Leimgruber, W., and Weigele, M. (1972), *Science 178,* 871.
Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245,* 3565.
Warren, S. G., Edwards, B. F. P., Evans, D. R., Wiley, D. C., and Lipscomb, W. N. (1973), *Proc. Nat. Acad. Sci. U. S.*
Weber, G. (1968a), *in* Molecular Associations in Biology, Pullman, B., Ed., New York, N. Y., Academic Press, p 499.
Weber, K. (1968b), *Nature (London) 218,* 1116.
Weber, K. (1968c), *J. Biol. Chem. 243,* 543.
Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239,* 1415.
Wehrli, W. E., Verheyden, D. L. M., and Moffatt, J. G. (1965), *J. Amer. Chem. Soc. 87,* 2265.
Weigele, M., Blount, J. F., Tengi, J. P., Czajkowski, R. C., and Leimgruber, W. (1972a), *J. Amer. Chem. Soc. 94,* 4052.
Weigele, M., DeBernardo, S. L., Tengi, J. P., and Leimgruber, W. (1972b), *J. Amer. Chem. Soc. 94,* 5927.
Wiley, D. C., Evans, D. R., Warren, S. G., McMurray, C. H., Edwards, B. F. P., Franks, W. A., and Lipscomb, W. N. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 285.
Winlund, C. C., and Chamberlin, J. J. (1970), *Biochem. Biophys. Res. Commun. 40,* 43.
Wu, C.-W., and Stryer, L. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1104.
Yates, R. A., and Pardee, A. B. (1956), *J. Biol. Chem. 221,* 757.
Yguerabide, J. (1972), *Methods Enzymol. 26,* 498.

Calorimetric Analysis of Aspartate Transcarbamylase from *Escherichia coli:* Binding of Cytosine 5′-Triphosphate and Adenosine 5′-Triphosphate†

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ABSTRACT: The binding of CTP and ATP to aspartate transcarbamylase at pH 7.8 and 8.5 at 25° has been investigated by equilibrium dialysis and flow microcalorimetry. The binding isotherms for CTP at both pH 7.8 and 8.5 and ATP at pH 8.5 can be fit by a model which assumes three tight, three moderately tight, and six weak binding sites. The binding isotherms for ATP at pH 7.8 are best fit by a model which assumes six tight and six weaker sites. Both $\Delta H_{\text{binding}}$ and $\Delta S_{\text{binding}}$ are negative for both nucleotides at both pH values, so that the binding is enthalpy driven. For both nucleotides, ΔH is the same for the first two classes of binding sites, implying that the difference in the dissociation constants of these two classes of sites is the result of entropic effects. Direct pH measurements and calorimetric measurements in two buffers with very different heats of ionization (Tris and Hepes) indicate that the binding of both nucleotides is accompanied by the binding of protons. In the pH range 6.7–8.4, the number of moles of protons bound per mole of nucleotide increases as the pH decreases.

The allosteric regulation of enzymatic activity is generally believed to require changes in tertiary and quaternary structure. However, very little quantitative information is available at the present time on the nature or magnitude of these conformational changes. Crystallographic investigations now in progress will undoubtedly provide a substantial body of detailed structural information. However, our ability to elucidate from a crystal structure the thermodynamic principles which underlie it is still very rudimentary. Moreover the question of the extent to which the conformation of a protein differs in solution and in the crystal will be very difficult to resolve for proteins which are known to be conformationally flexible.

Because of these considerations, we have undertaken a comprehensive calorimetric analysis of aspartate transcarbamylase from *Escherichia coli,* an enzyme which has been widely used as a model system for investigating the mechanism of allosteric regulation. We hope to (a) establish thermodynamic criteria against which models of the catalytic and regulatory mechanisms may be tested, (b) evaluate the magnitude of the various conformational changes which the enzyme undergoes, and (c) clarify the nature of the forces involved in binding small molecules to the enzyme and in the interactions of its subunits.

The properties of aspartate transcarbamylase have been summarized in recent review articles (Gerhart, 1970; Jacobson and Stark, 1973). The enzyme catalyzes the first metabolic reaction unique to the synthesis of pyrimidine nucleotides, the transfer of the carbamyl group of carbamyl phosphate to the α-amino group of L-aspartic acid. At nonsaturating substrate concentrations, CTP inhibits enzymatic activity, while ATP is an activator (Gerhart and Pardee,

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1962). The native enzyme contains six Zn(II) ions (Rosenbusch and Weber, 1971) and 12 polypeptide chains (Weber, 1968), and can be dissociated into two catalytic and three regulatory subunits. The catalytic subunits bind substrates noncooperatively and are catalytically active, while the regulatory subunits bind nucleotides but have no catalytic activity (Gerhart and Schachman, 1965). The crystal structure of the enzyme is under investigation (Evans *et al.*, 1973) and several models of its structure have been proposed (Markus *et al.*, 1971; Cohlberg *et al.*, 1972). Conformational changes accompanying the binding of effectors have beem demonstrated by ultracentrifugation (Gerhart and Schachman 1968), optical rotatory dispersion (Dratz and Calvin, 1966), and circular dichroism (Griffin *et al.*, 1972, 1973), and by their effects on sulfhydryl reactivity (Gerhart and Schachman, 1968; Markus *et al.*, 1971) and rates of tryptic digestion (McClintock and Markus, 1969).

We present here thermal titration curves, obtained by flow microcalorimetry, of the native enzyme with CTP and ATP at pH 7.8 and 8.5. Two different buffer systems with quite different heats of protonation were used at pH 7.8, making it possible to detect a change in the state of protonation of the enzyme which accompanies nucleotide binding. This effect was verified and studied as a function of pH by direct titration. Binding curves were obtained by equilibrium dialysis under the same conditions used in the calorimetric experiments and are compared with the results of other binding studies (Cook, 1972; Matsumoto and Hammes, 1973; Gray *et al.*, 1973).

Material and Methods

(*a*) *Materials.* CTP and ATP (sodium salts), carbamyl phosphate (dilithium salt), and L-aspartic acid were obtained from Sigma Chemical Co.; [^{14}C]CTP (350 Ci/mol) [^{14}C]ATP, (30–40 Ci/mol), and Aquasol were from the New England Nuclear Corporation; and scintanalyzed dioxane, naphthalene, and 2,5-diphenyloxazole were from Fisher Scientific Company.

The concentrations of solutions of CTP and ATP were determined spectrophotometrically using extinction coefficients of 1.54×10^7 cm^2/mol at 259 mμ and 9.00×10^6 cm^2/mol at 271 mμ, respectively.

(*b*) *Preparation and Characterization of Enzyme.* Aspartate transcarbamylase was prepared according to the procedure described by Gerhart and Houloubek (1967) and stored at 4° in 0.1 M Tris-acetate, 2×10^{-3} M 2-mercaptoethanol, and 2×10^{-4} M EDTA (pH 7.8).[1] The homogeneity of enzyme solutions was checked by polyacrylamide gel electrophoresis using the procedures of Weber and Osborn (1969) and Davis (1964). While the SDS gels showed only the bands corresponding to the C and R chains, the gels run under nondissociating conditions showed two minor bands, comprising approximately 5% of the total protein. One migrated just ahead of the main band, the other considerably more slowly.[2]

Enzyme concentrations were determined on a Beckman DU spectrophotometer, assuming an extinction coefficient of 0.59 cm^2 mg^{-1} and a molecular weight of 310,000 (Gerhart and Houloubek, 1967).

Enzymatic activity was routinely assayed in distilled water at 28° and pH 8.3 on a Metrohm pH-Stat using 0.03 M L-aspartate and 0.06 M carbamyl phosphate. Under these conditions, the turnover number of the enzyme was 1.2–1.4 $\times 10^5$ min^{-1}. Enthalpy measurements were made at 25° in a flow modification of the Beckman Instruments Model 190 microcalorimeter (Sturtevant, 1969; Sturtevant and Lyons, 1969).

Before each experiment, an aliquot of the stock enzyme solution was passed through a G-25 Sephadex column preequilibrated with the buffer to be used, diluted with buffer to a concentration of 16 mg/ml, and deaerated. Flow rates of 0.0441 ml/min were used for each syringe throughout, producing rates of heat change in the range of 140–160 μcal/min at saturating concentrations of nucleotides. Heats of dilution of the nucleotide and enzyme solutions into buffer were obtained in each experiment and used to correct the observed heats of binding.

(*c*) *Equilibrium Dialysis Measurements.* Equilibrium dialysis was carried out at 25° in lucite cells with a total volume of either 0.6 or 1.2 ml, using Sartorius regenerated cellulose membranes with a pore size of 10–20 nm^2. An equilibration time of 5 hr was used. This was sufficient for cells set up with protein in one side and ATP in the other to reach equilibrium since the initial ATP gradient is very steep, but was not sufficient for CTP. Consequently, in the CTP experiments, the cells were set up with protein on one side and CTP on both sides at concentrations which preliminary experiments had indicated were close to equilibrium. Under these conditions the changes in nucleotide concentration were never greater than 10%.

Early experiments were performed at protein concentrations of approximately 24 mg/ml. At this concentration, it was necessary to correct for the migration of solvent into the protein compartment, and protein concentrations were determined before and after equilibration using a biuret procedure (Goa, 1953). To ensure that the results were independent of concentration, control experiments were carried out at protein concentrations of 8 mg/ml (in the case of CTP) and 16 mg/ml (in the case of ATP). At these concentrations, the inbibition of solvent was much less severe.

Duplicate 100-μl samples taken from each cell compartment were counted in 15 ml of scintillation fluid in a Beckman LS-250 scintillation counter. For experiments with Tris as buffer, a dioxane–naphthalene–2,5-diphenyloxazole scintillation fluid was used. Aquasol was used in experiments with Hepes[3] since Hepes reacts with the dioxane-based fluid producing a small amount of a brown precipitate which produced erratic quenching of the counts.

As a check on the system, the total number of counts in each cell before and after equilibration were compared. The number of counts after equilibration was generally 2–3% less than before, suggesting that a small amount of the nucleotide may be adsorbed by the membrane.

(*d*) *Titrimetric Determination of Protein Uptake.* These experiments were performed with protein concentrations of 10–18 mg/ml and nucleotide concentrations of 1.1–3.7 mM (CTP) or 3.2–7.3 mM (ATP), in the presence of 0.07 sodium acetate and 2×10^{-4} M EDTA. The titrant was 10^{-3} M HCl.

[1] The facilities of the New England Enzyme Center were used for growing up the bacteria.

[2] Similar patterns have been reported [Griffin *et al.* (1972); Eckfeldt *et al.* (1970)]. The slower moving minor band has been isolated by Griffin *et al.* (1972) and by us and has the properties of the aggregate described by Nelbach *et al.* (1972). The second minor band was reported by Eckfeldt *et al.* (1970) and has been shown by H. K. Schachman (personal communication) to lack one regulatory subunit.

[3] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

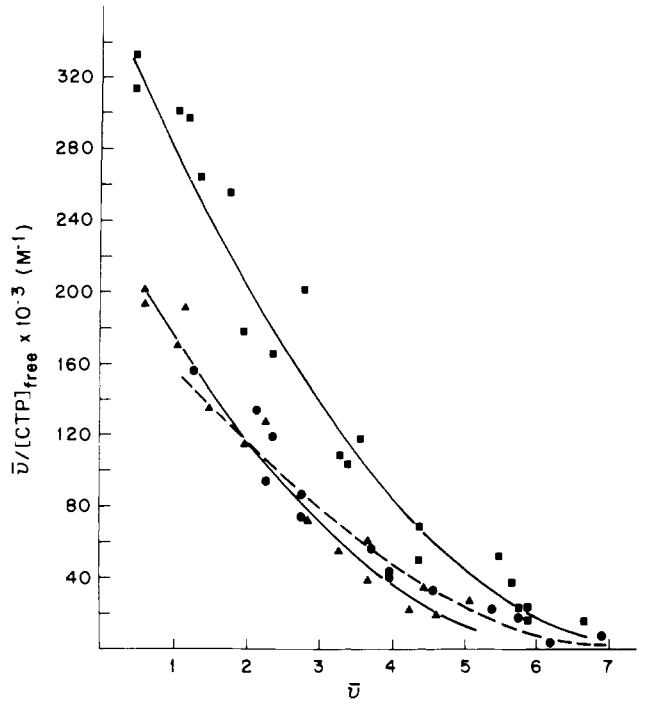

FIGURE 1: CTP binding isotherms at 25° where $\bar{\nu}$ is the number of moles of ligand bound per mole of enzyme of molecular weight 310,000 and $[CTP]_{free}$ is the concentration of free ligand: (●,- - -) 0.1 M Tris-acetate, pH 7.8, (▲,——) 0.1 M Tris-acetate, pH 8.5, (■) 0.1 M Na-Hepes (pH 7.8). All experiments were carried out in the presence of 2×10^{-3} M 2-mercaptoethanol, 2×10^{-4} M EDTA.

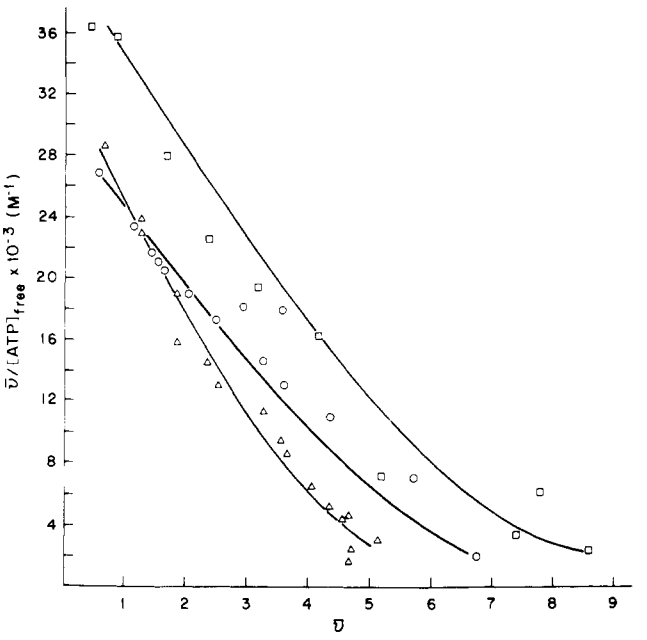

FIGURE 2: ATP binding isotherms at 25° where $\bar{\nu}$ is the number of moles of ligand bound per mole of enzyme of molecular weight 310,000 and $[ATP]_{free}$ is the concentration of free ligand: (○) 0.1 M Tris-acetate (pH 7.8), (△) 0.1 M Tris-acetate (pH 8.5), (□) 0.1 M Na-Hepes, (pH 7.8). All experiments were carried out in the presence of 2×10^{-3} M 2-mercaptoethanol, 2×10^{-4} M EDTA.

(*e*) *Data Analysis.* The nonlinear regression program of Cornish-Bowden and Koshland (1970) was used to obtain association constants from the equilibrium binding data.

Results

Scatchard plots of the binding data are given in Figures 1 and 2. These data are in good qualitative agreement with those of Cook (1972); Matsumoto and Hammes (1973); and Gray *et al.* (1973) for $\bar{\nu} > 0.5$. Quantitative agreement would not be expected because of differences in temperature, buffers, and pH. Moreover, we did not investigate binding at the very low CTP concentrations where positive cooperativity has been reported by Cook (1972).

The marked deviation from linearity in the range $\bar{\nu} < 6$ indicates that the association constants for nucleotide binding at the six regulatory sites differ. Both Winlund and Chamberlin (1970) and Matsumoto and Hammes (1973) concluded that the regulatory binding sites are divided into two classes of three each, a result which is consistent with the pairing of these sites in the regulatory subunits. Tondre and Hammes (1974) have recently presented kinetic evidence which suggests that the nonequivalence of the two classes of sites results from negative cooperativity in the binding of the second nucleotide to the regulatory dimer, rather than a difference in the two classes of sites in the free enzyme. $\bar{\nu}$ values greater than 6 at high nucleotide concentrations indicate that nucleotides bind weakly to sites other than the regulatory sites. This nonspecific binding apparently involves the carbamyl phosphate binding sites, since it is eliminated in the presence of carbamyl phosphate (Matsumoto and Hammes, 1973).

Three models were considered in fitting the binding data. The best fits of the CTP data at pH 7.8 were obtained assuming a model consisting of three classes of binding sites, with three sites in both classes of tighter binding sites and six in the third (model 1). Since the CTP and ATP data at pH 8.5 do not extend beyond $\bar{\nu} = 6$, these data were fit assuming two classes of binding sites, with three sites in each class (model 2). The ATP data at pH 7.8 could be fit satisfactorily only by assuming two classes of binding sites with six in each class (model 3). When model 2 was applied to these data, nearly equal values of K_1 and K_2 were obtained. from the linear regression program, so that the calculated Scatchard plots flattened at low $\bar{\nu}$ values. Values of K_1, K_2, and K_3 for model 2 can be found which fit the experimental curves ($K_1 = 10^4$, $K_2 = 2.7 \times 10^3$, $K_3 = 1.5 \times 10^2$); however, the calculated errors are unacceptably large.

In accordance with Tondre and Hammes' observation

Table I: Association Constants for the Binding of CTP and ATP to Aspartate Transcarbamylase.

Nucleotide	Buffer	K_1 (l./mol)	K_2 (l./mol)	K_3 (l./mol) $\times 10^2$
CTP	0.1 M Na-Hepes (pH 7.8)	$1.2 \pm 0.2 \times 10^5$	$1.6 \pm 0.2 \times 10^4$	3.8 ± 0.5
	0.1 M Tris-Ac (pH 7.8)	$6.6 \pm 1.9 \times 10^4$	$1.0 \pm 0.3 \times 10^4$	1.0 ± 0.4
	0.1 M Tris-Ac (pH 8.5)	$8.1 \pm 1.1 \times 10^4$	$7.0 \pm 0.9 \times 10^3$	
ATP	0.1 M Na-Hepes (pH 7.8)	$6.7 \pm 1.1 \times 10^3$	$6.7 \pm 1.1 \times 10^3$	2.6 ± 0.5
	0.1 M Tris-Ac (pH 7.8)	$5.0 \pm 0.2 \times 10^3$	$5.0 \pm 0.2 \times 10^3$	0.8 ± 0.2
	0.1 M Tris-Ac (pH 8.5)	$1.2 \pm 0.1 \times 10^4$	$1.2 \pm 0.1 \times 10^3$	

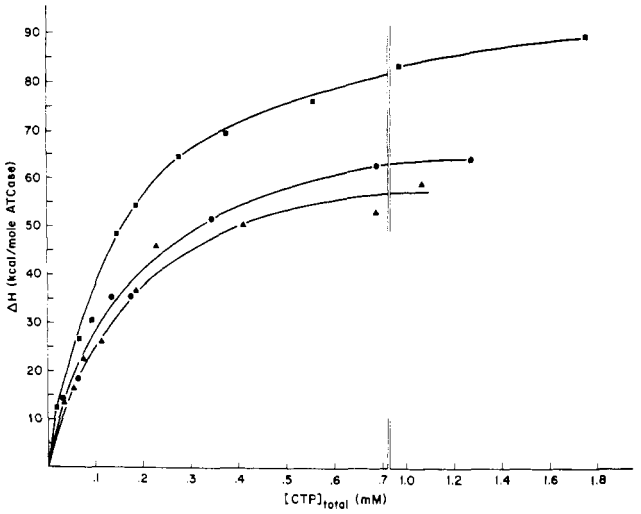

FIGURE 3: Thermal titrations of aspartate transcarbamylase with CTP at 25°. Enzyme concentrations 8 mg/ml (2.08×10^{-5} M). (●) 0.1 M Tris-acetate (pH 7.8), (▲) 0.1 M Tris-acetate (pH 8.5), (■) 0.1 M Na-Hepes (pH 7.8).

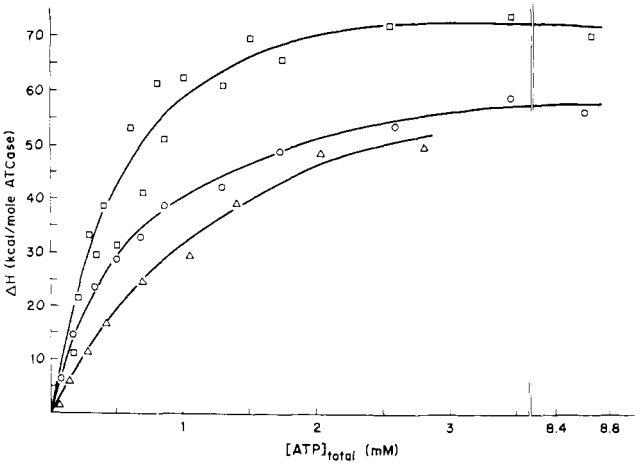

FIGURE 4: Thermal titrations of aspartate transcarbamylase with ATP at 25°. Enzyme concentrations 8 mg/ml (2.08×10^{-5} M) (○) 0.1 M Tris-acetate (pH 7.8), (△) 0.1 M Tris-acetate (pH 8.5), (□) 0.1 M Na-Hepes (pH 7.8).

(1974) the assumption was made for models 2 and 3 that binding at the two classes of regulatory sites is not independent; *i.e.*, that binding at the weaker binding site occurs only after the stronger binding site is occupied. Hence the equations giving $\bar{\nu}$ as a function of nucleotide concentration ([N]) for each of these models are

$$\bar{\nu} = \frac{3K_1[N] + 6K_1K_2[N]^2}{1 + K_1[N] + K_1K_2[N]^2} + \frac{6K_3[N]}{1 + K_3[N]} \quad (1)$$

$$\bar{\nu} = \frac{3K_1[N] + 6K_1K_2[N]^2}{1 + K_1[N] + K_1K_2[N]^2} \quad (2)$$

$$\bar{\nu} = \frac{6K_1[N]}{1 + K_1[N]} + \frac{6K_3[N]}{1 + K_3[N]} \quad (3)$$

The calculated values of the associated constants are tabulated in Table I. These values were used to derive the curves in Figures 1 and 2.

The slightly tighter binding of CTP at pH 7.8 in Na-Hepes than in Tris-Ac may result from the binding of anions of the buffer to the enzyme, since we find that, at non-saturating concentrations of L-aspartate, high concentrations of both acetate and Hepes inhibit enzymatic activity. (At pH 8.3, with saturating carbamyl phosphate and 0.01 M L-aspartate, both 0.1 M sodium acetate and 0.1 M Na-Hepes produce ~35% inhibition.) Inhibition is greater in the presence of CTP than in its absence, implying that this effect results from the binding of anions to the catalytic, rather than the regulatory, site. While the interaction of the protein with the buffer complicates the interpretation of the thermodynamic data to some extent, the wide variety of anions bound by the enzyme (Kleppe, 1966) makes it unlikely that a completely inert buffer could be found.

Thermal titration curves for the binding of CTP and ATP are presented in Figures 3 and 4. The binding of both nucleotides is exothermic for all of the conditions examined, with the heat effects for CTP about 30% larger than those for ATP. In both cases, the heat effects are greater at pH 7.8 than at pH 8.5, and greater in Hepes than in Tris.

Curves of ΔH *vs.* $\bar{\nu}$ are given in Figures 5–7. Values of $\bar{\nu}$ were obtained for each point on the thermal titration curves by varying $\bar{\nu}$ and calculating the concentration of free nucleotide until values which were consistent with the binding

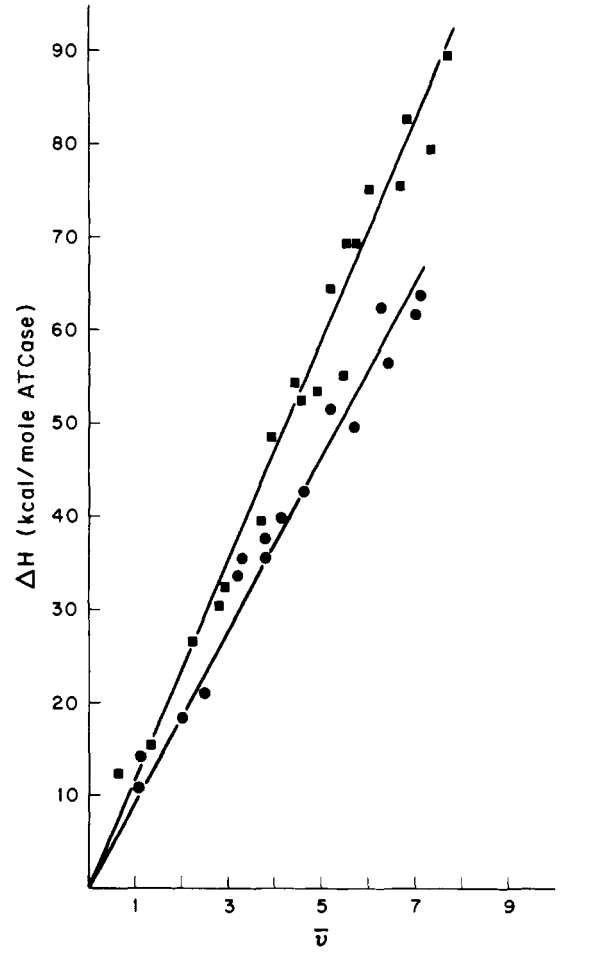

FIGURE 5: The dependence of $\Delta H_{binding}$ on the moles of CTP bound ($\bar{\nu}$) to aspartate transcarbamylase (pH 7.8). (○) 0.1 M Tris-acetate, (□) 0.1 M Na-Hepes.

data from the equilibrium dialysis experiments were obtained. In all cases the uncertainty in $\bar{\nu}$ is not greater than ±0.1. With the exception of the curves for ATP at pH 8.5, all of the curves are quite linear, indicating that there are no significant differences in the heat effects associated with binding to the various classes of sites. In the case of ATP binding at pH 8.5, the heat effects at low $\bar{\nu}$ appear to be significantly less than at high $\bar{\nu}$.

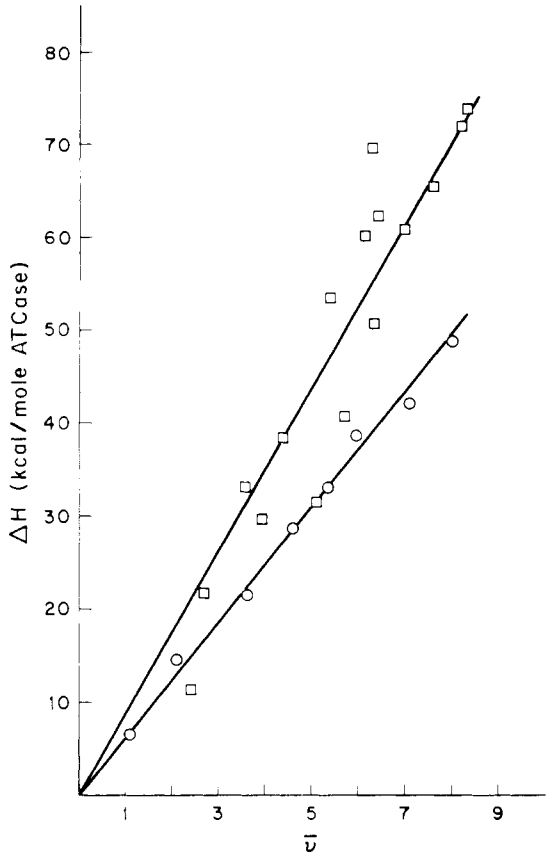

FIGURE 6: The dependence of $\Delta H_{\text{binding}}$ on the moles of ATP bound ($\bar{\upsilon}$) to aspartate transcarbamylase, pH 7.8. (○) 0.1 M Tris-acetate, (□) 0.1 M Na-Hepes.

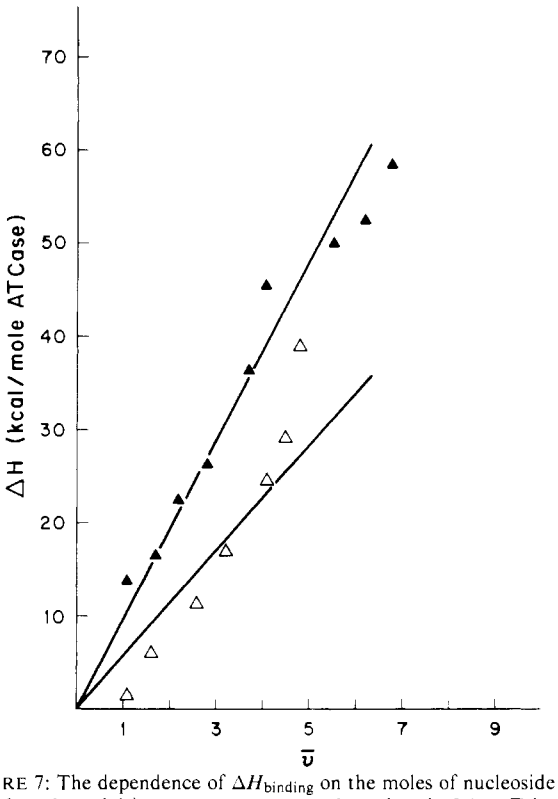

FIGURE 7: The dependence of $\Delta H_{\text{binding}}$ on the moles of nucleoside triphosphate bound ($\bar{\upsilon}$) to aspartate transcarbamylase in 0.1 M Tris-acetate (pH 8.5). (▲) CTP, (△) ATP.

Since the heat of ionization of Tris is 6.4 kcal/mol more positive than that of Hepes (Hinz *et al.*, 1971), the difference in the heat effects observed in the two buffers suggests that binding may be accompanied by the uptake of protons from the buffer. The magnitude of the difference is consistent with the uptake of 0.32 ± 0.03 mol of protons/mol of nucleotide bound. Direct pH-Stat titrations of proton uptake yielded values of 0.35 ± 0.03 and 0.28 ± 0.02 mol of protons/mol of nucleotide bound at pH 7.8 and 8.3, respectively. The magnitude of the effect increases as the pH is lowered; at pH 6.74 at a nonsaturating concentration of CTP (2.4×10^{-4} M), 4.1 mol of H^+ are taken up/mol of enzyme, implying that the pH of the group involved is less than 6.7.

The values of the thermodynamic parameters derived from the binding and calorimetric data are tabulated in Table II. The ΔH values were calculated from the slopes of the lines in Figures 5-7 and have been corrected for the heat effects resulting from the uptake of protons from the buffer. We estimate that these values have associated errors of 5-10%, as a result of the small heat effects being measured and variability in protein preparations. The cratic contribution to ΔS resulting from the binding of each mole of nucleotide is -8 cal deg^{-1} mol^{-1}.

Discussion

The most interesting aspect of these results is the similarity in the thermodynamic effects of CTP and ATP binding, in spite of their opposite effects on enzyme activity. The values of the thermodynamic parameters are the sum of the effects for two separate processes: the binding of nucleotides at the nucleotide binding site, and the conformational changes which are induced by their binding. If ATP and CTP bind to the same effector site and form the same bonds with the enzyme, as London and Schmidt (1972) have proposed, our data imply that the overall differences in $\Delta H_{\text{binding}}$ and $\Delta S_{\text{binding}}$ between the two conformations are small for both nucleotides. The similarity in the effects of ATP and CTP binding upon the rate of protease digestion (McClintock and Markus, 1968), sodium dodecyl sulfate induced dissociation (Coleman and Markus, 1972), and the mobility of a spin-label (Buckman, 1970) are consistent with this possibility. However, it is equally possible at this point that ATP and CTP interact differently with the effector site, and that the differences in the thermodynamic effects accompanying binding are cancelled out by reciprocal differences in the thermodynamic effects associated with the induced conformational change. Some difference in the interaction of CTP and ATP with the enzyme is indicated by our binding data; at pH 7.8, the values of K_1 and K_2 for the binding of ATP are very similar, while those for CTP are quite different. An analysis of the binding of nucleotides to the isolated regulatory subunit, in the absence of any changes in quaternary structure, should provide further insight into the extent of similarity between the binding of the two nucleotides.

The binding of CTP at both pH 7.8 and pH 8.5 and of ATP at pH 8.5 at both classes of binding sites is enthalpy driven, and is opposed by an unfavorable entropy change. In each of these cases, the difference in the tightness of the binding at the two classes of binding sites results from a difference in the magnitude of the entropy changes accompanying binding. The smaller decrease in entropy which accompanies binding at the class 1 sites may reflect the change in quaternary structure which results in weaker binding at the class 2 sites. In any case, the negative ΔS

Table II: Thermodynamic Parameters for the Binding of CTP and ATP to Aspartate Transcarbamylase.

		$\Delta G°$ kcal (mol of nucleotide)$^{-1}$	ΔH kcal (mol of nucleotide)$^{-1}$	$\Delta S°$ cal deg^{-1} (mol of nucleotide)$^{-1}$
Class 1 Sites				
CTP	0.1 M Na-Hepes (pH 7.8)	−6.9 ± 0.1	−13.5 ± 0.1	−22.1 ± 0.3
	0.1 M Tris-Ac (pH 7.8)	−6.6 ± 0.2	−13.3 ± 0.1	−22.6 ± 0.6
	0.1 M Tris-Ac (pH 8.5)	−6.7 ± 0.1	−12.8 ± 0.1	−20.5 ± 0.3
ATP	0.1 M Na-Hepes (pH 7.8)	−5.2 ± 0.1	−10.4 ± 0.1	−17.4 ± 0.3
	0.1 M Tris-Ac (pH 7.8)	−5.04 ± 0.05	−10.2 ± 0.1	−17.2 ± 0.2
	0.1 M Tris-Ac (pH 8.5)	−5.56 ± 0.05	−8.9 ± 0.1	−11.2 ± 0.2
Class 2 Sites				
CTP	0.1 M Na-Hepes (pH 7.8)	−5.7 ± 0.1	−13.5 ± 0.1	−26.1 ± 0.2
	0.1 M Tris-Ac (pH 7.8)	−5.5 ± 0.2	−13.3 ± 0.1	−26.3 ± 0.6
	0.1 M Tris-Ac (pH 8.5)	−5.24 ± 0.08	−12.8 ± 0.1	−25.4 ± 0.3
ATP	0.1 M Na-Hepes (pH 7.8)	−5.2 ± 0.1	−10.4 ± 0.1	−17.4 ± 0.3
	0.1 M Tris-Ac (pH 7.8)	−5.04 ± 0.05	−10.2 ± 0.1	−17.2 ± 0.2
	0.1 M Tris-Ac (pH 8.5)	−4.18 ± 0.05	−8.9 ± 0.1	−15.8 ± 0.2

values are consistent with a general decrease in the flexibility of the enzyme upon nucleotide binding, as suggested by McClintock and Markus (1968) to explain the inhibition by nucleotides of the tryptic digestion of the regulatory subunit.

The thermodynamic effects which accompany the binding of nucleotides to several other proteins have been examined. For example, Velick *et al.* (1971) obtained values of $\Delta G = -7.3$ kcal/mol, $\Delta H = -12.4$ kcal/mol, $\Delta S = -16.8$ eu, and $\Delta C_p = -517$ cal/(deg mol) for the binding of NAD^+ to yeast glyceraldehyde-3-phosphate dehydrogenase at 25° and pH 7.3, while, in an ultracentrifugation study of the binding of ATP to methemoglobin, Jänig *et al.* (1970) obtained values of $\Delta G = -2.67$ kcal/mol, $\Delta H = -6.85$ kcal/mol, and $\Delta S = -14.4$ eu at 17° and pH 7.2. Finally, the binding of 3′-CMP to RNase A has been examined calorimetrically by Bolen *et al.* who obtained values of $\Delta G = -5.11$ kcal/mol, $\Delta H = -9.2$ kcal/mol, and $\Delta S = -14.0$ eu in 0.5 M NaOAc (pH 5.5) at 25° (Bolen *et al.*, 1971).

Similarities in secondary structure at the nucleotide binding sites of several proteins whose crystal structures have been determined have been pointed out by Rossman and others (Hill *et al.*, 1972; Brandon *et al.*, 1973). The fact that in each of the cases discussed above ΔH and $\Delta S°$ are negative and $\Delta S°/\Delta H > 1 \times 10^{-3}$ °K^{-1} invites the speculation that there may be similarities in the interactions of these proteins with nucleotides. The signs and relative magnitudes of the thermodynamic parameters in each case are consistent with the formation of several hydrogen bonds; for hydrogen bond formation between the amide and carbonyl groups of urea at 25°, $\Delta G_{unitary} = -0.4$ (± 0.1) kcal/mol, $\Delta H = 1.4 \pm 0.1$ kcal/mol, and $\Delta S_{unitary} = -3.3$ (± 0.1) cal/(deg mol) (Kauzmann, 1959). The crystallographic analysis of the structure of the 3′-CMP–RNase complex (Richards *et al.*, 1970) has demonstrated the existence of several hydrogen bonds between the pyrimidine and ribose rings and the enzyme. It is also of interest that the binding of 3′-CMP to RNase is accompanied by the binding of protons to histidyl residues (Meadows and Jardetsky, 1968), since the pH dependence of proton uptake and the ΔH values for nucleotide binding to both aspartate transcarbamylase and methemoglobin are also consistent with the protonation of histidyl residues.

Several proteins are known for which the energetics of nucleotide binding are very different from those discussed above. However, in each of these cases binding is associated with substantial changes in quaternary structure. Examples are the binding of 5′-AMP to both glutamine synthetase and phosphorylase *b* at 18°. The ΔH values for these reactions are −2 kcal/mol of subunit and +2.6 kcal/mol, while the ΔS values are +11 cal/(deg mol) of subunit and 26.8 cal/(deg mol), respectively (Ross and Ginsberg, 1969; Ho and Wang, 1973). However, in the case of phosphorylase *b* at 25°, where the binding of 5′-AMP does not induce the association of the enzyme, the thermodynamic parameters for its binding resemble those of aspartate transcarbamylase.

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References

Bolen, D. W., Flögel, M., and Biltonen, R. (1971), *Biochemistry 10,* 4136.

Branden, C. I., Eklund, H., Nordström, B., Boiwe, T., Sudeerland, G., Zeppezauer, E., Ohlsson, I., and Åkeson, Å. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2439.

Buckman, T. (1970), *Biochemistry 9,* 3255.

Cohlberg, J. A., Pigiet, V. P., Jr., and Schachman, H. K. (1972), *Biochemistry 11,* 3396.

Coleman, P. D., and Markus, G. (1972), *J. Biol. Chem. 247,* 3829.

Collins, K. D., and Stark, G. R. (1969), *J. Biol. Chem. 244,* 1869.

Cook, R. A. (1972), *Biochemistry 11,* 3792.

Cornish-Bowden, A., and Koshland, D. G., Jr. (1970), *Biochemistry 9,* 3225.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121,* 404.

Dratz, E. A., and Calvin, M. (1966), *Nature (London) 211,* 497.

Eckfeldt, J., Hammes, G. G., Mohr, S. C., and Wu, C. W. (1970), *Biochemistry 9,* 3353.

Evans, D. R., Warren, S. G., Edwards, B. F. P., McMurray, C. H., Bethge, P. H., Wiley, D. C., and Lipscomb, W. N. (1973), *Science 179,* 683.

Gerhart, J. C. (1970), *Curr. Top. Cell. Regul. 2,* 275.

Gerhart, J. C., and Houloubek, H. (1967), *J. Biol. Chem. 242,* 2886.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237,* 891.
Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4,* 1054.
Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7,* 538.
Goa, J. (1953), *Scand. J. Clin. Lab. Invest. 5,* 218.
Gray, C. W., Chamberlin, M. J., and Gray, P. M. (1973), *J. Biol. Chem. 248,* 6071.
Griffin, J. H., Rosenbusch, J. P., Blout, E. R., and Weber, K. K. (1973), *J. Biol. Chem. 248,* 5057.
Griffin, J. H., Rosenbusch, J. P., Weber, K. K., and Blout, E. R. (1972), *J. Biol. Chem. 247,* 6482.
Hill, E., Tsernoglou, D., Webb, L., and Banaszak, L. J. (1972), *J. Mol. Biol. 72,* 577.
Hinz, H. J., Shiao, D. D., and Sturtevant, J. M. (1971), *Biochemistry 10,* 1347.
Ho, H. C., and Wang, J. H. (1973), *Biochemistry 12,* 4750.
Jacobson, G. R., and Stark, G. (1973), *Enzymes, 3rd Ed. 9,* 226.
Jänig, G. R., Gerber, G., Ruckpaul, K., Rapaport, S., and Jung, R. (1970), *Eur. J. Biochem. 17,* 441.
Kauzmann, W. (1959), *Advan. Protein Chem. 14,* 1.
Kirschner, M. W., and Schachman, H. K. (1971), *Biochemistry 10,* 1900.
Kleppe, K., (1966), *Biochim. Biophys. Acta 122,* 450.
London, R. E., and Schmidt, P. G. (1972), *Biochemistry 11,* 3136.
Markus, G., McClintock, D. K., and Bussel, J. B. (1971), *J. Biol. Chem. 246,* 762.
Matsumoto, S., and Hammes, G. G. (1973), *Biochemistry 12,* 1388.
McClintock, D. K., and Markus, G. (1968), *J. Biol. Chem. 243,* 2855.
McClintock, D. K., and Markus, M. (1969), *J. Biol. Chem. 244,* 36.
Meadows, D. H., and Jardetsky, O. (1968), *Proc. Nat. Acad. Sci. U. S. 61,* 406.
Nelbach, M. E., Pigiet, V. P., Gerhart, J. C., and Schachman, H. K. (1972), *Biochemistry 11,* 315.
Pigiet, V. (1971), Ph.D. Thesis, University of California, Berkeley.
Richards, F. M., Wyckoff, H. W., and Allewell, N. (1970), *in* the Neurosciences Second Study Program, Schmitt, F. O., Ed., New York, N. Y., Rockefeller Press, p 901.
Ross, P. P., and Ginsberg, A. (1969), *Biochemistry 8,* 4690.
Rosenbusch, J. P., and Weber, K. (1971), *J. Biol. Chem. 246,* 1644.
Sturtevant, J. M. (1969), *Fractions 1,* 1.
Sturtevant, J. M., and Lyons, P. A. (1969), *J. Chem. Thermodyn. 1,* 201.
Tondre, C., and Hammes, G. G. (1974), *Biochemistry 13,* 3131.
Velick, S. F., Baggott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10,* 779.
Weber, K. (1968), *Nature (London) 218,* 1116.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Winlund, C. C., and Chamberlin, M. J. (1970), *Biochem. Biophys. Res. Commun., 40,* 43.

Properties of the Highly Reactive SH Groups of Phosphorylase *b*†

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ABSTRACT: The reaction of rabbit muscle phosphorylase *b* with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) has been studied with stopped-flow spectrophotometry. Two highly reactive sulfhydryl groups per dimer reacted with Nbs_2 within a few seconds, while the remaining SH groups needed several minutes and hours. Decomposition of the time curve revealed that the highly reactive SH groups can be divided into two subclasses: a fast type which reacted with a rate constant of 3×10^3 M^{-1} sec^{-1} and a more slowly reacting type disappearing with a rate constant of 0.3×10^3 M^{-1} sec^{-1}. The reactivity of the slowly reacting type increased by a factor of about 2 in the presence of 1 mM AMP. Concurrently, the ratio between the fast reacting and the more slowly reacting subclasses decreased from 5.3 to 1.0. The AMP effect was greatly enhanced by glucose 1-phosphate. This enhancement was abolished in the presence of ATP. The finding that the ratio between the number of SH groups in the two subclasses of the highly reactive SH groups changed upon addition of ligand molecules indicates that the two subclasses reflect the different reactivities of the SH groups when the enzyme is present in different conformational states. It is suggested that the highly reactive SH group measured belong to the peptide: Gly-Cys-Arg-Asp.

Rabbit muscle phosphorylase *b* (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) possesses at least three types of SH-groups with widely different reactivity (Damjanovich and Kleppe, 1966; Gold, 1968; Kastenschmidt *et al.*, 1968a; Kleppe and Damjanovich, 1969; Avramovic-Zikic *et al.*, 1970; Zarkadas *et al.*, 1970; Hasinoff *et al.*, 1971). Two to four SH groups react very rapidly with most sulfhydryl reagents. Approximately four SH groups show a moderate reactivity. Finally, the enzyme has 10–12 SH groups which react very slowly with most re-

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