the sample.

Enzyme Activity. With Gdn·HCl and urea in the predenaturational concentration range, assays were carried out in the presence of denaturant. Irreversible changes in enzyme activity with concentrated urea, instead, were obtained after dilution of the incubating mixture by a factor of 1000, thus reducing the concentration of urea in the assay to ineffective levels. The assays were carried out in 0.03 M pyrophosphate buffer, pH 8.6.

Luminescence Measurements. Fluorescence and phosphorescence measurements were carried out with a conventionally designed instrument. Exciting light was provided by a 100-W high-pressure Hg arc lamp (Osram HBO 100W/2) filtered through a nickel sulfate–cobalt sulfate solution. The excitation centered at 297 nm was selected by a 250-mm grating monochromator (Jarrel-Ash) employing a band-pass of 2 nm for fluorescence and 10 nm for phosphorescence. The phosphorescence was isolated by a single chopping wheel intersecting alternatively the excitation and emission beams, thus blocking direct light and fluorescence from reaching the detector.

The emission was dispersed by a 250-mm grating monochromator (Jobin-Jvon H25) and detected with an EMI 9635 QB photomultiplier. The steady-state signal was finally amplified by a lock-in amplifier (Itaco, Dynatrac 393). Compensation for fluctuating lamp intensity was achieved by a reference photomultiplier whose output was used in a ratio mode to normalize the emission signal.

Phosphorescence decays were monitored by a double-shutter arrangement permitting the emission to be detected only 2 ms after the excitation cutoff. The decay signal was stored and on occurrence averaged in a Varian C-1024 time-averaging computer. For the weakest intensities, 20–30 decays were sufficient to give a good signal to noise ratio.

Circular dichroism measurements were carried out in a Jasco Model J-500 A recording spectropolarimeter utilizing a cell of 1-mm path length. The solutions incubated with urea prior to CD measurements were dialyzed for 7 h against 20 mM phosphate buffer, pH 8. This medium is highly transparent up to 190 nm. All experiments were conducted at 25 °C.

RESULTS

LADH Inactivation by Gdn·HCl. Changes in LADH enzyme activity in the presence of Gdn·HCl are completely reversible for concentrations up to 1 M (Brand, 1962). In the present work, reversibility was found to extend to 1.5 M for an incubation time of 10 min. In Figure 1, we observe a monotonic decrease in enzyme activity with denaturant concentration, and almost all activity is lost within the predenaturational range. A kinetic analysis of the mechanism of inhibition, shown in Figure 2, demonstrates that Gdn·HCl acts as a classical noncompetitive inhibitor. The binding affinity of LADH for both NAD^+ and ethanol remains unaltered, and the presence of denaturant affects exclusively the catalytic

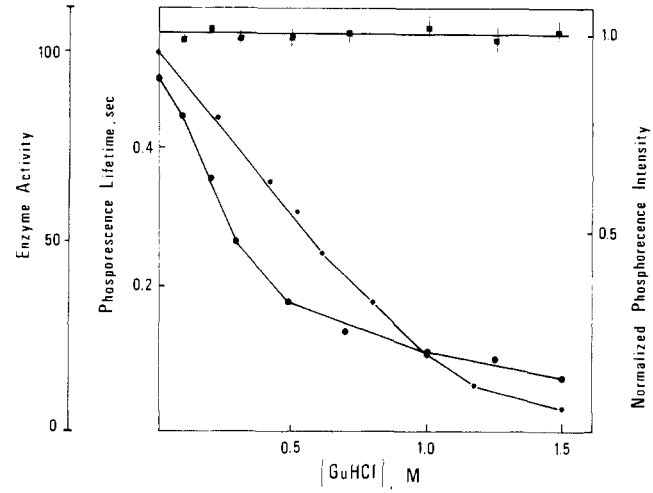

FIGURE 1: LADH phosphorescence lifetime (●), lifetime-normalized phosphorescence intensities of 440 nm (■), and relative enzyme activity for the oxidation of ethanol by LADH (asterisks) as a function of Gdn·HCl concentration. The buffer used was 0.03 M pyrophosphate, pH 8.6.

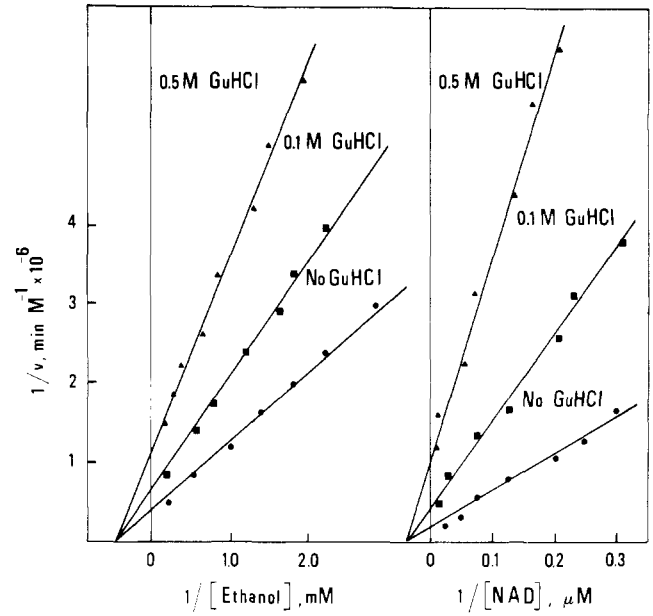

FIGURE 2: Gdn·HCl inhibition of the oxidation of ethanol by LADH. Variation of the reciprocal of the initial rate with the reciprocal of one substrate concentration (ethanol or NAD^+) in excess of the other substrate (1.5×10^{-2} M ethanol and 4.0×10^{-4} M NAD^+, respectively).

properties (V_{max}) of the enzyme.

The luminescence of LADH excited at 297 nm originates exclusively from the two tryptophan residues in each subunit. Trp-314 is deeply buried within the protein folds with practically no access to the solvent. It gives rise to a blue-shifted unrelaxed fluorescence spectrum (Ross et al., 1981), and it is the sole contributor to the room temperature phosphorescence (Saviotti & Galley, 1974). Trp-15, on the other hand, being close to the surface of the macromolecule, is solvent-exposed and gives rise to a red-shifted fluorescence emission. Protein unfolding or conformational changes which bring Trp-314 in contact with water would result in a red-shifted fluorescence with lower quantum efficiency followed by the complete disappearance of phosphorescence. The effects of Gdn·HCl on the luminescence of LADH are shown in Figure 3. The fluorescence spectrum is not affected by Gdn·HCl, and even at 1.5 M, the only visible change is an 8–10% decrease in the apparent quantum yield (to be corrected for

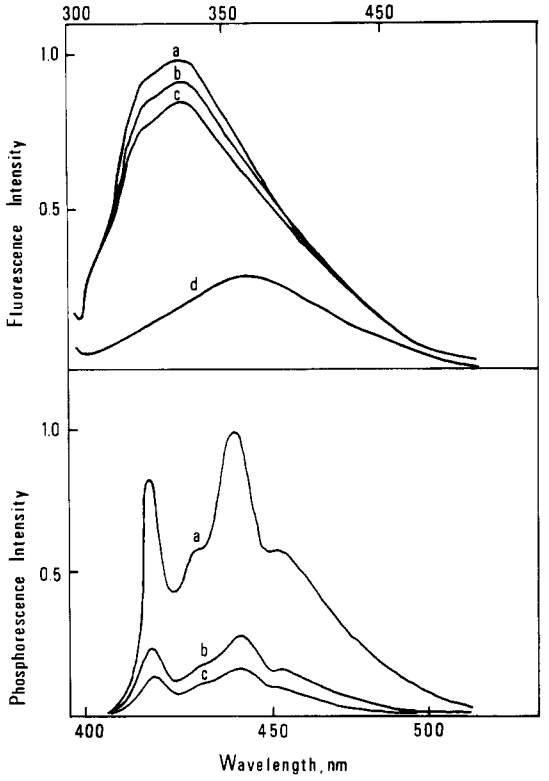

FIGURE 3: Fluorescence and phosphorescence spectra of LADH in 0.03 M pyrophosphate buffer, pH 8.6, in the presence of Gdn·HCl. (a) No Gdn·HCl; (b) 0.8, (c) 1.5, and (d) 6 M Gdn·HCl. Spectra are not corrected for instrumental factors.

changes in the refractive index and fluorescence yield of Trp in the presence of denaturant). Sharp vibrational bands are displayed by the phosphorescence spectrum, and no broadening or shifting of maxima is detected throughout the concentration range. The steady decrease in phosphorescence intensity does not mean that fewer LADH molecules are contributing to the emission. In fact, lifetime-normalized intensities are seen in Figure 1 to remain constant throughout.

LADH phosphorescence lifetime measurements show that the decay of about 95% of the emission is well represented by a single exponential function. This was also found to be the behavior in Gdn·HCl. Figure 1 shows that with increasing amounts of Gdn·HCl the tryptophan triplet-state lifetime, τ, becomes ever smaller and that τ is the only emission parameter really sensitive to the denaturant. Since Gdn·HCl is not a quencher of tryptophan phosphorescence, as confirmed in low-temperature experiments, the shortening of τ can only reflect a change in the dynamics of the LADH structure about Trp-314. The spectral properties of LADH emission, together with a monoexponential decay of the phosphorescence, are consistent with the presence in solution of a single conformation of the macromolecule similar to the native state and whose characteristics are modulated by the interaction with the denaturant. The loss of enzyme activity in Gdn·HCl would, then, reflect such changes in each protein rather than the complete denaturation of a part of the population.

LADH Conformational Changes Induced by Urea. Alterations in molecular structure induced by concentrated urea are clearly manifested by irreversible changes in enzyme activity. The rate of this process was found to be independent of protein concentration and to be governed by such factors as concentration of urea, time of incubation, and temperature. Figure 4 reports the activity recovered from an LADH solution incubated for 10 min at various urea concentrations. The time dependence of this process in 6 M urea at 25 °C is represented

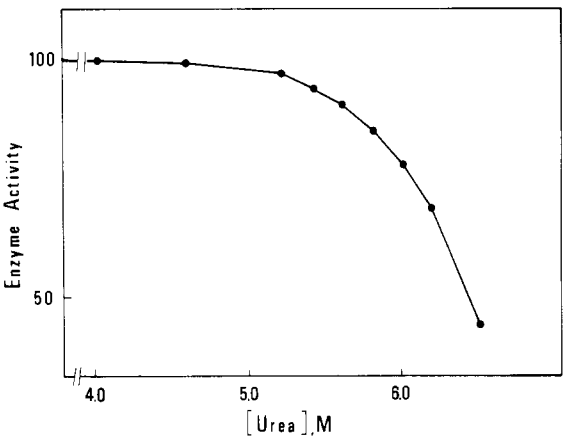

FIGURE 4: Irreversible loss of enzyme activity for the oxidation of ethanol by LADH in 0.03 M pyrophosphate buffer, pH 8.6, after 10-min incubation with urea.

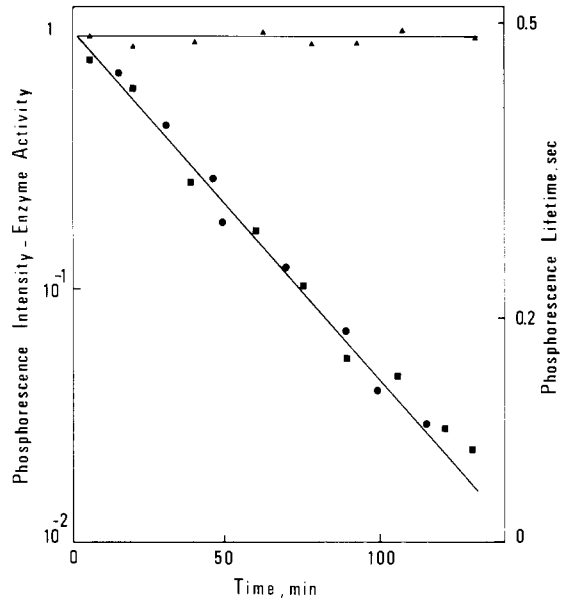

FIGURE 5: Phosphorescence intensity at 440 nm (■), lifetime (▲), and enzyme activity (●) of LADH with time of incubation in 6 M urea.

in Figure 5. The kinetics of inactivation follow an exponential law typical of simple two-stage unimolecular reactions. From this behavior, it may be inferred that the unfolding process responsible for the inactivation of LADH is characterized by a single rate-limiting step.

The luminescence changes accompanying the irreversible inactivation of LADH by 6 M urea are shown in Figure 6. As the reaction proceeds, we observe a progressive broadening of the fluorescence spectrum whose λ_{max} red-shifts from 335 to 357 nm. Concomitant to it is a reduction in fluorescence yield. These changes are over at complete inactivation and are consistent with the exposure of Trp-314 to solvent. Phosphorescence data confirm this picture and provide even greater detail. While the spectrum is unaltered with regard to the widths and maximum wavelengths of the main vibronic bands, the phosphorescence intensity drops with time of incubation in a fashion identical with the enzyme activity (Figure 5). Such parallelism has been verified across a range of temperatures and urea concentrations. This data will be reported elsewhere.

A salient feature of the phosphorescence in urea is the constancy of the triplet-state lifetime. The phosphorescence intensity decays exponentially with a τ of 0.40 ± 0.02 s at 25

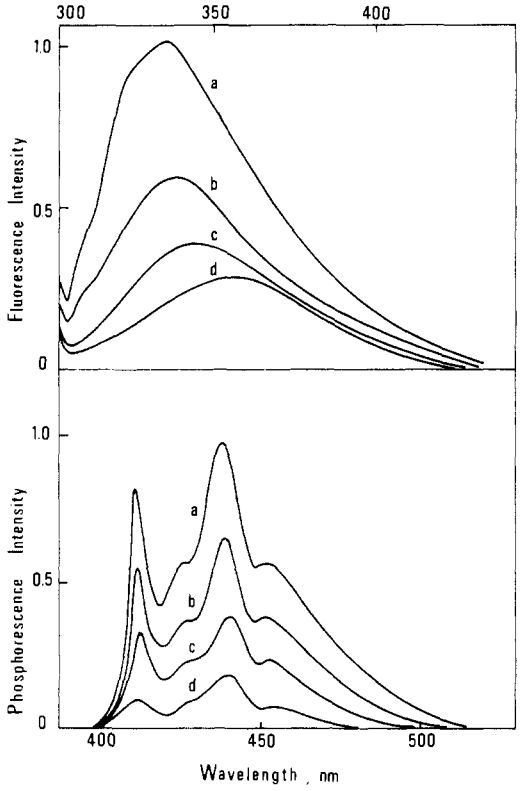

FIGURE 6: Fluorescence and phosphorescence spectra of LADH in 6 M urea after various periods of incubation: (a) 2, (b) 15, (c) 30, and (d) 70 min. Spectra are not corrected for instrumental response.

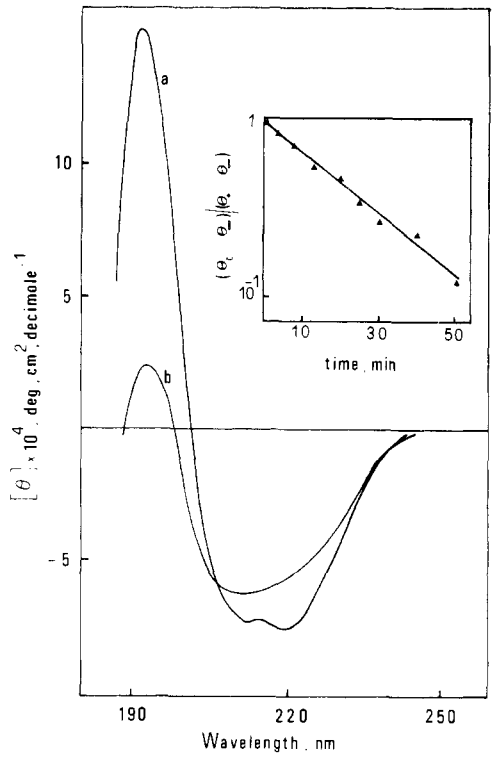

FIGURE 7: Circular dichroism spectra of LADH in the peptide absorption band. (a) Native enzyme; (b) after 24 h in 6 M urea. Inset: Plot of the relative change in molar ellipticity at 194 nm as a function of the time of incubation in 6 M urea.

°C for any concentration of urea examined and at any stage of the inactivation process. Because of the phosphorescence lifetime is the same throughout, the decrease in phosphorescence intensity is a direct measure of the number of protein molecules which contribute to the emission. As the fraction of LADH molecules which phosphoresce is at any stage identical with the fraction of remaining enzyme activity, it must be concluded that exposure of Trp-314 to the solvent and enzyme inactivation are due to the same unfolding process. Conversely, even in concentrated urea, those macromolecules which have not undergone such unfolding are fully functional.

To assess the structural changes ensuing from the interaction with urea, the circular dichroism (CD) spectrum was recorded at various stages of the process. Figure 7 displays the CD spectrum in the peptide absorption region for native LADH and for the final product of denaturation in 6 M urea. The native spectrum is typical of α/β-proteins (Parthasarathy & Curtis Johnson, 1983). The characteristic features are a negative band with a minimum at 220 nm more pronounced than the one at 208 nm and a positive band with a maximum at 193 nm. The unfolded protein shows a marked reduction in the 193-nm band accompanied by a decrease in the 220-nm band. The spectrum resembles that of all β-proteins. Unfolding of LADH produced by the interaction with urea would then seem to consist mainly in the loss of α-helical content. The time dependence of the relative change in molar ellipticity at 293 nm also follows an exponential law, yielding a rate constant, $k = 6.8 \times 10^{-4}$ s^{-1}, within experimental error equal to that observed with phosphoresence intensity and enzyme activity.

Discussion

On the basis of the irreversible loss of enzyme activity, a critical concentration of denaturant is found for Gdn·HCl and urea beyond which perturbations of protein structure lead to permanent unfolding. In the predenaturational range, while the effects of such interactions appear as a decreased functionality of the enzyme, they are hardly detectable by common physical methods. In this respect, the intrinsic phosphorescence of LADH, being particularly sensitive to the dynamic makeup about Trp-314, was found to be helpful in discerning the kind of alterations resulting from the interaction with Gdn·HCl and urea. While urea seems to have little if any effect on the native structure of LADH and the loss of enzyme activity is attributed to a specific interaction at the active center (Rajagopalan et al., 1961), even modest amounts of Gdn·HCl were seen to alter the internal dynamics of the macromolecule. Furthermore, such a change in flexibility corresponds to a change in catalytic activity which cannot be ascribed to a specific inhibition mechanism or to unfolding of the macromolecule.

Greater flexibility at the chromophore site may be brought about by Gdn·HCl in a number of ways. Two extreme situations may be envisaged: (1) The macromolecule may undergo a major reversible unfolding which brings Trp-314 in contact with the solvent. Schematically

$$\begin{array}{ccc} \text{Trp-314}_{\text{native}} & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & \text{Trp-314}_{\text{solvent}} \\ \downarrow & & \downarrow \\ \tau_n & & \tau_s \end{array} \qquad (1)$$

where $\tau_n = 0.4$ s and $\tau_s = 1.6 \times 10^{-5}$ s are the triplet lifetimes for the two conformations of LADH. Interaction with Gdn·HCl may lower the activation barrier such that when k_1 becomes of the order of $1/\tau_n$ the phosphorescence lifetime is governed by

$$1/\tau(\text{Gdn·HCl}) = 1/\tau_n + k_1(\text{Gdn·HCl}) \qquad (2)$$

(2) The substitution of intramolecular H bonding by Gdn·HCl may gradually loosen some segments of secondary and tertiary

structure to allow a greater flexibility for the unusually rigid environment of Trp-314 which then translates in a smaller τ value.

Although a rigorous discrimination between these proposals is not possible at this stage, some considerations support the second hypothesis as the more plausible one. Trp-314 as seen from the crystal structure (Branden et al., 1975) is placed in the inner core of the macromolecule boxed in by β-sheets and α-helical segments. This assembly of secondary structure also defined as a "knot" is characterized, according to H-exchange, neutron scattering, and NMR studies, by an exceptional rigidity (Gregory & Lumry, 1985). A major unfolding which exposes the aromatic side chain to the solvent is bound to be slow and of an irreversible nature. Further, since such a solvent-exposed conformer is not detected in fluorescence emission, we must have $k_2 > 10k_1$ in eq 1. From the kinetics of denaturation of small proteins at even higher Gdn·HCl content (Tanford, 1968), the rates of unfolding are typically of the order of minutes, the refolding being invariably slower. The corresponding values of $k_1 \sim 10\ s^{-1}$ and $k_2 > 10^2\ s^{-1}$ in 1.5 M Gdn·HCl would then be too large by at least 2 orders of magnitude.

It is not uncommon to find Gdn·HCl and urea grouped together for the mechanism of protein unfolding, namely, stabilization of the denatured state of the macromolecule by lowering the hydrophobic interaction. The greater effectiveness of Gdn·HCl has been attributed to a more pronounced reduction in free energy when amino acids are transferred from water to 6 M Gdn·HCl (8.3 cal/Å^2) compared to 8 M urea (7.1 cal/Å^2) (Creighton, 1979). The ability of Gdn·HCl, then, to affect the dynamic behavior of LADH may seem somewhat surprising when compared to the ineffectiveness of urea. It must be recalled, however, that unlike urea the former is a positively charged species which, as found for inorganic salts, shows a stabilizing effect of the peptide unit in water (Tanford, 1968). Thus, the greater affinity of Gdn·HCl for H bonding to the peptide unit may result in the partial disruption of internal H-bonded structures and be at the root of the dynamical changes observed. A similar interpretation was also given for the swelling of α-lactalbumin in predenaturational concentrations of denaturant (Kuwajima et al., 1976).

In the last few years, almost the entire arsenal of biophysical techniques has been tackling the question of how structural fluctuations might play a role in the functioning of biological macromolecules (Richards, 1983; Käiväräinen, 1985; Compiani et al., 1985). Protein hydration studies have provided evidence linking enzyme activity to the dynamics of protein structure, full activity in the lysozyme being achieved only after the flexibility of the macromolecule is restored (Rupley et al., 1983). The present findings of an increased flexibility of LADH in the presence of Gdn·HCl, without alteration of the overall three-dimensional structure, as judged by binding affinities and fluorescence spectra, constitute an example of how too great a flexibility may dampen the effectiveness of the biological catalyst. By means of the relationship found between triplet-state lifetime and microviscosity (Strambini & Gonnelli, 1985), the fall in enzyme activity is compared in Figure 8 to the increase in the effective viscosity experienced by the indole nucleus at the site of Trp-314. Although the meaning of such a correlation awaits for a proper model to be developed, it does nevertheless demonstrate that the dynamic behavior of this knot region of the macromolecule is either directly or indirectly involved in its capability to function.

The techniques normally employed to detect the transformations induced by denaturants or other effectors report on changes averaged over the entire sample. The main usefulness of the spectroscopic approach presented here comes perhaps from the ability of phosphorescence spectra together with yields and lifetimes to provide microscopic information about the population of macromolecules subject to the perturbation. Thus, it has been possible to attribute the loss of enzyme activity of LADH to the alteration of the characteristics of each macromolecule in the case of Gdn·HCl in contrast to full denaturation of only a fraction of the population in the case of urea. Furthermore, protein denaturation mechanisms usually treat the reaction as a two-stage process (Tanford, 1970; Privalov, 1979). While in many cases this may be a valid assumption, the present results indicate that at least with Gdn·HCl this is not justified.

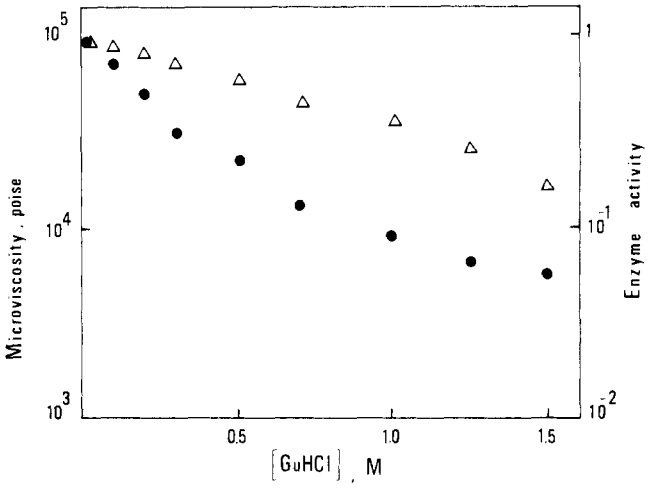

FIGURE 8: Lifetime-derived microviscosity at the site of Trp-314 (●) and LADH enzyme activity (△) in the presence of Gdn·HCl.

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Registry No. LADH, 9031-72-5; Gdn·HCl, 50-01-1; $(NH_2)_2CO$, 57-13-6.

REFERENCES

Brand, L., Everse, J., & Kaplan, N. O. (1962) *Biochemistry 1*, 423–434.

Brändén, C. I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed. 11A*, 103–190.

Calhoun, D. B., Vanderkooi, J. M., Woodrow, G. V., III, & Englander, S. W. (1983) *Biochemistry 22*, 1526–1532.

Compiani, M., Fonseca, T., Grigolini, P., & Serra, R. (1985) *Chem. Phys. Lett. 114*, 503–507.

Creighton, T. E. (1979) *J. Mol. Biol. 129*, 235–264.

Dalziel, K. (1957) *Acta Chem. Scand. 11*, 397–398.

Gregory, R. B., & Lumry, R. (1985) *Biopolymers 24*, 301–326.

Janin, J., & Wodak, S. J. (1983) *Prog. Biophys. Mol. Biol. 42*, 21–78.

Käiväräinen, A. I. (1985) in *Solvent-Dependent Flexibility of Protein and Principles of their Function*, D. Riedel Publishing Co., Dordrecht, The Netherlands.

Kishner, S., Trepman, E., & Galley, W. C. (1979) *Can. J. Biochem. 57*, 1299–1304.

Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359–373.

Parthasaraty, M., & Curtis Johnson, W. (1983) *Nature (London) 305*, 831–832.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.

Rajagopalan, K. V., Fridovich, I., & Handler, P. (1961) *J. Biol. Chem. 236*, 1059–1065.
Richards, F. (1983) *Ciba Found. Symp. 93*, 1–3.
Ross, J. B. A., Schmidt, C. J., & Brand, L. (1981) *Biochemistry 20*, 4369–4377.
Rupley, J. A., Gratton, E., & Careri, G. (1983) *Trends Biochem. Sci. (Pers. Ed.) 8*, 18–22.
Saviotti, M. L., & Galley, W. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4154–4158.
Strambini, G. B. (1983) *Biophys. J. 43*, 127–130.
Strambini, G. B., & Gabellieri, E. (1984) *Photochem. Photobiol. 39*, 725–729.
Strambini, G. B., & Gonnelli, M. (1985) *Chem. Phys. Lett. 115*, 196–201.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–281.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Theorell, H., & Yonetani, T. (1963) *Biochem. Z. 338*, 537–553.

Activity of Copper-Substituted Carboxypeptidase A toward Oligopeptides and Depsipeptides†

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ABSTRACT: Cu(II)-substituted carboxypeptidase A catalyzes the hydrolysis of oligopeptides and their depsipeptide (ester) analogues. Stopped-flow fluorescence assays demonstrate that relative to the zinc enzyme the Cu enzyme can have k_{cat}/K_m values up to 24% toward esters but only up to 2.5% toward the corresponding peptides. Adding Zn(II) to the copper enzyme reveals a slow exchange process that correlates with an increase in peptidase activity and with changes in the Cu(II) electron paramagnetic resonance spectra. Low concentrations of 1,10-phenanthroline (OP) (0.1–2.5 μM) markedly increase activity toward furanacryloyl-Phe-Phe (up to 8% of the zinc enzyme), but higher concentrations inhibit, resulting in complete inhibition at 0.8 mM OP. The non-metal-binding, hydrophobic analogues *m*- and *p*-phenanthroline are only activators of peptide hydrolysis, even at 1 mM. Activation is likely due to a modifier binding to a hydrophobic locus and either displacing an inhibitory peptide binding mode or inducing a conformational change in the active site.

The replacement of Zn(II) in several metalloenzymes by Cu(II) usually leads to nearly or totally inactive derivatives as is observed for carbonic anhydrase (Bertini et al., 1978), alkaline phosphatase (Lazdunski et al., 1970), liver alcohol dehydrogenase (Maret et al., 1983), and thermolysin (Holmquist & Vallee, 1974).

The activity of the mixed Cu–Zn derivative of *Aeromonas* aminopeptidase, on the other hand, is increased about 60-fold compared to that of the native 2Zn enzyme (Prescott et al., 1985), and Cu_2–Cu_2 superoxide dismutase is about as efficient a catalyst as the Zn_2–Cu_2 dimer (Fee, 1973; Fee & Briggs, 1975). Except for the reported hydrolysis of a thiol ester catalyzed by Cu-substituted carboxypeptidase A (Cu-carboxypeptidase A)[1] with a k_{cat} of 0.04 s^{-1} and a K_m of 94 μM (Schneider et al., 1976), no peptidase and esterase activity data are available for this metallo derivative. The reconstitution of apocarboxypeptidase A with all other VIIB–IIB (groups 7, 8, 9, 10, and 12 in 1985 notation) divalent metal ions of the first transition series (Mn, Fe, Co, Ni, Zn), however, restores the catalytic activity to different but significant extents (Coleman & Vallee, 1960, 1961; Auld & Vallee, 1970; Auld & Holmquist, 1974; King & Fife, 1983; Vallee et al., 1983; Dua & Gupta, 1984), esters generally being hydrolyzed more readily than peptides.

Since the electronic and paramagnetic spectral properties of Cu(II) (Bertini & Scozzafava, 1981) could render it a useful probe of carboxypeptidase for the investigation of its reaction mechanism, we reexamined the question of the activity of this metalloenzyme toward oligopeptides and their depsipeptide analogues under experimental conditions that we improved substantially compared to earlier studies (Coleman & Vallee, 1961).

Materials and Methods

Carboxypeptidase A was substituted with Cu(II) by suspending the affinity chromatography purified (Bicknell et al., 1985) enzyme crystals (Sigma Chemical Co., catalog no. C 0261) 4 times in 0.01 M 1,10-phenanthroline/0.01 M Mes, pH 7.0, buffer for 40 min and washing 5 times with metal-free buffer (Auld & Holmquist, 1974), followed by adding the desired amount of an aqueous solution of $CuSO_4$ (Puratronic, Johnson Matthey) to the dissolved enzyme. Solutions of the apoenzyme and of the metallo derivatives were prepared immediately before the experiments. Substrates were synthesized

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[1] Abbreviations: Cu(II)-carboxypeptidase A, Cu(II)-substituted carboxypeptidase A; RET, radiationless energy transfer; Dns or dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; FA, furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; OP, 1,10-phenanthroline; EPR, electron paramagnetic resonance; Bz, benzoyl; OPhe, L-β-phenyllactate.

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