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THE RATE OF EQUINE LIVER ALCOHOL DEHYDROGENASE DENATURATION BY UREA

DEPENDENCE ON TEMPERATURE AND DENATURANT CONCENTRATION

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The kinetics of the irreversible urea denaturation of equine liver alcohol dehydrogenase have been studied as a function of temperature and urea concentration. The unfolding of the macromolecule, monitored by means of the phosphorescence properties of a deeply buried tryptophan residue, was found to be strictly a two-state process over the entire temperature range. It is characterized by a steep dependence on urea concentration typical of highly cooperative transitions and below room temperature it possesses large negative activation energies. The reaction is comparatively slow, does not seem to be preceded by a fast phase, and the rate-limiting step does not have the characteristics of proline isomerization. When the data are analyzed in terms of binding equilibria the temperature dependence results from an anomalously large change in heat capacity. Although this is a property of strong hydrophobic interactions in model compounds the slow rates of denaturation are best understood with a model of protein stability which emphasizes the cooperative nature of intramolecular interactions such as hydrogen bonding.

1. Introduction

The interaction of equine liver alcohol dehydrogenase (LADH) with urea and guanidine hydrochloride (GuHCl), has recently been investigated utilizing a novel approach based on the intrinsic tryptophan phosphorescence of the enzyme at room temperature [1]. In this study advantage is taken of the particular sensitivity of the triplet state lifetime of the indole nucleus for the effective viscosity of its immediate environment [2]. Trp 314 in LADH is located in a highly structured region sandwiched between α-helices and β-sheets [3] whose remarkable rigidity is affected by even minor and otherwise undetectable structural changes [1]. Using the emission from the internal residue as a probe of the flexibility and integrity of protein structure it becomes possible to monitor subtle conformational changes all the way to major unfolding.

The former study of the interaction of LADH with these classical denaturants has emphasized the difference in the mechanism by which they destabilize the native conformation of the macromolecule. At predenaturational concentrations of GuHCl (less than 1 M) the loss of enzyme activity is not accompanied by visible unfolding of the native structure. The process is associated with a loosening of intramolecular interactions, resulting in a greater fluidity of the interior region of the macromolecule.

Urea, in contrast, even in large amounts, does not alter the dynamics of the native conformation, giving rise instead to a slow and extensive unfolding of the macromolecule.

For a number of small globular proteins denaturation was shown to exhibit complex kinetics with one or more fast phases accompanied by a slow rate-limiting phase [4]. There is growing consensus on the notion that unfolding of organized

protein structures can be a rapid process and the presence of a slow phase must be sought in processes such as the breaking of sulphur bridges and isomerization of the peptide bond of proline residues [5]. In view of the remarkable difference between GuHCl and urea in ability to affect the compactness of the native state of LADH it was deemed instructive to investigate the nature of the forces which stabilizes it and determines the slow irreversible unfolding in urea.

In the present study we follow the irreversible unfolding of LADH as a function of temperature and urea concentration. The finding of negative activation energies at temperatures below room temperature together with a steep dependence on denaturant concentration rule out proline isomerization as the mechanism for the slow unfolding. Since this protein possesses no sulphur bridges, the results suggest instead that large entropic barriers to unfolding may be provided by the high cooperativity of intramolecular hydrogen bonding.

2. Materials and methods

The LADH used in this study was the crystalline suspension supplied by Boehringer (Mannheim). Highest purity urea, from Carlo Erba (Milan), was 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 method of Dalziel [6], ranged between 130 and 145%. Active site concentrations were also determined by the spectrophotometric titration of LADH coenzyme-binding sites with NAD^+ in the presence of excess pyrazole [5]. Based on a molar extinction coefficient $E_{280} = 3.53 \times 10^4\ M^{-1}\ cm^{-1}$, the coenzyme-binding capacity was typically 95% or better.

2.1. Sample preparation and luminescence measurements

Since urea acts as a competitive inhibitor of this enzyme [1], irreversible changes in enzyme activity following incubation with concentrated urea were obtained after diluting the incubation mixture by a factor of 1000.

In emission studies the final concentration of LADH was typically $1.5\text{–}2 \times 10^{-5}$ M. Urea concentrations were determined by density measurements. Prior to phosphorescence measurements the samples were thoroughly deoxygenated following a procedure described elsewhere [1].

A check on the completeness of O_2 removal is obtained from the dependence of the phosphorescence lifetime on the amount of excitation absorbed by the sample [8]. A lifetime of 0.40 ± 0.02 s obtained for LADH at 25°C agrees with the oxygen-free value [1].

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/cobalt sulphate solution. The excitation centred at 297 mm was selected by a 250 nm grating monochromator (Jarrel-Ash) employing a band-pass of 10 nm for phosphorescence. The phosphorescence was isolated by a single chopping wheel intersecting alternately 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 monitorized by a double-shutter arrangement permitting the emission to be detected only 2 ms after the excitation cut off. The decay signal was stored and on occurrence averaged in a Varian C-1024 time-averaging computer. For the weakest intensities 10–20 decays were sufficient to give a good signal-to-noise ratio. Most experiments were repeated three times and the data reported refer to the mean values.

3. Results and discussion

The phosphorescence emission from LADH at room temperature is entirely due to Trp 314 [9] which in the native conformation of the macromolecule has an intrinsic lifetime (τ) of 0.4 s. Unfolding of the polypeptide resulting with the exposure of this aromatic residue to the solvent reduces its lifetime by at least 4 orders of magnitude [10]. As a result of the corresponding reduction in phosphorescence quantum yield the phosphorescence from denatured LADH is no longer detectable. The decrease in phosphorescence intensity with time is, therefore, a direct measure of the rate of LADH denaturation. Fig. 1 reports the change in phosphorescence intensity during the course of denaturation. The emission is found to decrease with time according to an exponential law independent of the protein concentration as would be expected for a pseudo-first-order reaction. The unimolecularity of the process is confirmed by the detection of a single phosphorescing molecular species throughout the course of the reaction. The absence of components with different lifetimes excludes, within the limits of detection, the existence of intermediate states in the structural change monitored by phosphorescence. The lifetime, even in the presence of urea, is identical to that of the native state. This finding is instructive because it implies that the environment of Trp 314 in the non-denatured fraction of macromolecules is structurally and dynamically identical to that in the native state. Furthermore, should the transition monitored by phosphorescence be preceded by a rapid phase, such partial unfolding would not affect the structure in the neighbourhood of this chromophore in any way.

In terms of the phosphorescence data the simplest general scheme for the overall irreversible process may be written (omitting denaturant) as follows:

$$\underset{\tau = 0.4}{N} \rightarrow \underset{\tau = 0.4}{D_1} \overset{k_{12}}{\rightarrow} \underset{\tau \leqslant 0.004}{D_2} \rightarrow \underset{\tau \leqslant 0.004}{D_3}$$

where the native state, N, may rapidly reach a

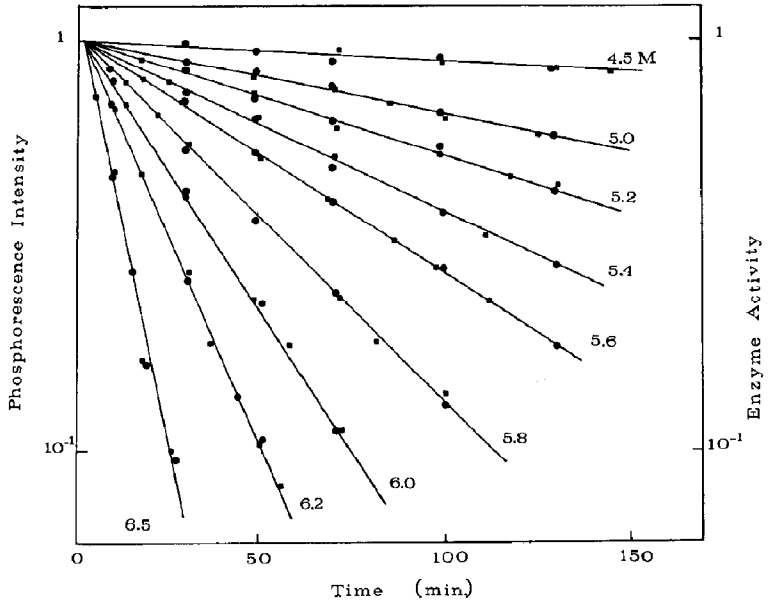

Fig. 1. Phosphorescence intensity at 440 nm (●) and enzyme activity (■) of LADH with time of incubation in various urea concentrations relative to the native state. Experiments were carried out at 25°C in 0.03 M pyrophosphate buffer (pH 8.6).

partially denatured form, D_1, which slowly undergoes a major unfolding to D_2. The final stage of the process, D_3, may then be obtained in subsequent steps. One can be more specific about such a scheme if the effects of denaturation on enzyme activity (fig. 1) and on the overall structure as revealed by circular dichroism [1] are also taken into account. Most important is the observation that both the rate of change in enzyme activity as well as of the circular dichroism signal are within experimental error identical to k_{12}. This implies that the same rate-limiting step, represented by k_{12}, also governs the unfolding responsible for the complete loss of activity and organized structure.

If subsequent steps of denaturation exist beyond D_2 they must be rapid compared to k_{12}. In addition, the finding that both the enzyme activity and circular dichroism signal extrapolate at time zero to the value of the native conformation argues against the existence of a fast irreversible phase preceding the rate-limiting step, i.e., state D_1 should on these accounts be identified with N. Indeed, considering the sensitivity of triplet state lifetimes, τ, in proteins to even slight structural perturbations [11] the constancy of τ in high concentrations of urea suggests the absence of even reversible changes prior to the slow step. The conclusion to be drawn from the three experimental approaches employed so far is that urea denaturation of LADH is a slow highly cooperative all-or-none two-state transition.

With the detection of rapid phases in the pathway of protein unfolding and refolding the notion has spread that slow phases with lifetimes of the order of minutes are invariably associated with particular processes such as proline isomerization and/or the making or breaking of sulphur bridges [12]. In the absence of the latter, as is the case with LADH, if proline isomerization were indeed the rate-limiting step the denaturation would be expected to show an activation energy within 6–19 kcal mol^{-1} depending on the particular environment of this residue [13]. The effect of temperature on the rate of LADH denaturation in 6 M urea is shown in fig. 2. The striking feature of the temperature dependence is the presence of a pronounced minimum in the velocity of the reaction around 25–30°C. Below this temperature the process has an apparently negative activation energy. However singular this phenomenon may seem, it is not the first example reported and closely resembles the denaturation of ovalbumin in urea observed by Simpson and Kauzmann [14].

In order to gain some understanding as to the molecular interactions at the root of such behaviour the dependence on urea concentration was studied. The concentration dependence of the rate constant for a number of protein denaturations is found to obey an empirical relationship of the type [15,16]:

$$k = (\text{constant}) \cdot C^{\nu}$$

where C is the concentration of denaturant. In our experiments we find that, over the limited range of concentrations employed, such a relationship is satisfactorily obeyed from 0 to 55°C (fig. 3). The values obtained for ν are reported in fig. 2 and compared to those found by Simpson and Kauzmann [14] for ovalbumin.

In the case of ovalbumin the authors have worked out a mechanism to account for the steep dependence of the rate constant on the concentration of urea. The basic idea was that the combination of denaturant with particular sites of the native protein destabilizes the native state and facilitates the transition to the activated state. In such a framework ν is simply the difference between the number of urea molecules bound to the activated complex and the native state. A fall in its value with increasing temperature is attributed to an increased dissociation of addition complexes. It is this decrease of ν with temperature that brings about the unusual negative activation energies at low temperatures. Such an interpretation, however, does not account for the present findings with LADH in that the native state does not seem to be affected by urea binding and furthermore ν is not a monotonic function of temperature.

A more general approach, based on binding equilibria, was applied by Tanford [15,16] for the interpretation of both kinetic and equilibrium data on protein denaturation. In this formalism:

$$\delta \ln k / \delta \ln a_x = \nu_{\text{pref.}}$$

where k is either a rate or equilibrium constant,

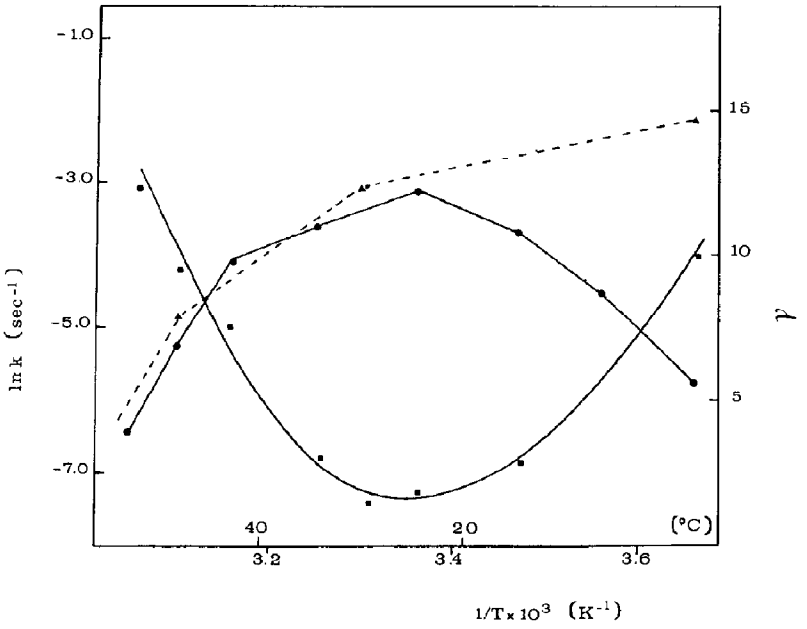

Fig. 2. Effect of temperature on both the rate constant (■) and the parameter υ (●) for LADH denaturation in 6 M urea. For comparison, values of υ found by Simpson and Kauzmann (▲) for ovalbumin are reported. The experiments were carried out in 0.03 M pyrophosphate buffer (pH 8.6).

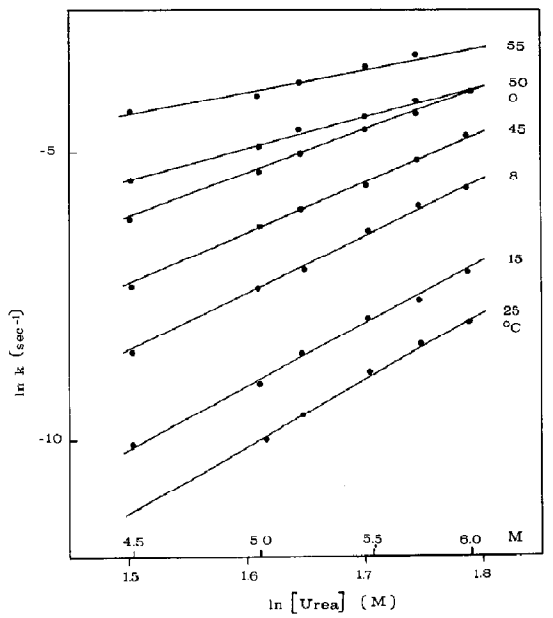

Fig. 3. Rate constant for LADH denaturation as a function of urea concentration at several temperatures. The data were obtained in 0.03 M pyrophosphate buffer (pH 8.6).

a_x the denaturant activity and $\upsilon_{pref.}$ represents the number of denaturant molecules preferentially bound to the activated complex or the denatured state, respectively. Since binding sites for denaturant are deemed to exist only after unfolding of the native structure, preferential binding refers in practice to the number of molecules bound to the activated complex or to the denatured state.

Fig. 4 shows the temperature dependence of the activation enthalpy and a comparison of selected thermodynamic parameters for the denaturation of ovalbumin and β-lactoglobulin is reported in table 1. We note a striking similarity in the behaviour of LADH with both the rate of denaturation of ovalbumin and the equilibrium constant for the denaturation of β-lactoglobulin. The main feature is an anomalously large change in heat capacity typical of strong hydrophobic interactions in model compounds. Because of this analogy to model compounds it has been proposed that the rate-limiting process in protein unfolding is dominated by strong hydrophobic interactions brought about by an extensively unfolded activated

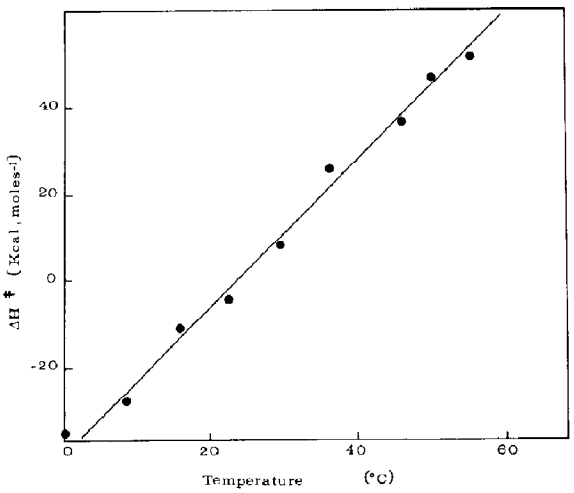

Fig. 4. Effect of temperature on the activation enthalpy for LADH denaturation in 6 M urea. Activation enthalpy was evaluated from $\Delta H^{\ddagger} = -R(\partial \ln k/\partial\ 1/T) - RT$ (R, gas constant).

complex [16]. However, such preponderance of hydrophobic interactions in stabilizing the native state of proteins has been severely questioned [17]. Quantitative estimations of hydrophobicity are unable to account for the stability of the folded state [16,18] and the hydrophobic effect in model compounds would predict, contrary to experimental evidence, increasing protein stability up to temperatures of about 60°C [16].

A better appreciation of the stabilizing forces in globular proteins has come with the recognition that intramolecular interactions, hydrogen bonds and salt bridges in particular, can be orders of magnitude stronger than intermolecular ones due to the large 'effective concentration' of the interacting groups in the former [17,19,20]. Large effective concentrations, whence compactness and stability of the folded state, rely entirely on the simultaneous presence of all interactions, and the breaking of the first bonds will have a large destabilizing effect due to the weakening of those remaining. Thus, the activated complex in the pathway to folding and unfolding would be high-energy distorted form of the native state. Furthermore, because of the cooperative nature of the process there exists a correlation between the stability of the native state and the kinetics of unfolding. This picture of protein stability provides a better framework within which to interpret urea denaturation of LADH. With LADH there is abundant evidence that the inner core forms a highly cooperative rigid structure dominated by hydrogen bonding. X-ray pictures of LADH show the coenzyme-binding domain to be formed by an

Table 1

Kinetic parameters for the activation process in urea denaturation of LADH and ovalbumin as compared to thermodynamic parameters for denaturation of β-lactoglobulin

		Activation N ⇌ X* LADH + urea	Activation [a] N ⇌ X* ovalbumin + urea	Equilibrium [b] N ⇌ D β-lactoglobulin + urea
$\upsilon = \delta \ln K/\delta \ln C$ ($T = 25°C$)		12	15	17
ΔC_p (cal/K per mol)		1740	1800	2150
ΔH (kcal/mol):	0°C	−34	−36	−75
	25°C	−4.8	+9	−21
	50°C	+54	+53	+33
ΔS [c] (cal/K per mol):	0°C	−154	−160	−260
	25°C	−55	−1	−72
	50°C	+137	+140	+100

[a] From the data of Simpson and Kauzmann [14].

[b] From the data of Tanford [16].

[c] ΔS was calculated from $R \ln Ah/ekT$ where: A = preexponential factor in Arrhenius equation; R, k and h are the gas, Boltzmann's and Planck's constant, respectively.

extensively hydrogen-bonded β-sheet which, judging by the long phosphorescence lifetime of Trp 314, must be exceptionally rigid in structure.

Indirect evidence that the compactness of this core is primarily due to hydrogen bonding and salt bridges comes from the differential effects of the interaction with urea and GuHCl. Both denaturants reduce the hydrophobic interaction but GuHCl, due to its charge, also has affinity for binding to the peptide unit and to substitute intramolecular hydrogen bonds with intermolecular ones. Accordingly, whereas urea is unable to alter the dynamics of this domain GuHCl, even at low non-denaturational concentrations, was found to increase drastically the flexibility of this region [1]. Using this model of cooperative interactions, where activation free energies go hand in hand with stability, the temperature dependence of the rate of LADH denaturation would reflect essentially the change in stability of the native state. It predicts a temperature of maximum stability around 25–30°C, a property shared by other globular proteins [21]. In addition, by linking stability to cooperativity this model anticipates a similar temperature profile for the degree of cooperativity of the unfolding transition. It is therefore significant that the coefficient υ, which is an indication of the degree of cooperativity of the reaction, does indeed vary with temperature in a fashion inverse to the rate.

In conclusion, the present results with LADH denaturation point out that slow unfolding of globular structures is not necessarily a manifestation of peptide isomerization or sulphur bridge rearrangement. The existence of compact structures with an extended network of hydrogen bonding may offer a sufficiently large entropic barrier to account for the slowness of the process.

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