Thermodynamic Interactions in the Glutamate Dehydrogenase-NADPH-Oxalylglycine Complex*

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The enzyme-reduced coenzyme-α-ketoglutarate ternary complex is a critical intermediate in the glutamate dehydrogenase-catalyzed reaction. Oxalylglycine, a structural analog of α-ketoglutarate which contains an amide carbonyl group in place of a reducible ketone group, is one of the few compounds known to compete with α-ketoglutarate itself. In order to examine the role of the ketone group of α-ketoglutarate in the ternary complex, we have carried out a calorimetric study of the corresponding oxalylglycine ternary complex, determining the complete $\Delta H^\circ$, $\Delta G^\circ$, $\Delta S^\circ$, and $\Delta C_p$ profiles and the corresponding interaction parameters for that complex and have compared the various parameters with the corresponding ones previously reported for the α-ketoglutarate ternary complex.

While the overall $\Delta G^\circ$ values of the two ternary complexes differ only slightly, the enzyme-NADPH-oxalylglycine ternary complex appears to achieve much of its stability from a very tight enzyme-oxalylglycine binary complex with little or no contribution from favorable interactions in the ternary complex, while the α-ketoglutarate ternary complex appears to achieve the same stability by a large interaction starting from a very weak enzyme-α-ketoglutarate binary complex. Consideration of the enthalpic profiles, however, show that this $\Delta G^\circ$-derived picture is deceptive. The excess binding energy which stabilizes the oxalylglycine ternary complex appears to be due to hydrogen bonding of the amide group of oxalylglycine to the enzyme; in forming the ternary complex, this hydrogen bonding is lost in favor of forming an oxalylglycine-NADPH interaction, which is very similar to the α-ketoglutarate-NADPH interaction which stabilizes the α-ketoglutarate ternary complex.

We conclude that the α-ketoglutarate-NADPH interaction must depend on either hydrogen bonding or steric hindrance by the ketone group and that the existence of this energetically large interaction cannot be ascribed to imine formation between the ketone group and enzyme. These findings also indicate the focus on the reaction coordinate where the reduced coenzyme plays a critical role, a role other than its obvious function as a hydride donor.

The enzyme-NADPH-α-ketoglutarate complex is an obligatory intermediate in the oxidative deamination of L-glutamate and in the reductive amination of α-ketoglutarate catalyzed by glutamate dehydrogenase (1). This complex (and its possible isomeric forms) appears to constitute a critical point in the reaction pathway. The rate of dissociation of this complex is the rate-limiting step in the forward direction (2); it is the site of ADP activation (3) and of GTP inhibition (4); it constitutes the lowest point on both the enthalpic and free energy reaction profiles (1, 5); and it provides the only crossover point between two parallel reaction sequences involving different enzyme forms (6, 7). Aside from these mechanistic features related to the reaction course, the enzyme-NADPH-α-ketoglutarate complex, as an entity in itself has a number of unusual properties: 1) its dissociation is accompanied by a substantial pH-independent D2O solvent isotope effect (2); 2) the dissociation of NADPH from the complex has a half-life of almost 2 s (2); 3) the binding mode of α-ketoglutarate in the enzyme-NADPH-α-ketoglutarate ternary complex has been shown to differ from that of other dicarboxylate compounds by competitive inhibition (8) and by its pH dependence (9); 4) the formation of the complex has a $\Delta H^\circ$ (enthalpy of interaction) at least twice as large as that of other corresponding dicarboxylate ternary complexes (10).

It is obvious that these differences between enzyme-NADPH-α-ketoglutarate and the other ternary complexes of glutamate dehydrogenase must be due in some way to the keto group of α-ketoglutarate, but there are at present no clues as to the chemical nature of the interactions of that group in the complex. Recently, Rife and Cleland have found oxalylglycine to be a very effective inhibitor competitive with α-ketoglutarate in the enzyme-catalyzed reaction (9). This compound, whose structure is shown in Scheme 1, is one of the few compounds found to show such competitive behavior. It may be noted that α-ketoglutarate and oxalylglycine each contain an α-carbonyl group, but that of α-ketoglutarate is aketone group and hence is reducible, while that of oxalylglycine is part of an amide group and, therefore, not reducible or otherwise reactive. Thus, the side-by-side comparison of this pair of compounds provides an opportunity to determine which of the various phenomena exhibited by the enzyme-NADPH-α-ketoglutarate complex must be attributed to the reactivity of a true ketone and which require only the hydrogen bonding ability possessed by any carbonyl group. We report here the results of such a comparative study of the detailed thermodynamics of the enzyme-NADPH-oxalylglycine and the enzyme-NADPH-α-ketoglutarate ternary complexes of glutamate dehydrogenase.

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Thermodynamics of Glutamate Dehydrogenase Complexes

MATERIALS AND METHODS

Beef liver L-glutamate dehydrogenase was purchased from Boehringer Mannheim as an ammonium sulfate dialyzed and charcoal filtered as previously described (3). Only preparations having a 280/260 nm ratio > 1.9 were used. Oxalylglycine was prepared by the method of Viscontini (11).

Enthalpies and equilibrium constants were determined with a LKB 10700-1 flow microcalorimeter equipped as previously described (12). Flow rates were determined by weighing the amount of water pumped through the calorimeter over a known amount of time. Pumping was accomplished with LKB Perpex peristaltic pumps with individual flow rates of about 4.3 μl s⁻¹. Electrical and chemical heat calibrations were done as previously described.

All experiments were carried out in 0.1 M potassium phosphate buffer at pH 7.6. Where NADPH was the saturating ligand, its final concentration was 800 μM. Where oxalylglycine was the saturating ligand, its final concentration was 3 nm. In flow calorimetry, known and approximately equal volumes of two solutions are mixed within the thermal sensing chamber. The observed heat, qobs, includes contributions from all interactions of the newly mixed components and from the heats of dilution of these components at the concentrations employed in any given experiment. Thus for the formation of a binary complex, E-L,

\[ q_F = \text{net}(\Delta H_{EL}) = q_{\text{obs}} - q_F - q_L \]  

where \( q_{\text{obs}} \) is the molarity of the E-L complex formed, \( \Delta H_{EL} \) is the standard enthalpy change for the formation of 1 mol of E-L complex from E and L, and \( q_F \) and \( q_L \) are the heats of the 2-fold dilution of E and L. Since \( q_F \) is usually much larger than \( q_L \), it is convenient in the measurement of \( \Delta H^\circ \) for the formation of a binary complex to establish a base-line by mixing a given concentration of ligand in buffer with an equal volume of buffer.

The quantities \( q_{\text{obs}}, q_F, \) and \( q_L \) are evaluated from the following series of experiments,

\[ (L, B) + (B) \rightarrow q_L \]
\[ (L, B) + (E, B) \rightarrow q_{\text{obs}} \]
\[ (B) + (E, B) \rightarrow q_F \]

where L, B, and E indicate the presence of ligand, buffer, and enzyme, respectively, in one of the two solutions to be mixed. The contents of each of the two solutions are defined by their exclusion in one of the two pairs of parentheses, and the mixing operation is represented by the symbol "+". \( q_F \) is then calculated from Equation 1. \( q_F \) is the experimental quantity used in Equation 5, as discussed under "Results."

An analogous procedure is used to measure the heat formation of a ternary complex from either of its two binary complexes,

\[ E - L_F + L_o = E - L_F - L_o \]

where \( L_F \) represents the ligand whose concentration is held at a fixed and saturating level, while \( L_o \) represents the other ligand whose concentration is varied.

Again, three experiments are carried out for each concentration of \( L_o \) to be used:

\[ (L_F, L_o, B) + (L_F, B) \rightarrow q_{L'o} \]
\[ (L_F, L_o, B) + (L_F, B, E) \rightarrow q_{\text{obs}} \]
\[ (L_F, B) + (L_F, B, E) \rightarrow q_F' \]

where the prime symbol indicates a value measured from a binary complex. The measured quantities are then treated as in the case of the binary complex as we have described.

In the case of the ternary complexes, the base-line was obtained by mixing a solution containing buffer and the saturating ligand (a) with a buffer (saturating ligand (a) and ligand (b)) solution. Measurements were carried out at both 15 and 25 °C. In both cases, with the exception of the enzyme-oxalylglycine binary complex at 15 °C, complete concentration curves were obtained.

RESULTS

The thermodynamics of the formation of the enzyme-NADPH-oxalylglycine ternary complex may be represented by the conventional "square."

\[ E \longrightarrow E-R \]
\[ E-Ox \longrightarrow E-R-Ox \]

where R is NADPH and Ox is oxalylglycine.

All thermodynamic parameters (\( \Delta G^\circ, \Delta H^\circ, \Delta S^\circ, \) and \( \Delta C_p^\circ \)) for step 1 have been previously reported (13). Corresponding measurements for any two of the remaining three steps completely define the system; measurements of a fourth step serve as a means of checking the internal consistency of the data.

We have carried out complete calorimetric titration curves for steps 2, 3, and 4.

A typical thermal titration curve is shown in Fig. 1A for the reaction,

\[ E-R + Ox = E-R-Ox \]  

measured at a saturating concentration of R and at 25 °C. The solid line of the figure represents the least squares fit of the data to the equation.

\[ q = \Delta H^\circ \left( \frac{[E_o] + [L_o] + [K_o]}{[E_o] + [L_o] + [K_o]^2 - 4[E_o][L_o]} \right) - \frac{1}{2} \]

where \( q \) is the heat evolved on complex formation, \( [E_o] \) is the total enzyme concentration, \( [L_o] \) is the total ligand concentration, and \( K_D \) is the dissociation constant for the measured process. Using a nonlinear regression fit to this equation, we calculate the values \( \Delta H^\circ = q_{\text{obs}}/[E_o], \Delta G^\circ = -RT \ln K_D \) and \( \Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T. \) Curve B of Fig. 1 represents the same reaction at 15 °C. \( \Delta C_p^\circ \) is calculated from the equation,

\[ \Delta C_p^\circ = (\Delta H^\circ - \Delta H^\circ)/10^6 \]

Calculated thermodynamic parameters obtained in this manner for steps 2, 3, and 4 are listed in Table I along with previously reported values for step 1.

Due to the very small \( \Delta H^\circ \) for step 4, the \( K_p \) for the enzyme-oxalylglycine binary complex could not be determined calori-
Thus, \( \Delta G' \), for the enzyme-NADPH-oxalylglycine complex is the difference in the \( \Delta G' \) of NADPH binding to the enzyme-oxalylglycine complex and the \( \Delta G' \) of NADPH binding to free enzyme. A mathematically equivalent statement is that \( \Delta G' \) is the difference between the \( \Delta G' \) of formation of the ternary complex and the sum of the \( \Delta G' \) values of formation of the two binaries. We define \( \Delta H' \), \( \Delta S' \), and \( \Delta C_p' \) in this value using the relationship,

\[
\Delta G' = \Delta H' - T \Delta S'
\]

We calculate \( K_D \) from data obtained from the other three legs of the thermodynamic square to be \( 142 \, \mu M \), a value consistent with the independence of the calorimetrically measured heat of formation of the complex over a concentration range of oxalylglycine from 0.24 to 6.2 mM. Using an equation analogous to Equation 5, we can also calculate \( \Delta H_2 \) from the three measured \( \Delta H' \) values to be \(-1.2 \) kcal. Since this is again in good agreement with the directly determined value of \(-0.5 \pm 0.5 \) kcal, we are assured that we are indeed measuring the properties of the active site of the enzyme-oxalylglycine binary complex without measurable contributions from extraneous binding sites.

The relationships between the various parameters of the four steps of the thermodynamic square are best visualized in the thermodynamic profile shown in Fig. 2A. This profile is based on \( 2^5 \) °C data alone, and the temperature dependences of the various \( \Delta H' \) values are shown in the \( \Delta C_p' \) profile at the bottom of Fig. 2A. The corresponding profiles for the enzyme-NADPH-\( \alpha \)-ketoglutarate complex (10) are shown in Fig. 2B for comparison.

In our previous thermodynamic studies of ternary complexes we have introduced the concept of "interaction parameters" to analyze the rather complex profiles typified by Fig. 2 (12, 13). Referring once more to the thermodynamic square of Equation 3, we define a "free energy of interaction," \( \Delta G_f \), as

\[
\Delta G_f = \Delta G' + \Delta G_{\text{ox}} - \Delta G_{\text{ox}}
\]

Thus, \( \Delta G_f \) for the enzyme-NADPH-oxalylglycine complex is the difference in the \( \Delta G' \) of NADPH binding to the enzyme-oxalylglycine complex and the \( \Delta G' \) of NADPH binding to free enzyme. A mathematically equivalent statement is that \( \Delta G_f \) is the difference between the \( \Delta G' \) of formation of the ternary complex and the sum of the \( \Delta G' \) values of formation of the two binaries. We define \( \Delta H_f \), \( \Delta S_f \), and \( \Delta C_p_f \) in an analogous manner. Interaction parameters for the enzyme-NADPH-oxalylglycine and enzyme-NADPH-\( \alpha \)-ketoglutarate complexes are given in Fig. 3.

**DISCUSSION**

Referring first to the thermodynamic profiles of Fig. 2, it can be seen that the overall \( \Delta G' \) for the reaction,

\[
E + R + Ox = E-R-Ox
\]

is only slightly smaller than that of

\[
E + R + K = E-R-K
\]

where \( K \) is \( \alpha \)-ketoglutarate and \( E-R-K \) is the \( \alpha \)-ketoglutarate ternary complex. Actual measurements show this difference to be about 0.5 kcal. Thus, the \( \alpha \)-ketoglutarate ternary complex is about twice as "tight" as that of oxalylglycine. This ratio of \( K_D \) values agrees with the ratio of competitive inhibition constants for \( \alpha \)-ketoglutarate and oxalylglycine determined by Rife and Cleland (9).

While the overall stabilities of the two complexes are quite similar, comparison of their free energy profiles and interaction parameters shows that they are achieved by two very different sets of interactions. The \( \alpha \)-ketoglutarate ternary complex, starting from an extremely weak enzyme-\( \alpha \)-ketoglutarate binary, achieves its ternary stability by virtue of a very large negative \( \Delta G_f \). The oxalylglycine ternary complex, on the other hand, begins with an unusually strong enzyme-oxalylglycine binary complex; the \( \Delta G_f \) is negligible, the \( \Delta G' \) of the formation of the ternary complex comprising essentially the simple sum of the \( \Delta G' \) values of formation of the two binary complexes.

From molecular models it can be seen that the structures of oxalylglycine and \( \alpha \)-ketoglutarate are geometrically very similar, oxalylglycine having a peptid—NH group in place of the \( \gamma \)-CH\(_3\) group of \( \alpha \)-ketoglutarate. The orientations of, and distance between, the carboxyl groups of the two molecules are almost identical. The observed differences in the mechanisms of binding of oxalylglycine and \( \alpha \)-ketoglutarate must, therefore, be due to differences in the functional group interactions available to the two ligands.

Oxalylglycine, for its part, contains a peptide bond whose NH group could quite conceivably engage in effective hydrogen bonding to the surface of the free enzyme, accounting for the existence of a tight enzyme-oxalylglycine binary.

\( \alpha \)-Ketoglutarate, on the other hand, lacks this feature and forms an enzyme-\( \alpha \)-ketoglutarate binary that is about 1.5 kcal weaker than the enzyme-oxalylglycine binary. It can be seen, however, from the large negative \( \Delta G' \) of the enzyme-NADPH-\( \alpha \)-ketoglutarate complex (Fig. 3) that the reducible ketone group of \( \alpha \)-ketoglutarate is able to engage in some productive interaction in its ternary complex which appears to be unavailable to oxalylglycine. This interaction adds about 2 kcal to the overall stability of the enzyme-NADPH-\( \alpha \)-ketoglutarate ternary complex, compensating for the relative weakness of the enzyme-\( \alpha \)-ketoglutarate binary and creating an overall stability which is slightly greater than that of enzyme-
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A. Thermodynamic profiles for the formation of the enzyme-NADPH-oxalylglycine (ER-Ox) complex (A) and of the enzyme-NADPH-α-ketoglutarate (ER-K) complex (B). It should be noted that $\Delta G^\circ$ and $\Delta H^\circ$ are plotted in the algebraic positive sense so that downward changes in parameters reflect contributions to increased binding. Data in the upper part were obtained at 25 °C; the $\Delta C_p$ data in the lower part were measured at 10 and 25 °C and were calculated from Equation 6. Data for B are taken from Ref. 10.

FIG. 2. Thermodynamic interaction parameters of: A, enzyme-NADPH-oxalylglycine complex, B, enzyme-NADPH-α-ketoglutarate complex, and C, enzyme-NADPH-α-glutamate complex. Parameters for the enzyme-NADPH-oxalylglycine complex are calculated from data of Table I. Those for the enzyme-NADPH-α-ketoglutarate complex are from Ref. 10. Those for the enzyme-NADPH-α-glutamate complex are from Ref. 13.

NADPH-oxalylglycine ternary complex.

The free energy parameters of the enzyme-NADPH-oxalylglycine complex just discussed are compatible with either of the two mechanistic classes shown schematically in Fig. 4. The “mechanism” for the formation of the enzyme-NADPH-α-ketoglutarate complex is shown in the upper part for comparison. The symbolism used is intended to imply only that the presence of a suitably located carbonyl group on the substrate is both necessary and sufficient for the occurrence of a large favorable interaction leading to the formation of the enzyme-NADPH-α-ketoglutarate complex. In the lower part, the enzyme-oxalylglycine binary complex is portrayed as engaging in some interaction involving its amide group in which α-ketoglutarate, lacking such a group, cannot engage. Such an enzyme-oxalylglycine binary complex could go on to form the enzyme-NADPH-oxalylglycine ternary complex by either of the two routes labeled A and B. Either of those routes would result in a net $\Delta G^\circ$ of nearly zero in agreement with the experimental results. In pathway A, oxalylglycine remains bound in the ternary complex as it is in the binary; $\Delta G^\circ$ is negligible simply because the interaction present in the enzyme-NADPH-α-ketoglutarate complex never occurs in the enzyme-NADPH-oxalylglycine complex. In pathway B the $-3$ kcal interaction occurs in the enzyme-NADPH-oxalylglycine complex just as it does in the enzyme-NADPH-α-ketoglutarate complex; but in order to engage in that interaction, the oxalylglycine molecule must break its $-2$ kcal amide interaction with the enzyme. The resultant overall $\Delta G^\circ$ observed, therefore, is $-1$ kcal, in good agreement with the observed value of $-0.8$ kcal.

While a comparison of the free energy profiles of the enzyme-NADPH-α-ketoglutarate and enzyme-NADPH-oxalylglycine complexes does not afford a means of distinguishing between the two possible routes of enzyme-NADPH-oxalylglycine ternary complex formation, consideration of the observed $\Delta H_f$ values does permit such a distinction. It can be seen from Fig. 4 that route A predicts a $\Delta H_f$ of zero for enzyme-NADPH-oxalylglycine formation, while route B predicts that $\Delta H_f$ would equal the difference between the 16 kcal $C = O \approx R$ interaction (observed in the enzyme-NADPH-α-
In the enzyme-oxalylglycine binary complex, the amide interaction is the same in both enzyme-α-ketoglutarate and enzyme-oxalylglycine binary complexes, which differs from that occupied by those dicarboxylate ligands which lack an α-carbonyl group.

Since oxalylglycine and α-ketoglutarate both induce this excess ΔHf in the enzyme-α-ketoglutarate complex, the effect cannot be attributed to the presence of a reactive ketone group. It must be ascribed to the one feature these two ligands have in common, an α-carbonyl group. Since these two α-carbonyl groups differ almost totally in their chemical reactivity (one being a reducible ketone while the other constitutes part of an amide) the only effect that they could both exert would be either a steric one or one due to carbonyl-hydrogen bonding. In either case, the result would be that these two ligands are bound in the ternary complex in the same general locus but with a geometric placement which differs from that occupied by those dicarboxylate ligands which lack an α-carbonyl group.

One of the motives in beginning this study on the comparison of the thermodynamics of formation of the enzyme-NADPH-oxalylglycine and enzyme-NADPH-α-ketoglutarate complexes was to distinguish between two proposed mechanisms for glutamate dehydrogenase catalysis. In the mechanism proposed by Rife and Cleland (9) the ketone group of α-ketoglutarate in the enzyme-NADPH-α-ketoglutarate complex remains intact, being hydrogen bonded only; while in the transmission scheme suggested by Smith (14) and elaborated by Fisher (1), that ketone group is converted into a mixture of an enzyme-bound carbonolamine in one enzyme-NADPH-α-ketoglutarate complex and an enzyme-bound imine in an isomeric enzyme-NADPH-α-ketoglutarate' complex. Rife and Cleland have suggested that the relatively tight binding of oxalylglycine argues against the necessity of covalent bonding of the α-ketoglutarate-ketone group to the enzyme. Fisher, on the other hand, has suggested that the ΔHf of −16 kcal could be explained by an enzyme-bound ketimine based on calorimetric measurements of formation of an oxime from pyruvate (1).

While the results described here do not categorically rule
out such a ketimine intermediate, we can say at this point that the mere existence of a large negative $\Delta H_f$ is not a viable argument to support the existence of such an entity.

There is, however, a more important conclusion to be drawn from these results. In all detailed mechanisms drawn for pyridine nucleotide dehydrogenase-catalyzed reactions, the role of the reduced coenzyme is limited to a single step, a hydride transfer from coenzyme to some substrate form. It is, of course, widely recognized that the reduced coenzyme must have some other important, albeit obscure, function; and for this reason NAD(P)H is customarily written as being present in the many other steps in which it plays no defined role. It is apparent that in the glutamate dehydrogenase-catalyzed reaction, we can pinpoint at least the site of one such obligatory nonreductive role for NADPH as being located at the step or steps involving the formation of the reactive enzyme-NADPH-$\alpha$-ketoglutarate complex.

We have previously reported the fact that the formation of this complex involves some nonreductive interaction, either direct or indirect, between the reduced coenzyme and $\alpha$-ketoglutarate at the active site of the enzyme and that this interaction involves a very large negative change in enthalpy. The findings presented in this work prove that this interaction requires the presence of a properly located carbonyl group on the substrate moiety of the ternary complex, but does not involve bonding changes typical of ketone groups; simple hydrogen bonding to, or steric consequences of, such a carbonyl group are sufficient to cause the phenomenon. Finally, it should be pointed out that while a $\Delta H_f$ of the magnitude observed here may be induced by the simple formation of a hydrogen bond, a phenomenon of this magnitude must involve some very major structural change in the complex itself. It is not unreasonable to believe, therefore, that the interaction observed in the active enzyme-NADPH-$\alpha$-ketoglutarate complex and identified in the closely analogous enzyme-NADPH-oxalylglycine complex may reflect the postulated but unidentified nonreductive role of the reduced coenzyme in the mechanism of the catalyzed reaction. Further studies of the phenomenon may clarify the nature of that role.

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