# Calorimetric Analysis of Aspartate Transcarbamylase from *Escherichia coli*. Binding of Substrates and Substrate Analogues to the Native Enzyme and Catalytic Subunit†

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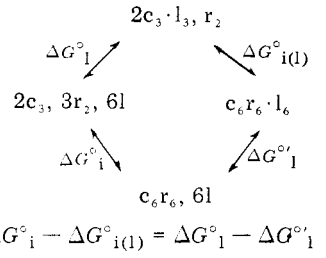
ABSTRACT: The energetics of binding of a series of substrates and substrate analogues to *Escherichia coli* aspartate transcarbamylase ($c_6r_6$) and its catalytic subunit ($c_3$) have been examined by flow microcalorimetry and difference spectroscopy. These measurements provide evidence for conformational transitions upon ligand binding and allow the energetics of these transitions to be characterized. Since various ligands have differing effects upon the subunit interaction energies, they also provide information on the interactions at the active site which are required to promote changes in the subunit interactions. The calorimetric data indicate that changes in the state of ionization of either the protein or its ligands occur upon complex formation. This effect is greatest for *N*-phosphonacetyl-L-aspartate (PALA) and carbamoyl phosphate (CP) + succinate and is maximal, for PALA and $c_6r_6$, between pH 7.8 and 8.3. Its significance in terms of the catalytic and regulatory mechanisms is considered. Although the binding of PALA and CP + L-malate produces substantial changes in the subunit interaction enthalpies, the enthalpic coupling between active and regulatory sites in the native enzyme appears to be less than 0.5 kcal/mol. Moreover, within the limits of precision of the measurements (±0.5 kcal/mol), $\Delta H_{binding}$ for PALA, at nonsaturating concentrations, is a linear function of its concentration. While this result does not rule out significant enthalpic coupling between active sites, it does eliminate the possibility that any conformational change resulting from the binding of the first molecules of PALA is accompanied by a large enthalpic effect.

A substantial body of evidence indicates the binding of ligands to either the catalytic or regulatory sites of *Escherichia coli* aspartate transcarbamylase induces transitions between conformational states. (For a recent review, see Jacobson & Stark, 1973.) While these transitions appear to be directly involved in both the catalytic and regulatory mechanisms, very little quantitative information on their nature is available at the present time. Important insights will undoubtedly come from crystallographic studies now in progress (Edwards et al., 1974). However, since both the catalytic and regulatory mechanisms depend ultimately upon free-energy differences between various states of the protein, an adequate understanding of both mechanisms will require not only structural but also thermodynamic information.

The allosteric properties of aspartate transcarbamylase do not appear to depend upon changes in the state of association of the subunits. However, the binding of effectors clearly alters the interactions between the subunits of the native enzyme (Jacobson & Stark, 1973). One measure of the effect of ligand binding on the energetics of these interactions is the difference between the energies of subunit association in the presence and absence of the ligand. (For recent discussions of energetic coupling, see Weber (1975) and Mills et al. (1976).) This difference can in turn be evaluated by comparing the energetics of binding of a particular ligand (l) to the native enzyme and its isolated subunits. Since the net energy change must be zero for the cycle:




$$\Delta G^\circ_i - \Delta G^\circ_{i(l)} = \Delta G^\circ_l - \Delta G^{\circ\prime}_l$$

The same relationships hold for $\Delta H$ and $\Delta S^\circ$.

In this study we have used this approach to evaluate the changes in the subunit interaction energies which accompany the binding of a series of substrates and substrate analogues. Enthalpies of binding, to both $c_6r_6$ and $c_3$, were determined calorimetrically for the series *N*-phosphonacetyl-L-aspartate (PALA),[1] carbamoyl phosphate (CP), phosphonacetic acid (PA), and the dicarboxylic acids, succinate, L-malate, and L-Asp (which bind productively only in the presence of CP (and possibly PA) (Jacobson & Stark, 1975)). In addition, for CP, PA, succinate, and L-malate (to $c_3$), $\Delta G^\circ_{binding}$ was determined by spectrophotometric titration. Substantial differences in the energetics of binding to $c_3$ and $c_6r_6$ were observed for several members of the series, from which the changes in the subunit interaction energies[2] in $c_6r_6$ which occur as a result of binding can be evaluated. Moreover, within the series, a spectrum of effects was observed, indicating differences in the ability of these ligands to promote conformational changes, and providing information on the coupling between interactions at the active site and between subunits.

Since the function of the conformational transitions induced

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[1] Abbreviations used are: $c_6r_6$, aspartate transcarbamylase; $c_3$, catalytic subunit; $r_2$, regulatory subunit; PALA, *N*-phosphonacetyl-L-aspartate; CP, carbamoyl phosphate; PA, phosphonacetic acid; Hepes, *N*-2-hydroxyethylpiperazineethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

[2] The observed changes in subunit interaction energies may include contributions both from changes in the contact regions between subunits, and from changes in the tertiary and quaternary structure of the subunits themselves.

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by ligand binding is to alter the binding properties of the catalytic and regulatory sites, it is of interest to compare the magnitudes of the changes produced by a particular ligand in subunit interaction energies and binding energies. Here we have evaluated the changes in nucleotide binding enthalpies which result from the binding of PALA to $c_6r_6$, and obtained preliminary information on the enthalpic coupling between active sites from the thermal titration curve for PALA. These results indicate that the changes in the enthalpies of the subunit interactions are much greater than the changes in binding enthalpies which result from them.

## Materials and Methods

(a) *Chemicals.* PALA was synthesized according to the method of Collins & Stark (1971). The purity of the product was assessed by titrating both $c_6r_6$ and $c_3$, using ultraviolet difference spectroscopy to monitor complex formation (Collins & Stark, 1968). Assuming the number of binding sites for $c_6r_6$ and $c_3$ to be 6 and 3, respectively, the expected sharp break in the plot of $\Delta OD_{290}$ vs. [PALA] was observed, since $K_d$ <<< [binding sites], when [PALA] = [binding sites]. (The data for $c_6r_6$ are shown in Figure 4.) In addition, identical difference spectra were obtained with our preparation and a sample of known purity (73%, established by elemental analysis) provided by Dr. George Stark, when the degree of purity of the Stark sample was taken into account.

PA was prepared from the triethyl ester by hydrolysis in 6 M HCl for 3 h under reflux, and purified by chromatography on Dowex 50W-X8, 200–400 mesh, with distilled water as the eluent. Difference spectroscopy in 0.1 M Tris-acetate, 2 mM $\beta$-mercaptoethanol, 0.2 mM EDTA, at pH 8.3 and 28 °C, yields a $K_d$ of 13.8 ± 0.2 $\mu$M for $c_3$, a value substantially lower than the $K_i$ obtained by enzymatic assay in 0.2 M Tris-acetate at pH 8 and 28 °C (320 $\mu$M; Porter et al., 1969).

All other chemicals were reagent grade. The CP, from Sigma, was of 97% purity, and was used without further purification.

(b) *Preparation and Characterization of Enzyme.* The enzyme was prepared, using the procedure of Gerhart & Houloubek (1967), from cells of the derepressed, diploid strain which were provided by Dr. Howard Schachman. The facilities of the New England Enzyme Center were used through the heat step. The original growth medium (Gerhart & Houloubek, 1967) was used, rather than the enriched medium generally used at the Center, to avoid possible heterogeneity in the CP binding sites (Ridge et al., 1976). The purified enzyme was stored at 4 °C under 2.2 M $(NH_4)_2SO_4$, 0.04 M Tris-acetate, pH 8.3, in the presence of 2 mM $\beta$-mercaptoethanol, 0.2 mM EDTA.

The homogeneity of the preparation was evaluated by polyacrylamide gel electrophoresis using the procedures of Weber & Osborn (1969) and Davis (1964). The sodium dodecyl sulfate gels showed only the bands corresponding to the c and r chains. The gels run under nondenaturing conditions showed, in addition to the major band, the normal pattern of two minor bands, corresponding to an aggregate of the native enzyme (Nelbach et al., 1972) and $c_6r_4$ (Yang et al., 1974), respectively. The concentrations of these impurities did not exceed 5%.

$c_3$ was prepared by dissociating the enzyme with neohydrin (1-(3-chloromercuri-2-methoxypropyl)urea) [6.3 mg of neohydrin/100 mg of $c_6r_6$ in 0.01 M Tris-acetate, pH 8.6; reaction time 10–15 min at room temperature (Ying Yang, personal communication)] and separating the subunits by chromatography on DEAE-cellulose (Gerhart & Houloubek, 1967). The purity of the preparation was confirmed by disc gel electrophoresis (Weber & Osborn, 1969). $c_3$ was stored at 4 °C as an $(NH_4)_2SO_4$ precipitate, with 2 mM $\beta$-mercaptoethanol and 0.2 mM EDTA present.

Protein concentrations were determined on a Varian 635 spectrophotometer assuming extinction coefficients of 0.59 and 0.72 $(mg/mL)^{-1}$ for $c_6r_6$ and $c_3$, respectively (Gerhart & Houloubek, 1967), and molecular weights of 310 000 and 100 000.

Enzymatic activity was routinely assayed in distilled water at 25 °C and pH 8.3 on a Brinkman pH stat, in the presence of 30 mM L-Asp and 4.8 mM CP. Under these conditions the turnover number of the enzyme was $1.3 \pm 0.1 \times 10^5$ $min^{-1}$.

(c) *Calorimetric Measurements.* Heats of binding were measured with the LKB flow microcalorimeter, LKB 10700-1, modified so that the calorimetric head, housed in a water-tight container (built by Mr. Earl Sandbeck of Johns Hopkins University, according to Zimmer's (1971) specifications), could be submerged in a Tronac 1005 water bath thermostated with a Tronac 1040 proportional temperature controller. A Keithley 150 B microvoltammeter was used to amplify the signal which was displayed on a Linear Instruments 252/MM chart recorder. The instrument was calibrated electrically from the LKB 10700 control unit; a calibration constant of 3.60 ± 0.01 $\mu$cal/(s $\mu$V) was obtained at 25 ° C.

Enzyme solutions to be used for calorimetric measurements were exhaustively dialyzed against the appropriate buffer. The dialysate was used to prepare the solution of the ligand, with the pH adjusted, if necessary, to within 0.02 pH unit of the enzyme solution with NaOH at 25 °C.

Solutions were pumped into the calorimeter by means of LKB 10200 Perpex peristaltic pumps fitted with silicone rubber pump tubing (i.d., 1.1 mm). Flow rates were determined from the time required to pump 4–5 mL of fluid from a microburet which was fitted with a vapor trap to prevent evaporation. The flow rates obtained varied from 1.86 to 2.02 $\mu$L/s, depending on the tubing used, but by less than 0.02 $\mu$L/s, for any particular length of tubing.

In a typical experiment, a baseline was first established by flowing a solution of the ligand through one channel of the mixing cell, and the dialysate through the second. When a satisfactory baseline had been obtained, the dialysate was replaced with the protein solution. After a new steady state had been achieved, the protein solution was replaced with dialysate so that any baseline drift could be detected and corrected for.

Heats of dilution of the protein, which were used to correct the experimentally determined heats, were determined in a similar sequence, with the exception that the solution of the ligand was replaced with dialysate. These heats were generally negligible.

(d) *Difference Spectra.* Difference spectra were recorded on a Varian Techtron 635 double beam spectrophotometer using quartz split cells. $\Delta OD_{290}$ was calculated relative to a baseline drawn from 310 to 285 nm (Collins & Stark, 1968).

(e) *Buffers.* All experiments were performed in 0.1 M Tris-acetate, $Na^+$-Hepes, or $Na^+$-Bicine, with the pH adjusted to 8.30 ± 0.02 at 25 °C, in the presence of 2 mM $\beta$-mercaptoethanol and 0.2 mM EDTA.

(f) *Analysis of Data.* (i) Calorimetric. The purpose of making measurements in three buffer systems is, first, to establish that specific ion effects do not significantly influence the observed heat effects, and, secondly, to evaluate the contribution of heat effects resulting from changes in the state of ionization of either the protein or the ligand, as a result of complex formation. Since $\Delta H_{obsd} = \Delta H_{binding} + \Delta\bar{\nu}_{H^+}\Delta H_{HB}$

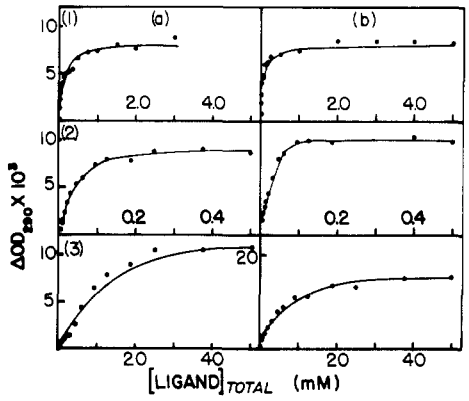



FIGURE 1: Spectrophotometric titrations of (a) $c_6r_6$ and (b) $c_3$ with CP, PA, and succinate (in the presence of 1 mM CP) in 0.1 M Tris-acetate, pH 8.3, 25 °C (2 mM β-mercaptoethanol, 0.2 mM EDTA present). Actual protein concentrations: $c_6r_6$, 2.4–2.6 mg/mL; $c_3$, 1.9–2.0 mg/mL. $\Delta OD_{290}$ normalized to 5 mg/mL for $c_6r_6$, 3.33 mg/mL for $c_3$: (1) PA; (2) CP; (3) succinate. The curves were generated from the dissociation constants derived by least-squares analysis, assuming a single class of binding sites. These values were: $c_6r_6$—PA, $5.2 \pm 1 \times 10^{-5}$ M; CP, $1.32 \pm 0.08 \times 10^{-5}$ M; succinate, $12.2 \pm 0.4 \times 10^{-3}$ M; $c_3$—PA, $1.38 \pm 0.2 \times 10^{-5}$ M; CP, $4.7 \pm 0.5 \times 10^{-7}$ M; succinate, $5.63 \pm 0.23 \times 10^{-3}$ M.

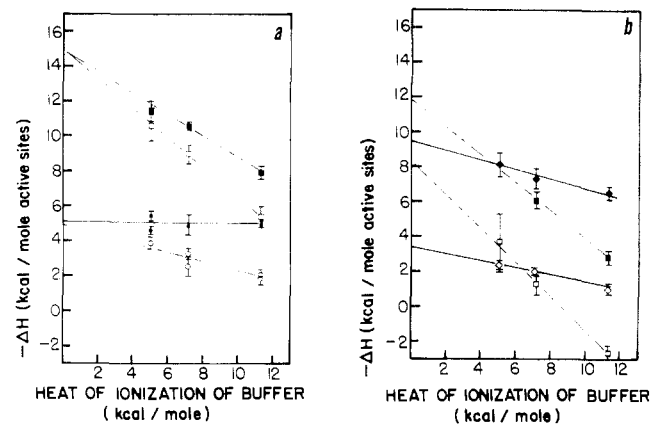



FIGURE 2: Apparent values of $\Delta H_{binding}$ in 0.1 M Hepes, Bicine, and Tris (25 °C) vs. the heat of ionization of the buffer. Protein concentration: 6.10 mg/mL. $\Delta H_{binding}$, corrected for ionization effects, is given by the $y$ intercept, the moles of proton consumed per mol of complex formed by the −(slope). The error estimates were calculated assuming a sensitivity of 0.1 μcal/s and the points fit by least-squares analysis. (a) (---) Succinate (60–95 mM) + CP (4.8 mM); (■) $c_3$; (□) $c_6r_6$; (—) CP (4.3–4.8 mM); (●) $c_3$; (○) $c_6r_6$. (b) (- - -) PALA (1.15–1.7 × concentration of active sites); (■) $c_3$; (□) $c_6r_6$; (—) L-malate (60–115 mM) + CP (4.8 mM); (♦) $c_3$; (◇) $c_6r_6$.

(where $\Delta H_{obsd}$ is the experimentally determined binding enthalpy, $\Delta H_{binding}$ is the true binding enthalpy corrected for ionization effects, $\Delta H_{HB}$ is the enthalpy of ionization of the buffer, and $\Delta\bar{\nu}_{H^+}$ is the number of protons transferred from the buffer to the complex), $\Delta H_{binding}$ and $\Delta\bar{\nu}_{H^+}$ can be determined from plots of $\Delta H_{obsd}$ vs. $\Delta H_{HB}$. $\Delta H_{HB}$ for Tris and Hepes were assumed to be $+11.30 \pm 0.01$ kcal/mol (Öjelund & Wadsö, 1968) and $+5.01 \pm 0.25$ kcal/mol (Beres & Sturtevant, 1971), respectively. $\Delta H_{HB}$ for Bicine was found experimentally to be $+7.16 \pm 0.25$ kcal/mol.

The points on these plots were fit by linear least-squares analysis, with each plot weighted by the inverse of the square of its estimated error, which in turn was determined by the sensitivity of the calorimeter: 0.1–0.2 μcal/s. The estimated errors in $\Delta H_{binding}$ and $\Delta\bar{\nu}_{H^+}$ correspond to the standard deviations.

(ii) Spectrophotometric. Dissociation constants were obtained from spectrophotometric titration curves by varying $\Delta OD_{max}$ and $K_d$ iteratively until the variance between the observed and calculated values of ΔOD was minimized.

## Results

(a) *Energetics of Binding of Substrate Analogues to $c_3$ and $c_6r_6$.* In the first phase of this study, heats of binding were determined by flow microcalorimetry, in three buffer systems, at concentrations which would be expected to saturate the primary binding sites.[3] (As noted in the Materials and Methods section the purpose of making measurements in three buffer systems was to verify that specific ion effects did not seriously bias the results, and to detect and correct for heat effects resulting from changes in states of ionization upon complex formation.) The concentrations used were 70–1000 × the estimated values of $K_d$ for CP and PA, and 2–15 × $K_d$ for the dicarboxylic acids. For PALA, which binds extremely tightly to both $c_6r_6$ and $c_3$,[4] concentrations 15–70% in excess of the concentrations of active sites were used. In order to verify that the primary binding sites were indeed saturated under these conditions, the initial values of $\Delta H_{binding}$ were compared with the values obtained at 75 and 125% of the initial concentration. In all cases, the differences between the three sets of measurements fell within the normal limits of experimental error (±0.5 kcal/mol).

The apparent values of $\Delta H_{binding}$ for PALA, CP, CP + succinate, and CP + L-malate in each buffer system, obtained by averaging all of the results for that system, are shown in Figure 2. (For the experiments with two ligands, the points shown correspond to the heats observed with saturating concentrations of both ligands.) For each of these cases, the points for the three buffer systems define a line, indicating that the differences in the observed heat effects are the result of proton uptake by the complex rather than specific ion effects.

PA was originally selected as part of the series with the hope that it could be substituted for CP in examining the binding of the second substrate, L-Asp. However, the results obtained indicate that this approach is not likely to be fruitful. First, in contrast to CP, PA binds endothermically to $c_6r_6$, in all three of the buffer systems examined. Secondly, its binding may be influenced by specific ion effects, since the apparent values for $\Delta H_{binding}$, when plotted against the heat of ionization of the buffer, do not define a line. Third, the heat effects observed for succinate (60 mM) and L-malate (100 mM) were small and endothermic, rather than large and exothermic as was the case in the presence of CP. (Those for L-Asp (60 mM) were essentially zero.) All of this tends to suggest, that, although PA binds tightly to both $c_6r_6$ and $c_3$, as indicated by difference spectroscopy, it does not promote the same conformational

[3] $K_d$ values for CP, PA, and succinate (in the presence of CP) binding to both $c_3$ and $c_6r_6$ were determined by spectrophotometric titration, making use of the difference spectrum resulting from binding (Collins & Stark, 1968). These curves are shown in Figure 1. L-Malate produced a typical difference spectrum when bound to $c_3$ and a $K_d$ of 63 ± 13 mM was estimated. No difference spectrum was observed for $c_6r_6$, although 24 mM L-malate has been reported to half-saturate $c_6r_6$ at pH 7.4 (relaxation measurements; Hammes & Wu, 1971). The binding of succinate, L-malate, and L-Asp in the presence of PA might be expected, a priori, to be similar to L-Asp in the presence of phosphonacetamide, for which $K_d$ has been estimated at 43 mM (Collins & Stark, 1968).

[4] $K_d$ of $7 \times 10^{-8}$ M has been estimated for $c_3$, under conditions similar to those used here (Jacobson & Stark, 1975). (This value is, however, very dependent on the assumptions made with respect to nonproductive binding of L-Asp.) The $K_i$ for the native enzyme determined by enzymatic assay in distilled water at pH 8.3, 25 °C, appears to be about 3 times as large (N. M. Allewell and B. L. Knier, unpublished results).

TABLE I: $\Delta H_{binding}$ and Proton Effects Accompanying the Binding of Ligands to $c_6r_6$ and $c_3$.

| Ligand | $c_6r_6$ | | $c_3$ | |
|---|---|---|---|---|
| (a) | $\Delta H_{binding}$ (kcal/mol) | $\Delta\bar{\nu}_{H^+}$/mol of ligand bound | $\Delta H_{binding}$ (kcal/mol) | $\Delta\bar{\nu}_{H^+}$/mol of ligand bound |
| PALA | −8.5 ± 1 | 0.98 ± 0.12 | −11.3 ± 0.7 | 0.75 ± 0.09 |
| CP | −4.0 ± 0.6 | 0.18 ± 0.07 | −3.6 ± 0.6 | −0.11 ± 0.15 |
| PA | 1.7 ± 0.4 | 0.0 ± 0.1 | | |
| CP + succinate | −14.9 ± 0.4 | 0.85 ± 0.13 | −14.9 ± 0.6 | 0.61 ± 0.07 |
| CP + L-malate | −3.4 ± 0.3 | 0.21 ± 0.03 | −9.2 ± 0.9 | 0.24 ± 0.10 |
| (b) | $\Delta\Delta H_{binding}$ (kcal/mol) | $\Delta\Delta\bar{\nu}_{H^+}$/mol of ligand bound | $\Delta\Delta H_{binding}$ (kcal/mol) | $\Delta\Delta\bar{\nu}_{H^+}$/mol of ligand bound |
| Succinate (CP) | −10.9 ± 1 | 0.57 ± 0.19 | −11.3 ± 1.2 | 0.5 ± 0.2 |
| L-Malate (CP) | +0.6 ± 0.9 | 0.0 ± 0.1 | −5.6 ± 1.2 | 0.35 ± 0.25 |
| L-Asp (PA) | −0.1 ± 0.6 | 0.02 ± 0.08 | | |
| L-Malate (PA) | +2.2 ± 0.6 | 0.04 ± 0.07 | | |
| Succinate (PA) | +2.3 ± 0.6 | 0.23 ± 0.08 | | |

TABLE II: Proton Uptake Associated with PALA Binding to $c_6r_6$ and $c_3$ (Titration Data, 0.07 M NaOAc, 25 °C).

| | $\Delta\bar{\nu}_{H^+}$/PALA | |
|---|---|---|
| pH | $c_6r_6$ | $c_3$ |
| 6.5 | 0.39 ± 0.06 | |
| 7.0 | 0.64 ± 0.05 | |
| 7.8 | 1.01 ± 0.09 | |
| 8.3 | 0.938 ± 0.07 | 0.780 ± 0.018 |
| 8.8 | 0.869 ± 0.002 | |

changes as CP, and in its presence dicarboxylic acids bind quite differently. Hence although the information obtained for $c_6r_6$ is summarized in Table I, comparable experiments were not performed with $c_3$.

Values for $\Delta H_{binding}$ and the stoichiometry of proton transfer, derived by least-squares analysis of the calorimetric data, are given in Table I. The first point of interest is the relationship between the magnitude of $\Delta H_{binding}$ and the structure of the ligand. For example, although PALA would be expected to form the same bonds with the active site as CP + succinate, the linking together of the functional groups within a single molecule in PALA reduced $\Delta H_{binding}$ for both $c_3$ and $c_6r_6$ by 3.6 ± 1.3 and 6.4 ± 1.4 kcal/mol, respectively. Similarly the introduction of the $\alpha$-hydroxyl group in L-malate reduces $\Delta H_{binding}$, relative to succinate, by 5.7 ± 2.4 and 11.5 ± 1.9 kcal/mol for $c_3$ and $c_6r_6$, respectively. These effects suggest a relationship between $\Delta H_{binding}$ and the steric constraints upon the bound ligand which will be considered further in the Discussion.

There are also significant differences in the enthalpies of binding of PALA, and of CP + L-malate, to $c_3$ and $c_6r_6$. These differences imply that the binding of these ligands in turn alters the subunit interaction enthalpies. In contrast, the enthalpies of binding of CP, and of CP + succinate, to $c_3$ and $c_6r_6$ are very similar.

Finally, the proton effects are noteworthy, since Schmidt and his coworkers (Beard & Schmidt, 1973; Ireland & Schmidt, 1977; Mosberg et al., 1977) and Roberts et al. (1976) have suggested that the conformational change accompanying the binding of dicarboxylic acids during catalysis requires protonation. While the proton effects are small for CP, PA, and L-malate, the binding of both PALA and CP + succinate to both $c_6r_6$ and $c_3$ is accompanied by the transfer of more than 0.6 mol of protons from the buffer to the complex. In each case, the magnitude of the effect is greater for $c_6r_6$ than for $c_3$.

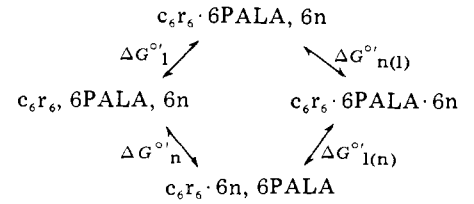
The pH dependence of the proton effects accompanying the binding of PALA to $c_6r_6$ was examined by direct titration (Table II). The results shown are averages of two-five determinations. The data at 8.3 agree with the calorimetric results, within the estimated error. As shown, the effect for $c_6r_6$ is maximal at pH 7.8–8.3.

In the case of CP, PA, succinate, L-malate, and PALA, values of $\Delta G°$, and hence $\Delta S°$, were derived either from the spectrophotometric titrations shown in Figure 1[5] or from literature values of $K_d$ obtained under conditions similar to those used here. These values, which are tabulated in Table III, reinforce the conclusions drawn from the calorimetric data. Again, structural alterations in the substrates result in substantial changes in the energetics of binding, and the effects of binding on the subunit interaction energies can be seen not only in $\Delta H$, but also in $\Delta S°$.

With respect to the subunit interaction energies, three patterns emerge. First, the binding of succinate alters neither the subunit interaction enthalpy nor entropy. CP, on the other hand, although generally considered to have only minor effects on the structure of both $c_3$ and $c_6r_6$ (Collins & Stark, 1968; Howlett & Schachman, 1977), produces a substantial change in the subunit interaction entropy, and hence free energy. Finally, L-malate and PALA, the two ligands which would be expected to be most sterically constrained when bound to the active site, alter both the subunit interaction enthalpy and entropy. The significance of these effects will be considered in the Discussion.

(b) *Enthalpic Coupling between Allosteric Sites.* Since the subunit interactions in $c_6r_6$ mediate interactions between allosteric sites, it is of interest to compare the changes in the subunit interaction energies produced by effector binding with the corresponding changes in binding energies.

Since the net energy change in the cycle:




[5] As noted in the figure legend, these data were fit to a model involving only a single class of binding sites. While the standard errors in $\Delta G°$ are not excessive, this can only be an approximation for the native enzyme, whose cooperative properties depend upon differences between classes of binding sites. A more detailed analysis is planned for the future, particularly for succinate, where there are clearly systematic differences between the observed and calculated values.

TABLE III: Thermodynamic Parameters for the Binding of Substrate Analogues to $c_6r_6$ and $c_3$.

| Ligand | $c_6r_6$ kcal (mol of ligand)$^{-1}$ $\Delta G°$ [a] | $\Delta H$ | $\Delta S°$ [a] (cal deg$^{-1}$ (mol of ligand)$^{-1}$) | $c_3$ kcal (mol of ligand)$^{-1}$ $\Delta G°$ [a] | $\Delta H$ | $\Delta S°$ [a] (cal deg$^{-1}$ (mol of ligand)$^{-1}$) |
|---|---|---|---|---|---|---|
| PALA | −9.1 ± 0.1 [b] | −8.5 ± 1 | 2 ± 4 | −9.7 [c] | −11.3 ± 0.7 | −5 ± 2 |
| CP | −6.63 ± 0.06 [d] | −4.0 ± 0.6 | 9 ± 2 | −8.6 ± 0.09 | −3.6 ± 0.6 | 16.7 ± 2.3 |
| PA | −5.82 ± 0.19 | 1.2 ± 0.6 | 24 ± 3 | −6.6 ± 0.1 | | |
| Succ (CP) | −2.60 ± 0.29 | −10.9 ± 1 | −27 ± 3 | −3.06 ± 0.04 | −11.3 ± 1.2 | −27.7 ± 4.2 |
| L-Malate (CP) | −2.2 [e] | 0.6 ± 0.9 | | −1.6 ± 0.1 | −5.6 ± 1.2 | −13 ± 4 |

[a] The differences between $\Delta S°_{unitary}$ and $\Delta G°_{unitary}$ and the values given here are +8 eu and −2.4 kcal/mol, respectively. [b] B. L. Knier and N. M. Allewell, unpublished results. Note that the $\Delta G°$ values for PALA are strongly dependent on the assumptions made with respect to nonproductive L-Asp binding. The values given here, for both $c_3$ and $c_6r_6$, are based on the analysis of Jacobson & Stark (1975). [c] Jacobson & Stark (1975). [d] Standard error. [e] pH 7.4, Hammes & Wu (1971). Calculated from the concentration required for half-saturation.

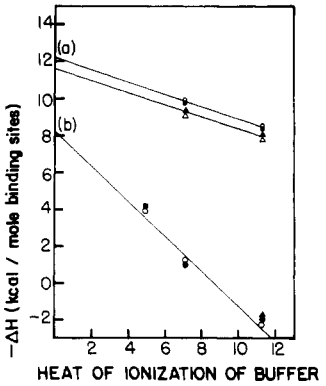



FIGURE 3: Enthalpic coupling between PALA and nucleotide binding. (a) Effect of PALA (750 $\mu$M) on $\Delta H_{binding}$ for CTP (0.7 mM) and ATP (5 mM) in 0.1 M Bicine and Tris, pH 8.3, 28 °C (2 mM $\beta$-mercaptoethanol, 0.2 mM EDTA present). The protein concentration was ~15 mg/mL for the ATP experiment, ~11 mg/mL for the CTP experiment: (▲) ATP, PALA present; (△) ATP, PALA absent; (●) CTP, PALA present; (○) CTP, PALA absent. Estimated error: ±0.5 kcal/mol. (b) Effect of CTP (0.7 mM) and ATP (5 mM) on $\Delta H_{binding}$ for PALA, in 0.1 M Tris, Bicine, and Hepes, pH 8.3, 28 °C (2 mM $\beta$-mercaptoethanol, 0.2 mM EDTA present). Protein concentration: 8 mg/mL. Total PALA concentration: 185–260 $\mu$M: (●) CTP present; (▲) ATP present; (○) no nucleotides present. Estimated error: Bicine, Tris, ±0.3 kcal/mol; Hepes, ±0.9 kcal/mol.

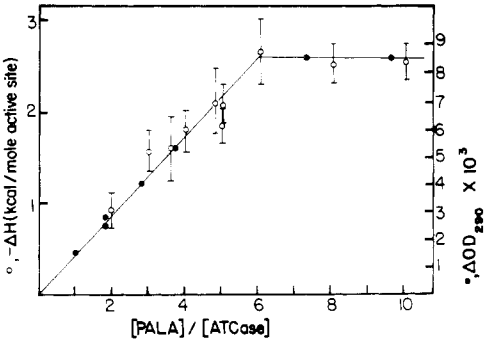



FIGURE 4: (○) Thermal titration of $c_6r_6$ (11.6–19.7 mg/mL) with PALA in 0.1 M Tris-acetate, pH 8.3, 2 mM $\beta$-mercaptoethanol, 0.2 mM EDTA, 25 °C. Error bars calculated from sensitivity (0.1 $\mu$cal/s). The lines, which were derived by least-squares analysis, intersect at [PALA]/[$c_6r_6$] = 5.95. (●) Spectrophotometric titration of $c_6r_6$ (1.87 mg/mL) under the experimental conditions described above.

(where n = CTP or ATP) must be zero, the energetic coupling between active and regulatory sites is given by either $\Delta G°'_n - \Delta G°'_{n(l)}$ or $\Delta G°'_{l(n)} - \Delta G°'_l$ (and the corresponding expressions for $\Delta H$ and $\Delta S°$). Gray et al. (1973) have already examined the binding of CTP and ATP in the presence and absence of PALA. The binding constants which they derive indicate that $\Delta G°$ for CTP binding decreases by no more than 0.4 kcal/mol in the presence of saturating PALA, and that $\Delta G°$ for ATP changes even less. This does not necessarily imply that the enthalpic coupling between the catalytic and regulatory sites is equally small. However, the data shown in Figure 3, which are in good agreement with our previous observations on CTP and ATP binding (Allewell et al., 1975), indicate that this is indeed the case and that $|\Delta H°'_{l(n)} - \Delta H°'_l|$ and $|\Delta H°'_n - \Delta H°'_{n(l)}|$ are significantly smaller than $|\Delta H°_i - \Delta H°_{i(l)}|$. In other words, a relatively large change in the subunit interaction enthalpies is required to produce a small, but functionally significant, change in binding enthalpies at the allosteric site.

(c) *Enthalpic Coupling between Active Sites.* In principle, the enthalpic coupling between catalytic sites in the native enzyme can be derived from the thermal titration curves for homotropic effectors. As shown in Figure 4, within the limits of precision of the calorimetric measurements, $\Delta H$ binding for PALA at nonsaturating concentrations is a linear function of its concentration. This result clearly rules out the possibility that the sole effect of the binding of the first molecule of PALA is to induce a concerted transition in which the conformation of $c_3$ shifts to that of isolated $c_3$ without complementary changes in $r_2$ (Changeux & Rubin, 1968). However, without information on the association constants for individual sites, required to determine to what extent the experimental values are averaged over various classes of sites, the possibility that there is significant enthalpic coupling between active sites cannot be excluded (see, for example, Atha & Ackers (1974)). The experimental uncertainty in the data is certainly large enough to allow for substantial differences in $\Delta H_{binding}$ to different classes of sites. Analogous experiments with other substrate analogues, which bind more weakly so that dissociation constants for individual sites can be determined, and with the more sensitive batch calorimeter, are required and are in progress.

It should, however, be noted that the heat effects associated with the absorption of protons by the PALA-$c_6r_6$ complex make a major contribution to the overall heat effects. Hence the linearity of the data indicates unambiguously that the proton effect is coupled directly to PALA binding. If the proton effects are also coupled to a conformational change in the protein, as Beard & Schmidt (1973) have suggested, it follows that this conformational change occurs as a direct consequence of PALA binding (that is, in a sequential, rather than concerted fashion).

## Discussion

This study is one of a series aimed at establishing thermodynamic criteria against which models of the catalytic and

regulatory mechanisms may be tested. In this discussion, we will consider the relationship between the thermodynamic data and the predictions of existing models.

The data given in Table III can generally be rationalized in terms of the model for the catalytic mechanism proposed by Stark and his coworkers (Collins & Stark, 1968). This model postulates that CP is bound to $c_3$ by (a) Coulombic interactions between its phosphate dianion and one or more cationic residues on the protein and (b) a hydrogen bond between its carbonyl group and a proton donor at the active site. Binding of CP is thought to result in a small conformational change, required for the productive binding of L-Asp, which is postulated to interact, via its carboxyl groups, with two positively charged groups on the enzyme. The binding of L-Asp results in a second conformational change, leading in turn to the formation of a hydrogen bond between its $\alpha$-amino group and a basic group in the enzyme, and, at the same time, bringing this $\alpha$-amino group into close contact with the carbonyl group of CP.

In terms of this model, the driving force for the binding of all of the ligands examined here would be expected to be a large positive entropy change, resulting from the formation of ionic bonds, aided by a small negative enthalpy change, from the formation of both ionic and hydrogen bonds (Kauzmann, 1959). These effects would be opposed by the negative entropic effect associated with the elimination of rotational degrees of freedom in either the ligand or groups at the binding sites (~4.5 cal/(deg mol df)$^{-1}$ (Page & Jencks, 1973)), and by the energetic changes accompanying local conformational changes in $c_3$ and more extensive conformational changes in $c_6r_6$.

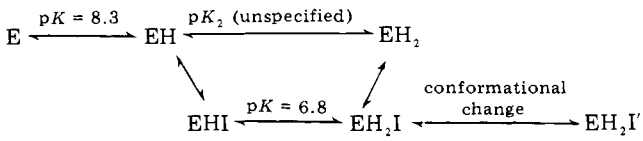
Considering first CP, the energetics of its binding to $c_3$ can be accounted for solely in terms of the formation of ionic and hydrogen bonds, without invoking conformational changes. In contrast, the reduced magnitude of $\Delta S°$ and hence $\Delta G°$ for $c_6r_6$, relative to $c_3$, suggests that significant restructuring of the native enzyme occurs upon binding. An alternative explanation is that the subunit interactions in $c_6r_6$ deform the active site so that CP is more constrained when bound to $c_6r_6$ than to $c_3$. The balance between these two effects would depend upon the relative deformability of the active site and the rest of the enzyme.

In contrast to CP, the binding of PALA, succinate, and L-malate, to both $c_3$ and $c_6r_6$, results not in the large positive entropy changes expected for the formation of hydrogen and ionic bonds, but in negligible or negative changes. As a result, the binding of these ligands is enthalpically driven. These effects can be rationalized in terms of the combined effects of the very tight steric constraints on bound succinate (Davies et al., 1970) and the conformational changes accompanying its binding. As above, the differences in the energetics of binding of L-malate and PALA, but not succinate, to $c_3$ and $c_6r_6$, are evidence that the first two ligands, but not succinate, alter the subunit interactions in $c_6r_6$. The more positive values of $\Delta H$ and $\Delta S°$ for $c_6r_6$, relative to $c_3$, would be consistent with either the formation of hydrophobic bonds or the breaking of hydrogen bonds in the protein upon binding of the inhibitor (Kauzmann, 1959).

The striking differences in the energetics of binding of PALA, CP + succinate, and CP + L-malate are among the most interesting results of the study, since in the past all three ligands have generally been considered to interact in very similar ways with the protein. The results presented here strongly suggest that the imposition of steric constraints upon the bound ligand (by the methylene group and the linkage between the two substrates in PALA, and by the $\alpha$-hydroxyl group in L-malate) has profound effects either on interactions at the active site, or on the conformational changes induced by binding. The effect of the $\alpha$-hydroxyl group in L-malate is particularly noteworthy. Although L-malate binds reasonably tightly to $c_3$, producing a normal difference spectrum, and has been reported to bind to and activate $c_6r_6$ in the pH range 7–7.4 (Hammes & Wu, 1971; Jacobson & Stark, 1975), we fail to observe a difference spectrum, a heat of binding, or a proton effect for $c_6r_6$ at pH 8.3. Either L-malate does not bind to the native enzyme under these conditions, or it interacts very differently with the native enzyme and catalytic subunit. Since this effect results from the presence of the $\alpha$-hydroxyl group, in place of the reactive $\alpha$-amino group of L-Asp, a more thorough understanding of its basis might shed considerable light on the catalytic mechanism.

A second point of interest is the difference in the energetics of binding of CP and PA. In contrast to CP, the binding of PA to $c_6r_6$ results in a small positive enthalpy change and a large positive entropy change. And the binding of dicarboxylic acids, in its presence, is endothermic. These results seem to indicate that PA is not able to bring about the conformational change required for productive binding by dicarboxylic acids. Instead, its two anionic groups may actually cause it to bind at the dicarboxylic acid site.

The data presented in Table II provide a test of models implicating ionizable groups in substrate binding and catalysis. Schmidt and his coworkers (Beard & Schmidt, 1973; Ireland & Schmidt, 1977; Mosberg et al., 1977) have recently proposed the following model for the binding of succinate to $c_6r_6$:




Roberts et al. (1976) have further suggested that part of the binding energy of either PALA, or L-Asp, is used to shift the p$K$ of the carbonyl group of either PALA, or CP, so that it becomes protonated at the time of the major conformational change.

As shown in Figure 5a, the proton uptake data for PALA can be fit reasonably well by a model involving only a single ionizable group, whose p$K$ shifts from 6.7 to 9.9 upon PALA binding, presumably because of electrostatic effects. This is a unique solution for a single ionizable group, since it can easily be shown that the p$K$ at which $\Delta\bar{\nu}_{H^+}$ is a maximum corresponds to the average of the two p$K$s, while the maximum value of $\Delta\bar{\nu}_{H^+}$ defines the difference between them.

Figure 5b shows the proton uptake effects predicted by the Schmidt model, for various values of p$K_2$. While none of the curves fits the PALA data, this is not sufficient to rule out the model, since the contribution from the CP moiety has not been included in the calculation. Rather, the model remains an intriguing one, because of the unexpected correspondence between the pH dependence of $\Delta\bar{\nu}_{H^+}$ and enzymatic activity (Weitzman & Wilson, 1966) for both $c_6r_6$ and $c_3$ (not shown). Measurements of the proton uptake effects which accompany CP and succinate binding are required and are in progress.

Turning now to the regulatory mechanism, the following thermodynamic information is available for $c_6r_6$. (1) Under conditions similar to those used in this study (0.1 M Tris-acetate, pH 8.5, 25 °C) $\Delta H_{binding}$ and $\Delta S°_{binding}$ for ATP are 3.9 ± 0.2 kcal/mol and 9.4 ± 0.5 eu, respectively, more positive than the corresponding quantities for CTP (Allewell et al., 1975). (2) $\Delta H_{binding}$ and $\Delta S°_{binding}$ for PALA are 2.8 ± 1.7 kcal/mol and 7 ± 6 eu, respectively, more positive than the corresponding quantities for $c_3$. (3) The enthalpic coupling

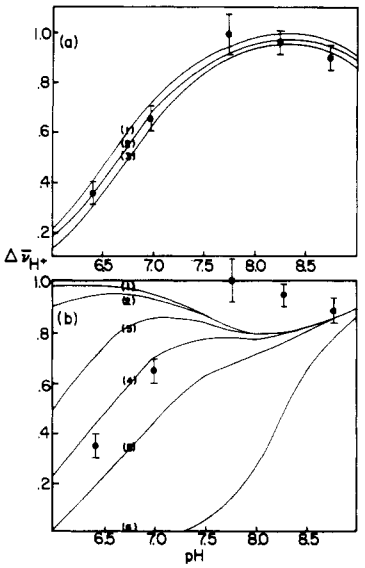



FIGURE 5: (a) Comparison of $\Delta\bar{\nu}_{H^+}$, the number of moles of protons absorbed per mole of PALA bound to $c_6r_6$, as a function of pH, with theoretical curves for proton uptake by a single ionizable group, whose p$K$ increases upon PALA binding: (1) initial p$K$, 6.6; final p$K$, 10.0; (2) initial p$K$, 6.7; final p$K$, 9.9; (3) initial p$K$, 6.8; final p$K$, 9.8. (b) Comparison of $\Delta\bar{\nu}_{H^+}$ with theoretical curves calculated from the model of Ireland & Schmidt (1977) using the equations for the distribution of the protein among various forms and the constants given by Mosberg et al. (1977); (1) p$K_2$ = 4.0; (2) p$K_2$ = 5.0; (3) p$K_2$ = 6.0; (4) p$K_2$ = 6.5; (5) p$K_2$ = 7.0; (6) p$K_2$ = 8.0.

between the catalytic and regulatory binding sites as determined by the change in $\Delta H_{binding}$ for PALA produced by nucleotides (or vice versa) is less than ±0.5 kcal/mol. (4) $\Delta H_{binding}$ for PALA, at nonsaturating concentrations, is a linear function of its concentration.

If, as has been suggested (London & Schmidt, 1972), ATP and CTP form the same bonds at the regulatory site, the differences in the energetics of their binding to the native enzyme would correspond to the differences in energies of the two conformational states to which they bind. The fact that these differences correspond approximately to the energetic differences for PALA binding to $c_3$ and $c_6r_6$ is consistent with a two-state model in which the properties of the relaxed form of the enzyme are similar to those of isolated $c_3$ (Changeux & Rubin, 1968). However, as discussed earlier, such a model is definitely ruled out by the negligible effects of PALA on $\Delta H_{binding}$ for nucleotides.

An alternative model for the regulatory mechanism has recently been proposed by Chan (1975), who defines c:r:r:c as the basic allosteric unit, and specifies that homotropic and heterotropic effects have different structural bases. The lack of enthalpic coupling between PALA and nucleotide binding is consistent with this model but does not constitute a rigorous test. Two other tests are planned for the future: a comparison of the energetics of binding of nucleotides to $c_6r_6$ and $r_2$ and an examination of the distribution of the changes in the subunit interaction energies over successive sites.

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