THERMODYNAMIC INTERACTION PARAMETERS OF A REACTIVE ENZYME TERNARY COMPLEX: GLUTAMATE DEHYDROGENASE–NADPH–α-KETOGLUTARATE

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1. Introduction

The recent advent of microcalorimetric measurements of the thermodynamics of enzyme–ligand complex formation has demonstrated quite clearly that in many cases enthalpies of formation of such complexes are larger, more varied, and, therefore, more indicative of differences in bond formation than are free energy measurements alone. Such effects are, of course, a manifestation of the phenomena of entropy–enthalpy compensation [1]. Thermodynamic interaction parameters of the glutamate dehydrogenase–NADPH-L-glutamate dead end inhibitor complex (a positively interacting system) [2] and the glutamate dehydrogenase–NADPH–ADP complex (a negatively interacting system) and a number of analogs of the two have been studied [3]. In both systems we observed a very large negative enthalpy of interaction, more or less offset by an opposing entropic term, resulting in free energies which were therefore quite small by comparison. In both systems large negative differential heat capacities were observed. It was of interest therefore to examine the interaction parameters of a reactive enzyme–coenzyme–substrate ternary complex which is an obligatory intermediate directly on the reaction path. This report is a study of the interaction parameters of the enzyme–NADPH–α-ketoglutarate complex, an entity whose properties are suspected to be intimately involved with the catalytic step of the enzyme-catalyzed reaction.

Thermodynamic parameters of the formation of this complex have been measured calorimetrically. ΔH°, ΔG°, ΔS° and ΔC_p are reported, along with the corresponding interaction parameters. The latter comprise an extreme example of enthalpy–entropy compensation. Possible causes of the value of ΔH_I° of −15.6 kcal are examined, including the possibility of covalent bond formation.

2. Materials and methods

Calorimetric measurements were made on a flow calorimeter as in [2,4]. For each step of the reaction, the enzyme, saturated with one of the two ligands, was thermally titrated with the other, and both ΔH° and ΔG° were determined from such curves as in [2]. In some cases (as noted in the text) where ΔG° could not be determined accurately from the thermal titration, spectroscopically determined values were used. ΔC°_p values were calculated from the difference between thermal titrations done at 25 and 15°C.

The enzyme–NADPH–α-ketoglutarate complex reacts rapidly with ammonium to form L-glutamate and NADP, which is, of course, the normal course of the reaction. To prepare a solution of this highly reactive complex which is sufficiently stable for quantitative measurements, it is necessary to eliminate even trace amounts of ammonia, a very difficult problem [5]. Enzyme was dialyzed overnight immediately before use to remove ammonium ions. The procedure was found to be essential since otherwise, heat generated by the enzymatic reaction itself would alter and thus obscure results. [The hydrolysis of amide groups from small amounts of denatured enzyme, for example, may be a principal contributor to these very low ammonia concentrations.] Ammonia levels of both enzyme and buffer solutions were continually checked using the Orion model 95-10 specific ion electrode as in [6]. Enzyme concentration, expressed as molarity of active sites assuming an equivalent

binding weight of 56 100, was 90 μM in all experiments. The buffer used was 0.1 M phosphate (pH 7.6). Where NADPH was the saturating ligand, its concentration was 600 μM. Where α-ketoglutarate at 30 mM was the saturating ligand.

3. Results

We may represent the formation of the ternary complex in the following conventional manner:

$$\begin{array}{ccc} & K & \quad\quad K \\ & + \quad 1 & + \\ NADPH + & E \rightleftharpoons & E\text{–}NADPH \\ & 3 \updownarrow & \updownarrow \quad 4 \\ NADPH + & E\text{–}K \rightleftharpoons & E\text{–}NADPH\text{–}K \\ & \quad 2 & \end{array} \qquad (1)$$

where E represents glutamate dehydrogenase, and K represents α-ketoglutarate.

The relationship between the thermodynamic parameters of the 4 enzyme forms (E,E–NADPH, E–K and E–NADPH) are shown in fig.1. Since all measurements are taken on systems at equilibrium, measurements of any given parameter for any 3 of the 4 equilibria involved suffice to determine the fourth. Values of ΔG°, ΔH°, ΔS° and ΔC°_p for reaction 1 have been published [2]. Values of ΔG° and ΔH° for reaction (4) and ΔH° for reaction (2) at 25 and 15°C are from thermal titrations reported here. Direct thermal titration of reaction (3) could not be done accurately due to the small magnitude of the enthalpy and to signals from the binding of α-ketoglutarate to a second site on the enzyme at the high concentrations necessary to saturate the weak E–K complex. Therefore, ΔH° for reaction (3) is calculated by difference from the 3 directly obtainable ΔH° values and ΔG° for that reaction is taken from spectrophotometric titration [5]. Finally, ΔG° for reaction (2) is calculated from the 3 known ΔG° values. [Cross [5] reported a K_d of 0.5 μM (ΔG° −8.4 kcal) for reaction (2) based on spectrophotometric titration. If this value were correct then K_d for reaction (4) would be 80 μM. The calorimetric titration of reaction (4), however, actually gave K_d 20 μM, requiring K_d 0.1 μM for reaction (2). Since Cross' spectrophotometric titration was carried out at 35 μM enzyme, that experiment could not distinguish between K_d 0.1 μM and K_d of 0.001 μM.

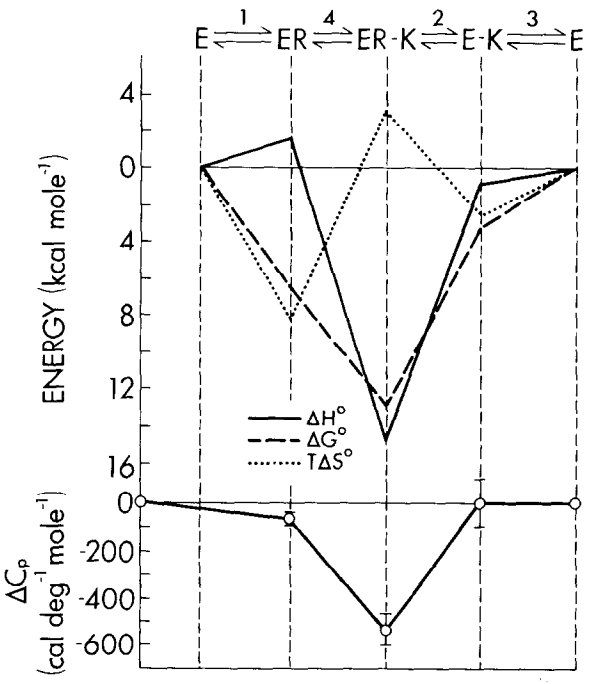

Fig.1. Thermodynamic profiles for the formation of the E–NADPH–α-ketoglutarate. R = NADPH. It should be noted that ΔG° and ΔH° are plotted in the positive sense so that downward changes in parameters reflect increased binding tendencies. Data in the upper panel were obtained at 25°C; the ΔC_p data in the lower panel were obtained from measurements at 15 and 25°C.

Therefore we use the larger (and therefore, more easily measured) K_d of reaction (4) (along with K_d values from reactions (1) and (3)) to calculate a maximum value for the very tight (and therefore inaccessible) K_d of reaction (2)]. The ΔC_p for the formation of the E–NADPH–K complex at 20°C is −500 ± 60 cal . deg.$^{-1}$M^{-1}.

4. Discussion

The course of the reaction in the direction of reductive amination of K may be written schematically in terms of the major known enzyme complexes as:

$$R + K + E \rightleftharpoons ERK \overset{N}{\rightleftharpoons} ERNK \overset{H_2O}{\rightleftharpoons}$$

$$EOG \rightleftharpoons O + G + E \qquad (2)$$

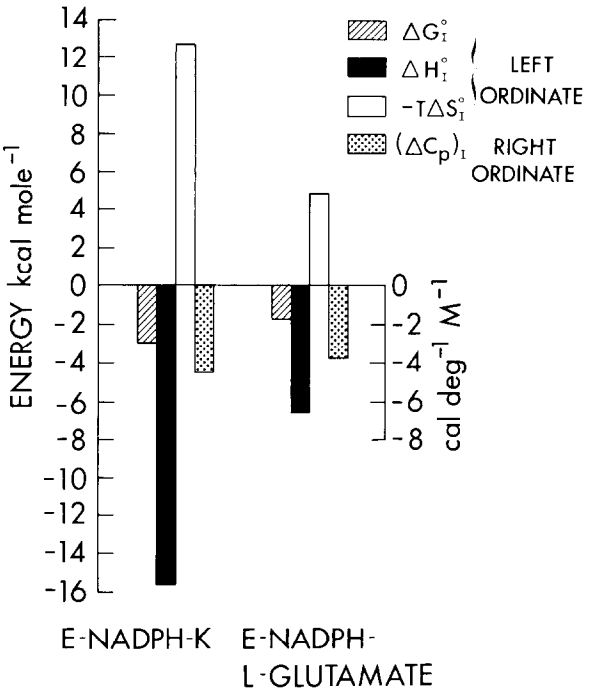

Fig.2. Thermodynamic interaction parameters for the E–NADPH–α-ketoglutarate complex. Parameters are plotted as described in fig.1. See text under 'Interaction parameters'.

where R = NAPH, O = NADP, G = L-glutamate, and N = ammonia [7]. This reaction has ΔH° −15.4 kcal [8]. It may be noted from fig.2 that the ΔH° for the formation of the E–R–K complex, which involves neither the transfer of the hydride ion nor the formation of the α-C–N bond of glutamate, corresponds to 96% of the total enthalpy drop of the overall reaction. This observation suggests that the formation of the E–R–K complex is by no means a simple passive binding of two potential reactants at an active site; but rather, that a considerable amount of rearrangement of chemical bonding has occured.

The rather complex energetic pattern seen in fig.2 is best analyzed in more detail in terms of the interaction parameters introduced in [2,3]. Referring to the 'thermodynamic square' [eq. (1)] we have defined the free energy parameter:

$$\Delta G_I^\circ = \Delta G_2^\circ - \Delta G_1^\circ = \Delta G_4^\circ - \Delta G_3^\circ \qquad (3)$$

An algebraically equivalent definition expresses the 'free energy of interaction' as the difference between the free energy of formation of the ternary complex and the sum of those of the two binary complexes. We can then define the other corresponding thermodynamic interaction functions, ΔH_I°, ΔS_I° and $(\Delta C_p)_I$ by analogy.

The interaction parameters for the formation of the E–NADPH complex calculated from the data of fig.1 are shown in the bar graph of fig.2, along with those of the E–NADPH–L-glutamate complex [2] for comparison.

The observed pattern of the E–NADPH–K complex resembles qualitatively those of the interaction parameters of the other ternary complexes of glutamate dehydrogenase which we have measured. It consists of a large favorable enthalpy almost completely compensated by an unfavorable entropic term and a very substantial negative $(\Delta C_p)_I$. The small favorable free energy of interaction appears as an almost trivial difference in the balance between the two dominant terms. [A detailed analysis of the relationships between the interaction parmeters of a number of ternary complexes of this enzyme will be published elsewhere.] The magnitude of the ΔH_I°, however, is unusually large compared to that of other ternary complexes of this enzyme; or, indeed, of most pyridine–nucleotide–dehydrogenase complexes [1]. It has been demonstrated by transient state kinetics [9] that two isomeric states of E–NADPH–K exist. We have suggested the possibility that α-ketoglutarate in such a complex forms a carbinolamine with a lysine residue on the enzyme which, in turn, loses water to form an enzyme-bound imine [7]. We reported transient state calorimetric measurements of the reaction between hydroxylamine and pyruvic acid, which involves precisely these same two steps, and found the ΔH° of the carbinolamine formation step to be −10 kcal, while that of the subsequent step added an additional −5 kcal [10]. It is at least conceivable, then, that the −15.6 kcal ΔH_I° may include some mixture of such steps involving covalent bond formation.

Another possibility which must be considered, however, is that of proton transfer. Since the reaction is measured in phosphate buffer, which has a very small heat of ionization, there can be no substantial enthalpic contribution from the buffer itself. However, transfer of H^+ to a group on the complex could make such a contribution. Further studies on the pH dependence of the ΔH° of this reaction will be required to establish this distinction.

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