Ligand-induced changes in the conformational stability and flexibility of glutamate dehydrogenase and their role in catalysis and regulation

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Abstract: Bovine glutamate dehydrogenase (GDH) is allosterically regulated and requires substrate-induced subunit interactions for maximum catalytic activity. Steady-state and presteady-state kinetics indicate that the rate-limiting step depends on the nature of the substrate and are likely associated with conformational fluctuations necessary for optimal hydride transfer. Deuterated glutamate shows a steady-state isotope effect but no effect on the presteady-state burst rate, demonstrating that conformational effects are rate limiting for hydride transfer while product release is overall rate limiting for glutamate. Guanidine hydrochloride unfolding, heat inactivation, and differential scanning calorimetry demonstrate the effects of alternative substrates, glutamate and norvaline, on conformational stability. Glutamate has little effect on overall stability, whereas norvaline markedly stabilizes the protein. Limited proteolysis demonstrates that glutamate had a variety of effects on local flexibility, whereas norvaline significantly decreased conformational fluctuations that allow protease cleavage. Dynamic light scattering suggests that norvaline stabilizes all interfaces in the hexamer, whereas glutamate had little effect on trimer–trimer interactions. The substrate glutamate exhibits negative cooperativity and complex allosteric regulation but has only minor effects on global GDH stability, while promoting certain local conformational fluctuations. In contrast, the substrate norvaline does not show negative cooperativity or allow allosteric regulation. Instead, norvaline significantly stabilizes the enzyme and markedly slows or prevents local conformational fluctuations that are likely to be important for cooperative effects and to determine the overall rate of hydride transfer. This suggests that homotropic allosteric regulation by the enzymatic substrate involves changes in both global stability and local flexibility of the protein.

Keywords: glutamate dehydrogenase; allosteric regulation; protein stability; local flexibility; ligand-induced changes

Introduction

Since the work of Monod *et al.*[1] followed by Koshland *et al.*[2] and Dalziel and Engel[3] introducing allosteric regulation concepts, allosteric models have been described as either a pre-existing equilibrium that shifts with ligand binding or a combination of different ligand-induced conformations only formed upon binding. The images of the "R" and "T" states of hemoglobin, with fixed conformations of active and inactive protein were followed by demonstrations of induced conformational changes in proteins and the thermodynamic arguments of Weber[4] that proteins

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must change conformation when a ligand binds. In the early 1970s, the first direct demonstrations that proteins have a dynamic structure[5] and that ligand-induced changes affect the active sites of other subunits in an oligomer[6] were established. Many groups documented that a variety of enzymes, receptors and other functional proteins exhibit allosteric phenomena, including the work on ATP synthesis.[7,8] Often aided by crystal structures of different conformational states, these studies solidified the concepts of allosteric proteins having discrete conformations: pre-existent, ligand induced, or resulting from post-translational modifications. Few studies, other than work on ATP synthesis, addressed how conformational changes were transduced in these proteins, and little is known of the detailed mechanisms of conformational change in allosteric proteins. Even in allosteric proteins as well studied as aspartate transcarbamoylase, the flexibility of the catalytic trimer is only suggested as a means of regulation.[9,10]

In recent years, a resurgence of interest in the dynamic properties of proteins has occurred, particularly concerning those proteins associated with catalytic events that might proceed via quantum tunneling mechanisms such as hydride or proton transfer. Research has focused on the experimental detection of tunneling using isotope effects,[11–14] and in combination with computational approaches[15] have provided reasonable agreement between experimental and computational analysis of the chemical mechanism for alcohol dehydrogenase. As emphasized in recent reviews,[16–23] one of the future challenges, in terms of understanding enzyme mechanisms, involves understanding the relationship of the dynamic properties of enzymes to catalysis. Beyond that lies an understanding of how dynamic properties of allosteric proteins are related to the complex events associated with homotropic and heterotropic regulation (V-type, involving rate-limiting steps in the reaction, or K-type, where regulation is at the level of ligand saturation).[24]

Bovine liver glutamate dehydrogenase (E.C. 1.4.1.3, GDH) catalyzes the oxidative deamination of L-glutamate and various monocarboxylic acid substrates. The enzyme also shows the unique ability, among mammalian dehydrogenases, of being able to utilize either NAD^+ or $NADP^+$ as cofactor with near equal affinity,[25] although NAD(H) is thought to have an additional binding site per subunit. The enzyme is a hexamer of chemically identical polypeptide chains[26] and exhibits negative cooperativity resulting from coenzyme-induced conformational changes.[3,6,27] It has been shown that coenzyme-induced conformational changes require a dicarboxylic acid substrate or analog with a 2-position substituent.[28] With alternative amino acid substrates such as norvaline, the manifestations of cooperative interactions between the subunits of the enzyme are absent.[3,29] Since the entire hexamer is required to give optimal activity of the enzyme[30] with glutamate as substrate, it is likely that the cooperative interactions between subunits in the hexamer are required for maximal activity. Glutamate dehydrogenase from mammalian sources is highly regulated by a diverse array of small molecules, with ADP, GTP, leucine, and the combination of malate and palmitoyl CoA being the most effective regulators of activity.[31,32]

The crystal structures of both bovine and human forms of the enzyme are available[33–37] and have led to considerable insight into the structural basis for subunit interactions and the mechanism of regulation by purine nucleotides. However, the crystal structures have not revealed a consistent picture of how ligand-induced effects alter activity of this complex regulatory enzyme.

In this study, stopped flow kinetic studies including isotope effects have established that conformational transitions are rate limiting for the hydride transfer phase of the reaction. We have examined the overall conformational stability of GDH using the techniques of heat inactivation, differential scanning calorimetry (DSC), and guanidine hydrochloride (GuHCl) unfolding. The effects on hexamer stability of glutamate or the alternative substrate, norvaline, as assessed using dynamic light scattering (DLS) in conjunction with GuHCl dissociation, are correlated with their ability to impact conformational flexibility, as indicated by limited proteolysis studies. Through these techniques, along with known catalytic and crystal structure data, a picture emerges where conformational flexibility is intimately tied to the ability of the glutamate dehydrogenase hexamer to exhibit subunit interactions necessary for efficient catalysis.

Results

Presteady-state kinetics and isotope effects

To investigate the various rate-limiting steps in hydride transfer, as well as the overall reaction catalyzed by glutamate dehydrogenase, presteady-state kinetic studies were used with rapid mixing of the enzyme with cofactor and either glutamate or norvaline. The reaction was followed by fluorescence of the reduced cofactor from the dead time up to 8 s, allowing both the presteady-state and the steady-state phases of the reaction to be tracked. As shown previously with GDH, the burst phase followed by a linear steady-state is indicative of the formation of NADH still bound to the protein followed by NADH release and steady-state cycling.[38] Experiments comparing glutamate and norvaline show a presteady-state phase with glutamate but not with norvaline [Fig. 1(A)]. When the steady-state rate is subtracted from the presteady-state phase, an exponential rise to a maximum is observed [Fig. 1(B)], which is fit to

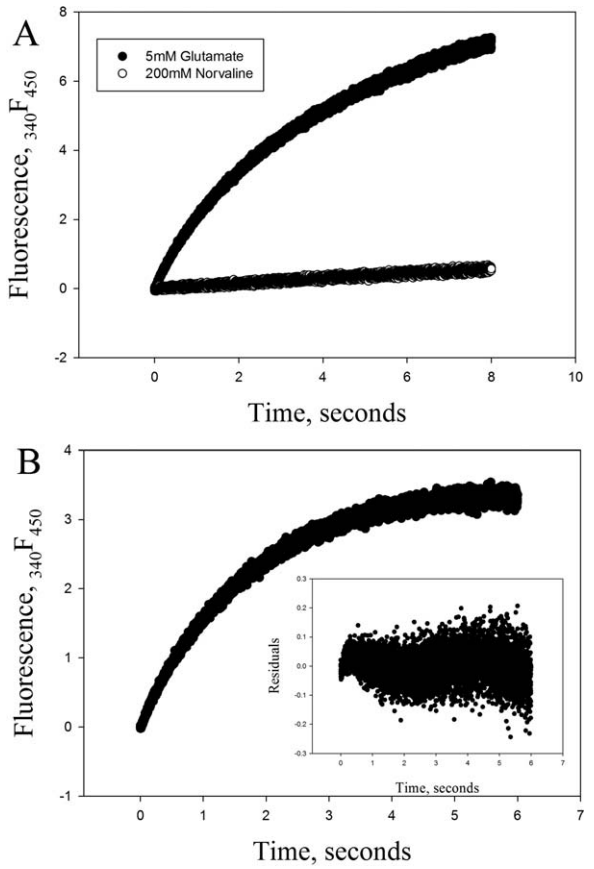

Figure 1. Stopped flow fluorescence measurements of GDH with glutamate and norvaline. Enzyme was rapidly mixed with cofactor and substrate; fluorescence (excitation at 340 nm and emission at 450 nm) was followed for a total of 8 s, collecting data every millisecond. Experiments were conducted with 1 m*M* NAD^+ and either 5 m*M* glutamate or 200 m*M* norvaline, in 0.1*M* phosphate buffer, pH 8.0. GDH concentration was 0.5 mg mL^{-1} after mixing. The primary data is shown in (**A**). **B**: The exponential rise to a maximum obtained by subtracting the steady-state rate from the burst phase. The inset in (B) shows the residuals obtained from fitting the data to a single exponential process.

a single exponential to give an amplitude and burst rate. The inset to Figure 1(B) shows the residuals for fit to a single exponential. In these experiments and others involving norvaline, concentrations of norvaline were 10 times those of glutamate since the K_m for norvaline is approximately 10 times that of glutamate.[39] The presence of the presteady-state phase with glutamate allowed three parameters to be determined: a presteady-state or "burst" rate, the amplitude of this presteady-state phase, and the overall steady-state rate. Isotope effects on these parameters were identified by using deuterated glutamate as the substrate. Figure 2 shows the isotope effect observed on the burst amplitude. The errors associated with the amplitude (and other parameters calculated here) are uniformly less than 1%. Figure 3 shows the lack of significant effect of isotope on the presteady-state rate constant, whereas Figure 4

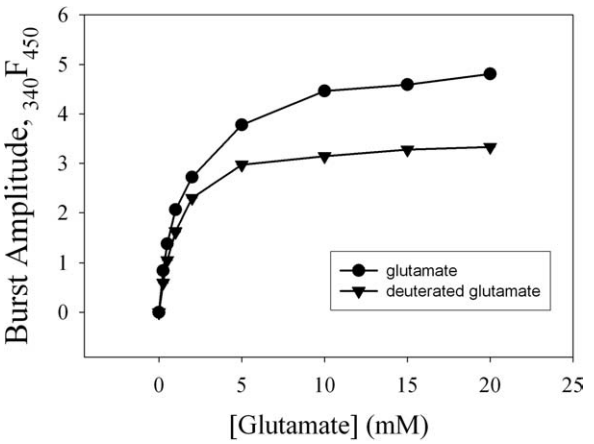

Figure 2. Effects of deuterated glutamate on the burst amplitude of the presteady-state phase: dependence on glutamate concentration. Experiments were performed as described in Figure 1 with either deuterated glutamate (triangles) or glutamate (circles). The burst amplitude was obtained by subtracting the steady-state rate from the presteady-state phase and fitting the resultant data to a single exponential process to obtain the amplitude. All other conditions were as in Figure 1. All errors were less than 1% and are not shown.

shows that there is an isotope effect observed for the steady-state rate. Isotope substitution is associated with a decreased steady-state rate along with a lower amplitude of the presteady state, but no significant effect on the presteady-state burst rate is observed (Figs. 2–4).

Heat inactivation of GDH with ligands

To investigate the effects of glutamate and norvaline on global stability of glutamate dehydrogenase, we compared the rates of inactivation of GDH when

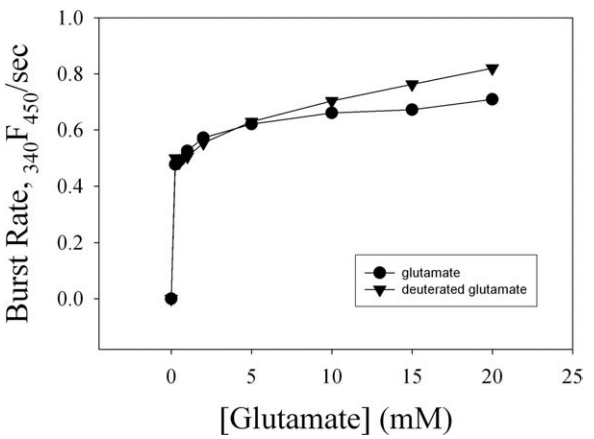

Figure 3. The effects of deuterated glutamate on the presteady-state "burst" rate. Experiments were conducted as described in Figure 2, and the burst rate was obtained from the fit of the data to a single exponential process with glutamate (circles) or deuterated glutamate (triangles). All other conditions were as in Figure 1. All errors were less than 1% and are not shown.

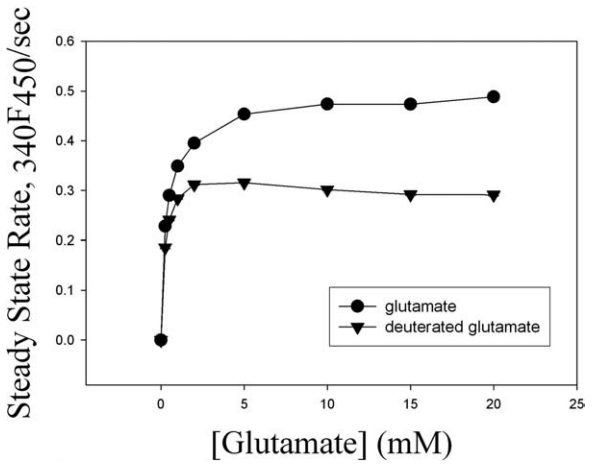

Figure 4. The effects of deuterated glutamate on the steady-state rate of the reaction. The steady-state rate was calculated from the stopped flow data between 4 and 8 s for glutamate (circles) or deuterated glutamate (triangles). All other conditions were as in Figure 1. All errors were less than 1% and are not shown.

incubated at 50°C in the presence of various substrates and substrate analogs. Heat inactivation of the native enzyme occurred with a rate constant of inactivation of 0.252 min^{-1} (Fig. 5). Addition of dicarboxylic acid substrate, L-glutamate or α-ketoglutarate, had a negligible effect on the rate (Table I). When the monocarboxylic acid substrate norvaline is present, the rate of inactivation slows significantly (Fig. 5) and a rate constant of 0.0049 min^{-1} is obtained. Two substrate analogs, glutarate and 3,3-dimethylglutarate, also slowed the rate of inactivation but not as dramatically as norvaline (Table I).

Differential scanning calorimetry of GDH

DSC experiments build on the global stability data from heat inactivation experiments, by providing a highly replicable means of determining the temperature of protein denaturation. The high level of reproducibility of DSC data is shown by a number of independent runs under each condition over a 2-week period which gave virtually identical transition temperature (T_t) values (±0.2°C for all measurements). Native GDH, in the absence of ligands, gave a T_t of 58.3°C. Incubation with glutamate had little effect (T_t of 59.4°C); however, the addition of norvaline (Fig. 6) dramatically affected T_t (T_t of 67.5°C), indicating norvaline greatly stabilized the overall structure of GDH.

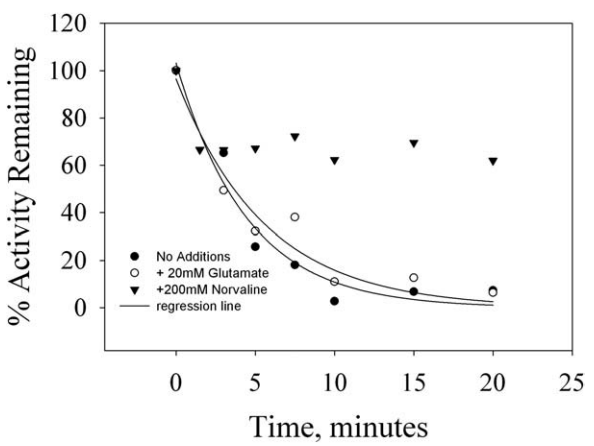

Figure 5. Heat inactivation curves of native GDH (black circle) and GDH enzyme associated with 20 m*M* glutamate (white circles) or 200 m*M* norvaline (black triangles).

Table I. *Effects of Carboxylate Ligands on the Heat Stability of GDH*

Ligand	Average rate constant	Standard error
None	0.252	0.048
20 m*M* glutamate	0.194	0.055
20 m*M* α-ketoglutarate	0.168	0.027
20 m*M* L-hydroxyglutarate	0.159	0.031
200 m*M* norvaline	0.0049	0.0034
20 m*M* glutarate	0.064	0.017
20 m*M* 3,3-dimethylglutarate	0.074	0.026

Guanidine hydrochloride unfolding of glutamate dehydrogenase in the presence or absence of ligands

Previous work using DLS[40] has demonstrated that increasing concentrations of GuHCl result in a hexamer–trimer transition followed by a trimer–monomer transition and rapid, irreversible denaturation of the monomer. Figure 7(A) shows the representative tryptophan fluorescence emission spectrum of GDH incubated in various concentrations of GuHCl. When the fluorescence maximum at each GuHCl concentration is plotted against the GuHCl concentration

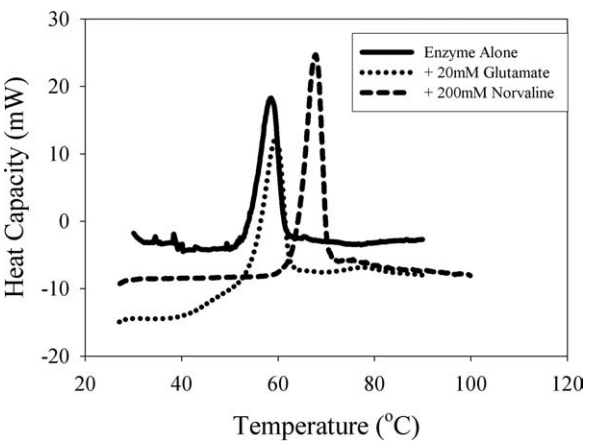

Figure 6. Differential scanning calorimetry thermograms of 2 mg mL^{-1} GDH in 0.1*M* phosphate buffer, pH 7.0. Separate baselines were subtracted from each thermogram with scan rates of 1°C min^{-1}. Thermograms were recorded in the absence of ligands (solid line) or in the presence of 20 m*M* glutamate (dotted line) or 200 m*M* norvaline (dashed line).

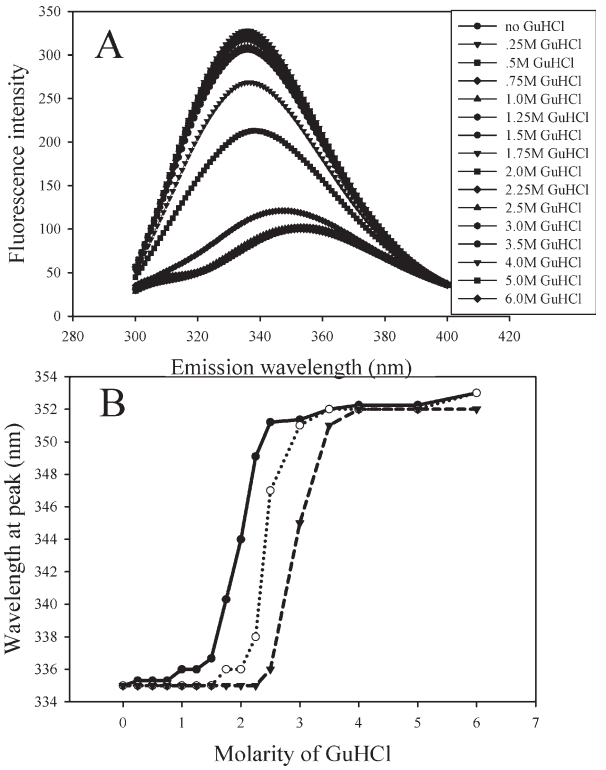

Figure 7. Guanidine hydrochloride unfolding of GDH in the presence and absence of ligands. **A**: A representative tryptophan fluorescence emission spectrum of GDH incubated in various concentrations of guanidine hydrochloride. **B**: The fluorescence emission maximum at each guanidine hydrochloride concentration is plotted to generate unfolding curves of native GDH (black circles) and GDH associated with 20 m*M* glutamate (white circles) or 200 m*M* norvaline (black triangles). Experiments use 1 mg mL^{-1} glutamate dehydrogenase in 0.1*M* phosphate buffer, pH 7.0.

[Fig. 7(B)], two transitions are consistently seen for GDH in the absence of any ligand. We attribute the first, small, transition at 1*M* GuHCl to the breakdown of the hexamer into two trimers (as has been suggested by previous DLS experiments[40]), and the second, major, transition starting at 1.75*M* is indicative of the breakdown of trimer into monomers which rapidly unfold. When norvaline is added to the enzyme, two effects on this profile are observed: there is no distinct first transition, and the second, major, transition is shifted to a higher GuHCl concentration (3.00*M*). Similar experiments with glutamate show a shift in the first transition to 1.625*M* GuHCl, whereas the second transition shifts to 2.40*M* GuHCl. The effects of other ligands used in this study on the major transition are summarized in Table II.

Effects of glutamate and norvaline on the strength of subunit interactions

DLS was also used to monitor hexamer dissociation, reflected by the weight-average molecular weight in the presence of increasing GuHCl concentrations. The weight-average molecular weights obtained in the DLS experiments are consistent with both a hexamer–trimer and a trimer–monomer transition occurring at the same time. The concentration of GuHCl required to observe these transitions is clearly shifted by binding of substrates (Fig. 8).

Table II. *Effect of Ligands on GDH During the Major Unfolding Transition as Identified by Guanidine Hydrochloride Unfolding*

Ligand	Concentration of guanidine hydrochloride at the mid-point of the unfolding transition
No additions	1.95*M*
Glutamate	2.4*M*
Norvaline	3.0*M*
α-Ketoglutarate	2.10*M*
3,3-Dimethylglutarate	1.75*M*
Glutarate	1.60*M*
L-α-Hydroxyglutarate	2.10*M*

Circular dichroism of GDH with glutamate and norvaline

Circular dichroism data (not shown) were collected but showed no significant changes when the enzyme was associated with either glutamate or norvaline. This indicates that there are no major changes in the α-helical structure of GDH induced by either glutamate or norvaline.

Effects of glutamate or norvaline on limited proteolysis of GDH by immobilized trypsin

To look at regions of the protein that may be flexible or that have altered accessibility, GDH was incubated

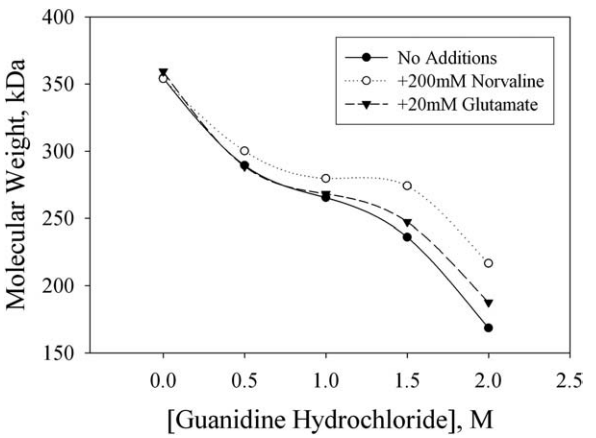

Figure 8. Subunit dissociation induced by guanidine hydrochloride followed by dynamic light scattering. The average molecular weight, as determined by dynamic light scattering, was followed as a function of guanidine hydrochloride concentration in the absence of ligands (filled circles) or in the presence of glutamate (filled triangles) or norvaline (unfilled circles).

Table III. *Effect of Glutamate or Norvaline on Trypsin Cleavage of GDH*

Location	Residue #	Relative rate	Glutamate[a]	Norvaline[b]
	Trimer interface I			
Trimer interface on extended loop	R35	xxxxx	+	---
Trimer interface, near dimer interface	R146	x	++	ND
Trimer interface, end of helix	R174	xx	++	----
Top of antenna	R419	xx	++	---
Base of Antenna region	R439	xx	++	----
	Dimer interface			
Dimer interface, end of helix	K155	xx	++	----
	Active site region			
Active site region	K114	x	++	ND
	Solvent exposed			
On loop before β-strand	K245	xxx	++	---
Center of helix	R261	xxx	++	---
On loop before β-strand	K342	xxx	+++	ND
End of helix	R363	xxx	+++	ND
Base of antenna	R403	xx	++	---
Top of antenna	K423	xx	++	---

In the absence of a coligand, the relative rate of cleavage is denoted on a 1-5 scale by x (slow) to xxxxx (fast). In the presence of coligands, +, promotes trypsin cleavage; - slows trypsin cleavage (magnitude of effect on 1-5 scale). ND denotes not detectable.

[a] Addition of 20 m*M* glutamate prior to trypsin cleavage.

[b] Addition of 200 m*M* norvaline prior to trypsin cleavage.

for different lengths of time with immobilized trypsin; protein fragmentation was then detected by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. Experiments were conducted with enzyme alone or in the presence of glutamate or norvaline. Using Protein Prospector, a variety of the fragments obtained were uniquely identified indicating cleavage at certain residues at different relative rates, as shown in Table III. Glutamate has varied effects on different regions of the protein, whereas norvaline very significantly slows all of the proteolysis observed with the GDH in the absence of ligands. The regions of the protein affected are shown in Figure 9.

Discussion

Before discussing the overall conclusions that can be drawn from the studies reported here, it is important to note that the techniques in this study investigate different aspects of protein stability and flexibility, and it is only when they are used in conjunction with each other that a thorough picture of ligand-induced changes in conformational stability and local flexibility or accessibility can be seen. These studies suggest that in glutamate dehydrogenase, there is a direct correlation between conformational flexibility and activity. Moreover, allosteric interactions require sufficient conformational flexibility in the protein so that homotropic cooperativity can be mediated by alterations in conformational flexibility rather than structural transitions between relatively fixed conformational states.

Glutamate dehydrogenase shows negative cooperativity in both kinetic and cofactor binding studies when glutamate is used as substrate. With the alternative substrate, norvaline, no cooperativity is found in either initial rate studies[3,29,38] or in oxidized cofactor binding studies.[41] Because the burst rate is not significantly affected by deuteride versus hydride transfer, the chemical transfer step itself must be fast relative to some conformational rearrangement step following substrate binding but before chemical catalysis (hydride transfer). The isotope effects reported here demonstrate that with glutamate as substrate, a conformational step is rate limiting to hydride transfer although product release is overall rate limiting. In experiments with norvaline, catalysis (hydride transfer) is overall rate limiting and is much slower than observed with glutamate. Similar conclusions have been reached previously based on isotope exchange studies.[42] It is not possible (because of the uncertainty of the fluorescence properties of the central complexes in the reaction) to quantitate the number of subunits that may be operating during the burst phase. The major point is that the isotope does not affect the presteady-state rate and hence bond breaking events are not rate limiting for the burst phase of the reaction. This demonstrates that a conformational change, preceding the actual hydride transfer step is rate limiting for the presteady-state phase of the reaction. The fact that the presteady-state phase, under the conditions reported, can be adequately described by a single exponential suggests that all operative subunits are behaving similarly although, as discussed earlier, it is not possible to determine the number of catalytically active subunits in the hexamer. A correlation between the isotope effect of deuterated glutamate on the steady-state rate and the presteady-state burst amplitude was unexpected,

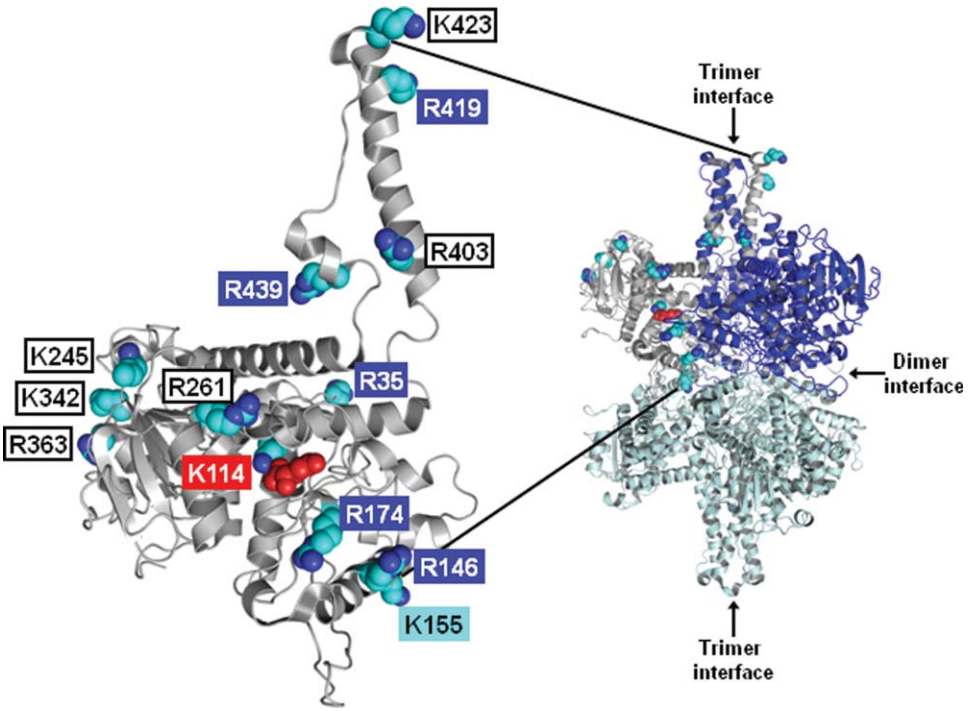

Figure 9. Sites of trypsin cleavage observed in limited proteolysis experiments in the presence or absence of carboxylic acid ligands. Dark blue indicates trimer interface; light blue indicates dimer interface; red indicates active site region; and white indicates solvent exposed. An interactive view is available in the electronic version of the article.

but it suggests that the isotope effect is on the equilibrium constant of the central complexes in the reaction. Such effects were predicted by Hartshorn and Shiner[43] and have been observed in several other systems and are predicted to be more widespread than recognized.[44]

Significantly, norvaline dramatically enhances stability, as measured by heat inactivation, GuHCl unfolding, and DSC, and appears to affect conformational flexibility or equilibrium surface accessibility as measured in the limited proteolysis experiments. Furthermore, these effects are correlated with the ability of a given ligand to support or impact negative cooperativity of the enzyme. The ligands used in this study can be grouped into the following categories:

1 Substrates (glutamate, α-ketoglutarate) and the substrate analog L-α-hydroxyglutarate promote negative homotropic interactions within GDH; the substrates are also subject to extensive heterotropic regulation.
2 Substrate analogs (glutarate and 3,3-dimethylglutarate) that do not support negative cooperativity.
3 Norvaline, an alternative amino acid substrate, shows no signs of negative cooperativity or heterotropic allosteric regulation.

Of the substrates and substrate analogs used in this study, there is a clear division between those that support full activity and trigger intersubunit allosteric regulation versus those that merely bind at the active site. Specifically, glutamate, α-ketoglutarate, and L-α-hydroxyglutarate, all of which support subunit interactions, have little impact on the overall conformational flexibility of the protein. Glutarate and 3,3-dimethylglutarate, which do not support subunit interactions, have a slight, but significant stabilizing effect as judged by the heat inactivation studies. It would be possible for the binding of a ligand to stabilize an individual subunit which then negatively affects the ability of that stabilized subunit to interact with other subunits. What stands out from these findings is the classification of flexibility following already existing classification regarding a second-position substituent, in conjunction with two carboxyl groups and negative cooperativity.[28] In addition, this class of ligands does affect the hexamer–trimer transition, where they may trigger intersubunit contacts that might prime the interface for communication.

Norvaline, which lacks a second carboxyl group, produced the most dramatic effect on protein stability in each of the experimental approaches. In the heat inactivation, DSC, and GuHCl studies presented here, norvaline induced a distinct increase in the stability of GDH, and we speculate that it decreases the flexibility options GDH has in its native form.

This study used DLS to look at intersubunit stability, using GuHCl to shift the hexamer–trimer equilibrium (measured as the average molecular weight by DLS) and probing the effects of the

substrates glutamate and norvaline on this equilibrium. Glutamate had little effect on the GuHCl profile measured by DLS (Fig. 8), suggesting little effect on the hexamer–trimer interactions. Norvaline significantly shifted the GuHCl profile to the right, indicating that norvaline binding strengthens trimer–trimer interactions and, hence, there is more hexamer at any given GuHCl concentration. These results demonstrate the dramatic increase in overall stability that norvaline causes in GDH.

Limited proteolysis experiments described here give further insight into the local conformational flexibility seen in GDH. While one can interpret proteolysis experiments to reveal information about either increased exposure to proteases or greater flexibility, we prefer the flexibility interpretation because of the location of protease sites on the three-dimensional structure of GDH (Fig. 9). The limited proteolysis experiments demonstrate that both glutamate and norvaline cause changes in local flexibility or accessibility, but in opposite effects, glutamate in general increases proteolysis while norvaline dramatically slows proteolysis. It is interesting that many of the changes are regions located either at the base of the antennae region of the molecule or at subunit interfaces. This suggests that norvaline causes conformational effects that interfere with the normal transmission of subunit interactions within the hexamer and involve "flex points" at the back of the glutamate-binding domain near residue 35 and within the GTP binding site.[35] GDH can be thought of as inherently having sufficient conformational flexibility to allow for cofactor-induced subunit interactions. We hypothesize that the ligands that stabilize the protein, such as norvaline, block such interactions and effectively slow the overall reaction.

Overall, these studies suggest that in glutamate dehydrogenase, there is a direct correlation between conformational flexibility and activity and that allosteric interactions require sufficient conformational flexibility in the protein such that homotropic cooperativity can be mediated by alterations in conformational flexibility rather than transitions between discrete conformational states.

Materials and Methods

Preparation of glutamate dehydrogenase

Bovine liver GDH was obtained as a glycerol solution from Sigma Chemical Co. Enzyme solutions were prepared using 0.1*M* phosphate buffer at pH 7.0, containing 10 μ*M* EDTA. Enzyme preparation was shown to be a single species of appropriate molecular mass using MALDI-TOF mass spectrometry. All solutions were made up with distilled, deionized water from a 4-bowl Milli Q system. Enzyme concentrations were determined spectrophotometrically using the absorbance at 280 nm, with an extinction coefficient of 0.93 for a 1 mg mL^{-1} solution. Coenzyme concentrations were also determined spectrophotometrically using absorbance measurements at 260 nm and a millimolar extinction for $NAD(P)^+$ at 260 nm of 15.9 cm^{-1} mM^{-1} or at 340 nm using an extinction coefficient for NAD(P)H of 6.22 cm^{-1} mM^{-1}.

Rapid reaction studies

Using the fluorescence of NAD(P)H to follow the oxidative deamination reaction, stopped flow studies were conducted using an SLM-Aminco Bowman fluorometer fitted with a Milliflow Stopped Flow Reactor rapid mixing attachment with a deadtime of 1–2 ms. Enzyme, at twice the final concentration desired, was loaded into one syringe and the appropriate mixture of substrates into the other. Ten time traces up to 8 s were collected and averaged to allow both a presteady-state rate and amplitude (where appropriate) and the overall steady-state rate to be obtained for the same reaction mix. Where a presteady-state phase was observed, the steady-state rate was subtracted and the remaining data fit to a single exponential function to give the amplitude and rate constant as appropriate. In all cases, the errors associated with the parameters calculated from the data were less than 1%.

Heat inactivation studies

Measurements were made by preincubating buffer to 50°C and at time zero adding GDH to obtain a final concentration of 1 mg mL^{-1}; no more than 100 μL of a concentrated stock solution was added to 3 mL of preheated buffer. An aliquot was immediately withdrawn for an enzyme assay. Further aliquots were withdrawn over a 20-min period and assayed under standard assay conditions: 0.1*M* phosphate buffer, pH 7.0, 20 m*M* glutamate, and 500 μ*M* NAD^+ using a thermospectronic dual beam UV 500 spectrophotometer. The resultant data were analyzed according to:

$$\frac{\mathrm{enz}_t}{\mathrm{enz}_0} = e^{-k_{\mathrm{inact}}t},$$

where enz_t is the activity at time t after introduction of the enzyme, and enz_0 is the activity at time equal zero. Replicate determinations of the rate constant for inactivation, k_{inact}, were made and averaged. When ligands were included in the inactivation incubation, they were added to the buffer at the appropriate concentration prior to the preincubation. Control experiments showed that inclusion of ligands in the preincubation buffer had no effect on the measured rate when an aliquot of enzyme was removed.

Differential scanning calorimetry

Calorimetric curves were obtained using a Microcal DSC. GDH was dialyzed a minimum of two times for 12 h using a 500-fold excess of 0.1*M* phosphate

buffer, pH 7.0, containing the appropriate ligand. Samples were exhaustively degassed and then injected into the calorimetric cell. A baseline scan was completed with 0.1*M* phosphate buffer, pH 7.0 (with ligand as appropriate), in both reference and sample cells. For the sample run, glutamate dehydrogenase (2 mg mL^{-1}) was used in sample cell, with 3 atm of pressure and a temperature range of 25–85°C. Data were analyzed by using a sigmoidal curve through CPCalc software.

Guanidine hydrochloride unfolding

In the experiments described here, we have incubated protein (1 mg mL^{-1}) in a given concentration of GuHCl for 1 h before taking a fluorescence emission spectrum, with excitation at 280 nm. Data were recorded using a thermospectronic Aminco-Bowman spectrofluorimeter and the appropriate blanks were subtracted. If a protein has several domains each contributing to the parameter being followed, a multitransitional denaturation curve will be obtained depending on the method of following the effects of unfolding, reflecting the variable stabilities of the different domains. If a ligand is present that stabilizes the overall structure of the protein, the denaturation curve will be shifted to higher concentrations of guanidine hydrochloride, whereas the opposite will be true of a ligand that destabilizes the protein.

Circular dichroism

The α-helical structures of the protein were investigated with a Jasco J-720 CD spectrometer. GDH at a concentration of 0.2 mg mL^{-1} was exhaustively degassed and scanned at wavelengths of 280–180 nm in a 1-mm quartz cuvette. Replicate scans were completed at a rate of 20 nm min^{-1} and a response time of 4 s. The appropriate baselines were subtracted and samples with ligands were compared with the native enzyme.

Dynamic light scattering

GDH was diluted in 0.1*M* phosphate buffer, pH 7.0, with varying concentrations of GuHCl (0, 0.5, 1.0, 1.5, and 2.0*M*) to a final protein concentration of 0.15 mg mL^{-1}. Light scattering measurements were carried out using a miniDAWN Tristar laser photometer with a Wyatt Quasi Elastic Light Scattering attachment (Wyatt Technology, Santa Barbara, CA). The instrument measured the amount of laser light at a wavelength of 690.0 nm, which was scattered by a protein sample. Solutions were introduced into the system using 10-mL syringes with a single inorganic membrane filter (0.2 μm, Whatman) attached. Light scattering data were collected and processed using the Astra V (5.1.9.1) software package (Wyatt Technology). Within this package, molecular weights were determined from a Zimm plot.

Limited proteolysis

To perform limited proteolysis, GDH was incubated at a concentration of 2 mg mL^{-1} (0.1*M* phosphate buffer, pH 8.0) with immobilized trypsin; preliminary experiments established a suitable ratio of GDH to protease to give limited proteolysis over a 1-h time course. Each time course was repeated three times with similar results. The digestion was "limited" by removing, at 15-min intervals, a sample from the digestion mix and centrifuging for 1 min to remove the immobilized protease. On completion of limited proteolysis, samples were analyzed by MALDI-TOF. For MALDI-TOF calibration purposes, bovine serum albumin was used as a standard and was diluted from 2 to 0.5 mg mL^{-1} using 6*M* guanidine hydrochloride. The cleavage sites were analyzed using Protein Prospector; a program made available by the University of California, San Francisco. The program finds all theoretical cleavage sites and determines the masses of potential fragments. By comparing the predictions with the experimental results, the most likely locations of cleavage can be determined.

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