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Calorimetric Investigation of Inhibitor Binding to Rabbit Muscle Aldolase*

Hans J. Hinz,† Daniel D. F. Shiao,‡ and Julian M. Sturtevant§

ABSTRACT: The enthalpies of binding, ΔH_B, of the substrate-analogous inhibitor D-hexitol 1,6-diphosphate to rabbit muscle aldolase have been measured at different temperatures at pH 7.5, employing a flow microcalorimeter. By using four buffer systems with different heats of ionization, ΔH_i, it has been shown that 1.4 ± 0.2 moles of H^+ per mole of enzyme is absorbed when the enzyme is saturated with inhibitor irrespective of the temperature at which the reaction takes place. The temperature variation of ΔH_B corresponds to a temperature independent heat-capacity change, ΔC_p, of −1100 ± 200 cal deg^{-1} (mole of enzyme)$^{-1}$ at saturation. Calorimetric titration data at 25° have been interpreted on the assumption of independent binding sites to yield a value for the intrinsic binding constant, K_B. The temperature variation of K_B calculated using the observed values of ΔH_B, including ΔC_p, is consistent with that deduced from enthalpy titration curves at other temperatures and is very similar to that reported by Lehrer and Barker (Lehrer, G. M., and Barker, R. (1970), *Biochemistry 7*, 1533) for D-arabinitol 1,5-diphosphate. These results emphasize that the explanation offered by Lehrer and Barker *et al.*, for nonlinear van't Hoff or Arrhenius plots, based on an assumed temperature-dependent equilibrium between two forms of the enzyme, can *in general* be replaced by the simpler assumption of a nonvanishing ΔC_p or $\Delta C_p^{\ddagger}$.

In a recent publication, Lehrer and Barker (1970) reported that various binding and kinetic parameters of rabbit muscle aldolase show a strong dependence on temperature. The parameters studied were the Michaelis–Menten constant, K_m, and the maximum velocity, V_{max}, for the splitting of D-fructose 1,6-diphosphate, and the equilibrium constant, K_I, for the dissociation of an inhibitor, D-arabinitol 1,5-diphosphate. Lehrer and Barker proposed an explanation for the observed temperature effects based on a temperature-dependent transition, centered at approximately 30°, of the enzyme between two forms having markedly different binding and catalytic properties. A similar proposal has been made by Massey *et al.* (1966) for the case of D-amino acid oxidase.[1]

Data such as those reported by Lehrer and Barker (1970) can always be rationalized in a very reasonable way on the basis of heat capacity changes accompanying binding processes or the formation of activated complexes, without any assumption of multiple forms of the native enzyme. This is illustrated in Figure 1, in which the data given by Lehrer and Barker for the dissociation constant of the aldolase–arabinitol diphosphate complex are reproduced (within the accuracy of reading their Figure 1). The solid curve in the figure was calculated on the basis of the assumptions that the apparent heat

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[1] In calculating the temperature variation of the *equilibrium* distribution of the enzymes between the two hypothesized forms, both Lehrer and Barker (1970) and Massey *et al.* (1966) used assumed enthalpy values which they termed enthalpies of *activation* and represented by the symbol $\Delta H^{\ddagger}$. It seems unfortunate at this late date to use this terminology, which has for decades been reserved exclusively for the analysis of the variation of reaction rates with temperature, for the standard enthalpy change which controls the temperature variation, as expressed in the van't Hoff equation, of an equilbrium constant.

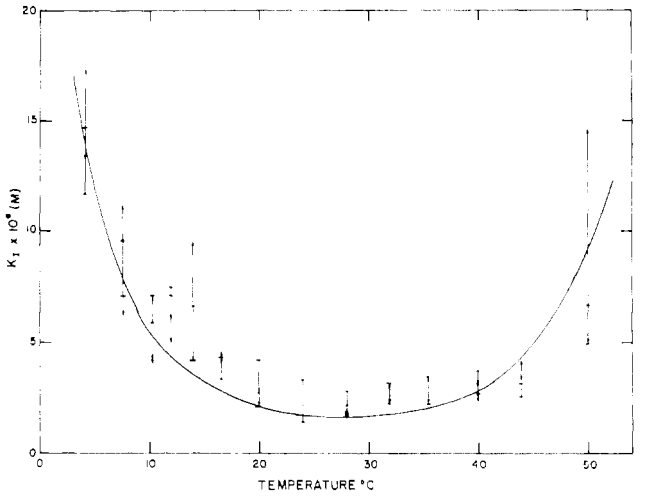

FIGURE 1: Illustration of the use of $\Delta C_p^\circ \neq 0$ to fit experimental data. The experimental data are those of Lehrer and Barker (1970) for the dissociation of D-arabinitol 1,5-diphosphate from its complex with muscle aldolase; the indicated error limits, ↕ and **I**, are for two sets of experiments with ligand concentrations differing by a factor of two. The solid curve is drawn with ΔC_p° for the dissociation process assumed to be +1200 cal deg^{-1} mole^{-1}, $\Delta H = 0$ at 28°, and $K_I = 1.7 \times 10^{-6}$ M at 30°.

capacity of the products of the dissociation is 1200 cal deg^{-1} mole^{-1} larger than that of the complex at all temperatures, that the slope of the pK_I *vs.* $1/T$ (van't Hoff) plot is zero at 28°, and that K_I has the value 1.7×10^{-6} M at 30°. On this basis the enthalpy of the dissociation process is

$$\Delta H_I = -361{,}400 + 1{,}200T \quad (1)$$

where T is the absolute temperature, and the ratio of the equilibrium constant at T_1 to that at T_2 is given by

$$\log \frac{K_{I_1}}{K_{I_2}} = 79{,}040\left(\frac{1}{T_1} - \frac{1}{T_2}\right) + 604.2 \log \frac{T_1}{T_2} \quad (2)$$

It is seen that the experimental data can be adequately fitted with a value for ΔC_p of approximately 1200 cal deg^{-1} mole^{-1}. Recent thermochemical investigations of macromolecular systems have shown that this is a not unreasonable value.

In similar manner it can be shown that the curved Arrhenius plot given by Lehrer and Barker (1970) (their Figure 4) for the specific activity of aldolase can be accounted for by a relatively small heat capacity change on activation, $\Delta C_p^\ddagger$, amounting to −140 cal deg^{-1} mole^{-1}.

In this paper we report the results of direct calorimetric experiments which show that there is indeed a large decrease in apparent heat capacity when another inhibitor, hexitol 1,6-diphosphate (a mixture of D-mannitol and D-sorbitol 1,6-diphosphates), is bound to aldolase, and which thus indicate that all the abnormalities reported by Lehrer and Barker (1970) can be attributed to the existence of significant ΔC_p terms in the aldolase system.

Materials and Methods

Rabbit muscle aldolase was purchased from Sigma Chemical Co., St. Louis, Mo., as a 1% crystalline suspension in ammonium sulfate and was used without further purification. Fructose 1,6-diphosphate tetracyclohexylammonium salt·$10H_2O$ was supplied by Boehringer und Soehne, Mannheim, Germany, and was used without further purification. Hexitol

TABLE 1: Enthalpy of Association of Hexitol 1,6-Diphosphate with Rabbit Muscle Aldolase at pH 7.5 in Various Buffers at 25, 18, and 8°.

$(A)_0$[a] (μM)	$(I)_0$[b] (mM)	ΔH_c[c] (kcal/ Mole of Enzyme)	$(A)_0$[a] (μM)	$(I)_0$[b] (mM)	ΔH_c[c] (kcal/ Mole of Enzyme)
0.1 M Tris, 8°			0.1 M Hepes, 8°		
18.5	1.20	41.2	22.7	2.44	28.7
18.5	0.80	38.0	32.4	1.39	30.1
18.5	0.60	37.0	37.8	1.812	28.7
18.5	0.30	35.4	40.0	0.574	29.3
18.5	0.15	32.1	41.2	0.443	26.9
18.5	0.075	21.9	0.1 M Hepes, 18°		
18.5	0.0375	12.3	20.9	0.973	14.5
18.5	0.0188	8.8	37.0	0.810	15.4
18.5	0.0094	3.5	39.1	0.570	14.5
0.1 M Tris, 18°			40.3	0.441	14.8
23.4	2.92	31.2	41.0	0.360	15.0
23.4	1.46	27.1	0.01 M Hepes, 25°		
23.4	0.73	25.6	32.1	3.0	10.5
23.4	0.365	25.9	25.7	1.27	9.2
23.4	0.150	21.8	27.5	1.0	7.6
23.4	0.075	16.4	32.1	0.623	8.3
23.4	0.0375	8.3	28.8	0.380	8.2
23.4	0.0188	2.4	23.1	0.338	8.2
23.4	0.0094	1.5	41.2	0.218	9.1
0.1 M Tris, 25°			50.8	0.0895	5.5
13.4	3.34	20.0	52.5	0.0690	4.5
43.3	1.43	18.8	41.2	0.0725	6.1
50.0	0.834	16.8	48.1	0.0422	3.7
53.4	0.588	17.5	50.8	0.0298	0.9
56.0	0.376	16.8	52.5	0.023	1.8
30.3	0.250	15.9	0.1 M Glycylglycine, 25°		
43.3	0.143	14.4	24.5	6.7	24.3
50.0	0.0834	10.0	36.1	5.0	20.9
53.4	0.0588	5.1	40.7	4.4	21.9
55.0	0.0455	4.0	51.3	2.9	20.4
56.0	0.037	3.3	60.2	1.67	18.0
0.1 M Pipes, 8°			24.2	2.0	19.5
23.4	2.44	24.3	24.2	1.0	14.3
25.9	2.16	26.2	24.2	0.50	15.3
33.4	1.39	23.8	24.2	0.25	14.2
38.9	0.812	24.3	24.2	0.125	15.7
41.2	0.573	25.7	24.2	0.0625	9.6
0.1 M Pipes, 18°			24.2	0.0313	5.8
35.8	2.43	11.6	24.2	0.0156	4.0
39.7	2.22	11.3			
50.5	1.39	10.8			
59.5	0.81	11.1			
63.0	0.56	11.3			
0.1 M Pipes, 25°					
30.9	4.00	5.0			
30.9	2.00	4.7			
30.9	1.00	5.8			
30.9	0.50	4.5			
30.9	0.25	4.9			

[a] Total enzyme concentration. [b] Total inhibitor concentration. [c] Enthalpy, per mole of enzyme, accompanying the binding of the inhibitor.

TABLE II: Enthalpies of Ionization of Various Buffer Acids.

Buffer	Temp (°C)	ΔH_i (kcal mole^{-1})
Pipes	25	2.74 ± 0.06[a]
Hepes	25	5.01 ± 0.25[a]
Tris	25	11.34[b]
Glycylglycine (pK_2)	25	10.6[c]
Pipes	5	2.7 ± 0.3[d]
Hepes	5	4.7 ± 0.3[d]
Tris	5	11.44 ± 0.20[d]

[a] Beres and Sturtevant (1971). [b] Öjelund and Wadsö (1968). [c] Izatt and Christensen (1968). [d] This work.

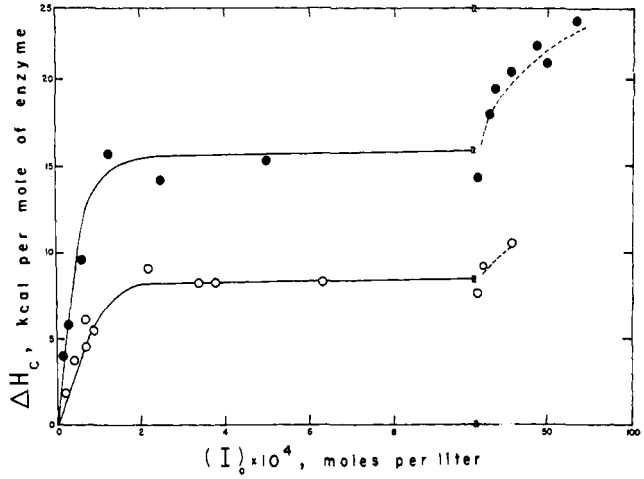

FIGURE 2: Calorimetric titration curves of muscle aldolase with hexitol 1,6-diphosphate at pH 7.5, 25°. The heat absorbed, corrected for dilution heats, when the inhibitor is bound is plotted as a function of the total inhibitor concentration. (○) Titration in 0.01 M Hepes buffer; enzyme concentrations, 26–52 μM. (●) Titration in 0.1 M glycylglycine buffer; enzyme concentrations, 24–60 μM. All solutions contained 0.001 M EDTA. The solid curves are calculated on the basis of $K = 1.8 \times 10^5$ M^{-1} and the observed value for ΔH_c at inhibitor saturation ($(I)_0 \approx 0.6$ mM), with the assumption of 2.7 combining sites per molecule of enzyme (Ginsburg and Mehler, 1966).

1,6-diphosphate was prepared from fructose 1,6-diphosphate according to the procedure described by Ginsburg and Mehler (1966). All other chemicals employed were commercial preparations of reagent grade. The water used for preparing the buffer solutions was demineralized and glass distilled.

Measurement of aldolase activity was based on the spectrophotometric hydrazine trap procedure (Jagannathan *et al.*, 1956). Assays were performed at ambient temperatures (about 22–23°) in a Cary 14 recording spectrophotometer. Immediately prior to each assay, hydrazine sulfate dissolved to a final concentration of 0.0035 M at pH 7.5 in the buffer solution employed for the corresponding calorimetric measurements was mixed in the volume ratio 2:1 with a 0.012 M fructose 1,6-diphosphate solution in the same buffer. Three ml of the resultant solution was added to both sample and reference cells (1-cm light path). Small volumes (5–10 μl) of enzyme solution were added to yield a final enzyme concentration in the range from 1 to 15 μg (ml)$^{-1}$, in order to obtain a rate lower than 0.03 optical density unit/min (Drechsler, 1959). The enzyme solutions were prepared immediately prior to the measurements from the ammonium sulfate suspension by centrifugation and dialysis against the appropriate buffer solution. Only enzyme solutions yielding a higher activity than 22 ΔOD_{245} ml min^{-1} (mg protein)$^{-1}$ were used for the calorimetric investigations.

The concentration of the enzyme was determined spectrophotometrically assuming an absorption coefficient of 9.38 for a 1% protein solution at 280 nm in a 1-cm light path (Donovan, 1964).

The calorimetric measurements were performed with a flow modification of the Beckman Model 190 microcalorimeter (Sturtevant and Lyons, 1969; Velick *et al.*, 1971). With one exception all buffer solutions used were 0.1 M in the buffering component and 0.001 M in EDTA. All measurements were made at pH 7.5, the pH being adjusted with a Radiometer pH meter (type TTT 1a), at the same temperature as that of the calorimetric experiment.

Results

Enthalpy of Binding of Hexitol Diphosphate to Aldolase. The enthalpy change accompanying the binding of hexitol 1,6-diphosphate to aldolase was determined as a function of inhibitor concentration at several different temperatures and in the presence of various buffers, with the results shown in Table I. The enthalpies of binding, ΔH_c, have been corrected for the heats of dilution of the inhibitor and the protein determined in blank experiments, which were in all cases very small or negligible, The buffer systems employed were 0.1 M Tris, 0.1 M Hepes[2] (0.01 M in one experiment), 0.1 M Pipes, and 0.1 M glycylglycine, all adjusted to pH 7.5 with HCl, and containing 0.001 M EDTA. The values for ΔH_c are based on a molecular weight of 150,000 (Morse and Horecker, 1968).

Heats of Ionization of the Buffers. It will be shown that the protein undergoes a change in extent of protonation on binding the inhibitor. It is therefore essential, in interpreting the values of ΔH_c in Table I, to know the heats of ionization of the buffers, ΔH_i, used at the various temperatures employed. These quantities are known from previous work at 25°, as recorded in Table II, and were determined with sufficient accuracy for our purposes at 5° in the flow calorimeter using the method outlined by Beres and Sturtevant (1971), except that there was no added salt present. As the results given in Table II show, the values of ΔH_i at 18° could be obtained by interpolation with adequate accuracy.

Enthalpies of Binding under Saturating Conditions. As shown in Figures 2 and 3, when ΔH_c is plotted as a function of inhibitor concentration in a particular buffer and at a constant temperature, a typical saturation curve is obtained, with indication of a small additional, perhaps nonspecific, binding at very high inhibitor concentrations. From the relatively horizontal portions of such titration curves, values for the limiting enthalpy change, ΔH_{sc}, have been estimated. These values are plotted as functions of the buffer heats of ionization, ΔH_i, in Figure 4.

Variation of Binding Enthalpies with Buffer Ionization Heats. The linearity of the variation of ΔH_{sc} with ΔH_i shown in Figure 4 indicates that there is a change in protonation, undoubtedly of the protein, when the inhibitor is bound. The interaction between inhibitor and enzyme at saturation, in an

[2] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid).

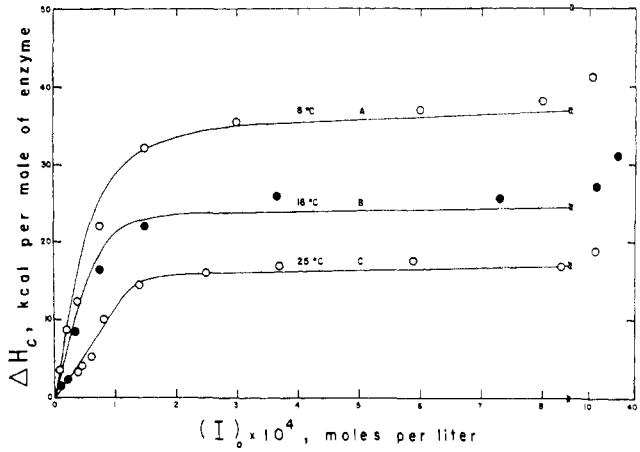

FIGURE 3: Calorimetric titration curves of muscle aldolase with hexitol 1,6-diphosphate at pH 7.4 in 0.1 M Tris-HCl buffer. The heat absorbed when the inhibitor is bound is plotted against the total inhibitor concentration at three different temperatures. All solutions contained 0.001 M EDTA. The solid curves are calculated using values for the equilibrium constant derived by means of the van't Hoff equation from the value at 25° and the observed values for ΔH_c at inhibitor saturation ($(I)_0 \approx 0.6$ mM), with the assumption of 2.7 combining sites/molecule of enzyme (Ginsburg and Mehler, 1966).

adequately buffered solution, can be formally separated into the two processes (eq 3 and 4), where E, I, and B refer, respec-

$$E + mI + nH^+ \rightleftharpoons EI_mH_n^{n+}; \Delta H_B \quad (3)$$

$$nBH^+ \rightleftharpoons nB + nH^+; n\Delta H_i \quad (4)$$

tively, to enzyme, inhibitor, and unprotonated buffer. ΔH_B is the enthalpy of binding in a hypothetical buffer with zero heat of ionization. Then, since

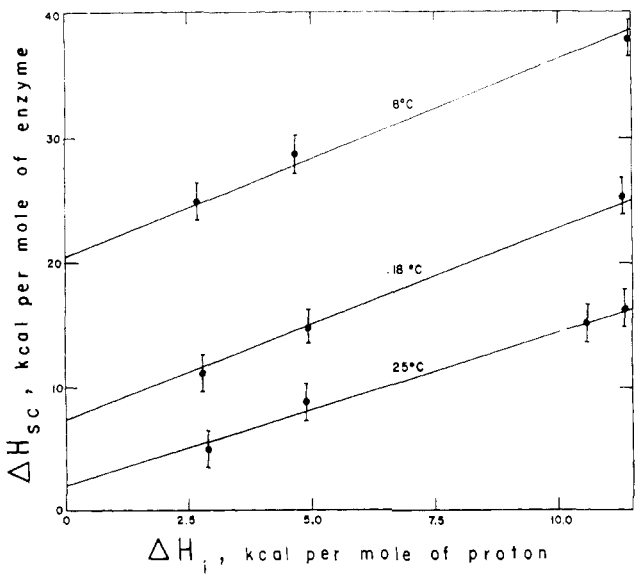

FIGURE 4: The variation with ΔH_i, the heat of ionization of the buffer, of ΔH_{sc}, the enthalpy change on saturating muscle aldolase with hexitol 1,6-diphosphate at pH 7.5. The values for ΔH_{sc} for those cases not shown in Figures 2 and 3 were taken as the mean of several values determined at inhibitor concentrations of 0.4–2.4 mM.

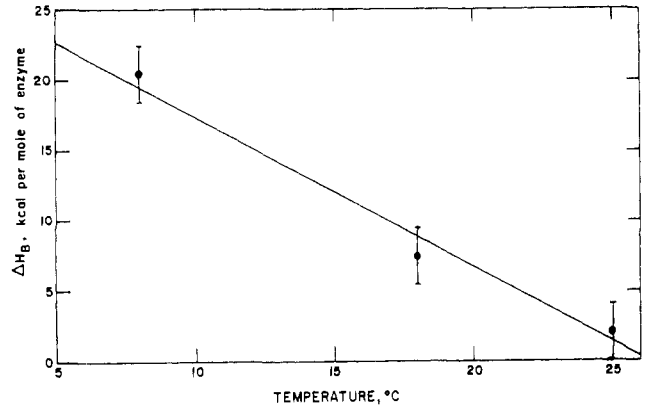

FIGURE 5: The variation with temperature of ΔH_B, the enthalpy change on saturating muscle aldolase at pH 7.5 with hexitol 1,6-diphosphate in a hypothetical buffer of zero heat of ionization. The values for ΔH_B are the intercepts of the lines in Figure 4.

$$\Delta H_{sc} = \Delta H_B + n\Delta H_i \quad (5)$$

the value of n is given by the slope of a plot of ΔH_{sc} *vs.* ΔH_i, and ΔH_B is the intercept. The lines in Figure 4 give $n = +1.4 \pm 0.2$ moles of H^+ per mole of enzyme, independent of temperature. Thus protons are absorbed when the binding takes place.

Variation of Binding Enthalpies with Temperature. The values of ΔH_B obtained by extrapolation of the data plotted in Figure 4 are presented in Figure 5 as a function of temperature. Within experimental uncertainty, ΔH_B decreases linearly with temperature, the decrease corresponding to a constant value for the change in heat capacity, ΔC_p, of -1.1 ± 0.2 kcal deg^{-1} (mole of enzyme)$^{-1}$.

Discussion

Analysis of Calorimetric Titration Curves. The titration data at 25°, shown in Figure 3, can be utilized, on the basis of certain assumptions, to obtain estimates of equilibrium constants. The most important assumption is that there are, on the average, 2.7 independent binding sites/molecule, as reported by Ginsburg and Mehler (1966). It is further assumed that these sites are saturated at inhibitor concentrations corresponding to the plateau regions of the titration curves, and that the enthalpy change per ligand bound is independent of the extent of binding. The expression for the intrinsic binding constant, K_B, is then

$$K_B = \frac{(EI)}{[(E)_0 - (EI)][(I)_0 - (EI)]} \quad (6)$$

where $(E)_0$ is the total concentration of binding sites, $(I)_0$ is the total inhibitor concentration, and (EI) is the concentration of bound inhibitor. Obviously

$$\Delta H_c = \frac{(EI)}{(E)_0}\Delta H_{sc} \quad (7)$$

where, as defined previously, ΔH_c is the observed enthalpy per mole of enzyme and ΔH_{sc} is the value at inhibitor saturation. A value of K_B for the titration at 25° in Tris buffer (Figure 3, curve C) was selected by trial and error to minimize the squares of the deviations of observed values of ΔH_c from the

calculated values, the resulting value being 2.0×10^5 M^{-1}. It should be added that the titration curves could equally well be fitted on the assumption of three binding sites per molecule, but that four sites would lead to a poorer fit.

From the value of K_B at 25°, the expected values at 8 and 18° were calculated by the van't Hoff expression, taking account of the observed temperature variation of ΔH_{sc}. Curves A and B in Figure 3 were calculated using these predicted values of K_B. It is seen that they fit the experimental data well, showing that the set of titration curves is fully internally consistent.

Titrations were also performed in other buffers at 25°, as shown in Figure 2. The curves in the figure were drawn using the value for K_B at 25° given above and the observed ΔH_{sc} in each case. It is evident that there are no specific buffer effects on the equilibrium.

The assumption of independent binding sites gains support from a recent study (Eagles *et al.*, 1969) in which evidence for the absence of site interaction is derived from kinetic investigations. However, the same study provides chemical as well as crystallographic evidence for a tetrameric structure of the protein, with two pairs of nonidentical subunits. A possible reconciliation of the apparent existence of only about three binding sites per molecule with the tetrameric structure may be given by the tight binding of approximately 1 mole of organic phosphate in the native enzyme (Kobashi *et al.*, 1966; Morse and Horecker, 1968). On the basis of our calculations, we can only state that the existence of 2.7 to 3 binding sites is consistent with our calorimetric results.

It is evident in Figures 2 and 3 that the enthalpy values at high inhibitor concentrations tend to increase; whether this is due to a fourth binding site becoming available or is to be explained by nonspecific binding cannot be decided on the basis of the available evidence.

Ginsburg and Mehler (1966) reported a value for K_B at 25° for hexitol diphosphate of 8.3×10^5 M^{-1} determined by equilibrium dialysis at pH 7.8, which is considerably higher than our value of 2.0×10^5 M^{-1} at pH 7.5.

Thermodynamic Parameters for the Binding of Hexitol Diphosphate to Aldolase. The variation of ΔH_{sc} with the heat of ionization of the buffer, illustrated in Figure 4, leads to the conclusion, as shown above, that 1.4 ± 0.2 moles of H^+/mole of protein are absorbed when hexitol diphosphate is bound to aldolase at each of the temperatures employed. Extrapolation of the lines in Figure 4 gives values for ΔH_B, the heat of binding in a buffer having a vanishing heat of ionization.

The outstanding feature of the results presented in this paper is the large variation of ΔH_B with temperature, shown in Figure 5. We believe that this variation is best interpreted as resulting from a large decrease in apparent heat capacity accompanying the binding of the inhibitor to a single form of the enzyme. It is admittedly impossible on the basis of presently accessible evidence to prove that the variation is not due to a temperature-dependent equilibrium between two forms of the enzyme having markedly different but temperature-independent values of ΔH_B, as assumed by Lehrer and Barker (1970) in the case of arabinitol diphosphate. For example, if the transition between the two forms involved an absorption of as little as 20–30 kcal mole^{-1} in a molecule as large as that of aldolase, it would be impossible to detect by direct transition calorimetry using equipment of the sensitivity currently available (Danforth *et al.*, 1967; Tsong *et al.*, 1970), whereas a transition enthalpy of no more than 10 kcal mole^{-1} is required to account for the experimental data. In spite of this ambiguity we shall continue with discussion of our results in terms of a nonvanishing change in apparent heat capacity, ΔC_p.

TABLE III: Thermodynamic Parameters for the Binding of Hexitol 1,6-Diphosphate to Rabbit Muscle Aldolase at pH 7.5.

Temp (°C)	$\Delta G°_B$[a] (kcal (Mole of Enzyme)$^{-1}$)	ΔH_B[b] (kcal (Mole of Enzyme)$^{-1}$)	$\Delta S°_B$[c] (cal deg^{-1} (Mole of Enzyme)$^{-1}$)
5	−5.91	23.3	104.8
10	−6.38	17.8	85.2
15	−6.75	12.3	65.9
20	−7.03	6.8	47.0
25	−7.23	1.3	28.4
30	−7.31	−4.3	10.1
35	−7.32	−9.8	−7.9
40	−7.23	−15.3	−25.6
45	−7.05	−20.8	−43.1

[a] Calculated from the value of $\Delta G°_B = -RT \ln K_B$ obtained at 25°, using the Gibbs-Helmholtz equation and the enthalpies given in column 3. [b] Calculated from the value at 25° given in Figure 5 and $\Delta C_p = -1100$ cal deg^{-1} (mole of enzyme)$^{-1}$. [c] $\Delta S°_B = (\Delta H_B - \Delta G°_B)/T$.

The slope of the plot in Figure 5 gives $\Delta C_p = -1100 \pm 200$ cal deg^{-1} (mole of enzyme)$^{-1}$ or -410 ± 70 cal deg^{-1} (mole of ligand)$^{-1}$ on the basis of 2.7 moles of inhibitor bound per mole of enzyme at saturation. The enthalpy data together with the value $K_B = 2.0 \times 10^5$ M^{-1} at 25° lead to the thermodynamic parameters given in Table III. It should be noted that extension of these quantities to temperatures above 25° represents an extrapolation which seems fairly safe in view of the experimental observations of Lehrer and Barker (1970), and the success of our interpretation of their data in terms of ΔC_p. Adoption of the value for K_B given by Ginsburg and Mehler (1966) would make $\Delta G°$ more negative by about 0.9 kcal mole^{-1} and $\Delta S°$ more positive by about 3 cal deg^{-1} mole^{-1} at each temperature.

The thermodynamic quantities in Table III refer to the single pH value, 7.5. Application of the relation (Alberty, 1969)

$$\left(\frac{\partial \Delta G°}{\partial \mathrm{pH}}\right)_T = -2.3RTn \qquad (8)$$

where n is the number of protons absorbed in a reversible process (eq 3), shows that at least over a short range of pH in the vicinity of pH 7.5, increase of pH will make $\Delta G°_B$ more negative by 1.9 kcal mole^{-1} per unit of pH change. This factor thus reduces the disagreement between our value for $\Delta G°_B$ and that of Ginsburg and Mehler (1966) to about 0.3 kcal mole^{-1} instead of 0.9 kcal mole^{-1}. Similarly, since n is experimentally found to be independent of temperature, the relation

$$\left(\frac{\partial \Delta H°}{\partial \mathrm{pH}}\right)_T = -2.3RT^2\left(\frac{\partial n}{\partial T}\right)_{\mathrm{pH}} \qquad (9)$$

shows that ΔH_B is independent of pH in the vicinity of pH 7.5.

Nature of the Binding of Hexitol Diphosphate to Muscle Aldolase. The value of ΔC_p for the binding process, amounting to -410 cal deg^{-1} per mole of ligand bound, compares with the large negative value, -520 cal deg^{-1} (mole of ligand)$^{-1}$, which has recently been reported for the binding of NAD by yeast glyceraldehyde 3-phosphate dehydrogenase (Velick *et al.*, 1971). In view of the complete lack of any similarity between the structures of the two ligands, hexitol diphosphate and NAD, and the sheer magnitude of the ΔC_p values, it appears that alterations in protein structure must be considered as the major source of the heat capacity changes, although it is also probable that smaller contributions arise in the ligands themselves. In the case of the dehydrogenase, binding of the coenzyme stabilizes the protein against denaturation (Furfine and Velick, 1965), a result which might be due to a stiffening of the protein structure, with concomitant loss of excitable internal degrees of freedom. Similarly, in the case of aldolase, binding of the inhibitor appears to promote a "tightened" structure of the enzyme (Adelman *et al.*, 1968). However, if we take as a very rough average the value of R cal deg^{-1} mole^{-1} as the heat capacity contribution of an internal weak vibration or rotation, we would have to postulate the loss of some 200 such degree of freedom per molecule of ligand bound in order to account fully for the observed values of ΔC_p. This appears to be an unreasonably large number.

Very large increases in apparent heat capacity have recently been found to accompany the reversible thermal unfolding of such proteins as chymotrypsinogen (Jackson and Brandts, 1970), ribonuclease (Tsong *et al.*, 1970) and myoglobin (Privalov *et al.*, 1971). These increases are currently attributed in large part to the exposure of hydrophobic groups to the solvent with a resulting increase in the ordering of the neighboring water molecules (Kauzmann, 1959; Tanford, 1970). The heat capacity increases encountered in these denaturation processes amount to roughly 0.15 cal deg^{-1} (g of protein)$^{-1}$, more than an order of magnitude larger than the decrease observed with aldolase at inhibitor saturation. It is thus not at all unlikely that a significant contribution to ΔC_p stems from a decrease in the solvent exposure of hydrophobic groups of the protein when the inhibitor is bound.

Since the range of ΔC_p values for the ionization of groups occurring in proteins is of the order of ± 50 cal deg^{-1} mole^{-1} (Edsall and Wyman, 1958), and only 1.4 protons are absorbed per molecule of enzyme at inhibitor saturation, it is evident that no significant fraction of the ΔC_p observed with aldolase can be due to this cause.

Although it is obviously impossible at present to apportion observed values of ΔC_p among the three effects mentioned here, namely, loss of degrees of freedom by the ligand and by the protein, and decrease in the exposure of hydrophobic groups, it seems likely that these are the main effects which contribute.

In discussions of the nature of processes occurring in macromolecular systems, the partition of the standard free-energy change between $\Delta H°$ and $-T\Delta S°$ has frequently been employed as a diagnostic indicator. At least in cases such as the present one which exhibit large values of ΔC_p, consideration of the thermodynamic relations: $(\partial \Delta H/\partial T)_P = \Delta C_p$ and $(\partial \Delta S/\partial T)_P = \Delta C_p/T$ makes it evident that simple conclusions based on the relative magnitudes of ΔH and ΔS are not very enlightening. Thus, both the binding of hexitol diphosphate to muscle aldolase and of NAD to yeast glyceraldehydephosphate dehydrogenase are "entropy driven" at low temperature and "energy driven" at high temperature. In the former case, the crossover point ($\Delta G° = 2\Delta H$) is 29.4° and in the latter case 8°. There is no reason to suppose that in either of these binding processes the relative importance of hydrophobic, hydrophilic, hydrogen bonding, electrostatic, or other types of interaction is strongly dependent on temperature.

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