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COMPARISON OF VAN 'T HOFF AND CALORIMETRICALLY DETERMINED ENTHALPIES OF BINDING OF N-PHOSPHONACETYL-L-ASPARTATE TO E. COLI ASPARTATE TRANSCARBAMYLASE *

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A comparison has been made of the values obtained by direct calorimetric measurements and van 't Hoff analysis, under similar conditions, for the enthalpy of binding of the bisubstrate analog N-phosphonacetyl-L-aspartate (PALA) to E. coli aspartate transcarbamylase and its catalytic subunit. In the case of the catalytic subunit, data were obtained at both saturating and non-saturating concentrations of L-Asp, and at two ionic strengths. Despite a 1000-fold difference in protein concentrations, and the obligatory omission of carbamyl phosphate in the calorimetric experiments, the values obtained by the two methods are shown to agree to within 15% when appropriate corrections are made. These results suggest that subunit dissociation is not a significant factor at the low protein concentrations used in the van 't Hoff analysis, and, conversely, that aggregation of the protein is negligible at the high protein concentrations used in the calorimetric experiments. They also imply that, at pH 8.3, the enthalpic difference between the two conformational states of the enzyme which exist in the presence and absence of substrates is less than 2.5 kcal/mol. In addition, the trends in the three sets of data for the catalytic subunit indicate that ionic bonds are involved in binding PALA to the active site, and that non-productive binding by L-Asp is negligible under these experimental conditions.

1. Introduction

In analyzing the energetics of a protein-ligand interaction, it is frequently desirable to determine the enthalpy of binding both by direct calorimetric measurements and by a van 't Hoff analysis, since the limitations of one method are often the strong points of the other. The principal limitation of calorimetry is the high concentrations of protein which must be used in order to produce a signal which is detectable with current instrumentation. This, in turn, may limit either the extent to which the linkage between subunit association and ligand binding is accessible to analysis or the solvent conditions which can be used. Furthermore, when the net state of ionization of the complex is different from that of the dissociated species and high concentrations of buffer are used to maintain constant pH, the observed heat effects will include a substantial contribution from the heat effects associated with binding or release of protons by the buffer.

In contrast, the data for a van 't Hoff analysis of ligand binding can often be obtained with microgram quantities of protein and measurements made over a wide range of experimental conditions. Furthermore, the heat effects associated with binding or release of protons by the buffer do not contribute to the van 't Hoff enthalpy. However, while a single calorimetric measurements yields $\Delta H_{binding}$ directly (when corrections for buffer effects have been made), the van 't Hoff analysis requires that a set of data, over a range of temperatures, be interpreted in terms of a model. This requirement not only makes the van 't Hoff analysis more time-consuming, but also, more importantly, imposes the restriction that the van 't Hoff enthalpy is only as good as the model on which it is based. This apparent disadvantage can, however, actually be

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a most useful feature in that the degree of agreement between the results of the van 't Hoff analysis and other studies can be used to test the validity of various plausible models. The discrepancy between ΔH_{vH} and ΔH_{cal} for the polymerization of bacterial flagellin [1], β-lactoglobulin [2], tubulin [3] and D-amino acid oxidase [4] have been considered from this point of view.

We have recently reported the results of a calorimetric study of the binding of the bisubstrate analog N-phosphonacetyl-L-asparate (PALA) ‡ to E. coli aspartate transcarbamylase (c_6r_6) and its catalytic subunit (c_3) [5]. These measurements, which were made at pH 8.3, protein concentrations of 5–10 mg/ml and buffer concentrations of 0.1 M, yielded values for $\Delta H_{binding}$ of −8.5 ± 1 and −11.3 ± 0.7 kcal/mol for c_6r_6 and c_3, respectively. They also indicated that complex formation requires that protons be transferred from the buffer to the complex: 0.98 ± 0.12 mol H^+/mol active site in the case of c_6r_6, and 0.75 ± 0.09 mol H^+/mol active site in the case of c_3.

We have now carried out a van 't Hoff analysis of the binding of PALA to c_6r_6 and c_3, using the inhibition of the enzyme-catalyzed reaction to measure PALA binding. In the case of c_3, measurements were made at two concentrations of L-Asp and at two ionic strengths. At the same time, the apparent heats of binding and the magnitude of the proton effects accompanying complex formation were determined directly, under nearly identical conditions, by flow microcalorimetry and potentiometry, respectively. These measurements allow the effects of the high protein and buffer concentrations used in the previous study [5] to be evaluated. They also make it possible to compare values for $\Delta H_{binding}$ obtained in the presence and absence of substrates, and hence provide an estimate of the enthalpic difference between the corresponding conformational states of the enzyme. Finally, they allow the question of nonproductive binding by L-Asp [6] to be addressed, and provide new evidence for the involvement of ionic interactions in binding.

‡ The following abbreviations have been used: PALA, N-phosphonacetyl-L-aspartate; c_6r_6, native E. coli aspartate transcarbamylase; c_3, catalytic subunit; CHES, 2-(N-Cyclohexylamino)ethanesulfonic acid.

2. Materials

The enzyme and its catalytic subunit were prepared from the derepressed, diploid strain provided by Dr. Howard Schachman (Virus Laboratory, University of California, Berkeley) and grown up at the New England Enzyme Center as previously described [5]. Protein concentrations were determined assuming extinction coefficients of 0.59 and 0.72 $(mg/ml)^{-1}$ for c_6r_6 and c_3, respectively, [7] and molecular weights of 310 000 and 100 000 [7]. The purity of both proteins, as determined by SDS-PAGE [8], and their specific activities, assayed by pH stat [5], were comparable to those of previous preparations [5,9].

PALA was the generous gift of Dr. George Stark (Department of Biochemistry, Stanford University). Its purity, as determined by ultraviolet difference spectroscopy [10], was found to be greater than 90%. The concentrations reported have been corrected for the impurities present. L-aspartate, carbamyl phosphate (97% pure, dilithium salt) and 2-[N-Cyclohexylamino] ethane sulfonic acid (CHES) were purchased from Sigma. Certified 1N HCl was obtained from Fisher Scientific. All other chemicals were reagent grade and were used without further purification.

3. Methods

The release of protons during the enzyme-catalyzed reaction was monitored on a Brinkmann pH stat, with the temperature maintained to within ±0.25°C with a Haake FE constant temperature circulator. Typically, 5 ml of L-Asp in distilled water containing the desired concentration of PALA (0–12 μM) was added to solid carbamyl phosphate (to give 0.5–11 mM carbamyl phosphate) and adjusted to pH 8.3. 50 μl of enzyme in distilled water was added, and the rate of proton release followed by titration with 10 mM NaOH. (In some experiments the final concentrations of c_3 was less than 1 μg/ml, concentrations at which a loss of activity with time has been reported [11]. However, under our conditions (reaction time ≤2 min), the activity was found to be constant, over the range 0.29–1.77 μg/ml.)

All calorimetric measurements were made with a

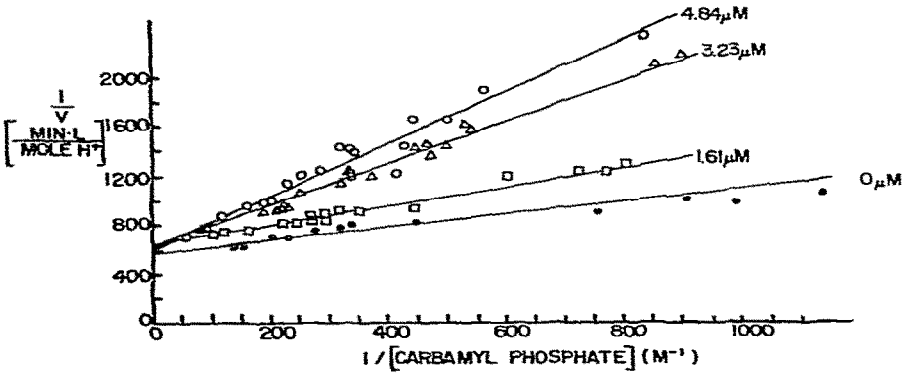

Fig. 1. Lineweaver-Burke plots of the initial velocities obtained with c_6r_6, pH 8.3, 25°C, with 30 mM L-Asp, and the concentrations of carbamyl phosphate and PALA indicated. Enzyme concentration 4 μg/ml.

LKB flow microcalorimeter submerged in a Tronac 1005 bath, as described previously [5]. ΔH_{ion} of both L-Asp and CHES was determined by both acid and base titration. In either case, 0.01–0.025 M solutions, the pH of which had been accurately determined, and which were 0.1 M in NaCl, were mixed with equal volumes of 0.005–0.010 M HCl or NaOH containing 0.1 M NaCl in the calorimeter. The heat evolved was measured, and the pH of the effluent solution determined. For the acid titrations, the initial pH ranged from 8–9.5, the final pH from 7–8.15. For the basic titrations, the initial pH was 7.5, the final pH 9.5–9.9. The pK values cited by Sigma for CHES (9.36) and by Sober [12] for L-Asp (10.00) and the initial and final pHs were used to calculate the changes in concentrations of the acidic and basic forms, which were in turn used to calculate ΔH_{ion} from the measured heat effect. For the titrations with NaOH, this required subtracting out the heat of formation of H_2O (–13.34 kcal/mol) [13]. Within the limits of precision of the measurements, the values obtained appeared to be independent of the method used and the initial and final pH values.

Proton uptake accompanying PALA binding was determined by back titration with 5×10^{-4} M HCl on a Brinkmann pH meter, with the temperature controlled to 25 ± 0.25°C.

4. Data analysis

Lineweaver-Burke plots of 1/v (in units of (mol H^+ released/hr-mol binding sites)$^{-1}$) versus 1/[carbamyl phosphate] were fit by linear least-squares analysis. The procedure described by Bevington was used to calculate the error in the slope [14]. K_i was also determined by linear least-squares analysis, from the dependence of the slope of the Lineweaver-Burke plots on the concentration of PALA, with each point weighted by the error in the slope. The error in K_i was determined by standard procedures [15] from the errors in the Y-intercept and slope. ΔH_{vH} was similarly determined from plots of ln K_i versus $1/T$ (K)$^{-1}$. Errors associated with ln K_i are necessarily non-symmetric about the mean because the function is logarithmic.

5. Results

Since the kinetic mechanism of the enzyme catalyzed reaction is bi-bi [16], with carbamyl phosphate binding first [11,17], and PALA has been shown to compete only with carbamyl phosphate [10], ΔH_{vH} for PALA binding can in principle be calculated from the temperature dependence of its K_i with respect to carbamyl phosphate. The measurements reported here were made at pH 8.3 at a fixed concentration of L-Asp, and varying concentrations of carbamyl phosphate, over the temperature range 20–35°C. Four sets of data were obtained: three sets for c_3 at (a) a saturating concentration of L-Asp (30 mM) (b) non-saturating L-Asp (5 mM), in the presence of 25 mM CHES to maintain the ionic strength (CHES was chosen because its pK is close to that of L-Asp, making the buffering effects of both species similar, and be-

cause it is a relatively large molecule which would not be expected to bind at the active site.) and (c) elevated ionic strengths (30 mM L-Asp + 70 mM NaBr). (NaBr was chosen to maintain the ionic strength because it does not appear to inhibit enzymatic activity [18].) In addition, one set of data was obtained for c_6r_6 at saturating L-Asp (30 mM), for purpose of comparison with (a).

Fig. 1 shows a typical Lineweaver-Burke plot, in this case for the set of c_6r_6 data obtained at 30 mM L-Asp and 25°C. It will be noted that the inhibition is largely competitive, and that, in spite of the well-known cooperative properties of this enzyme, the curves are essentially linear. This is not surprising in the case of c_3, for which cooperative binding of substrates has never been observed under any circumstances. In the case of c_6r_6, linear Lineweaver-Burke plots have been reported previously [19] at high concentrations of L-Asp (>8 mM at pH 7) and have been attributed to complete conversion of the enzyme to the R (relaxed) state.

Given the information which is presently available, the following would appear to be a reasonable model to use in analyzing these data:

$$E + \text{L-Asp} \overset{K_{ib}}{\longleftrightarrow} E \cdot \text{L-Asp} \quad (1)$$

$$E + \text{PALA} \overset{K_i}{\longleftrightarrow} E \cdot \text{PALA} \quad (2)$$

$$E + CP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot CP \quad (3)$$

$$E \cdot CP + \text{L-Asp} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E \cdot CP \cdot \text{L-Asp} \overset{k_5}{\longrightarrow} \text{Products} \quad (4)$$

The model assumes that the binding of substrates is ordered [17], and that PALA competes only with carbamyl phosphate [10]. Since nonproductive binding by L-Asp (reaction 1) has been considered as a possible basis for the increased values of K_d for PALA at high concentrations of L-Asp, it has been included at this point in order to assess its effect on the calculations. Finally, in spite of the oligomeric structure of both c_6r_6 and c_3, all binding sites have been assumed to be equivalent. This assumption is clearly well justified for c_3, which exhibits simple Michaelis-Menten kinetics under all conditions. It is a reasonable assumption for c_6r_6 at the high substrate concentrations used in this study, where the principal reactions will be the binding of carbamyl phosphate, L-Asp, or PALA to active sites on molecules on which all other binding sites are occupied:

$$\begin{array}{ccccc} & & & & c_6r_6 \cdot 5CP \cdot 6\text{L-Asp} \\ & & & & \uparrow\downarrow \text{(possible nonproductive binding by L-Asp)} \\ c_6r_6 \cdot 5CP \cdot 5\text{L-Asp} \cdot PALA & \overset{PALA}{\longleftrightarrow} & c_6r_6 \cdot 5CP \cdot 5\text{L-Asp} & & \end{array}$$

$$\overset{CP}{\longleftrightarrow} c_6r_6 \cdot 6CP \cdot 5\text{L-Asp} \overset{\text{L-Asp}}{\longleftrightarrow} c_6r_6 \cdot 6CP \cdot 6\text{L-Asp}$$

Defining

$$K_{ia} = k_2/k_1, \quad K_a = k_5/k_1, \quad K_b = (k_4 + k_5)/k_3,$$

$$v = k_5[E \cdot CP \cdot \text{L-Asp}],$$

making use of the steady state assumptions,

$$d[E + E \cdot \text{L-Asp} + E \cdot \text{PALA}]/dt = d[E \cdot CP]/dt$$

$$= d[E \cdot CP \cdot \text{L-Asp}]/dt = 0$$

and setting $E_0 = [E] + [E \cdot CP] + [E \cdot CP \cdot \text{L-Asp}] + [E \cdot \text{L-Asp}] + [E \cdot \text{PALA}]$, and $V_{max} = k_5 E_0$, it can be shown that:

$$\frac{1}{v} = \frac{K_b + [\text{L-Asp}]}{[\text{L-Asp}]V_{max}} + \frac{K_{ia}K_b + K_a[\text{L-Asp}]}{[\text{L-Asp}]V_{max}} \times \left(1 + \frac{[\text{PALA}]}{K_i} + \frac{[\text{L-Asp}]}{K_{ib}}\right)\frac{1}{[CP]}$$

$$= \alpha + \beta\left(1 + \frac{[\text{PALA}]}{K_i} + \frac{[\text{L-Asp}]}{K_{ib}}\right)\frac{1}{[CP]} \quad (5)$$

where

$$\alpha = \frac{K_b + [\text{L-Asp}]}{[\text{L-Asp}]V_{max}}, \qquad \beta = \frac{K_{ia}K_b + K_a[\text{L-Asp}]}{[\text{L-Asp}]V_{max}}$$

Hence, m, the slope of a Lineweaver-Burke plot at a fixed concentration of PALA, is equal to $\beta(1 + [\text{PALA}]/K_i + [\text{L-Asp}]/K_{ib})$ and a plot of m versus [PALA] has an x-intercept ($-K_i$(app)) of $-K_i(1 + [\text{L-Asp}]/K_{ib})$. (Since $m = \beta(1 + [\text{PALA}]/K_i + [\text{L-Asp}]/K_{ib})$,

$$[\text{PALA}]_{m=0} = -K_i(1 + [\text{L-Asp}]/K_{ib}) = -K_i(\text{app}). \quad (6)$$

If L-Asp were able to bind nonproductively, the term $[\text{L-Asp}]/K_{ib}$ in (6) would require that information on the temperature dependence of K_{ib} be obtained

Table 1
Comparison of apparent K_i values (μM) derived from Lineweaver-Burke plots, for PALA and c_3, at 30 mM L-Asp and 5 mM L-Asp + 25 mM CHES, as a function of temperature

Temp. (°C)	[L-Asp] (mM)	
	5	30
20		0.46 ± 0.37
21	0.59 ± 0.62	
25	0.80 ± 0.44	1.15 ± 0.16
30	1.89 ± 0.25	0.97 ± 0.12
34	3.63 ± 0.22	
35		3.49 ± 1.56

before ΔH_{vH} for PALA could be calculated. The evidence for nonproductive binding is, however, tenuous at this point; Jacobson and Stark [6] have simply shown that 200 mM L-Asp, in the absence of carbamyl phosphate, reduces the apparent K_i for PALA by two orders of magnitude. They point out that acetate has very similar effects, and that nonspecific effects due to increasing ionic strength may be a major factor.

Two lines of evidence suggest that nonproductive binding by L-Asp need not be considered under the conditions used in this study. First, the calorimetrically determined heat of binding of L-Asp to c_6r_6, in the absence of carbamyl phosphate, is negligible (Allewell, unpublished). Secondly, the values of $K_{i(app)}$ for c_3 at 5 mM and 30 mM L-Asp do not show the differences which would be expected if L-Asp bound nonproductivily with the reported K_{ib} of 3.3 mM [6]. These data are shown in table 1. While nonproductive binding would be expected to reduce K_i(app) at 5 mM L-Asp relative to K_i(app) at 30 mM L-Asp by approximately a factor of 6 (9.09/1.51), the average value of this ratio over the temperature range 20–35°C is in fact 1.2 ± 0.3. The apparent absence of nonproductive binding permits reaction (1) to be dropped from the kinetic scheme, and the term $[\text{L-Asp}]/K_{ib}$ to be deleted from eqs. (5) and (6).

Van 't Hoff plots of the four sets of data are shown in fig. 2, and the values of ΔH_{vH} derived from these plots are given in table 2. Three points are worth noting. First, the magnitude of ΔH_{vH} for c_3 is substantially larger (12.9 ± 8.2 kcal/mol) than for c_6r_6, determined under the same conditions. Since the difference in $\Delta H_{binding}$ between the subunit and native enzyme is equal to the change in the subunit interaction enthalpy produced by the binding of that ligand [5], this result indicates that the binding of PALA reduces the subunit interaction enthalpy substantially, and is consistent with the hypothesis that homotropic effectors exert their effects by reducing the strength of the subunit interactions. Secondly, the fact that the magnitude of ΔH_{vH} is reduced by 14.4 ± 10.4 kcal/mol when the ionic strength is increased by the addition of 70 mM NaBr suggests that the energetics of binding are highly dependent upon ionic strength and that nonspecific ionic strength effects may indeed account for some of the effects of high concentrations of L-Asp or acetate upon PALA binding [6]. Third, the agreement between the values of ΔH_{vH} at 5 mM and 30 mM L-Asp is consistent with our previous conclusion, that non-specific binding by L-Asp is not significant under these conditions.

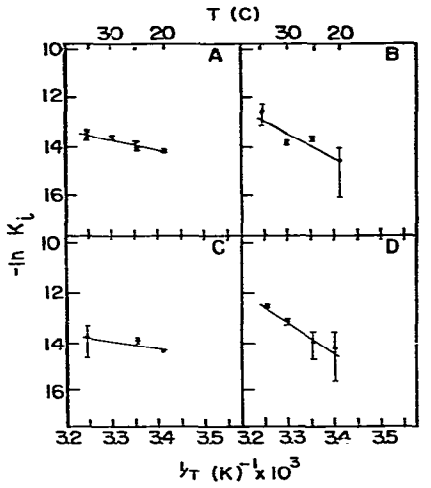

Fig. 2. Van 't Hoff plots of ln K_i versus $1/T$ (K)$^{-1}$, pH 8.3. (a) c_6r_6, 30 mM L-Asp; (b) c_3, 30 mM L-Asp; (c) c_3, 30 mM L-Asp + 70 mM NaBr; (d) c_3, 5 mM L-Asp + 25 mM CHES.

It was next of interest to evaluate the apparent enthalpy of binding (ΔH_{cal}) by calorimetry, under similar conditions. These results are shown in table 2. Although the only differences in the conditions used in the van 't Hoff and calorimetric experiments were the omission of carbamyl phosphate (to eliminate the enzyme-catalyzed reaction) and the elevated protein concentrations in the latter, the two sets of results are strikingly disparate; in all cases $\Delta H_{cal} \geqslant -4$ kcal/mol.

Table 2
$\Delta\bar{\nu}_{H^+}$, and a comparison of ΔH_{cal}, ΔH_{vH}, and corrected values of $\Delta H_{binding}$ (kcal/mol active sites)

Conditions	Protein	ΔH_{vH} a)	ΔH_{cal} b)	$\Delta\bar{\nu}_{H^+}$ c)	$\Delta H_{binding}$ d)
30 mM L-Asp	c_6r_6	−8.2 ± 1.4	1.43 ± 0.05	1.08 ± 0.08	−8.6 ± 0.8
30 mM L-Asp	c_3	−21.1 ± 6.8	−2.60 ± 0.25	1.01 ± 0.06	−12.0 ± 0.6
5 mM L-Asp, 25 mM CHES	c_3	−25.7 ± 2.8	−3.67 ± 0.16	1.67 ± 0.09	−20.1 ± 1
30 mM L-Asp, 70 mM NaBr	c_3	−6.7 ± 3.6	0.00 ± 0.25	0.66 ± 0.03	−6.3 ± 0.3

a) Binding enthalpy for PALA, derived by van 't Hoff analysis.
b) Apparent binding enthalpy, derived calorimetrically at 25°C, uncorrected for heat effects associated with the transfer of protons from solution to the protein-PALA complex.
c) Moles of protons transferred from solution to the protein-PALA complex, per mole active site at 25°C.
d) Binding enthalpy, derived calorimetrically, corrected for buffer effects, 25°C.

Since the formation of PALA-protein complexes is accompanied by proton binding [5], this discrepancy is to be expected, because the heat effects associated with removal of protons from solution contribute to ΔH_{cal} but not to ΔH_{vH}. Values for $\Delta\bar{\nu}_{H^+}$ (mol H^+ transferred from solution to the protein-PALA complex/per mol active site), obtained by potentiometric back titration, are given in table 2. As shown, $\Delta\bar{\nu}_{H^+} \geqslant 0.66$ in each case, increasing as the concentration of L-Asp and/or the ionic strength is reduced.

In order to correct ΔH_{cal} for buffer effects, the proton donating species must be identified and their enthalpies of ionization (ΔH_{ion}) determined. The contribution from ionizable groups on the protein will be negligible in all cases, since its concentration is less than 10^{-4} M. Hence, at 30 mM L-Asp and pH 8.3, the principal proton donor will be the α-amino group of L-Asp. Our calorimetrically determined value of ΔH_{ion} for this group is 9.30 ± 0.03 kcal/mole, at 25°C, in the presence of 0.1 M NaCl, in reasonable agreement with previously reported values [12]. When the concentration of L-Asp is reduced to 5 mM and 25 mM CHES is added, a contribution from CHES must also be considered. By manipulating $d[H^+] = d(([AH]/[A^-])K_d^A] = d(([BH]/[B^-])K_d^B)$, it can be shown that, in general, the ratio of the protons donated by two buffering species, A and B, is given by the relationship:

$$\frac{d(\ln [AH])}{d(\ln [BH])} = \frac{N_{AH}K_d^A}{N_{BH}K_d^B}, \qquad (7)$$

where

$$N_{AH} = \frac{[AH]}{[AH] + [A^-]}, \qquad N_{BH} = \frac{[BH]}{[BH] + [B^-]},$$

and K_d^A, K_d^B are the acid dissociation constants of the two species. Since the pK values of L-Asp [12] and CHES (Sigma) are 10.00 and 9.36, respectively, in the case being considered here, 4.8% of the protons will be donated by L-Asp, 95.2% by CHES. Our calorimetrically derived value of ΔH_{ion} for CHES is 9.87 ± 0.12 kcal/mol, a value significantly larger than would be expected from the value of ΔpK/°C (−0.009) given by Calbiochem.

Values for $\Delta H_{binding}$ derived by correcting ΔH_{cal} for the buffer effects are given in table 2. The agreement between the calorimetric and van 't Hoff values is excellent for c_6r_6, for the single set of experimental conditions examined, and for c_3, for the data obtained with 30 mM L-Asp and 70 mM NaBr. For the remaining two data sets, the discrepancy between the outer limits of the values set by the error estimates is approximately 3 kcal/mol.

6. Discussion

The general picture which emerges from this study is that the values of $\Delta H_{binding}$ derived by direct calorimetric measurements and by van 't Hoff analysis are in reasonable agreement, given the experimental complexity of the procedures used to derive ΔH_{vH} and the number of assumptions made in deriving the

two set of values. It appears, then, the model used to derive ΔH_{vH} is consistent with the data, that neither c_6r_6 nor c_3 dissociate at the low protein concentrations used for the van 't Hoff measurements, and, conversely, that the proteins do not associate at the high protein concentrations used in the calorimetric experiments (unless changes in states of aggregation occur with zero enthalpy changes). Furthermore, the values for $\Delta H_{binding}$ obtained in this study for both c_3 and c_6r_6, in the presence of 30 mM Asp, are in excellent agreement with those obtained previously [5] in the presence of 0.1 M Tris, Hepes, or Bicine. The agreement in $\Delta\bar{\nu}_{H^+}$ is equally good for c_6r_6, although somewhat less satisfactory for c_3. Hence neither L-Asp, nor any of the three buffers, appears to exhibit strong specific ion effects.

The degree of agreement between $\Delta H_{binding}$ and ΔH_{vH} for c_6r_6 is particularly noteworthy in view of the fact that carbamyl phosphate was present only for the van 't Hoff measurements. Since the binding of substrates is ordered [11], the difference in these quantities should correspond to the difference in enthalpy between the two conformational states of the enzyme which exist in the presence and absence of substrate. If the presence of substrates causes a complete shift from one state to the other, the fact that ΔH_{cal} and ΔH_{vH} agree within 0.4 ± 2 kcal/mol necessarily implies that the enthalpic difference between the two states at pH 8.3 is less than 2.4 ± 12 kcal/mol (6 × 0.4). In contrast, as discussed previously, the presence of PALA shifts the total subunit interaction enthalpy by several kcal/mol. Both of these observations are consistent with our previous work [5]. Together, they support Chan's contention, that c_3 is not a good model for the relaxed form of the enzyme [20].

Surprisingly, the two cases in which the discrepancy between ΔH_{vH} and $\Delta H_{binding}$ is larger than would be predicted from the estimated errors occur with c_3, for the measurements made in 30 mM L-Asp and in 5 mM L-Asp + 25 mM CHES. Since c_3 is generally believed to be noncooperative, these differences cannot be rationalized in terms of a difference in conformational states of the protein. While the relatively large error associated with ΔH_{vH} for the measurements made in 30 mM L-Asp suggests that the estimated value may be somewhat inflated, the 1000-fold difference in protein concentration may also contribute. Either a change in the properties of the solvent, or weak protein-protein interactions at the elevated concentrations used in the calorimetric experiments could account for the differences observed.

The trends in the data reported here provide some intriguing clues about the chemistry of the interaction between PALA and the active site. First, the strong dependence of $\Delta\bar{\nu}_{H^+}$ on the ionic composition of the medium is noteworthy; $\Delta\bar{\nu}_{H^+}$ ranges from 0.66 ± 0.03 in 30 mM L-Asp, 70 mM NaBr to 1.67 ± 0.09 in 5 mM L-Asp, 25 mM CHES. We have now carried out a detailed study of the pH dependence of proton binding [21], the results of which strongly suggest that these effects arise from electrostatic interactions at the active site which shift the pK values of both the phosphonate group and sidechains of residues on the protein by several units. If this is indeed the case, the variation in $\Delta\bar{\nu}_{H^+}$ with ionic conditions could be interpreted in terms of differences in the electrostatic shielding efficiencies of various ions, with L-Asp and CHES being less effective than Br^- because they are zwitterionic and larger. On the other hand, more complex possibilities, involving both specific ion binding and conformational transitions of the protein, such as appear to occur with ribonuclease [22], cannot be ruled out at this point.

A second point worth noting is the strong correlation between $\Delta\bar{\nu}_{H^+}$ and $\Delta H_{binding}$, which implies that the heat released in protonating the complex makes a substantial contribution to the overall enthalpy of binding. The results of the proton binding study [21] strongly support this conclusion, in that they indicate that, depending upon the ligand, 50–70% of $\Delta H_{binding}$ results from the protonation of groups on the protein.

However, values of $\Delta S_{unitary}$ calculated from the values of K_i and $\Delta H_{binding}$ given in tables 1 and 2 imply that ionic interactions cannot totally account for the energetics of binding. While interactions which are exclusively ionic would be expected to result in larger positive values of $\Delta S_{unitary}$, in two cases, $\Delta S_{unitary}$ is actually negative. (For c_3 in 30 mM L-Asp, $\Delta G = -14.0 \pm 0.3$ kcal/mol, $\Delta S_{unitary} = -4 \pm 1$ e.u.; in 5 mM L-Asp + 25 mM CHES, $\Delta G = -13.67 \pm 0.08$ kcal/mol; $\Delta S_{unitary} = -27 \pm 1$ e.u.) The most likely explanation of these values, and of the relatively small magnitude of the positive values obtained for the other two cases, is in terms of contributions from

either conformational changes induced in the protein by ligand binding, or the elimination of rotational degrees of freedom in PALA. A similar energy balance has been noted previously for succinate binding [5].

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