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pH-DEPENDENT THERMODYNAMIC PARAMETERS OF THE GLUTAMATE DEHYDROGENASE-α-KETOGLUTARATE-NADPH COMPLEX

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Summary

The enthalpy of formation of the reactive bovine glutamate dehydrogenase-α-ketoglutarate-NADPH complex has been measured as a function of pH, at two temperatures and in two buffer systems having different enthalpies of ionization. The results demonstrate the existence of an extensive two-way traffic of protons between the buffer and the complex itself. While the pattern in a single buffer is too complex to resolve within the experimental limitations imposed by the system, we develop a theory which shows that the difference in $\Delta H^{\circ}_{H^+}$ between buffers of different enthalpies of ionization has a simpler, more easily resolvable form. From such a relationship we show that in the process of binding of a molecule of NADPH to the enzyme-α-ketoglutarate complex, a proton has been shifted from the buffer to the complex and that this is due to the shift of the pK of an ionized group on the enzyme to a much higher value when the complex is formed. The ΔC_p of formation of the complex is −450 cal · deg.$^{-1}$ · M^{-1}, and is pH independent.

Implications of these results for the interpretation of enthalpies of enzyme-complex formation obtained at a single pH, and at one temperature, with one buffer system are mentioned.

The complexity of the pattern of the dependence of ΔH°, taken together with the magnitudes of the individual features of these dependencies, suggest that a number of large pK shifts occur when the enzyme-NADPH-α-keto-glutarate complex is formed. It is quite probable that these shifts represent a sizeable fraction of the machinery of the catalytic mechanism.

Introduction

Over the past few years a number of calorimetric measurements of the thermodynamic parameters of a variety of pyridine-nucleotide dehydrogenase complexes have been reported [1]. These direct measurements of ΔH° values and particularly of ΔC_p values have begun to reveal interesting new features involving interactions which are not readily discernible from the conventional analysis of the temperature dependence of equilibrium constants. Most of these studies have necessarily involved the formation of either simple binary or inactive ternary complexes. We report here a study of a reactive complex of L-glutamate dehydrogenase (E) and two of its three required substrates.

The enzyme catalyzes the reaction:

$$\underset{(G)}{\text{L-glutamate}} + \underset{(O)}{\text{NADP}} + \text{H}_2\text{O} \overset{\text{E}}{\rightleftharpoons} \underset{(K)}{\alpha\text{-ketoglutarate}} + \underset{(R)}{\text{NADPH}} + \underset{(N)}{\text{NH}_3} + \text{H}^+ \qquad (1)$$

We have previously shown that the formation of the E-R binary complex of glutamate dehydrogenase is endothermic and has only a small negative heat capacity (in contrast to the binary complexes of most pyridine nucleotide linked dehydrogenases) [2]. The formation of the dead-end-inhibitor complex E-R-G, however, does have the large negative ΔH° of formation and large negative ΔC_p, characteristic of dehydrogenase E-R binary complexes.

Our initial study of the reaction, E + R + K $\rightleftharpoons$ E-R-K (pH 7.6, 25°C), showed a ΔH° of formation of −15.1 kcal (Fischer, H.F. and Stickel, D.C., unpublished results). The magnitude of this value suggested that covalent bond formation between K and E might be occurring. We have previously postulated carbinolamine formation followed by imine formation for this reaction on other grounds [3], and indeed we have shown in nonenzymatic model reactions that carbinolamine formation from a keto acid and an amine has a ΔH° of −10 kcal and that its dehydradation to an imine contributes another −5 kcal [4]. Before jumping to the conclusion that we are really dealing with covalent bond changes, however, it is necessary to rule out possible enthalpic contributions from proton transfer to nitrogen containing basic residues on the protein, since such neutralization processes could contribute as much as 9—10 kcal to the observed ΔH° values. For this reason we undertook the following study, measuring complete calorimetric titrations of saturated enzyme-α-ketoglutarate complexes with NADPH, as a function of pH, in two buffer systems of differing ΔH° of ionization, at two different temperatures. While the results described below do not answer the question originally posed, they do shed some new light on the complexity of the complex formation reaction itself, and suggest some cautions to be observed in the interpretation of more limited observations of this kind.

Methods and Materials

Beef liver glutamate dehydrogenase was a product of Boehringer Mannheim. The enzyme was prepared for use as previously described [5] and displayed a 280/260 nm absorbance ratio of 1.90 or greater. Enzyme was dialyzed over-

night immediately before use to remove NH_4^+. The procedure was found to be essential since otherwise, heat generated by the enzymatic reaction itself would alter and thus obscure results. NH_4^+ levels of both enzyme and buffer solutions were continually checked using the Orion model 95-10 specific ion electrode. Enzyme ammonia was determined as described by Proeless and Wright [6], in which protein is separated from the test sample with perchloric acid. NH_4^+ levels in other solutions were determined directly.

NADPH, Tris and α-ketoglutaric acid were purchased from Sigma Chemical Company. α-Ketoglutaric acid was used without further purification and its solutions were adjusted to the experimental pH with KOH. KOH, KCl and potassium phosphate buffer salts were analytical grade products from Mallinckrodt. Binding studies were carried out at various pH values, in either 0.1 M potassium phosphate or 0.1 Tris-acetate, both of which contained 0.1 M KCl to maintain a more nearly constant ionic strength.

Enthalpies were measured on an LKB 10700-1 flow calorimeter equipped as previously described [5,2]. Flow rates were determined by measuring the volume of water pumped, over a period of time. Flow rates varied from about 8 $\mu l \cdot s^{-1}$ to 25.64 $\mu l \cdot s^{-1}$, corresponding to residence times of 46.9—14.6 s [4]. Flow rates were varied intentionally to make sure that all reactions had gone to completion. The reaction pH was determined by measuring the pH of the calorimeter effluent.

The experiments were carried out in one of two ways. In one case data were collected at the dialysis pH. In other experiments data were accumulated by mixing an enzyme solution at a given pH with a solution of the ligand dissolved in buffer at an alternate pH. The experiments were so designed that the pH range of points taken at the dialysis pH overlapped that of points determined by pH jump. Neither the manner in which data was collected nor the flow rate affected the ΔH° of the signals observed.

The calorimeter baseline was always obtained by mixing a solution containing enzyme and α-ketoglutarate with a buffer/α-ketoglutarate solution. This arrangement negated the heat due to change in pH and α-ketoglutarate dilution. Measurements of ternary complex formation were obtained by reacting with a solution containing α-ketoglutarate and saturating amounts of NADPH. Enzyme concentrations were maintained at about 90 μM; α-ketoglutarate at 30 mM. Complete concentration curves (varying [NADPH]) were carried out at varous pH values covering the entire pH range to ensure that we were in all cases measuring a true q_{max} from which a valid ΔH° could be calculated; and that no extraneous binding of NADPH at the higher concentrations was contributing to the measured signals.

Enzymatic reaction rates of diluted aliquots of the solutions emerging from the calorimeter were determined to ensure that the activity of the enzyme had not undergone pH-dependent degradation, particularly near the borderline pH regions. Conditions for determining such rates were 5 mM α-ketoglutarate, 50 mM NH_4Cl, and 100 μM NADH, with enzyme concentration adjusted to provide spectrophotometric changes between 0.01 A and 0.04 A per min on a Gilford 2000 Recording Spectrophotometer equipped with a Gilford Rapid Sampler.

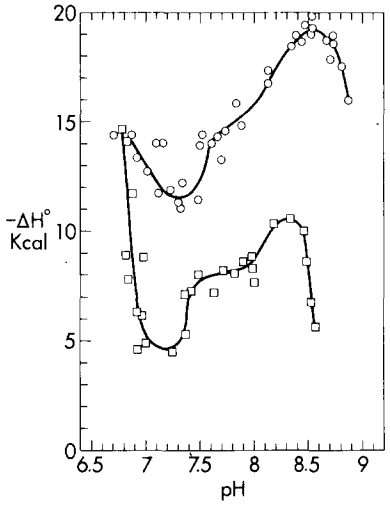


Fig. 1. The pH dependence of the enthalpy of reaction: E-K + R ⇌ E-R-K. ○———○, phosphate buffer; □———□, Tris-acetate buffer. See text for experimental details.

Results

ΔH° of the reaction EK + R ⇌ E-R-K, plotted as a function of pH in both potassium phosphate and Tris-acetate buffers at 25°C, is shown in Fig. 1.

In the presence of NH_3, the E-R-K complex reacts to form L-glutamate and NADP, the reverse of the reaction shown in Eqn. 1. To the extent that such a reaction occurs within the sensing chamber of the calorimeter, heat may be evolved, and in any case, some decrease in the amount of the E-R-K complex itself will ensue [7]. Despite all precautions taken to eliminate traces of NH_3, (and despite NH_3 electrode measurements of all solutions to identify results from contaminated experiments) such traces frequently exist * and they are the largest contributor to the scattering of points observed in the Fig. 1.

Discussion

It is obvious from the complexity of the pH curves, and from the magnitude of the effects observed, that in the formation of the E-R-K complex there is a considerable traffic of protons being both released and absorbed by the functional groups on the enzyme, its ligands, the complex and buffer. The accessible pH range is too small and the scatter in the data is too large to permit a valid resolution into component proton reactions. Yet, the experimental variations are sufficient to permit us to draw some interesting conclusions even without a dissection.

* The hydrolysis of amide groups from small amounts of denatured enzyme, for example, may be a principal contributor to these very low NH_3 concentrations.

We may represent the formation of the complex by the following scheme.

$$\begin{array}{ccc} R & & R \\ + & (\Delta H_1^\circ - \Delta H_B^\circ) & + \\ BH + E & \rightleftharpoons & EH + B \\ & K & \\ \Delta H_4^\circ \Big\downarrow \Big\uparrow & & \Big\downarrow \Big\uparrow \Delta H_2^\circ \\ & (\Delta H_3^\circ - \Delta H_B) & \\ BH + ER & \rightleftharpoons & EHR + B \\ & K' & \end{array} \qquad (2)$$

where E and EH represent unprotonated and protonated forms of the enzyme-α-ketoglutarate complex respectively, and ER and EHR indicate the presence of R in those forms. BH and B designate the protonated and unprotonated species for a given buffer. It will be noted that the ΔH° values on the two horizontal legs of the square indicate the heat of ionization of the buffer explicity (since that is a determinable quantity). ΔH_2° and ΔH_4° designate the enthalpy of the combination of R with the protonated and unprotonated E-K complex forms, respectively.

In an experiment at any given pH, because we have extrapolated our data to $[R] \to \infty$, the system is driven completely from one specific distribution of protonation states of free enzyme (the forms on the upper line) to a second (and generally different) specific distribution of protonation states of the E-R complex (the forms on the lower line).

The measured ΔH°, then, in any given buffer, as a function of pH is given by:

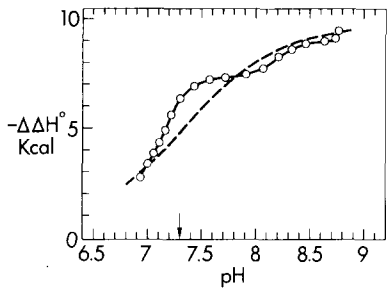
$$\Delta H_{B,H^+}^\circ = \frac{\Delta H_3^\circ - \Delta H_B^\circ}{K'/[H^+] + 1} - \frac{\Delta H_1^\circ - \Delta H_B^\circ}{K/[H^+] + 1} + \Delta H_4^\circ \qquad (3)$$

The difference between the molar enthalpies of formation of the same complex measured in two buffers of differing heats of ionization is given by:

$$\Delta H_{H^+}^\circ = (\Delta H_{B_2}^\circ - \Delta H_{B_1}^\circ) \frac{(K - K')[H^+]}{[H^+]^2 + (K + K')[H^+] + KK'} \qquad (4)$$

It will be noted that the equation for the difference in ΔH° for two buffers is a much simpler expression than that for a single buffer: all ΔH° values of complex formation and of enzyme ionization have been removed, and only the enzyme ionization constants and the difference in buffer heats of ionization (a directly measurable constant) remain.

In the derivation of Eqn. 4 it is necessary to assume that the reaction in two different buffers is identical in all respects except for the difference in heat of ionization. This condition is, in fact, seldom met; shifts in pK values of a few tenths of a pH unit in two chemically dissimilar buffers is quite common. Indeed, it is apparent from Fig. 1, that there is a striking correlation of features of the two curves all along the pH range, but in each case those of the Tris-acetate curve occur at a pH of 0.3 lower than those of the phosphate curve. Fig. 2 represents the difference between the two buffer curves of Fig. 1 after shifting the Tris-acetate curve to make the features coincide. Such a difference



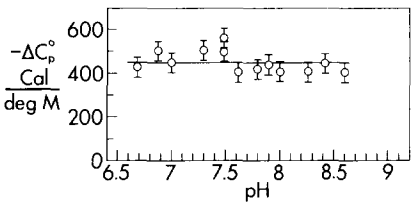


Fig. 2. The difference in enthalpy of formation of the E-R-K complex (from E-K) in phosphate and Tris-acetate buffers. o———o, calculated from Fig. 1 assuming a shift in pH of the entire Tris-acetate curve, as described in the text. ------, a theoretical component calculated from Eqn. 4, assuming ($\Delta H^{\circ}_{B_2} - \Delta H^{\circ}_{B_1}$) = 9.3, and with a p$K$ of 7.3 (indicated by arrow on abscissa).

Fig. 3. The pH dependence of ΔC°_p of formation of the E-R-K complex calculated from enthalpies measured at 25 and at 15°C.

curve is appropriate for analysis according to Eqn. 4, using the measured value, $\Delta H^{\circ}_{B_2} - \Delta H^{\circ}_{B_1} = 9.3$ kcal. Whilst the resulting difference curve is still quite complex, a reasonable major component may now be resolved based on the more stringent requirements of Eqn. 4: that such a component must constitute a bell shaped curve; each segment of which is a sigmoid curve whose ordinate starts at 0 kcal and reaches a maximum (in this case) of 9.3 kcal (assuming $K > K'$). The dashed line in Fig. 2 shows that such a process with a pK of about 7.0—7.4 does indeed account for the major portion of the buffer difference heats from pH 6.7 to 8.4 *.

The existence and sign of this component permits us to conclude that, in the process of binding of NADPH to an E-K complex, a proton has been transferred from the buffer to the E-R-K complex (i.e. $K >> K'$). This implies that the pK of a basic group on the enzyme or coenzyme is shifted at least 2 pH units to a higher pK as the E-R-K complex is formed.

The difference between the ΔH° of formation of E-R-K from E-K + R in phosphate buffer at 25°C, and that of the same reaction at 15°C is shown in Fig. 3. (The ordinate has been divided by 10°C so that the data are presented directly as ΔC°_p in cal · deg^{-1} · M^{-1}.) Since the ΔC_p is independent of pH within the experimental error (±50 cal · deg^{-1} · M^{-1}) over the entire measured pH range, it is clear that the large ΔC_p of —450 cal · deg^{-1} · M^{-1} is not due to proton ionization.

We may now consider the general conclusions to be drawn from the study as a whole. Because of the magnitude and complexity of the pH dependent enthalpic changes shown in Fig. 1, the data require that no less than three (and more probably four) functional groups undergo major changes in their states of protonation upon formation of the enzyme-NADPH-α-ketoglutarate complex. From Fig. 3 we can conclude that at least two (and possibly three) of these groups are involved in the transfer of protons to or from the buffer.

* It is also apparent that a second major process occurs below pH 7 involving the transfer of a proton from the complex to the buffer. The small sinusoidal deviation from the theoretical component observed in Fig. 3 can be attributed to the effect of the pH shift operation employed operating on a small bell-shaped feature which does not actually undergo the presumed pK shift in Tris-acetate buffer.

Any reasonable detailed mechanism for the reversible enzymatic catalysis of the oxidative deamination of L-glutamate by NADP must involve changes in the state of protonation of a number of functional groups as the course of the reaction proceeds. Such changes, in order to form the effective parts of a functional mechanism, must involve the shift of pK values more or less totally into or out of the relatively narrow pH range over which a given enzymatic reaction proceeds at a measurable rate.

The effects seen in Fig. 2 do indeed cover the pH range over which catalytic activity is significant; moreover, they are clearly of such magnitude as to require major pK shifts for their explanation. The data suggest that, in the enzyme-catalyzed reductive amination of α-ketoglutarate by NADPH and NH_3 to form L-glutamate and NADP, much of the bond rearrangement necessary for that reaction occurs in the formation of the enzyme-NADPH-α-ketoglutarate complex, and that these changes in bonding precede the formation of the αC-N bond and the subsequent hydride transfer step. This distribution of the number of observable functional pK values occurring before the introduction of NH_3 and hydride transfer may serve as a useful criterion to be met by mechanisms proposed to account for the reaction.

Finally we may note that most reports of the values of enthalpies of enzyme complex formation have been based on measurements carried out at a single temperature, at a single pH, and in a single buffer system. The results of the studies reported here strongly suggest that considerable caution should be exercised in the interpretation of thermodynamic parameters of enzyme complexes based on such limited measurements.

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