Relationship between the Conformation of Glutamate Dehydrogenase, the State of Association of Its Subunit, and Catalytic Function

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ABSTRACT: The fluorescence and phosphorescence properties of the tryptophan residues in glutamate dehydrogenase were utilized to probe the conformation of the macromolecule at various states of aggregation of its subunits (hexamer, trimer, and monomer) in guanidine hydrochloride. According to the phosphorescence lifetime no gross alteration in the conformation of the protein follows from complete dissociation of the hexamer into native monomer, implying that the native fold is stabilized exclusively by intrasubunit bonding. Although modest concentrations of denaturant induce a change in configuration in the enzyme, a comparison with the macromolecule cross-linked into the hexameric form by glutaraldehyde confirms that this alteration in structure is not the result of subunit dissociation. Inhibition of catalysis by the denaturant is found to be considerably smaller than anticipated from the extent of hexamer dissociation. Furthermore, this inhibition is in no way prevented by cross-linking the enzyme in its hexameric form. This finding together with the ability of the trimer to bind the coenzyme and to undergo the characteristic structural changes induced by the effectors ADP and GTP suggests that, contrary to what is generally believed, the smallest functional unit of glutamate dehydrogenase is not the hexameric form.

A general question with oligomeric enzymes is the possible role that the association of subunits might play in stabilizing particular functional states of the macromolecule (Jaenicke, 1984). With bovine liver glutamate dehydrogenase (GDH),[1] which is composed of six identical subunits, this question assumes even greater significance since the interaction among subunits is clearly responsible for a number of cooperative phenomena (Bell & Dalziel, 1973; Sund et al., 1977; Smith & Bell, 1982; Bell et al., 1985). Recent advances in the field of renaturation/reactivation of oligomeric enzymes have pointed out that most enzymes can renature from the unfolded state, the association among subunits occurring after the monomer has achieved a quasi-native conformation. The concentration dependence of the kinetics of reactivation has further emphasized that catalytic activity is in general restored *after* the association step, even if exceptions exist where the monomer or incomplete states of aggregation exhibit similar potentialities (Jaenicke, 1984). Unfortunately, the reactivation approach cannot be applied with GDH since for this enzyme, as opposed to many other dehydrogenases investigated, any attempt of renaturation has failed (Muller & Jaenicke, 1980).

In the presence of moderate, nondenaturing amounts of Gdn-HCl the protein dissociates first into trimers and finally into monomers (Tashiro et al., 1982; Inoue et al., 1984). The process is accompanied by a reversible loss of catalytic activity which apparently goes hand in hand with the dissociation of hexameric GDH into smaller units. Because along with inactivation of the enzyme there is no apparent change in secondary/tertiary structure (in circular dichroism), this observation has suggested that the catalytic function is relegated to the hexameric assembly (Bell & Bell, 1984; Fukushima et al., 1985).

Recently, we have reported tryptophan phosphorescence at room temperature from GDH, the emission arising from a single residue in each subunit of the enzyme (Strambini et al., 1987). Subsequently, the sensitivity of the triplet-state lifetime to the dynamical structure of the macromolecule has been instrumental in demostrating the changes in conformation induced by the binding of allosteric effectors (Cioni & Strambini, 1988). In this work we extend the phosphorescence approach to the study of GDH and GDH cross-linked in the hexameric form by glutaraldehyde, in the presence of various amounts of Gdn-HCl. Advantage is taken of the sensitivity of the emission properties of tryptophan to even subtle changes in structure in order to determine to what extent the interactions between the subunits of GDH are responsible for maintaining the native configuration of the enzyme as well as its catalytic and regulatory properties. The findings of the present investigation lead to the conclusion that both catalytic activity and the action of allosteric effectors are not exclusive of the hexameric species of GDH in that the trimer in part possesses these potentialities. Low concentrations of denaturant affect the conformation of the enzyme irrespective of the state of aggregation of its subunits; its inhibitory action however is complex, ionic strength alone accounting for a good portion of it.

Materials and Methods

All chemicals employed were of the highest purity grade available from commercial sources. Gdn-HCl was from Merck (Darmstadt). Its final concentration in the protein samples was determined by density measurements. Beef liver glutamate dehydrogenase was supplied by Boehringer (Mannheim) as a crystalline suspension in ammonium sulfate. Prior to use the enzyme was throughly dialyzed at 4 °C against 0.2 M potassium phosphate, pH 7.3, and then centrifuged for 20 min at 15 000 rpm to remove any precipitate. Water doubly distilled over quartz was employed throughout.

Enzyme concentrations were measured spectrophotometrically by using $E_{280} = 0.97$ mg^{-1} mL cm^{-1} (Olson & Anfinsen, 1952). The enzyme activity was determined from the absorption at 340 nm by following the oxidation of NADH with α-ketoglutarate and ammonia at 25 °C. The reaction was carried out in 0.1 M imidazole buffer at pH 7.9 in the presence of 13 mM α-ketoglutarate, 210 mM NH_4Cl, 0.1 mM NADH,

[1] Abbreviations: GDH, glutamate dehydrogenase from beef liver; Gdn-HCl, guanidine hydrochloride.

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and 0.9 mM EDTA. Under these conditions, the specific activity was 65 ± 2 units/mg of protein as specified by the supplier. In order to compare specific activities to the state of dissociation of GDH in Gdn-HCl, reported by Inoue et al. (1984), enzymatic assays were also conducted in 0.2 M phosphate buffer, pH 7.3.

Cross-linking of GDH by glutaraldehyde at a molar ratio of 1:200 was conducted for 2 h (Zeiri & Reisler, 1978) at an enzyme concentration of 1 mg mL^{-1} in 0.1 M phosphate buffer, pH 7.5 at a temperature of 22 °C. GTP (1 mM) and NADH (1 mM) were added to the reaction mixture because under these conditions the macromolecule is cross-linked in the hexameric form. The extent of cross-linking was verified by electrophoresis on SDS–PAGE gels and substantially confirmed the pattern reported in the literature (Josephs et al., 1973). As the modified protein eluted as a single band on gel filtration chromatography, no further purification was attempted.

Luminescence Measurements. Fluorescence spectra and quantum yields were obtained with a commercial fluorometer (Jasco FP-770). A conventional homemade instrument was employed for all phosphorescence measurements (Strambini, 1983). In the latter the excitation was selected by a 250-nm grating monochromator (Jarrel-Ash) employing a band-pass of 10 nm. The emission was dispersed by a 250-mm grating monochromator (Jobin-Yvon H25) and detected with an EMI 9635 QB photomultiplier. Phosphorescence decays were monitored at 440 nm by a double-shutter arrangement permitting the emission to be detected 2 ms after the excitation cutoff. The decaying signal was digitized by an Applescope system (HR-14, RC Electornics) and then transferred to an Apple II computer for averaging and subsequent exponential decay analysis by a least-squares method.

To obtain reproducible phosphorescence data in fluid solutions, it is of paramount importance to remove thoroughly all dissolved oxygen. The procedure followed to obtain satisfactory deoxygenation was described in a previous report (Strambini et al., 1987).

Light Scattering. The intensity of light scattered at 90° from the incident beam was measured in a fluorometer (Jasco FP-770). The wavelength of the incident light was 430 nm, and the band-pass employed in the excitation and emission monochromators was 1.5 nm. The sample cell (fluorescence cell 1 cm × 1 cm in cross section) was cleaned with dust-free water and the protein solution passed through 1-μm pore membrane filter. Since the sensitivity and precision of this apparatus for molecular weight determinations are not comparable to those achieved with dedicated instrumentation, we have not attempted to estimate molecular weight distributions from our data. We have obtained the fraction of hexameric GDH at various concentrations of Gdn-HCl relying on the refined data of Inoue et al. (1984). If α and β represent the degree of hexamer–trimer and trimer–monomer denaturant-induced dissociation, respectively, then the equilibrium constant governing the hexamer–trimer equilibrium is given by

$$K_{6,3} = 4c_0\alpha^2(1 - \beta)/(1 - \alpha)$$

where c_0 is the total protein concentration expressed as moles of hexamer per liter. Since experimentally little monomer formation is observed in the 0–1.5 M Gdn-HCl range ($\beta \sim 0$) this expression simplifies to

$$K_{6,3} = 4c_0\alpha^2/(1 - \alpha)$$

The fraction of macromolecules in the hexameric state, $f_H = 1 - \alpha$, in this range of denaturant concentrations was derived by using the above expression and the dependence of $K_{6,3}$ on [Gdn-HCl] given by Inoue et al. (1984), namely

$$\log K_{6,3} = 6 \log [\text{Gdn-HCl}] - 5.7$$

In our work light scattering intensities were monitored only as a control measure to verify how cross-linking and the addition of various constituents to the protein solution affected the average molecular weight. Scattered intensities were always compared between samples having the same protein concentration and refractive index so that under these conditions their ratio, according to Parr and Hammes (1975), is proportional to their respective molecular weight.

The enzyme concentration was typically 0.5 mg/mL in luminescence and light scattering experiments and 3.4×10^{-4} mg/mL in the assays of enzymatic activity. The temperature was 20 °C.

Results

Luminescence Spectra and Quantum Yields as a Function of Gdn-HCl Concentration. Up to a concentration of 1.5 M Gdn-HCl the steady-state fluorescence and phosphorescence properties of the tryptophan residues in GDH are practically unaffected by the denaturant. At larger concentrations both emissions become progressively quenched with time (Figure 1). The fluorescence intensity decreases in a biphasic way, and during the slow phase the spectrum undergoes a red shift, λ_{max} changing from 332 nm to a final value of about 355 nm. While the phosphorescence spectrum remains unaltered throughout, the phosphorescence intensity drops to zero following a time dependence similar to the slow phase in fluorescence. In 6 M gdn-HCl no phosphorescence is detectable and the fluorescence quantum yield is reduced to about 25% of the initial value. According to molecular weight determinations (Tashiro et al., 1982; Inoue et al., 1984) in 1.5 M Gdn-HCl the subunits of GDH are associated almost exclusively into trimers, larger concentrations of denaturant leading to dissociation into monomers and successive unfolding. The fluorescence and phosphorescence spectra and yields of the tryptophanyl side chains then point out that changes in the environment of these chromophores occur only upon monomer formation and/or its unfolding. The constancy of the emission properties at concentrations of Gdn-HCl covering the hexamer to trimer transition implies that the dissociation into trimers probably does not involve alteration of secondary structure nor does it lead to the solvent exposure of chromophores that might have been at the trimer–trimer interface.

Phosphorescence Lifetime τ as a Function of Gdn-HCl Concentration. The triplet-state lifetime of the single tryptophan residue phosphorescing at room temperature is an internal local probe of the dynamical structure of the polypeptide chain. At 20 °C the phosphorescence of the protein decays, as shown in Figure 2, in a strictly monoexponential fashion both in the presence and in the absence of denaturant. Thus any heterogeneity that might exist in the structure of GDH in terms of conformers with different triplet-state lifetimes must be averaged out in a time interval shorter than τ.

The values of τ obtained at various Gdn-HCl concentrations up to 3.4 M are shown in Figure 3. Any effect of Gdn-HCl on τ is restricted to the 0–1.5 M range over which τ drops from 1.6 ± 0.05 s in the absence of denaturant to a limiting value of 1.1 ± 0.05 s. Because the greater flexibility embodied in the decrease of τ is concomitant to trimer formation, the possibility exists that this alteration of protein structure reflects both/either the breaking of intersubunit bonds across the trimer interface and/or specific interactions between denaturant and polypeptide leading to a new and looser configuration. The subsequent constancy of τ at higher Gdn-HCl

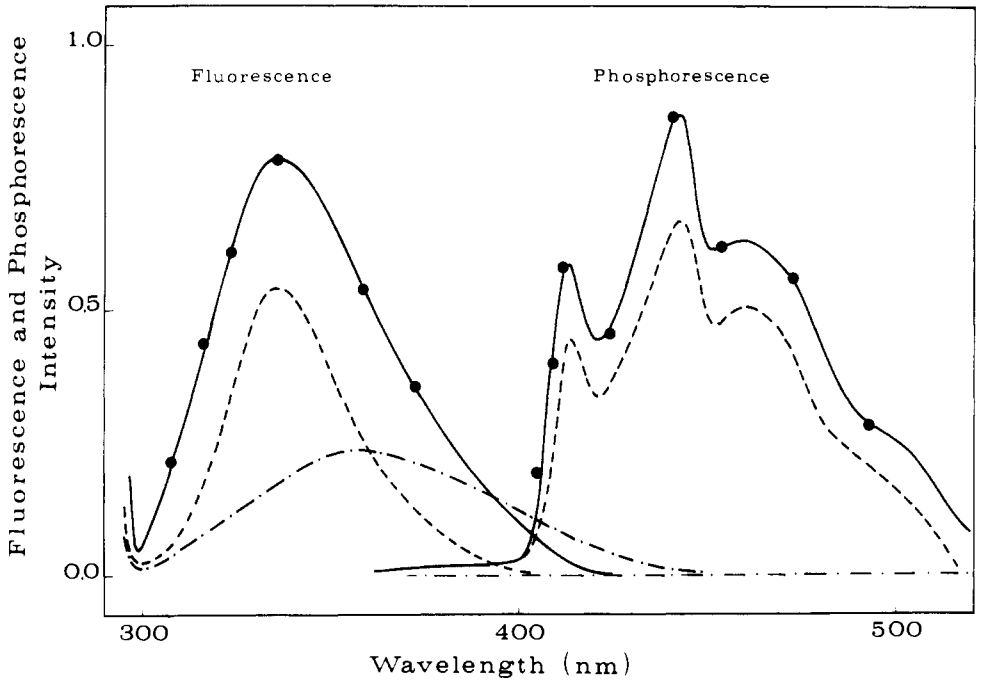

FIGURE 1: GDH fluorescence and phosphorescence spectra (λ_{ex} = 295 nm) in 0.1 M phosphate buffer, pH 7.5 at 20 °C (—); in the presence of 1.5 M Gdn-HCl (●); and in the presence of 3.2 M Gdn-HCl after 3 min of incubation (---) and after 30 min of incubation (-·-). Relative to fluorescence the phosphorescence intensity is roughly 20 times weaker, and it has been normalized for the change in triplet lifetime.

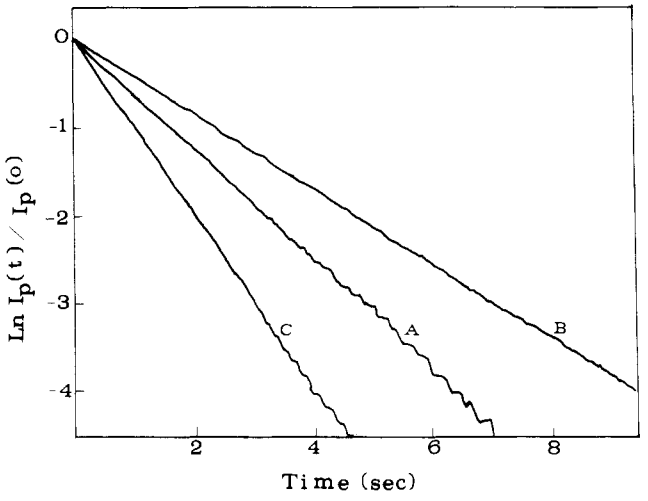

FIGURE 2: Decay of phosphorescence intensity with time (λ_{ex} = 290 nm; λ_{em} = 440 nm) in 0.1 M phosphate buffer, pH 7.5 at 20 °C: (A) GDH; (B) its binary complex with ADP (1 mM); (C) its complex with GTP (1 mM) or in the presence of 1.5 M Gdn-HCl. As shown by the lifetimes in Table II, the decay properties are identical also for the cross-linked enzyme.

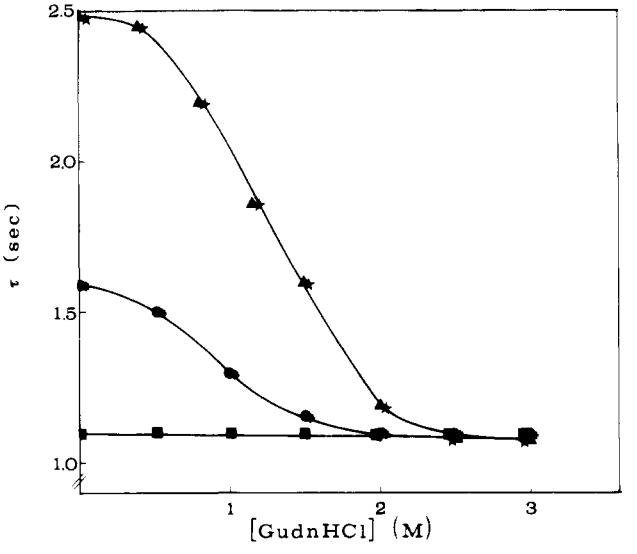

FIGURE 3: Phosphorescence lifetime as a function of Gdn-HCl concentration. The experimental conditions are the same as in Figure 2. (●) GDH; (*) GDH cross-linked by glutaraldehyde; (▲) GDH–ADP; (★) cross-linked GDH–ADP; (■) GDH–GTP. The concentration of ADP and GTP were 1 mM.

concentrations indicates that neither the successive dissociation of trimers into monomers nor the higher activity of the denaturant are able to affect the folded, still native-like conformation of the polypeptide. Unfolding of monomeric GDH, which occurs in few seconds beyond 3.4 M (Fukushima et al., 1985), causes the phosphorescence intensity to be completely quenched. In this state τ is shorter than 1–2 ms, the detection limit of the apparatus.

The monoexponentiality of phosphorescence decay and the constancy of τ during the course of denaturation show that increasingly flexible metastable states of the monomer are not detected in emission. This implies that major unfolding of the structured core hosting the chromophore occurs essentially as a one-step process. Such extensive unpacking and loss of secondary structure correlate well with the results of circular dichroism measurements (Tashiro et al., 1982).

Table I: Light Scattering Intensities at 430 nm from Solutions of GDH (E) and GDH Cross-Linked by Glutaraldehyde (EX)[a]

sample	light scattering intensity
E + 1.5 M NaCl	100 ± 3
E + 1.5 M Gdn-HCl	49 ± 2
EX + 1.5 M Gdn-HCl	97 ± 4
E + 1.5 M Gdn-HCl + ADP (1 mM)	51 ± 2
E + 1.5 M Gdn-HCl + GTP (1 mM)	49 ± 2
E + 1.5 M Gdn-HCl + substrates in assay mixture	50 ± 2

[a] The values reported are averages of four independent measurements. The protein was in 0.2 M phosphate buffer, pH 7.3, at a concentration of 0.5 mg/mL.

To address the question as to whether the change in τ at low Gdn-HCl concentrations reflects a dissociation-induced

Table II: Specific Activity and Phosphorescence Lifetime of Native and Cross-Linked GDH in the Absence and in the Presence of ADP and GTP[a]

	% sp act.	τ (s)		% sp act.	τ (s)
GDH native	100	1.58	GDH fixed	85	1.51
+ADP (1 mM)	215	2.4	+ADP (1 mM)	158	2.3
+GTP (1 mM)	9	1.1	+GTP (1 mM)	12	1.1

[a] Experimental conditions for emission work were as reported in Figure 2. The precision of activity and lifetime measurements is typically around 5%.

conformational change, we have investigated GDH cross-linked in the hexameric form by glutaraldehyde. The results of light scattering intensities in 1.5 M Gbdn-HCl (Table I) show unequivocably that, unlike for the native protein, effectively no dissociation is detectable with the chemically modified enzyme. The interaction with glutaraldehyde in the presence of GTP and NADH does in no way impair either catalytic or regulatory properties of the enzyme. In Table II we note that both specific activity and its regulation by the allosteric effectors ADP and GTP are hardly changed by the chemical modification. Similarly, all spectroscopic controls on the conformation of the cross-linked macromolecule (emission spectra, yields, and τ) report no change with respect to the native protein. The results of phoshorescence lifetime measurements with the cross-linked enzyme at various Gdn-HCl concentrations (Figure 3) are within expermental error coincident with those reported for the unmodified protein. Thus, if we assume that cross-linking maintains intact the association between trimers, then we are to conclude that the change in conformation of GDH at low Gdn-HCl concentrations is due primarily to specific interactions between denaturant and polypeptide.

Phosphorescence Lifetime and Binding of the Allosteric Effectors GTP and ADP. The phosphorescence lifetime of GDH has revealed marked changes in the conformation of the macromolecule upon binding of allosteric effectors. Moreover, the influence exerted by a member of one class (say activators) is often neutralized when a member of the opposite class (inhibitors) is also bound (Cioni & Strambini, 1988). To inquire on the nature of the conformational change induced by low concentrations of Gdn-HCl and on whether molecular species smaller than the hexamer are able to undergo the structural changes elicited by allosteric effectors, we have monitored the phosphorescence lifetime in Gdn-HCl with saturating amounts of ADP and GTP. Gdn-HCl affects τ to the same extent as does the inhibitor GTP. Should the denaturant behave similarly to an allosteric inhibitor, then binding of ADP would be expected to reverse the structural transition. Alternatively, in the presence of GTP addition of Gdn-HCl would have no further effect on the lifetime.

The lifetime profiles of the GDH–ADP and GDH–GTP complexes over the usual range of Gdn-HCl concentrations are displayed in Figure 3. As the complexes possess lifetimes distinct from that of the unliganded protein, we conclude that the denaturant does not displace the ligands from their binding sites. The results of τ measurements confirm our anticipations: in the presence of GTP τ is constant throughout. Thus, for the GDH–GTP complex trimer formation has apparently no influence on the dynamical structure about the triplet probe. For the GDH–ADP complex τ decreases upon addition of denaturant, but its value in the hexamer–trimer range is always larger than that of the free protein. Indeed at 1.5 M, where light scattering data (Table I) confirm the trimeric state, τ is about 1.6 s, the same value of the protein in buffer. According to the phosphorescence lifetime ADP interacts with the GDH trimer and, what is more significant, displays the mutual neutralization effect observed with combinations of opposite effectors. Similar results were also obtained with the cross-linked enzyme for which dissociation is not apparent in light scattering measurements. In the light of these findings, low concentrations of Gdn-HCl would seem to influence the structure of GDH in a fashion analogous to allosteric inhibitors and regardless of the state of association of its subunits.

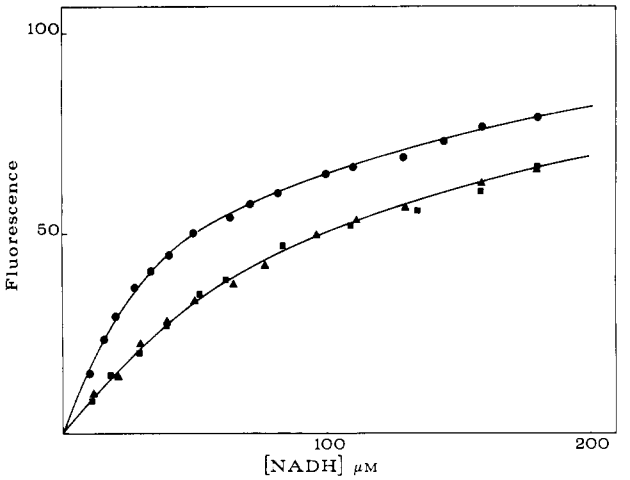

FIGURE 4: Fluorescence titration of glutamate dehydrogenase (6.6 μM) with NADH in 0.1 M phosphate buffer, pH 7.5 (●), and upon the addition of 1.5 M Gdn-HCl (▲) or NaCl (■). When a fluorescence cell 4 × 4 mm² in cross section was employed, the correction for inner-filter effects at the larger NADH concentrations was small.

Coenzyme Binding and Enzyme Activity in the Presence of Gdn-HCl. NADH binding studies with GDH were conducted by monitoring the increase in nicotinammide fluorescence emission at 450 nm (λ_{ex} = 340 nm) that accompanies complex formation (Bell et al., 1985). As shown in Figure 4, upon addition of 1.5 M Gdn-HCl, the enzyme–coenzyme binary complex is formed equally well, the dissociation constant increasing by roughly a factor of 2. Further, control experiments in which the ionic strength is held constant by NaCl indicate that this increase is not due to a specific action of denaturant.

Enzyme activity measurements were carried out in the presence of Gdn-HCl with native- and glutaraldehyde–cross-linked enzyme in addition to their respective complexes with ADP. The catalytic rates for the reduction of α-ketoglutarate at 0.5, 1.0, and 1.5 M Gdn-HCl are shown in Table III. Also reported in the same table are the specific activities found when NaCl was added in place of denaturant. Activity data confirm the inhibitory action of the denaturant although to a much less extent than reported previously by Bell and Bell (1984) and Fukushima et al. (1985) for the oxidation of glutamate. The comparison between native and cross-linked enzyme emphasizes that cross-linking of the macromolecule into its hexameric form does not reduce the inhibitory effects of the denaturant, meaning perhaps that factors other than hexamer dissociation are responsible for inhibition. NaCl controls do show that a substantial decrease in activity may be attributed simply to ionic strength effects. What is more, from the comparison between residual activity and fraction of GDH molecules estimated to be in the hexameric form (Table III) it is evident that at the present enzyme concentrations the activity in 1 M Gdn-HCl and above is due entirely to the trimeric and possibly monomeric species. The addition of the activator ADP, contrary to the expectations raised by lifetime measurements, does not reverse the inhibition by Gdn-HCl. At this high ionic strength, however, activation by

Table III: Residual Catalytic Activity and Fraction of Hexameric GDH in the Presence of Increasing Amounts of GdnHCl or NaCl: E (Native Enzyme), EX (Enzyme Fixed by Glutaraldehyde), and Their Respective Binary Complexes with ADP

		Gdn-HCl		NaCl	
	Gdn-HCl/ NaCl (M)	catalytic act.[a] (%)	f_H[b] (%)	catalytic act. (%)	f_H[c] (%)
E	0.5	76	12.5	91	100
	1.0	20	0.19	50	100
	1.5	16 (17)	0.017 (0.18)	38	100
EX	0.5	80		89	
	1.0	24		57	
	1.5	16 (15)		35	
E-ADP (1 mM)	0.5	95		107	
	1.0	32		62	
	1.5	15 (17)		41	
EX-ADP (1 mM)	0.5	75		90	
	1.0	23		61	
	1.5	15 (18)		38	

[a] Activity measurements were conducted in 0.2 M phosphate buffer, pH 7.3, at an enzyme concentration of typically (3.3–3.5) × 10^{-4} mg/mL (1 nM in hexamers). Moreover, to test for a possible protein concentration dependence, the specific activity in 1.5 M Gdn-HCl was determined also at 10 nM, and the value obtained is reported in parentheses. The enzyme, prior to activity measurements, was incubated in the reaction mixture (lacking α-ketoglutarate) for 10 min. No influence was found on incubation time. The value reported for the specific activity is the average of three independent determinations. [b] From the hexamer–trimer equilibrium constant determined by Inoue et al. (1984) which has a value of 3.1 × 10^{-8} M, 2.0 × 10^{-6} M and 2.27 × 10^{-5} M for 0.5, 1.0, and 1.5 M Gdn-HCl, respectively. Light scattering control experiments show that the addition of substrates does not alter the molecular weight distribution. [c] From our light scattering data.

Table IV: Kinetic Constants for the Irreversible Loss of Fluorescence/Phosphorescence Intensity and Catalytic Activity in the Presence of Gdn-HCl for the Native Enzyme (E), for Enzyme Cross-Linked by Glutaraldehyde (EX), and for the Binary Complex with ADP[a]

	Gdn-HCl (M)	$k \times 10^3$ (s^{-1})		
		fluorescence	phosphorescence	activity
E	3.2	3.5	3.2	3.4
	2.8	1.3	1.2	1.2
EX	3.2	2.9	2.6	2.7
E-ADP (1 mM)	3.2	2.7	2.6	2.8

[a] Total fluorescence and phosphorescence intensities (λ_{ex} = 295 nm) were evaluated from the areas under the respective spectra.

ADP is negligible even in absence of denaturant.

Kinetics of GDH Denaturation by Gdn-HCl. At larger concentrations of Gdn-HCl, above 2.8 M, GDH undergoes irreversible denaturation within several minutes. The kinetics of this process was characterized by three independent approaches: irreversible loss of enzymatic activity, decrease in fluorescence, and decrease in phosphorescence quantum yields. The time dependence of these quantities in 3.2 M Gdn-HCl at 20 °C is shown in Figure 5 and Table IV. The overall change in fluorescence intensity does not obey an exponential law. A rapid phase, with a rate constant of 5.5 × 10^{-2} s^{-1}, can be distinguished during which the total intensity drops by about 30% (with undetectable changes in spectrum). By means of light scattering data [ours and those reported by Tashiro et al. (1982)] we can identify this initial phase with the dissociation of the trimer into monomers. The subsequent slower phase is characterized by a rate constant of 3.5 (±0.2) × 10^{-3} s^{-1}, an additional drop of about 40% of the initial intensity, and a red shift in the spectrum. A further 6–8% of the

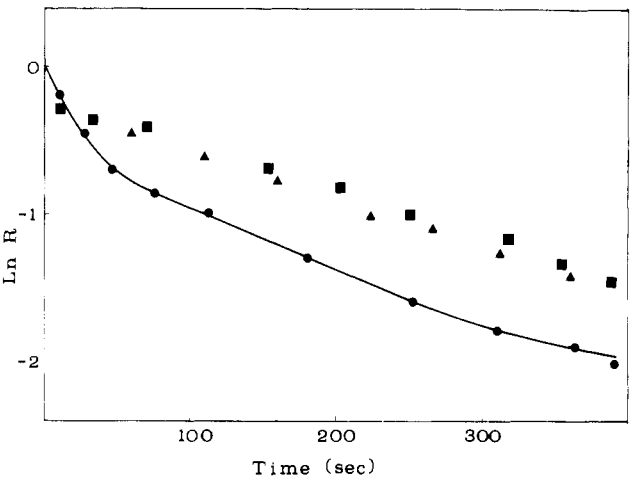

FIGURE 5: Decay of fluorescence, phosphorescence intensities, and catalytic activity with time in the presence of 3.2 M Gdn-HCl: (●) $R = (F(t) - F_\infty)/(F_{max} - F_\infty)$; (▲) $R = P(t)/P_{max}$; (■) $R = A(t)/A_{max}$. Here, F_{max}, P_{max}, and A_{max} refer to the value of fluorescence, phosphorescence, and catalytic activity in the absence of denaturant; F_∞ is the limiting value of fluorescence intensity in the presence of denaturant.

fluorescence intensity decays at yet slower rates. The phosphorescence intensity decreases exponentially with time, and the rate constant obtained for this process is 3.2 (±0.3) × 10^{-3} s^{-1}. Because mixing in the phosphorescence cell is more laborious, it is not possible to determine the kinetics during the first minute. Intensity data extrapolated to time zero reveal a loss in this quantity at short times. Relative to the value in buffer the intensity during the initial period drops by about 20–25%. Although there is no basis for assigning this rapid drop in phosphorescence intensity to a particular event, changes in fluorescence quantum yield of the phosphorescing residue during the initial phase could account for the observed effect. Strikingly similar to the loss in phosphorescence intensity is the kinetics of irreversible inactivation of GDH, the calculated rate constant being indistinguishable within the precision of activity measurements. Again, extrapolation of activity data to time zero shows that there is an initial loss of 15–20% occurring within the first 5 s and for which we have no explanation. The substantial agreement in the rates of decay of fluorescence (during the slow phase), phosphorescence, and catalytic activity stands to indicate that all three parameters are governed by processes having the same rate-limiting step. According to fluorescence and phosphorescence data this step involves profound structural changes in the macromolecule whereby even the buried Trp side chain becomes exposed to the solvent. In previous studies (Inoue et al., 1984; Fukushima et al., 1985) it was shown that concomitant to GDH inactivation circular dichroism spectra manifest an extensive unfolding of the protein secondary structure. To compare our results with these studies, rate constants were determined also at 2.8 M Gdn-HCl. The rate constant of 1.25 (±0.07) × 10^{-3} s^{-1}, again in fair agreement among the three approaches, is reasonably close to the value of 1.4 × 10^{-3} s^{-1} reported in the literature (Fukushima et al., 1985). Cross-linking of GDH and binary complex formation with ADP both induce a modest but distinct reduction in the rate of denaturation/inactivation by Gdn-HCl (Table III). The rate constant decreases roughly by 20%, the effect being manifested similarly on emission intensities and catalytic activity. Another noteworthy difference in the kinetics of the cross-linked enzyme is the almost complete lack of the initial rapid drop in fluorescence that accompanies monomer formation. Thus, cross-linking besides reducing the rate of unfolding of the polypeptide slows down

considerably the dissociation of trimers into monomers, a finding that reflects a stabilizing effect of the chemical modification toward trimeric and possibly hexameric states of GDH.

Discussion

A monomer of GDH possesses three tryptophan residues; there is evidence that only Trp-72 within the β-sheet of the catalytic coenzyme-binding domain phosphoresces at room temperature (Strambini et al., 1987). The long lifetime of this emission attests to an exceptional rigidity of the protein structure around the chromophore, a compactness of the polypeptide that practically prevents this residue from coming into direct contact with solutes in solution during the lifetime of the excited triplet state. Thus, any external agent or specific interactions such as subunit association and ligand binding that influence the decay kinetics of this chromophore do it only indirectly by modulating the protein structure. In other words, a change in phosphorescence lifetime signals an alteration of the dynamical structure of this core region of the macromolecule as it evolves into a new configuration.

In this investigation the phosphorescence lifetime of tryptophan reports on the flexibility of the coenzyme-binding domain, at various levels of subunit aggregation and unfolding in response to increasing amounts of Gdn-HCl. Relatively low concentrations of denaturant, within the range of the hexamer–trimer transition, induce a change in conformation in the enzyme. The structural alteration is similar to that induced by an assortment of allosteric inhibitors of which GTP is the most representative. This similarity with effector molecules is not limited to the actual value of τ, but as with GTP, the binding of the nucleotide activator ADP reverses the effect of Gdn-HCl. Consistent with this interpretation is also the observation that the denaturant has apparently no influence on the GDH–GTP complex. Finally, although the conformational change occurs at Gdn-HCl concentrations in which the hexamer dissociates into trimer, parallel studies with cross-linked hexamer would exclude a direct connection between conformational change and the dissociation process.

Within the 0–3.4 M concentration range of Gdn-HCl in which full dissociation of the subunits is achieved, there is no further influence of the denaturant on the flexibility of the core enclosing the chromophore. The compactness and integrity of the coenzyme-binding domain would appear then to rely solely on strong intrapeptide bonding, the interaction among subunits playing no major role in stabilizing its conformation. The change in environment of one or more tryptophan residues causing the decrease in fluorescence upon trimer to monomer dissociation may, on the other hand, indicate one of two things: either that the aromatic residues are located at the subunit interface or, more likely, that the structure of certain regions of the macromolecule, peripheral to the coenzyme-binding domain, is affected by the interaction among subunits.

The general trend emerging from renaturation/reactivation studies of a number of oligomeric proteins (mostly NAD-dependent dehydrogenases) is that the secondary and tertiary structure of the polypeptide follows, to a large extent, from purely intramolecular bonding whereas the native functional state is restored only subsequent to the correct association of the subunits (Jaenicke, 1984). In some cases of biphasic aggregation, even the smaller, higher affinity assemblies (dimer, trimer) acquire an almost full functionality (lactate dehydrogenase-M4, aldolase). It is not possible to say how far GDH follows this general scheme. The spectroscopic properties reported for the folded monomer suggest that if association confers any additional stabilization to the structure, such a reshuffling occurs upon trimer formation and involves only peripheral regions of the polypeptide.

In some enzymes nonspecific binding of Gdn-HCl results in a gradual loosening of the native structure which is attended by a greater flexibility and an increased hydrodynamic volume (Kuwajima et al., 1976; Strambini & Gonnelli, 1986). An example that is relevant in a comparison with GDH is the coenzyme-binding domain of horse liver alcohol dehydrogenase. In spite of the phylogenetic similarity of this domain among dehydrogenases phosphorescence lifetimes in the alcohol enzyme attest to a marked increase in flexibility upon addition of nondenaturating amounts of Gdn-HCl (Strambini & Gonnelli, 1986) that contrasts with the imperviousness displayed by GDH. A possible rationalization for this distinct behavior may lie on the fact that in alcohol dehydrogenase, unlike GDH (Hucho et al., 1975) and most other dehydrogenases, the coenzyme-binding domain is formed at the subunit interface. Increasing solvation of the overlapping region by the denaturant would interrupt the extended H bonding across β-sheets of different subunits, a network whose cooperative nature is the key to the stability of the structure. If such analysis were to be confirmed, the alcohol enzyme would certainly represent an example among oligomeric proteins for which the quaternary structure would be essential for acquiring the native fold.

From comparing the kinetics of denaturation in phosphorescence and in circular dichroism it appears that the disruption of the structural "knot" embedding the chromophore is critical for unlocking the native conformation to extended unfolding. The important role that tight agglomerates of β/α secondary structure play in stabilizing the conformation of proteins in solution has been stressed before (Lumry, 1986). The observation that the loosening of such a "knot" in GDH leads to irreversible denaturation suggests that perhaps exceptionally rigid cores may in general be kinetically inaccessible from the extended configuration of the polypeptide.

A question that bears heavily on detailing the mechanism of action of GDH is the functional significance attached to the association of its subunits into a hexamer. Although in buffer smaller assemblies of its subunits are not found, determination of the smallest critical size for enzymatic activity will identify at which level of quaternary structure activity and regulation are relegated. In the range 0–1.5 M Gdn-HCl there is, concomitant to hexamer dissociation, a drastic inhibition of catalytic activity. Although, as mentioned above, the phosphorescence lifetime shows that the denaturant affects the conformation of GDH, eliciting the same structural changes of allosteric inhibitors, circular dichroism data report a native-like conformation for the trimer (Tashiro et al., 1982). A lower sensitivity of the absorption method relative to the triplet emission was encountered also in other occasions (Strambini & Gonnelli, 1986, 1988; Gonnelli & Strambini, 1988). The notion that the trimer is native-like has led some workers (Bell & Bell, 1984; Fukushima et al., 1985) to interpret the parallelism between drop in activity and dissociation to mean that the hexameric assembly is indispensable for catalysis. It is, however, not clear if an adequate analysis of their data or a more comprehensive investigation would support such a conclusion. For example, both research groups find that at 1.5 M Gdn-HCl the oxidation of glutamate is totally inhibited. Bell and Bell (1984), after demonstrating that ionic strength alone accounts for the loss of 65% in activity, assume no inhibitory effects of Gdn-HCl and attribute the remaining loss to trimer formation (although at their enzyme concentration roughly 2% of GDH molecules are still hexameric).

Instead, according to Fukushima et al. (1985), the denaturant acts as a competitive inhibitor. When, in the limited range of 0–0.6 M Gdn-HCl, specific activities were corrected for competitive inhibition, values are obtained that fall rather approximately on the curve describing the hexamer fraction. The coenzyme binding capacity and the kinetics of α-ketoglutarate reduction (the reverse reaction) reported in this paper argue in favor of a catalytically competent trimer. Indeed, at 1.5 M Gdn-HCl in spite of the fact that the fraction of GDH molecules in the hexameric state is practically zero the residual activity is 16%. A few percent activity is measurable even at 2.2 M where a good portion of the protein is monomeric. Further, cross-linking of the enzyme in its hexameric state does not reduce the inhibitory action of the denaturant. It may be argued that in this circumstance the "artificial" hexamer may not possess intact intersubunit connections relevant to catalysis. Whatever the case may be, cross-linking certainly antagonizes the dissociating action of the denaturant (as evidenced by the much slower rate of trimer dissociation), and a shift in the equilibrium in favor of the "truly" hexameric species is therefore anticipated. That the inhibitory effects of Gdn-HCl are not to be correlated with the breaking down of the hexamer into trimers is also emphasized by control experiments with NaCl in which the catalytic rate is shown to become strongly depressed at larger ionic strengths. All together, GDH seems to resemble to the tetrameric enzyme lactate dehydrogenase LDH-M4) for which the structural and functional similarity between the dimeric and tetrameric states has been elegantly demonstrated.

The interaction between glutamate dehydrogenase and the allosteric activator ADP causes a conformational change in the protein whereby the coenzyme-binding domain acquires a considerably greater rigidity. The effect of ADP is manifest even in the presence of amounts of Gdn-HCl sufficient to induce trimer formation. If the conformational change induced by ADP is at the basis of its activation, then we should conclude that also the regulatory function of ADP, in terms of promoting a favorable conformational state of the enzyme, does not require the full complement of six subunits. Unfortunately, attempts to demonstrate this activation by ADP in the trimeric state have failed since at this ionic strength, and even lower, ADP does not longer function as an activator. Under these conditions decoupling of the regulatory function from the conformational change could arise simply from a change in the rate-limiting step in the pathway of catalysis.

In summary, the results of the present investigation underline that the integrity of the secondary/tertiary structure of the coenzyme-binding domain, and possibly the native conformation, relies mostly on intrasubunit rather than intersubunit interactions. Finally, although the hexameric assembly of the enzyme could confer to it potentialities for sophisticated regulation, this unit is not strictly necessary either for catalysis or for the macromolecule to undergo the conformational changes elicited by allosteric effectors.

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