Calorimetric Studies of the Binding of Ligands to Aldolase†

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ABSTRACT: Binding enthalpies of D-arabinitol 1,5-diphosphate, 1,5-pentanediol diphosphate, 1,5- and 2,7-naphthalene diphosphates, and inorganic phosphate to rabbit muscle aldolase (EC 4.1.2.7) have been measured at 37° and pH 7.5. In combination with kinetically determined K_I values the enthalpies yield complete sets of thermodynamic parameters for the binding process. The thermodynamic parameters for the binding of D-arabinitol 1,5-diphosphate, a potent inhibitor of the enzyme, can be expected to approximate those of the natural substrate, D-fructose 1,6-bisphosphate, as it has the same configuration (arabino) and can achieve a very similar conformation. For all ligands tested binding is accompanied by proton uptake which ranges from 0.3 to 0.7 mol/mol of ligand. The relative magnitudes of the thermodynamic parameters are discussed in terms of ligand structure and the changes in physical properties that occur on binding. Competition between buffer anions and ligands for the binding sites is also considered.

Enzyme action has been the subject of innumerable kinetic studies directed toward understanding the details of catalysis. None of these studies has progressed to the point that the complete process can be described in quantitative terms. A set of thermodynamic parameters for all steps in the process would provide a framework within which kinetic parameters must fit. In the present studies the enthalpies of binding of a series of substrate analogs to rabbit muscle aldolase were determined under a single set of conditions. These values when combined with kinetically determined dissociation constants for the inhibitor yield a complete set of thermodynamic parameters for the binding process. The binding of close structural analogs of the substrate can serve as a model for the first step in catalysis. Estimates are made of the contributions of various structural features of the ligand to binding and the extent to which each can serve as a model for the normal substrate, FrP_2.[1]

Calorimetric experiments were performed at 37° in order to obtain the thermodynamic parameters under approximately physiological conditions. This also allows a check of the van't Hoff enthalpy values determined by Lehrer and Barker (1971) wherein $\Delta H°$ of binding $AraP_2$ was found to be positive below 28° and negative above. This change in ΔH with temperature was also predicted by Hinz *et al.* (1971) from calorimetric studies of the binding of hexitol-P_2 to rabbit muscle aldolase between 8 and 25°. From measurements of ΔH and ΔC_p for binding they calculated that above 28° ΔH for the process would be negative.

Materials and Methods

The sources and purity of reagents and enzymes used in this study were described previously (Crowder *et al.*, 1973). In addition, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), was purchased from the Sigma Chemical Co.

The tetracyclohexylammonium salts of Nap-1,5-P_2 and Nap-2,7-P_2 were the gifts of Dr. Byungse Suh and were pure according to the criteria used for the other phosphates.

Specific activities of aldolase preparations ranged from 10 to 16 units as determined by the method of Richards and Rutter (1961) as modified by Lehrer and Barker (1970). For calorimetric experiments the stock enzyme suspension was centrifuged at 27,000*g*; the pellet was dissolved in 0.15 M glycylglycine buffer, pH 7.5, and made to 1.8 M in ammonium sulfate and centrifuged at 27,000*g*. This solution was dialyzed exhaustively against the appropriate buffer, centrifuged to clarify, and then stored at 4°. The final dialysate was also centrifuged and stored for use as a reference solution.

Inhibitor constants were determined kinetically at pH 7.5 in 0.02 M Tris at 37° using a Cary Model 15 spectrophotometer interfaced with a Linc 8 computer programmed to analyze progress curves as described by Martin (1972).

Calorimetric measurements were made at 37° with a batch microcalorimeter (Crowder, 1972) constructed according to the design of Wadso (1968). Output from the transducing units, which were Cambion Thermoelectric Corporation No. 3951-8 thermoelectric modules, was amplified by a Keithley Model 147 Nanovolt Null detector. The signal from the amplifier was recorded using a Varian Model 20 10-mV recorder.

Electrical calibration heaters, in contact with the cells, were used to standardize the calorimeter. The electrical heaters were standardized against two chemical reactions, the neutralization of Tris (Grenthe *et al.*, 1970) and the dilution of sucrose (Gucker *et al.*, 1939), for which the heats have been accurately established. The heats of these reactions at 37° were obtained by linear extrapolation of literature data.

Quartz reaction cells were purchased from Helma Scientific Corporation. The cells (4.5 × 4.8 × 1.2 cm) have two compartments separated by a partition such that mixing of the contents occurred when the cells were inverted. Each compartment has a filling port and a maximum capacity of 5.0 ml. The use of quartz instead of the usual metal for the cells had little effect on the equilibrium time for the calorimeter.

The calorimeter has two quartz cells and two sets of transducing units wired in opposition to compensate for one of the dilution heats. In a typical experiment 2.5 ml of aldolase (20–30 mg/ml) in buffer was mixed with inhibitor solution on one

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[1] Abbreviations used are: $FruP_2$, D-fructose 1,6-bisphosphate; $AraP_2$, D-arabinitol 1,5-diphosphate; $PenP_2$, 1,5-pentanediol diphosphate; Nap-2,7-P_2, 2,7-naphthalenediol diphosphate; Nap-1,5-P_2, 1,5-naphthalenediol diphosphate; hexitol-P_2, the mixture of D-glucitol and D-mannitol 1,6-diphosphates produced by the reduction of D-fructose 1,6-bisphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2'-ethanesulfonic acid.

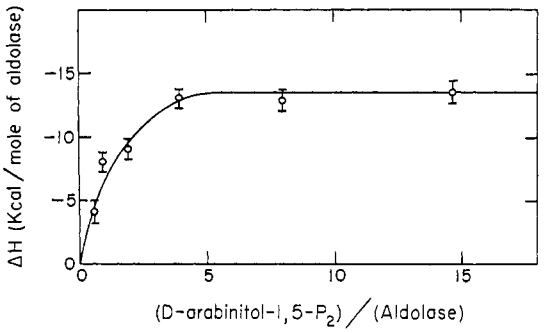

FIGURE 1: A plot of ΔH of binding of $AraP_2$ to aldolase *vs.* the concentration of ligand divided by the aldolase concentration (1.4×10^{-4} M).

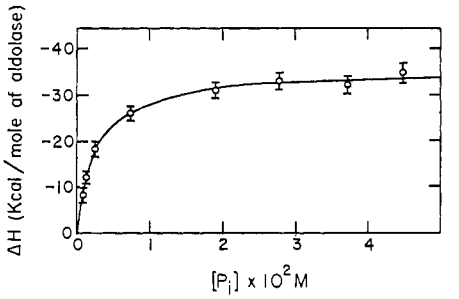

FIGURE 2: A plot of the ΔH of binding of P_i to aldolase *vs.* the concentration of inorganic P. The aldolase concentration was 1.8×10^{-4} M.

side while inhibitor was diluted by buffer on the other. Heats of dilution of the protein, which are small, were determined independently.

Results

Calorimetric measurements were made at 37° and pH 7.50 using three buffers: 0.02 M Tris, 0.018 M Hepes, and 0.008 M diethylmalonic acid. Heats of ionization for these buffers are 11.15, 5.2 (Beres and Sturtevant, 1971), and −1.0 kcal mol^{-1} (Crowder, 1972), respectively.

A typical enthalpic titration for ligand binding to rabbit muscle aldolase at 37° in 0.008 M diethylmalonic acid, pH 7.5, is shown in Figure 1. The limiting enthalpy value for saturating the ligand sites, ΔH_{sc}, is -13.0 ± 0.5 kcal mol^{-1} of aldolase and is obtained from the horizontal portion of the curve.

The binding of P_i at 37° in 0.008 M diethylmalonic acid at pH 7.5 is shown in Figure 2. From the titration data for the aldolase–P_i complex, the apparent dissociation constant (K_I) of $2.5 \pm 0.2 \times 10^{-3}$ M was obtained in good agreement with values obtained kinetically.

Binding enthalpies (ΔH_{sc}) at saturating concentrations of ligand in Tris, Hepes, and diethylmalonic acid buffers were determined from titration curves for all ligands except P_i. Enthalpies for P_i binding were measured at 2.5×10^{-2} M P_i (90% saturation) and corrected to values for complete saturation. In Figure 3 we plotted values for ΔH_{sc} *vs.* ΔH_i as suggested by Beres and Sturtevant (1971). Enthalpies of binding, ΔH_B, in a hypothetical buffer of zero heat of ionization are obtained from the ordinate intercept, and n_B, the number of protons involved in the binding, is obtained from the slope.

In making calorimetric measurements of the binding of ionic ligands (binding) to the macromolecule, competition by buffer ions for binding sites should be considered. Although this factor is often ignored it can significantly change the measured thermodynamic parameters. Two of the buffers used in this study, Tris and Hepes, have monovalent anions which typically have binding constants to the enzyme of approximately 10^2 M^{-1}. Thus, at the buffer concentrations used 8–12% of the aldolase binding sites will have anion bound and the measured heats (ΔH_{sc}) will include the heat of unbinding of the anion. Although the thermodynamic parameters may not be the same, the heats of dissociation of specifically bound chloride ion from bovine serum albumin as measured by Lovrien and Sturtevant (1971) can be used to make an estimate of the magnitude of this effect. Using the largest heat obtained by them we calculate that ΔH_B in these two buffers may be low by 1 kcal mol^{-1} of ligand. With the dianion, diethylmalonic acid, the binding constant is 10^3 M^{-1} and the problem is more pronounced; in this buffer approximately 90% of the sites would contain diethylmalonic acid anion and the measured thermodynamic parameters reflect the unbinding of diethylmalonic acid and the binding of ligand. Large effects could be observed, particularly when the binding constant for the ligand is not much larger than that of the buffer anion. This is the case for $PenP_2$ and P_i as the enzyme could not be saturated at ligand concentrations which did not exceed the ionic strength used in this study. Two observations suggest that the heat of binding of diethylmalonic acid is small. First, the heats of ionization of carboxylic acids are typically small (ΔH_i = 0.4 kcal/mol for acetic acid (Izatt and Christensen, 1968) and ΔH_i = −1.0 kcal mol^{-1} for diethylmalonic acid (Crowder, 1972). Second, for the strong binding ligands the experimental points fall on the same line as measurements in other buffers. Corrections were not made for buffer anion binding in calculating ΔH_B and n_B from the data in Figure 3; however, the experimental measurements in diethylmalonic acid were not used for $PenP_2$ and P_i.

The thermodynamic parameters determined as described above are presented in Table I. For diphosphates the stoichiometry used is 3.3 mol of ligand (mol of aldolase)$^{-1}$ (Castellino and Barker, 1966); for P_i the stoichiometry is assumed to be 6.6. The kinetically determined binding constants (K_B) are presented in Table I, column 6. These values were used to calculate the $\Delta G°$ values presented in column 5. The values of the thermodynamic parameters in Table I refer to a standard

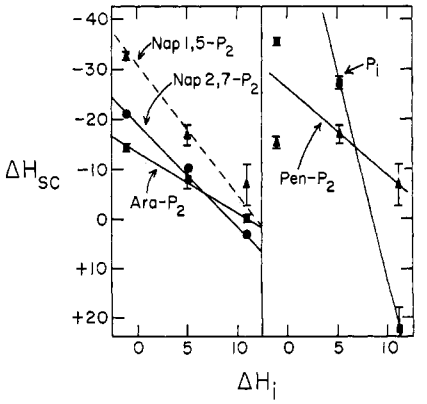

FIGURE 3: The variation of ΔH_{sc}, the heat of association of ligand, with ΔH_i, the heat of ionization of buffer, for saturating levels of five ligands with rabbit muscle aldolase. Values shown are the means of several determinations made with inhibitor concentrations of $100 \times K_I$.

TABLE I: Thermodynamics Parameters/Mole of Binding Sites for Ligand Binding to Aldolase at 37°, $\Gamma/2 = 0.013$, and pH 7.5.

Ligand	n_B (mol of H^+ mol^{-1})	ΔH_B[a] (kcal mol^{-1})	ΔS_B (cal deg^{-1} mol^{-1})	ΔG_B (kcal mol^{-1})	K_B (M^{-1})
Nap-1,5-P_2	0.73 ± 0.12	−9.5 ± 0.9	3.9 ± 3.5	−10.7 ± 0.2	1.1 ± 0.3 × 10^7
$AraP_2$	0.33 ± 0.06	−3.8 ± 0.4	19.9 ± 2.0	−10.0 ± 0.2	3.3 ± 0.7 × 10^5
Nap-2,7-P_2	0.60 ± 0.03	−5.9 ± 0.2	10.7 ± 1.5	−9.2 ± 0.2	3.3 ± 0.8 × 10^7
$PenP_2$	0.33 ± 0.10	−8.4 ± 2.5	−1.94 ± 8.1	−7.8 ± 0.2	2.9 ± 0.8 × 10^6
P_i	1.22 ± 0.20	−21.2 ± 3.6	−56.5 ± 11.6	−3.7 ± 0.1	4.0 ± 0.3 × 10^2

[a] Calculated from the experimental data assuming 3.3 binding sites/mol of aldolase.

state of 1.0 mol $l.^{-1}$ except for H^+ which is considered to be in a standard state at pH 7.5.

Discussion

We have studied the binding to rabbit muscle aldolase of $AraP_2$, a ligand which closely approximates the structure of the natural substrate, $FruP_2$, to obtain a complete set of thermodynamic parameters that may be of value in modeling the catalytic process. In addition, an attempt was made to determine the contributions to the binding of some structural features of the ligand by obtaining the thermodynamic parameters for several structurally dissimilar ligands. Since electrostatic interactions are important in binding ligands to aldolase (Mehler, 1963; Hartman and Barker, 1965; Ginsberg and Mehler, 1966) a single ionic strength was used for all measurements.

If electrostatic interactions were solely responsible for the stabilization of the ligand–aldolase complex, the thermodynamic quantities for binding 1 mol of a diphosphate ligand would be similar and approximately twice those for binding P_i. Although the binding free energy for pentanediol diphosphate is approximately twice that of inorganic phosphate the agreement is fortuitous and the contributions of ΔH and ΔS to the binding processes are very different (Table I).

Comparing the pairs of ligands, $AraP_2$ *vs.* $PenP_2$ and $PenP_2$ *vs.* $2P_i$, which differ, respectively, by three hydroxyl groups and five methylene groups, demonstrates the changing roles of enthalpy and entropy in the binding of ligands to aldolase. The binding of P_i is due principally to a decrease in enthalpy in the aldolase–P_i complex, whereas a decrease in enthalpy and an increase in entropy are important with $AraP_2$ and $PenP_2$. In addition, the tighter binding of $AraP_2$ as compared to $PenP_2$ can be attributed to a larger increase in entropy when $AraP_2$ is bound.

The thermodynamic parameters for binding naphthalenediol diphosphates (Nap-1,5-P_2 and Nap-2,7-P_2) also show large differences in the partition of energy between ΔH_B and ΔS_B. Fluorescence enhancement studies with rat muscle aldolase (Suh and Barker, 1971) showed that the organic portion of Nap-1,5-P_2 enters a hydrophobic region when it is bound. Neither the differences in thermodynamic parameters between Nap-1,5-P_2 and Nap-2,7-P_2 nor the differences between the thermodynamic parameters for these compounds and those of the other ligands examined are interpretable on the basis of this type of interaction.

Large changes in the contributions of ΔH and ΔS to the binding of hexitol-P_2 to aldolase as the temperature varies were also predicted by Hinz *et al.* (1971) from the large value of ΔC_p for the reaction (−410 cal deg^{-1} (mol of $ligand^{-1}$). Earlier studies, in which van't Hoff enthalpies were obtained (Lehrer and Barker, 1970), also indicated that ΔH_B was positive below 28° and negative above that temperature for the binding of $AraP_2$. This is confirmed by the data presented here. At pH 7.5 and 37°, for $AraP_2$, $\Delta H_B = -3.8$ kcal (mol of ligand)$^{-1}$ in good agreement with the value obtained in glycylglycine buffer at pH 7.5 by Lehrer and Barker (1970) of −4.2 kcal (mol of ligand)$^{-1}$ and that computed for the binding of hexitol-P_2 from the data of Hinz *et al.* (1971) of −3.6 kcal (mol of ligand)$^{-1}$. Thus, there is little doubt that ΔH_B for aldolase varies with temperature as predicted by earlier studies. As recently pointed out by Sturtevant (1973), this variation cannot be explained entirely by a large constant difference in heat capacity between the enzyme and the enzyme–substrate complex as initially proposed by Hinz *et al.* (1971).

The structural features that cause the thermodynamic parameters to be different for various ligands cannot be specified precisely. It is worth noting, however, that $AraP_2$ binding to aldolase causes tryptophyl residues to move into the solvent and tyrosyl residues to move into the protein fabric (Lehrer and Barker, 1970, 1971; Crowder *et al.*, 1973). The thermodynamic contribution of these movements can be computed from data obtained with model compounds (Nozaki and Tanford, 1965, 1970; Némethy and Scheraga, 1962, 1963; Kauzmann, 1959) and a good agreement with the observed differences in ΔG_B, ΔH_B, and ΔS_B between $AraP_2$ and $PenP_2$ is obtained (−1.5 kcal mol^{-1}, +2 kcal mol^{-1}, and +11 cal deg^{-1}, respectively). This agreement must be entirely fortuitous, however, since the binding of ligands probably results in the relocation of groups other than those that can be observed spectrophotometrically.

Calorimetric studies of the binding of charged ligands to enzymes have shown consistently that proton exchanges with the solvent are involved (Bolen *et al.*, 1971; Hinz *et al.*, 1971; Hunt *et al.*, 1972). The uptake of protons upon ligand binding to aldolase probably involves groups on the enzyme that increase in pK_a. It is less likely that the phosphate groups of ligands are involved as they probably increase in acid strength on binding. Conversely, groups on the enzyme, particularly those adjacent to the anion binding site, should become less acidic. Although lysine, cysteine, histidine, and N-terminal α-amino side chains could be involved, the imidazole groups of histidine are the most probable candidates (Hoffee *et al.*, 1967). If this is assumed to be the case, the thermodynamic parameters for protonation can be calculated to give an estimate of its contribution to the binding process. The measured values of n_B can be used with the molar values of imidazole protonation ($\Delta H_i = -8.6$ kcal mol^{-1} and $\Delta S_i = 2.6$ cal deg^{-1} mol^{-1}) to show that the contribution of protonation to

binding represents a large fraction of ΔH_B ($\geq 70\%$ for all ligands except for P_i and $PenP_2$, for which it is 35 and 50%, respectively). On the other hand, this contribution of protonation to ΔS_B is small ($\leq 20\%$) for all ligands except $PenP_2$ for which it is 40%. No further attempt to quantitate the process is justified as it is apparent that numerous compensating effects are involved which make contributions but cannot be estimated, such as solvation of both ligand and enzyme and hydrophobic interactions. The peculiar effectiveness of phosphate in causing proton binding cannot be rationalized.

Proton transfers are important in the catalysis of aldolization–dealdolization reactions and the alteration of the pK_a of a group that occurs on binding ligands to aldolase could facilitate these exchanges if the group is at the active site. At present, however, it appears that the proton binding or unbinding coupled with ligand binding is purely an electrostatic phenomenon. When positive ligands are bound, protons are released (Hunt *et al.*, 1972) and when negative ligands are bound they are absorbed (Bolen *et al.*, 1971; Hinz *et al.*, 1971).

In summary, data are presented that support earlier studies indicating that the enthalpies of binding of $AraP_2$ to muscle aldolase are negative at 37° and pH 7.5. Complete sets of thermodynamic parameters are presented for several inhibitors that demonstrate that significant differences exist in the contribution of ΔH_B and ΔS_B to binding and that these cannot be related easily to differences in structure of the ligands or to their known interactions with the enzyme. In addition, the binding of protons that occurs with ligand binding is shown to be sensitive to ligand structure. It is clear that, at least in the case of aldolase, a detailed interpretation of the ΔG_B values (or K_I values) of ligand binding in terms of structural differences will not often be warranted since significant differences in the relative contributions of enthalpy and entropy underlie very similar free-energy values.

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