The Direct Measurement of Thermodynamic Parameters of Reactive Transient Intermediates of the L-Glutamate Dehydrogenase Reaction*

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In a previous report (Fisher, H. F., Maniscalco, S. J., and Tally, J. (2002) Biochem. Biophys. Res. Commun. 287, 343–347) we demonstrated the capability of the “Le Chatelier forcing method” of producing stable solutions containing substantial amounts of transitory enzyme intermediate complexes that can otherwise be observed only fleetingly in the millisecond time range. The method requires nothing more than running an enzyme reaction using forcing concentrations of reactants against an equally forcing concentration of products until equilibrium is attained. Here we have applied this approach to the measurement of the thermodynamics of several such reactive (and normally transient) intermediate complexes of the bovine liver L-glutamate dehydrogenase-catalyzed reaction. At pH 9.5 and 20 °C, we observe both the enzyme-NADPH-α-iminoglutarate and enzyme-NADPH-α-carbinolamine complexes at concentrations whose sum accounts for 70% of the total enzyme. The pH dependence of these two complexes under equilibrium conditions provides thermodynamic parameters for both the protonated and the unprotonated forms of each of these two entities as well as those of the enzyme-NADP-L-glutamate complex. The equilibrium concentrations of each of these reactive complexes are compared with their corresponding transient steady-state values.

Establishing structure-function relationships is a focal point in the determination of mechanisms of enzymatic catalysis. The measurement of thermodynamic differences between related enzyme complexes is an important step in such endeavors. Such measurements have been necessarily limited to nonreactive complexes; the structures of the mechanistically and energetically vital reactive intermediate complexes have been typically derived solely on the basis of chemical intuition. Two recent experimental developments now provide the basis for the direct measurement of differences in thermodynamic parameters of spectroscopically identified reactive intermediate complexes whose kinetic competence has been verified. These developments are 1) the transient-state multiwavelength spectroscopic approach, which can produce resolved component reaction time courses of enzyme reactions (1, 2); and 2) the Le Chatelier forced equilibrium approach, which produces significant concentrations of reactive transient intermediate complexes in solution under true thermodynamic equilibrium conditions (3, 4). Here, we report the results of the first set of such thermodynamic measurements on reactive intermediates of the bovine liver L-glutamate dehydrogenase (bLGDH)3 reaction.

The Experimental System—The stoichiometry of the glutamate dehydrogenase reaction is shown in Equation 1. The capital letter below each reactant or product species is used to designate that entity in the various enzyme (E) complexes to be discussed.

\[ \text{NADP}^{+} + \text{L-glutamate} + \text{H}_{2}\text{O} \rightleftharpoons \text{NADPH} + \text{H}^{+} + \alpha\text{-ketoglutarate} + \text{ammonia} + \text{H}^{+} \] (Eq. 1)

The generally accepted mechanism of the amino acid dehydrogenase-catalyzed oxidative deamination reactions is shown in Scheme 1, which portrays the chemical state of the α-carbon atom of the substrate in the sequence of central complexes. In the bLGDH-catalyzed reaction we have shown that the nicotinamide moiety of the coenzyme has the following spectroscopic properties relative to those of the 340-nm peak of free NADPH:HEOG is colorless, EOG+ is a weakly absorbing, red-shifted highly fluorescent charge transfer complex (5), ERI (enzyme-NADPH-α-iminoglutarate) is highly blue-shifted, ERC (enzyme-NADPH-α-carbinolamine) is highly red-shifted (1), and ERK (enzyme-NADPH-α-ketoglutarate) is slightly blue-shifted.

The Le Chatelier Force (Ammonia Dam) Approach to the Study of Reactive Central Complexes—Here, we allow an amino acid dehydrogenase reaction containing high concentrations of the enzyme, the amino acid substrate, NAD(P), and a product, ammonia, to reach the equilibrium shown in Scheme 2, a process which is usually complete in 3–40 min.

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1 The abbreviations used are: bLGDH, bovine liver GDH; GDH, glutamate dehydrogenase; E, enzyme; O, oxidized coenzyme; I, iminoglutarate; R, reduced coenzyme; G, L-glutamate; C, carbinolamine; EOG, the sum of the protonated and unprotonated EOG; K, α-ketoglutarate; EOG+, isomerized EOG; HEOG, the protonated form of EOG; ER, the enzyme-NADPH complex; ERC, ER-α-carbinolamine; ERI, ER-α-iminoglutarate; MES, 2-(cyclohexylamino)ethanesulfonic acid; CHES, 2-(morpholino)ethanesulfonic acid; 5-enolpyruvoyl shikimate 3-phosphate.

2 Subsequent to publication of our paper (3) in which we introduced what we presumed to be a novel method and during the process of review of this paper, it came to our attention that the approach had in fact already been utilized successfully and elegantly in a paper from the laboratories of K. Johnson and K. Anderson in which they applied 13C NMR to a forced equilibrium system to identify and characterize an enzyme-bound tetrahedral intermediate in the 5-enolpyruvoylshikimate 3-phosphate synthase reaction (4).
The filtration experiment was carried out by loading an equilibrated solution into distinct components is shown in Fig. 1. It can be seen that the only R-containing complexes present at measurable concentrations are the highly blue-shifted ER and ERC. The pH dependence of resolved reactive intermediate complexes. The open triangles (△) show the relative concentrations of the ERC complex. Each point represents the average of 3–20 experimental values. The open squares (■) represent the relative concentrations of the ERC complex. All concentrations are expressed as fractions of the total enzyme [E]. The solid lines represent the best overall fit of the data to Equation 1. The vertical lines through each symbol show the estimated probable error. [E], varied from 5–40 μM.

Evidence that the Le Chatelier forced systems we have used in this work are at (or at least very close to) true thermodynamic equilibrium is provided by the following experimental observations: 1) The equilibrium constant for the O + G = R + N + K + H can be expressed as $K_{eq} = [R][N][H^+] / ([O] - [R])$ for a reaction started with O, G, and N and an amount of enzyme too low to permit the absorption of enzyme complexes to contribute to the observed signal. The components indicated by the superbar are considered to be “buffered”; that is, their concentrations remain constant throughout the reaction. The value of the equilibrium constant was measured over a range of very low enzyme concentrations. The observed pH-dependent value of $5.3 \times 10^{-11}$ m$^2$ obtained at 20 °C agrees...
very well with that of $7.3 \times 10^{-14}$ M$^2$ reported by Subramanian (6) for the same reaction run at 25 °C, corrected for the Δ$H^0$ of 17 kcal/mol$^{-1}$ reported in the same study. Fig. 3 shows the values of Δ$G^0$ calculated from $K_{eq}$ values from the same reaction at high enzyme concentration and varying pH using values of [RI]$_{low}$ obtained from the spectral component analysis. The variations from the theoretical value (indicated by the arrow in the figure) are not sufficiently large enough to effect our conclusions materially. 2) Addition of small amounts of $\alpha$-keto-glutarate to a presumably equilibrated reaction mixture rapidly reverses the reaction. 3) Forcing an equilibrated solution through a 0.22-μm filter in a syringe fitted with a Swinney adaptor produced a filtrate containing the essentially same concentration of free R as that calculated by spectroscopic resolution in the original equilibrated solution. 4) Raising or lowering the temperature of a high enzyme equilibrated reaction mixture substantially and reversibly changes both the concentration of free R in accordance with the positive Δ$H^0$ of the chemical reaction and alters the relative concentrations of the ERI and ERC components (data not shown). The reversal of temperature changes from 0 to 35 °C showed only a small hysteresis.

Given the observations and assumptions described above, the system can now be described by Scheme 3, which can be evaluated by Equations 2–7.

$$HERC = \frac{E}{1 + \frac{1}{K_1} + \frac{1}{K_3} + \frac{1}{K_5} + \frac{1}{K_7} + \frac{1}{K_9} + \frac{1}{K_{11}}} \left(1 + \frac{1}{K_2} \frac{1}{K_4} + \frac{1}{K_6} \frac{1}{K_8} \frac{1}{K_10} \frac{1}{K_{12}} \right) \frac{1}{K_2} \frac{1}{K_4} \frac{1}{K_6} \frac{1}{K_8} \frac{1}{K_{10}} \frac{1}{K_{12}} (Eq. 2)$$

$$EOG = \frac{K_1 \cdot HERC}{K_3 \cdot HERC + K_5} (Eq. 3)$$

$$HEOG = \frac{HERC}{K_1 \cdot K_3} (Eq. 4)$$

$$ERI = \frac{HERC \cdot K_5}{K_3 \cdot HERC} (Eq. 5)$$

$$HERI = \frac{HERC}{K_5} (Eq. 6)$$

$$ERC = \frac{HERC}{K_3 \cdot HERC} (Eq. 7)$$

The values of each of these pH-independent equilibrium constants were obtained from a simultaneous fit of the data to the whole set of Equations 2–7. The resulting values, along with those of the standard free energies (calculated from Δ$G^0$) are provided in Table I. The solid lines in Fig. 2 represent the fit with these parameters to the pH dependence of the concentrations of $ERI_{total}$ and $ERC_{total}$. The results shown in Fig. 5 lead to the potentially important conclusion that both HERI and ERI are thermodynamically highly unfavored structures, whereas the unprotonated ERC structure is only slightly less stable than the HEOG and EOG complexes. We now interpret the data of Fig. 2 in the context of Scheme 3. The data show that at pH values at or above 8, 70% of the total enzyme is tied up as ERI or ERC complexes under the conditions of the experiment and that throughout the pH range, the concentration of the carbinolamine complex exceeds that of the $\alpha$-imin complex by at least 6-fold. It is also apparent from Fig. 2 that the pH dependence of both the reactive intermediate complexes observed in this system involves the ionization of a single proton. Because the apparent $pK$ values of these complexes differ, it is conceivable that we could be observing the ionization of a different functional group in each case. However, evidence from other studies argues strongly that, to the contrary, we are titrating the same functional group in all three complexes and that the $pK_{app}$ differences reflect differences in the local interactions of that group with the varying chemical bond makeup of each individual complex. Piszczewicz et al. (6) showed that bGDH could be inactivated by pyridoxal or pyridoxal phosphate and that at pH values of less than 10 only one molecule of either reacted rapidly. They also showed that incorporation of the pyridoxyl moiety into the protein involved imine formation with (and only with) lysine 126 of the enzyme. They found an apparent $pK = 8.0$ for the reaction. Subsequent studies have found this particular residue to be conserved in all the pyridine nucleotide-linked $\alpha$-amino acid dehydrogenases (7), and its unmodified presence is an obligatory requirement for catalysis.

Assuming then that the marked differences in the $pK$ values observed represent only shifts in the $pK$ of the same residue, it follows that they must be because of corresponding differences in the bonding state of that residue in each of the three different complexes. The reaction shown in Equation 8 results in Equations 9 and 10.

$$K_1 \quad K_2$$

$$H^+ + K_3 + K_5 + K_7 + K_9 + K_{11} \quad (Eq. 8)$$

$$K_1 \quad K_2$$

$$pK_{hs} = -\log \left( \frac{K_1 \cdot K_2}{1 + K_1 + K_2} \right) (Eq. 9)$$

$$pK_{hs} \neq pK_{app} (Eq. 10)$$

The feature shown in Equation 8 occurs repeatedly throughout Scheme 3 on which we have based our interpretation of the observed pH dependences of the EOG, ERI, and ERC com-
plexes and would appear to provide a sound basis for explaining our results. It may also be noted that the apparent pK values defined by the inflection points of the ERI and ERC pH dependence curves shown in Fig. 2 do not correspond to any single pK value obtained from our analysis. Thus, such a phenomenological pK value has no real physical significance and represents only the result of the assembly of relationships of the type shown in Equation 8, which occur in a system described by the model shown in Scheme 3.

A comparison of both the absolute and relative concentrations of the ERI and ERC complexes at equilibrium, shown as dashed horizontal lines, and those in the transient local steady state, shown as solid curved lines in Fig. 6, reveals some striking differences between the compositions of the thermodynamic and kinetic states. At equilibrium, the concentration of ERC is far greater than that of ERI at all pH values. In the transient state, on the other hand, ERI dominates the early burst phase and remains at higher concentrations than ERC throughout the major portion of the reaction time course. The figure shows that at pH 7.6 the transient-state concentration of EOG is about twice that of its equilibrium concentration. This situation may be expressed in any of three equivalent sets of conceptual terms. 1) In terms of classic chemical kinetics, EOG exhibits some degree of kinetic rather than purely thermodynamic control. 2) In the language of absolute reaction rate (or

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**Table 1**

| Step  | Reaction                        | $K$          | $\Delta G^\circ$ | pK   |
|-------|--------------------------------|--------------|-----------------|------|
| 1     | EOG $\rightleftharpoons$ ERC   | (5.3 ± 0.1) $10^{-4}$ M² | 17.84 ± 0.02    |      |
| 2     | HEOG $\rightleftharpoons$ EOG   | (3.6 ± 0.3) $10^{-6}$ M | 10.0 ± 0.1      | 7.45 ± 0.08 |
| 3     | HERI $\rightleftharpoons$ ERI   | (5.0 ± 2) $10^{-5}$ M | 6 ± 0.2         | 4.3 ± 0.2  |
| 4     | HERC $\rightleftharpoons$ ERC   | (1.2 ± 0.4) $10^{-6}$ M | 7.9 ± 0.2       | 5.9 ± 0.2  |
| 5     | EOG $\rightleftharpoons$ ERI   | (2.4 ± 0.8) $10^{-4}$ M | 4.8 ± 0.2       |      |
| 6     | HEOG $\rightleftharpoons$ HERI  | (2.1 ± 0.3) $10^{2}$ M | -3.1 ± 0.2      |      |
| 7     | HERI $\rightleftharpoons$ ERC   | (1.7 ± 0.3) $10^{-7}$ M | 9.1 ± 0.2       |      |

*Step numbers refer to Scheme 3.*
activated complex) theory, EOG formation is preceded by a low transition state barrier and followed by a relatively high barrier. 3) In steady-state kinetic terms, EOG itself has a low forward commitment factor and must be preceded by a complex having a relatively high commitment factor.

The transient local steady-state concentration of the ERI complex is nearly identical to its equilibrium value. Therefore, ERI is 1) under thermodynamic control, 2) lies between low transition barriers, and 3) its forward commitment factor is effectively balanced by those of the preceding and following complexes.

The transient local steady-state concentration of the ERC complex is 10-fold lower than its equilibrium value. Therefore, 1) its concentration is dominated by kinetic control, 2) the transition state barrier preceding its formation is higher than that following it, and 3) its forward commitment factor is substantially greater than that of the preceding complex.

It can be seen from Fig. 5 that the unprotonated forms of both the imino and carbinolamine complexes are thermodynamically more stable than their corresponding protonated forms at pH ≈ 7.6. Although high reactivity of minor species is always a possibility, the predominance of unprotonated post-hydrate transfer species fits in well with the postulated kinetic mechanism (9).

Non-observed Phenomena—The lack of appearance of any of the relatively tight blue-shifted ERK complex from the equilibrium mixture is easily explained by the driving of the ERK + N ⇄ ERI far to the ERI form because of the presence of the high concentration of ammonium. The absence of any trace of the red-shifted ERG complex is somewhat more difficult to account for. This dead-end inhibitory complex, although slow to form, is so very tight that it constitutes the final complex in the transient-state reaction, and the release of free NADPH from the ERG complex is the rate-limiting step in the steady-state forward reaction. Although the inhibition of the GDH reaction by ammonia is quite complex, we will show elsewhere that the high concentration of ammonia is successful in competing with the α-amino group of l-glutamate at the ammonia binding site of the enzyme, forcing the substrate to bind in a mode in which hydride transfer is precluded.4

In a previous report on the transient-state resolved component time courses of the Clostridium symbiosum glutamate dehydrogenase (csGDH) reaction, we have demonstrated the occurrence of isomeric forms of ERI, ERC, and ERK complexes (3). These isomeric forms differ from the previously identified entities in that their spectral 340-nm peak is unshifted and therefore spectrascopically indistinguishable from that of free NADH. Their existence has been established and their location on the reaction coordinate has been determined by the kinetic competence of this anomalous free R to participate actively in the reaction at several mechanistically separate points. We have ascribed the characteristic of an unshifted spectra to open forms of the enzyme complexes in which the reduced nicotinamide moiety is exposed to a largely aqueous environment, in contrast to the hydrophobic environment of the closed forms. “pH jump” experiments performed on the csGDH transient-state reaction confirmed an obligatory alternation between open and closed forms along the reaction pathway (11). Had such open unshifted complexes been present in significant concentrations in the equilibrated blGDH solutions we have described here, they would necessarily have led to anomalously high values for the stoichiometric equilibrium constant K_eq. No such effect was observed in any of our blGDH experiments.

Furthermore, pH jump experiments on blGDH transient-state studies reported elsewhere (4) showed the occurrence of measurable amounts of open forms only in the prehydride transfer phase of the reaction. The lack of appearance of open forms in the equilibrated blGDH solutions reported here suggests that the basis of this aspect of the difference in kinetic behavior of the two GDH species is thermodynamic in nature; the "open = closed" equilibria lie much farther to the right in the blGDH reaction.

We have recently shown the presence of a generally conserved active site motif in this class of enzymes (12). It consists of a close-packed atomic tetrad made up of the α-amino nitrogen atom of lysine 126, an oxygen atom of the carboxylate group of aspartate 168,5 the nitrogen atom of the α-amino group of the substrate, and the oxygen atom of a water molecule hydrogen bonded to lysine 126. Superposition of x-ray crystal structures of different complexes of a variety of these dehydrogenases showed that these tetrads very nearly, but not quite exactly, mapped each other. Although the atom-to-atom center distances around the tetrad averaged about 2.8 Å, they differ individually by as much as 1 Å. Computer modeling of these structures correspondingly predicted a wide variety of hydrogen bonding patterns in this motif. Fig. 5 indicates ΔG° differences between the EOG, ERI, and ERC structures of the order of 1–5 kcal/mol⁻¹. This is about the range of values expected for either the formation of a strong H-bond or the difference between the breaking of one weak bond and the formation of a different strong H-bond. Although still a matter of conjecture, this agreement may not be coincidental.6

Finally we note that although the thermodynamic characterization of the transient ERI and ERC complexes provided here presents an essential but very elementary step in the full understanding of the structural and chemical nature of these catalytically important entities, the approach whose application we have demonstrated may contribute certain features that could be quite useful in extending studies of enzymatic catalysis to a deeper level of understanding. At the present time, the methods available for determining the structures of enzymes in various complexes (such as x-ray crystallography, neutron diffraction, NMR, IR) all require substantial periods of time for their completion. Their application therefore has been restricted to unreactive complexes. On the other hand, as seen in Fig. 1, the ERC and ERI complexes remain intact for at least one month. Enzyme assays conducted on aliquots of the equilibrated solution show no loss or enzyme activity during that time period. Thus, the Le Chatelier forced equilibrium approach permits the application of such time-requiring methods to the examination of the transient reactive intermediate complexes that constitute the heart of an enzymatic reaction.

REFERENCES
1. Maniscalco, S. J., Saha, S. K., and Fisher, H. F. (1998) Biochemistry 37, 14585–14590
2. Tally, J. F., Maniscalco, S. J., Saha, S. K., and Fisher, H. F. (2002) Biochemistry 41, 11284–11293
3. Fisher, H. F., Maniscalco, S. J., and Tally, J. (2001) Biochem. Biophys. Res. Commun. 287, 343–347
4. Anderson, K. S., Sammons, R. D., Leo, G. C., Sikorski, J. A., Benesi, A. J., and Johnson, K. A. (1990) Biochemistry 29, 1460–1465
5. Saha, S. K., Maniscalco, S. J., Singh, N., and Fisher, H. F. (1994) J. Biol. Chem.

5 Although residue 168 was assigned as an Asn in the blGDH crystal structure reported by Peterson and Smith (13), it is in fact a highly conserved Asp residue in all other GDH structures. Dr. T. Smith has informed us that the Asn assignment was based on very early amino acid–sequence studies (7) and that residue 168 is most probably an Asp.

6 A detailed description of the relationships between the geometric pattern of active site residues and the corresponding catalytic and thermodynamic properties in the various complexes identified here will be presented elsewhere.

S. K. Saha, J. F. Tally, S. J. Maniscalco, S. A. Adediran, and H. F. Fisher, unpublished data.
269, 29592–29597
6. Subrananian, S. (1978) Biophys. Chem. 7, 375–378
7. Piszkewicz, D., Landon, M., and Smith, E. L. (1970) J. Biol. Chem. 245, 2622–2626
8. Brunhuber, N. M., and Blanchard, J. S. (1994) Crit. Rev. Biochem. Mol. Biol. 29, 415–467
9. Maniscalco, S. J., Saha, S. K., Vicedomine, P., and Fisher, H. F. (1996) Biochemistry 35, 89–94
10. Rife, J. E., and Cleland, W. W. (1980) Biochemistry 19, 2328–2333
11. Saha, S. K., and Fisher, H. F. (1999) Biochim. Biophys. Acta 1431, 261–265
12. Fisher, H. F., and Maniscalco, S. J. (2002) Bioorg. Chem. 30, 199–210
13. Peterson, P. E., and Smith, T. J. (1999) Structure 7, 769–782
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