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NADPH Binding Induced Proton Ionization as a Cause of Nonlinear Heat Capacity Changes in Glutamate Dehydrogenase†

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ABSTRACT: Functional group interactions involved in the formation of the glutamate dehydrogenase–NADPH binary complex have been studied by three independent but complementary approaches: (1) the pH dependence of the overall dissociation constant measured by an improved differential spectroscopic technique; (2) the pH dependence of the enthalpy of complex formation measured by flow calorimetry; and (3) the pH dependence of the number of protons released to, or taken up from, the solvent in the complex formation reaction, measured by titration. We conclude that the coenzyme binds to the enzyme through three distinguishable interactions: a pH-independent process involving the binding of the reduced nicotinamide ring; a relatively weak "proton-stabilizing" process, occurring at low pH involving the shift at a pK of 6.3 in the free enzyme to 7.0 in the enzyme–NADPH complex; and a stronger "proton-destabilizing" process, occurring at a higher pH involving a shift of a pK of 8.5 in the enzyme down to 6.9 in the enzyme–NADPH complex. The proton ionization of the free enzyme involved in this third interaction exhibits some unusual thermodynamic parameters, having $\Delta G^\circ = +11.5 \pm 0.1$ kcal mol^{-1}, $\Delta H^\circ = +19 \pm 1$ kcal mol^{-1}, and $\Delta S^\circ = +23$ eu. We show here that this proton ionization step is directly related to and indeed constitutes the "implicit" shift in enzyme macrostates which we have shown to be responsible for the existence of large highly nonlinear ΔC_p° effects in the formation of this complex [Fisher, H. F., Colen, A. H., & Medary, R. T. (1981) *Nature (London) 292*, 271–272].

Most of the detailed chemical mechanisms currently written to explain the enzymatic catalysis of specific reactions feature the transfer of protons between the solvent and functional groups located on the various components of enzyme–substrate–(coenzyme complexes). Experimentally, this proton "traffic" expresses itself in the form of observed ligand-induced pK shifts of enzyme functional groups. Recent papers on the mechanisms of reactions catalyzed by L-glutamate dehydrogenase (Srinivasan & Fisher, 1984; Rife & Cleland, 1980) fit this description. In a previous report on calorimetric measurements of the enthalpies of formation of a ternary complex of L-glutamate dehydrogenase, we observed a complex pattern of just such proton transfer (Fisher et al., 1980). In order to determine the precise nature of these events in the active ternary complexes, and to explore their possible involvement in the catalytic process, it is first necessary to obtain a thorough understanding of protonic events in the enzyme–NADPH binary complex, whose formation precedes those of the higher order complexes. To this end, we have undertaken such a study using three complementary but independent experimental approaches: differential spectroscopy, calorimetry, and displaced-proton titration.

In an earlier study (Fisher et al., 1981), we had looked at the thermodynamics of the formation of this complex from a rather different point of view. The focus of that study was the unusual nonlinear temperature dependence of the enthalpy of the process. We concluded that the basis of the nonlinear ΔC_p° was the inclusion of a hidden step in the reaction course

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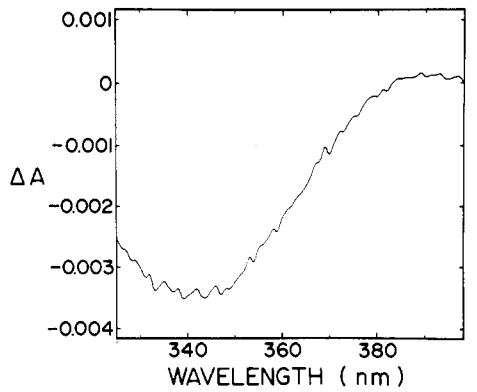


FIGURE 1: Typical difference spectrum of the glutamate dehydrogenase–NADPH complex; [(enzyme, NADPH)] – [(enzyme)(NADPH)]. The enzyme concentration was 5.38 μM; the NADPH concentration was 23.4 μM, pH 7.50.

and that this hidden step must involve a poised equilibrium between two macrostates of the free enzyme. The elucidation of the relationship between the nature of that poised equilibrium, of the identity of the two macrostates themselves, and of the proton ionization effects which are shown in this current work forms an additional point of consideration.

Materials and Methods

β-NADPH (Boehringer Mannheim), potassium phosphate, and potassium acetate (Mallinckrodt) were used without further purification. An ammonium sulfate suspension of bovine liver L-glutamate dehydrogenase (Boehringer Mannheim) was prepared as previously described (Colen et al., 1974). The prepared enzyme is dialyzed before each experiment in the buffer of that experiment. Enzyme concentrations were calculated by using $\epsilon_{280} = 0.97$ mg^{-1} mL cm^{-1} and $M_w = 56\,100$; NADPH concentrations were calculated by using $\epsilon_{340} = 6.22$ mM^{-1} cm^{-1}.

Dissociation constants of the E–NADPH complex were determined at 25.0 ± 0.05 °C by difference spectroscopy in a Hewlett-Packard 3450A spectrophotometer adapted with temperature control and continual stirring. This diode-array instrument measures a complete spectrum at 1-nm resolution each second. Each spectrum collected is an average of 15 individual spectra obtained over a period of 15 s. The enzyme spectrum was obtained before and after each determination. A series of NADPH dilutions (at least 10) were prepared, in the experimental buffer, and then pipetted, using the same 2-mL pipet, into matched sample and reference cells. The base-line spectrum was then collected. Enzyme was added to the sample solution, and an equivalent amount of buffer was added to the reference solution with Lang–Levy micropipets. The spectrum was collected after the contents of both cells were mixed. The base-line spectrum and an average enzyme spectrum were subtracted from this resulting spectrum. A typical example of such a difference spectrum is shown in Figure 1. We have found that at any given pH and buffer composition the *shape* of the difference spectra obtained in the manner described above is independent of the concentrations of NADPH and enzyme over the wavelength range 325–398 nm. The work reported here required an unusually high degree of accuracy in the determination of tight binding constants measured at relatively low enzyme concentrations. Therefore, we have departed from the customary practice of using a peak – trough absorbance difference as a signal proportional to the concentration of the enzyme–NADPH complex. We have instead used the entire difference spectrum from 325 to 398 nm (comprising some 74 individual absorbance values) for this purpose. Since such spectra show small (but quantitatively significant) differences in the various solvent conditions used, we found it necessary to develop separate model spectra for each individual set of experimental conditions. The method of determining the K_D at a given pH and buffer composition is as follows. From the set of difference spectra obtained at various concentrations of NADPH, a single spectrum representing a moderate degree of enzyme saturation is chosen as a "model" spectrum. This choice is not at all critical. The rest of the set of spectra are then each analyzed by a multicomponent analysis algorithm built into the HP-8450 to provide the concentration of enzyme–NADPH complex relative to that of the model spectrum (along with a small wavelength-independent constant used to correct for minor cell differences). Typical results from such a set of calculations, plotted vs. total NADPH concentration, are shown in Figure 2. The E–NADPH concentrations are at this point still in arbitrary units. The solid curve in Figure 2 shows the best fit to the equation:

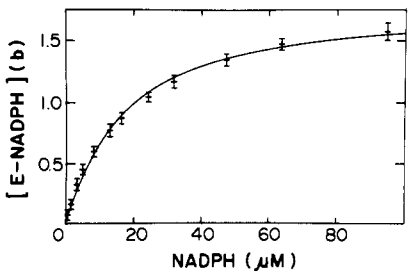


FIGURE 2: Typical binding constant determination. Enzyme concentration was 5.38 μM, pH 6.82; C is the nominal concentration of the binary complex relative to the concentration of an arbitrarily chosen model, as described in the text. The solid line is the best fit to eq 1 (see text). In this particular case, $K_D = 23 \pm 1.5$ μM. The error is estimated from a χ^2 calculation.

$$C/b = [(R_T + E_T + K_D) - \sqrt{(R_T + E_T + K_D)^2 - 4R_TE_T}]/2 \quad (1)$$

where R_T is the total concentration of NADPH, E_T is the total concentration of enzyme, K_D is the dissociation constant of the E–NADPH complex, C is the relative concentration of E–NADPH as determined by the component resolution, and b is a constant determined by the fit, such that C/b = [E–NADPH]. In all cases E_T was kept below K_D. Figure 2 shows a typical fit of eq 1 to experimental measurements. The error represented by the vertical bars is the standard deviation of individual measured points.

Enthalpy measurements were carried out in a flow microcalorimeter as previously described (Subramanian et al., 1978) using enzyme dialyzed at the pH indicated.

Proton flux measurements were carried out by a back-titration procedure [similar to that described by Lewis et al. (1976)] using a Radiometer TTT80 titrator, an ABU 80 autoburet, a PHM 84 pH meter, and a GK 23226 combined electrode. The titration vessel was thermostated at 25 °C and was continuously purged with a flow of argon gas directed over the surface of the titration solution. In a typical experiment, 760 μmol of enzyme in 0.1 M Na_2SO_4 was placed in the titration vessel.[1] The pH was lowered to 5.6 by addition of

[1] Sulfate is used in the titration experiments to replace phosphate, whose buffer capacity would reduce the sensitivity of the method. While the presence of anions does indeed affect the binding of NADPH to this enzyme, we have found that the data shown in Figure 3 are indistinguishable from those obtained in 0.1 M sulfate. The effect of specific anions on the formation of this complex will be described in a forthcoming paper to be described elsewhere.

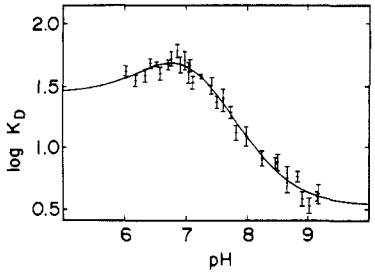


FIGURE 3: pH dependence of the K_D of the enzyme–NADPH complex. The solid line is the best fit to the logarithmic form of eq 3. K_D's are expressed in micromolar units.

Scheme I

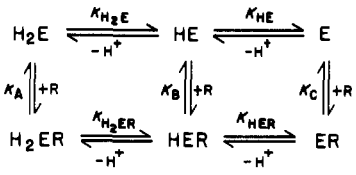


Table I: Equilibrium Constants of Scheme I As Evaluated by the pH Dependence on K_D, $\Delta H°$, and Δn_{H^+}

	K_D	$\Delta H°$	Δn_{H^+}
pK_{HE}	8.46 ± 0.05	8.54 ± 0.05	8.52 ± 0.05
pK_{HER}	6.99 ± 0.05		6.86 ± 0.05
pK_{H_2E}	6.3 ± 0.2		6.2 ± 0.2
pK_{H_2ER}	6.9 ± 0.2		6.9 ± 0.2
$1/K_A$ (μM)	27 ± 5		
$1/K_B$ (μM)	99 ± 13		
$1/K_C$ (μM)	3 ± 1		

0.01 M HCl to the rapidly stirred solution. As the CO_2 was carried out of the solution by continued gas purging, the pH was maintained by operation of the titrator in the pH-stat mode, until a stable state was reached (<0.02 pH change in 5 min without further acid addition). Typically, this condition was reached in 15–30 min. At this point, the pH was raised to the set point of the individual experiment, and 0.4 mL of a solution of 1.14 mM NADPH in 0.1 M Na_2SO_4 (previously degassed at pH 6.9 and then raised to the experimental set point) was added to the titration vessel. The solution was then back-titrated to the original set point with 0.1 M KOH or 0.1 M HCl. The number of moles of protons released to, or removed from, the solution was calculated on the basis of the molarity of enzyme binding sites in the solution. Corrections were carried out on the basis of control experiments in which either enzyme or NADPH was omitted from the appropriate solution. These corrections were found to be almost negligible.

RESULTS

Free Energy Measurements. The logarithmic dependence of the K_D of the enzyme–NADPH complex on pH is shown in Figure 3. The minimal scheme which can accommodate these data (Scheme I) requires the existence of three states of ionization where K_{H_2E}, K_{HE}, K_{H_2ER}, and K_{HER} are defined as proton dissociation constants; K_A, K_B, and K_C are defined as ligand binding constants; and R represents NADPH. The solid line in Figure 3 represents the best fit of the data to the logarithmic form of eq 2 where K_D is the observed dissociation

$$K_D = K_B\left(\frac{1 + K_{HE}/[H^+] + [H^+]/K_{H_2E}}{1 + K_{HER}/[H^+] + [H^+]/K_{H_2ER}}\right) \quad (2)$$

constant at any given pH and each of the five phenomenological parameters are defined as one of the equilibrium

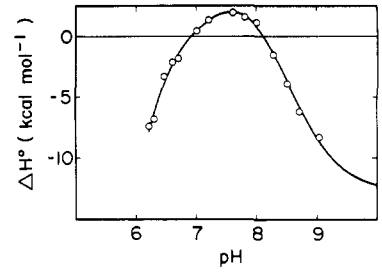


FIGURE 4: pH dependence of the $\Delta H°$ of formation of the enzyme–NADPH complex. The solid line is the best fit of the data to eq 4 using pK values from the results shown in Figure 3 (listed in Table I).

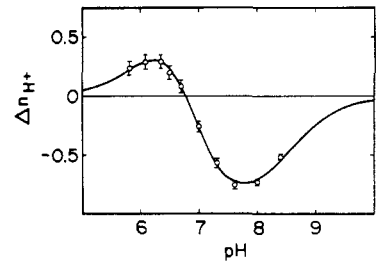


FIGURE 5: Number of protons per enzyme subunit removed from the buffer as a function of pH.

constants in Scheme I.[2] The values for the individual parameters are listed in Table I.

Enthalpy Measurements. The dependence of the observed standard enthalpy, $\Delta H°$, of formation of the enzyme–NADPH complex on pH is shown in Figure 4. Again, enzyme and the enzyme–NADPH complexes distribute themselves among the various ionic species available as in the K_D measurements, and that distribution must follow the same pH dependence as in the previous case. The mathematical equation describing that dependence for the $\Delta H°$ measurements, however, is quite different from eq 2 due to the nature of the experiment itself. The data plotted in Figure 4 are obtained from enthalpy measurements which have been extrapolated to an infinite NADPH concentration. Therefore, at any given pH, we are driving the system from one particular distribution of the forms H_2E, HE, and E to some generally different distribution of the forms, H_2ER, HER, and ER, as indicated by the upper and lower lines of Scheme I. Therefore, the data in Figure 4 are properly fitted to the equation:

$$\Delta H° = \frac{\Delta H°_{H_2E} + \Delta H°_{HE} + \Delta H°_{HE}K_{H_2E}/[H^+]}{1 + K_{H_2E}/[H^+] + K_{H_2E}K_{HE}/[H^+]^2} - \frac{\Delta H°_{H_2ER} + \Delta H°_{HER} + \Delta H°_{HER}K_{H_2ER}/[H^+]}{1 + K_{H_2ER}/[H^+] + K_{HER}K_{H_2ER}/[H^+]^2} + \Delta H°_C \quad (3)$$

[2] Equation 2 only distinguishes between reactant and product pK's, and the fit of the data to that equation does not in itself, therefore, permit the assignment of a given pK to any particular reactant species. In Scheme I, we have assumed that K_{H_2E} and K_{HE} both represent ionization constants of enzyme functional groups for the following reasons: pK_{HE} can be assigned to the free enzyme unambiguously because neither NADPH nor the phosphate buffer has any pK's near the observed value of 8.5. The assignment of K_{H_2E} is somewhat less certain, since the pK of the C-2′ phosphate group of NADPH (determined in this laboratory to be 5.96 ± 0.03 in 0.1 M sodium sulfate) is rather close to the pK determined experimentally for the ionization of the entity we have labeled H_2E, 6.33 ± 0.2. While the "best fit" of the data to eq 2 thus indicates that the pK of NADPH differs from the experimental value by an amount that appears to be slightly larger than the experimental error, we must regard the assignment of this pK to be quite uncertain at this time.

Using the values for K_{H_2E}, K_{HE}, K_{H_2ER}, and K_{HER} determined from the free energy measurements of Figure 3 in eq 3, we can fit the enthalpy data of Figure 4 quite satisfactorily, as shown by the solid line in that figure. However, because of the opposing pK shifts of H_2E and HE caused by NADPH binding, we are unable to provide a unique solution for all five $\Delta H°$ values. [Indeed, we can determine accurate values only for $\Delta H°_{HE}$ (+19 ± 1 kcal mol^{-1}) and $\Delta H°_{C}$ (−13 ± 1 kcal mol^{-1}).]

Proton Transfer Measurements. The number of protons transferred to or from the solvent per molecule of NADPH bound as a function of pH is shown in Figure 5. Again, as in the case of the enthalpy measurements, we are using saturating concentrations of NADPH, and therefore we are again driving the system from one particular distribution of ionic forms of enzyme to a different distribution of ionic forms of enzyme–coenzyme complexes. The number of protons, n, removed from the solution when NADPH binds to the enzyme at any given pH will be equal to the difference between the number of protons on all forms of the enzyme–NADPH complex and the number of protons on all forms of the free enzyme at that pH.

The equation expressing n as a function of pH for the system shown in Scheme I is

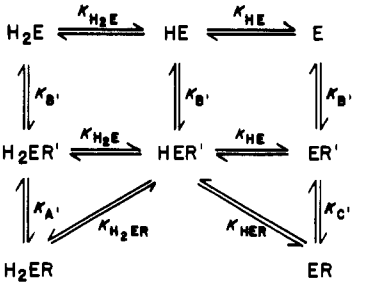
$$n = \frac{2}{1 + \frac{K_{H_2ER}}{[H^+]} + \frac{K_{HER}K_{H_2ER}}{[H^+]^2}} + \frac{1}{1 + \frac{[H^+]}{K_{H_2ER}} + \frac{K_{HER}}{[H^+]}} - \frac{2}{1 + \frac{K_{H_2E}}{[H^+]} + \frac{K_{HE}K_{H_2E}}{[H^+]^2}} - \frac{1}{1 + \frac{[H^+]}{K_{H_2E}} + \frac{K_{HE}}{[H^+]}} \quad (4)$$

The solid line in Figure 5 represents the best fit of eq 4 to the titration data. The parameters obtained from that fit are listed in Table I. The data themselves show that protons are released *to* the solvent at high pH and are abstracted *from* the solvent at lower pH, as would be predicted from Scheme I for the case where complex formation shifts the higher pK of the free enzyme *down* and shifts the lower pK of the free enzyme *up*. The very good fit of eq 4 to the data further assures us that each of the three ionic species of free enzyme and each of the three ionic species of enzyme–NADPH complex do indeed differ from the adjacent form by a single proton, as is assumed in Scheme I.

Discussion

Free Energy Considerations. Since the binding data shown in Figure 3 require a minium of three ionic species to account for the pH dependence of K_D, we can infer the existence of at least three distinguishable functional groups, each of which may be located on either reactant (enzyme or coenzyme) and each of which has at least one attainable ionic state which can engage in some enzyme–reduced coenzyme interaction. We label the interactions involving these groups A, B, and C, respectively, following the phenomenological parameters of Scheme I and eq 2. While the data presented thus far are perfectly consistent with Scheme I (three independent binding modes, each corresponding to one of three pH-dependent functional group interactions), other evidence suggests a mathematically identical but mechanistically different scheme. Resolution of binding difference spectra (such as that shown in Figure 1) into their spectral shift and hypochromicity components showed that the actual magnitude of the red shift of the reduced nicotinamide peak did vary considerably with pH, indicating pH-dependent variations in the degree of

Scheme II



surface interactions of the ring. However, the 340-nm peak was significantly red-shifted (2–4 nm) in the enzyme–NADPH complex over the entire pH range, indicating that the reduced nicotinamide ring is in at least some substantial contact with the enzyme surface at all pHs studied. Furthermore, in a previously published study of the transient-state kinetics of the formation of the glutamate dehydrogenase–NADPH–ADP ternary complex (Colen et al., 1974), we showed that under the same conditions as those in the present work, the formation of the E–NADPH complex proceeds in two steps: the initial formation of a relatively weak "encounter" complex followed by an isomerization leading to a 7-fold tighter complex. ADP interferes only with the isomerization step, producing a weaker complex in which the 340-nm band of the reduced nicotinamide ring is still red-shifted and in which, therefore, the reduced nicotinamide ring itself must remain bound. Since the K_D of NADPH from the E–NADPH–ADP complex of 109 μM is the same as that of interaction B in the present work, we conclude that interaction B, the binding of NADPH to the HE form of the enzyme, constitutes a pH-independent interaction, contributing equally to the two other pH-dependent interactions.

It is appropriate, therefore, to express the ER binding mechanism as shown in Scheme II. We may now consider the nature of the additional contributions to the overall binding provided by the two pH-dependent binding modes, A′ and C′, as shown by the magnitudes and directions of the respective pK shifts they exhibit.

Interaction A′ involves the shift of a reactant group having a pK of 6.3 to a value of 7 in the H_2ER complex. The *upward* direction of the pK shift indicates that the more protonated form of the complex, H_2ER, is more stable than the HER form. Therefore, this interaction will extract protons from the solvent in the region of its pK as required by eq 4, and as physically demonstrated by the titration data shown in Figure 5.

Since $K_{A'} = K_{H_2ER}/K_{H_2E}$

$$-\Delta G° = RT \ln K_{A'} = RT(\ln K_{H_2ER} - \ln K_{H_2E}) \quad (5)$$

and

$$-\Delta G° = -1358(pK_{H_2ER} - pK_{H_2E})$$

it follows that a pK shift of 0.7 will confer a $-\Delta G°$ increment of about 1 kcal mol^{-1} to the stability of the complex. Both the direction of the shift and the relationship between its magnitude and the resulting free energy increment are, of course, implicit in eq 2 itself; the titration results merely demonstrate these phenomena explicitly and provide an independent experimental confirmation of the validity of the treatment.

Interaction C′ involves a *downward* shift of pK from reactant to product, indicating that the *less* protonated form of the product complex, ER, is more stable than the HER form. The resulting displacement of protons into the solvent in the region

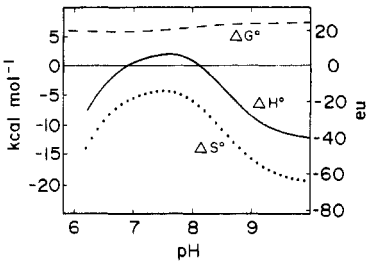


FIGURE 6: Dependence of the observed thermodynamic parameters of formation of the glutamate dehydrogenase–NADPH complex on pH. The solid line represents $\Delta H°$, the dotted line, $\Delta S°$, and the dashed line, $\Delta G°$. All values are calculated from the data shown in Figures 3 and 4.

of its pK is again demonstrated in Figure 5. The $\Delta G°$ of this proton-destabilizing interaction is −2 kcal mol^{-1}, corresponding to a pK shift of 1.46 pH units.

Thus, the formation of the E–NADPH complex is shown to involve one proton-destabilizing interaction, one proton-stabilizing interaction, and one proton-indifferent interaction.

Enthalpic Considerations. While the individual calorimetric measurements shown in Figure 4 are inherently much more precise than the equilibrium constant measurements of Figure 3, the effect of the opposing pK shifts makes it impossible to provide a unique fit of eq 3 to the enthalpy data. Indeed, we are able to determine the $\Delta H°$'s of only two distinct processes: one corresponding to the sum of the two sequential steps defined by $K_{B'}$ and $K_{C'}$ in Scheme II and a second process corresponding to K_{HE} in that scheme. For the sum of steps B′ and C′, we can do no more than list the overall thermodynamic parameters: $\Delta G°$ = −7.5 kcal mol^{-1}; $\Delta H°$ = −13 ± 1 kcal mol^{-1}; $\Delta S°$ = −18 ± 2 eu. The properties of the second process, however, corresponding to the reaction $HE^+ \rightleftharpoons E + H^+$, are much more interesting and will provide the basis for the remaining discussion.

While this phenomenon is quite easily explained on a simple and straightforward basis without introduction of any additional data or concepts, we will beg the reader's indulgence while we engage in what may appear to be a rather arcane detour. Our reason for doing so is that the observation cited above provides a very direct link to a group of heretofore puzzling thermodynamic phenomena and indeed leads to a simple chemical explanation of those phenomena for the particular case at hand.

In Figure 6, we show the pH dependence of the thermodynamic parameters of the overall process E + R ⇌ ER. Following the procedure of Eftink and Biltonen (1983), we then use these data to produce the $\Delta H°$ vs. $\Delta S°$ plot shown in Figure 7. It is important to note that the solid line in the figure has been drawn by taking points sequentially from low pH to high pH; this direction is indicated by the arrowheads on the curve. The peculiar hair-pin loop so obtained has a striking resemblance to those produced in corresponding plots based on the elegant theory of Eftink and Biltonen (1983) and to the experimental results they show for a number of enzyme–ligand binding reactions. According to that theory, such a feature is a clear indication of a hidden or implicit step in an overall observed reaction. We have reported previously (Fisher et al., 1981) not only that the observed $\Delta H°$ for the binding of NADPH to glutamate dehydrogenase is highly temperature dependent but also that this dependence is itself strikingly nonlinear; the observed $\Delta C_p°$ decreases from a value of about zero at 0 °C by at least 500 cal K^{-1} mol^{-1}. We could account for this behavior in a quantitative manner in the

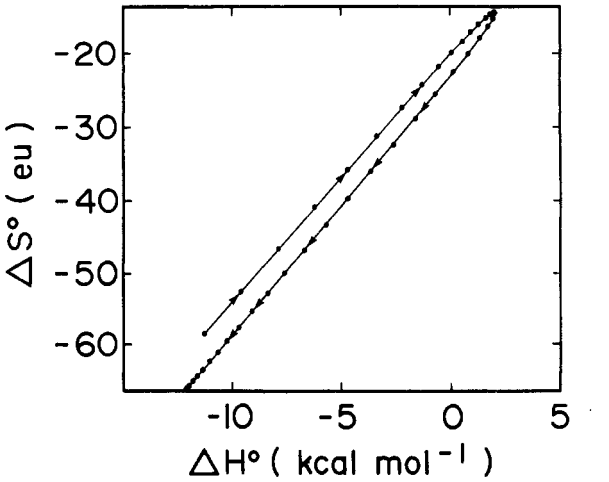


FIGURE 7: Dependence of the $\Delta H°$ vs. $\Delta S°$ relationship on pH. The arrows on the curve indicate the direction of increasing pH. The points are plotted at 0.1 increments of pH, starting at pH 6 and ending at pH 10. The extremum at the upper right-hand corner of the "loop" occurs at pH 7.55.

following way. If the reaction written and measured as

$$R + E \overset{K}{\rightleftharpoons} ER \tag{6}$$

really consists of the coupled reaction

$$\begin{array}{ccc} E & & \\ \uparrow\downarrow K_0 & & \\ R + E' & \overset{K_1}{\rightleftharpoons} & ER \end{array} \tag{7}$$

and if the E ⇌ E′ step has a very large $\Delta H°$ and is so poised that K_0 = 1 at some temperature within or at least near the experimental temperature range, then the $\Delta H°$ measured for reaction 6 will produce the strikingly nonlinear temperature dependence which we observe. For the reaction shown in eq 7, this observed $\Delta H°$ will be given by

$$\Delta H°_{obsd} = \Delta H°_1 + \Delta H°_0/(1 + K_0) \tag{8}$$

where $K_0 = \exp[\Delta H°_0(T - T_0)/(RTT_0)]$, T is the temperature, and T_0 is that temperature at which K_0 = 1 ($\Delta G°_0$ = 0). For the binding of NADPH to glutamate dehydrogenase measured as a function of temperature at pH 7.6, we were able to fit the enthalpy measurements using eq 8, with a $\Delta H°_0$ = 22 kcal mol^{-1} and a T_0 of 43 °C.

The work presented here, in which we have measured the thermodynamic parameters for E–NADPH formation as a function of pH at constant temperature, now provides a very simple explanation of the nature of the implicit E ⇌ E′ step inferred in the previous work. At pH 7.6, the formation of the E–NADPH complex does indeed involve an extra step due to the large downward shift in the pK of HE and the large driving force of K_C. At this pH, the binding of NADPH to the enzyme consists in large part of the conversion of HE to ER and the release to the medium of about 0.8 mol of protons. It is this enzyme–proton release step then which constitutes the hidden step in the reaction, and we may now write E ⇌ E′ as $EH^+ \rightleftharpoons E + H^+$. The physical meaning of the parameter T_0 in eq 8 now also can be established. Expressing K as $K_0[H^+]$, and taking the log of both sides of the definition of K_0 in that equation, we find that

$$\Delta pK_0 = \Delta H°_0 \Delta T_0/2.303RTT_0 \tag{9}$$

where ΔpK_0 is the difference between the actual pK and the experimental pH, and ΔT_0 is the difference between T_0 and the experimental temperature.

Taking the value from Fisher et al. (1981) of 22 kcal mol^{-1} for $\Delta H°$, at temperatures in the range of 25–35 °C:

$$\Delta T_0 \simeq 20\Delta pK \quad (10)$$

using the value of T_0 of 43 °C reported in that same paper in eq 9, we calculate a ΔpK_0 of 0.90, corresponding to a pK_{HE} = 8.50 in excellent agreement with the value observed here derived from free energy studies at a constant temperature. It is clear then that the implicit two-state process responsible for the unusual temperature-dependent $\Delta C_p°$ reported in our earlier paper is fully accounted for by a step in which the binding of NADPH drives a proton off of a functional group on the enzyme.

One major problem does remain. It is by no means easy to account for an ionization of an amino acid residue having either a $\Delta H°$ of 19 kcal mol^{-1} or a $\Delta S°$ of +23 eu regardless of its pK. The excessively large $\Delta H°$ could be accounted for by the forced ionization of *two* protons from highly basic amino acid residues, one proton being released to the solvent and the other being transferred intramolecularly to a residue having a low ionization enthalpy. Since amino acid residues which have ionization $\Delta H°$'s of 11–13 kcal mol^{-1} typically have small negative ionization $\Delta S°$'s, while those of low ionization enthalpy can have positive $\Delta S°$'s of neutralization of about +20 eu, the unusual combination of a large positive enthalpy change and a large positive entropy change could be accommodated by such a mechanism.[3] In any case, it would appear that the process we designate as $EH^+ \rightleftharpoons E + H^+$ must involve the ionization of a protonated enzyme group which is very tightly coupled to some other process having very large energetic properties. Any such change must in all probability involve very substantial alterations in protein structure.

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[3] Since ionizable groups in proteins are not infrequently found to have pK's which differ considerably from those of the corresponding free amino acids, perhaps we should not be astonished at finding "unusual" values for $\Delta H°$ and $\Delta S°$ for ionizable groups on enzymes.

Ionization State of the Coenzyme 5′-Phosphate Ester in Cytosolic Aspartate Aminotransferase. A Fourier Transform Infrared Spectroscopic Study†

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ABSTRACT: In order to determine the ionization state of the 5′-phosphate of bound pyridoxal phosphate, a Fourier transform infrared spectroscopic study of cytosolic aspartate aminotransferase has been carried out. Dianionic and monoanionic phosphate monoesters give rise to two bands each in the infrared spectrum [Shimanouchi, T., Tsuboi, M., & Kyogoku, Y. (1964) *Adv. Chem. Phys. 8*, 435–498]. These bands can be identified in infrared spectra of the free coenzyme in solution. Due to interfering bands arising from the protein, only the band assigned to the symmetric stretching of the dianionic phosphate is observed in holoenzyme solutions. The integrated intensity of this band does not change with pH in the range 5.3–8.6, while for free pyridoxal phosphate, the integrated intensity of the same band changes with pH according to the pK value expected for the 5′-phosphate group in solution. Moreover, the value of the integrated intensity for the bound cofactor is close to the value given by free cofactor at pH 8–9. These results suggest that the 5′-phosphate of the bound cofactor remains mostly dianionic throughout the investigated pH range and disfavor other interpretations in terms of ionization of the phosphate group on the basis of the nuclear magnetic resonance ^{31}P chemical shift–pH titration curve of holoenzyme [Schnackerz, K. D. (1984) in *Chemical and Biological Aspects of Vitamin B_6 Catalysis* (Evangelopoulos, E. A., Ed.) Part A, pp 195–208, Alan R. Liss, New York]. Instead, it seems likely that the ^{31}P chemical shift may sense the ionization of the pyridoxal phosphate–Lys-258 Schiff's base, as proposed for the mitochondrial isozyme [Mattingly, M. E., Mattingly, J. R., & Martinez-Carrion, M. (1982) *J. Biol. Chem. 257*, 8872].

A large variety of enzymes contain a bound phosphoryl group, usually attached to a serine or histidine residue, or as part of substrates or cofactors. A knowledge of the ionization state of the bound phosphoryl group is essential to understand the possible roles played by this group in catalytic events or in protein structure. In a given phosphoryl-containing enzyme, it is desirable to know if the phosphoryl group is dianionic or monoanionic, if the ionization state is affected in the steps of the catalytic process, or by the presence of ligands, substrates, and pH, and, in this last case, if its pK is perturbed by the protein environment.

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