Interactions of Ionizable Groups in *Escherichia coli* Aspartate Transcarbamylase with Adenosine and Cytidine 5′-Triphosphates†

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ABSTRACT: The energetics of binding of adenosine and cytidine mono-, di-, and triphosphates to *Escherichia coli* aspartate transcarbamylase (c_6r_6) and its catalytic (c_3) and regulatory (r_2) subunits have been studied from pH 7 to 10. Measurements with c_3 and c_6r_6 were made in both the presence and absence of the bisubstrate analogue *N*-phosphonoacetyl-L-aspartate (PALA). Free energies, enthalpies, and entropies of binding were evaluated by nonlinear least-squares analysis of a series of calorimetric titration curves. Linkages between proton and nucleotide binding were analyzed by potentiometry. We find the following: (a) Under all conditions examined, binding is enthalpically driven and accompanied by binding of protons. Increases in $\Delta\bar{\nu}_{H^+}$ (change in moles of protons bound per mole of ligand) are invariably correlated with decreases in both $\Delta H_{binding}$ and $\Delta S°_{binding}$. The consistency of this pattern suggests that nucleotide binding, structural changes in the protein, and proton binding are tightly coupled. (b) The free energy of the c–r interaction in c_6r_6·6PALA is affected only minimally by binding of either CTP or ATP. Both the subunit interaction enthalpy and entropy are, however, altered substantially when nucleoside triphosphates bind, indicating changes in specific bonding interactions between subunits. The changes appear similar for both nucleoside triphosphates. (c) There are major differences in the interactions of ATP and CTP with ionizable groups in the protein. Both $\Delta\bar{\nu}_{H^+}$ and the variation in the thermodynamic parameters with pH and with the number of phosphate residues are consistently greater for adenine nucleotides than for cytosine nucleotides, except at pH 10, where activity is not regulated. Moreover, while $\Delta\bar{\nu}_{H^+}^{c_6r_6}$ for CTP is similar to the appropriately weighted sum for the subunits ($2\Delta\bar{\nu}_{H^+}^{c_3} + 3\Delta\bar{\nu}_{H^+}^{r_2}$), $\Delta\bar{\nu}_{H^+}^{c_6r_6}$ for ATP is substantially less than the corresponding sum for the subunits. This result suggests that there are three or more ionizable groups, probably at the subunit interface, which are perturbed by ATP but not CTP.

Feedback regulation of the activity of aspartate transcarbamylase by the activator ATP and the inhibitor CTP is one of the principal means by which intracellular concentrations of purine and pyrimidine nucleotides are regulated in *Escherichia coli* (Yates & Pardee, 1956). Despite numerous studies, the molecular mechanism of allosteric regulation by nucleoside triphosphates is still poorly understood. The changes in physical properties which occur upon binding appear smaller than those produced by substrate analogues (Griffin et al., 1973; Honzatko et al., 1979; Moody et al., 1979; Lennick & Allewell, 1981). When changes are detected, they are sometimes parallel (Buckman, 1970; Colman & Markus, 1972; McClintock & Markus, 1968) and sometimes opposed (Howlett et al., 1977; Wang et al., 1981). While the elegant binding studies of Suter & Rosenbusch (1977) appear to establish unequivocally that there is one tight nucleoside triphosphate binding site per regulatory subunit and one per catalytic subunit, they raise new questions. What accounts for the disparate affinities of binding sites on identical polypeptide chains? Does tight binding of nucleoside triphosphates by the catalytic subunits play a role in the allosteric mechanism? Another question is raised by the results of Gray et al. (1973), which indicate that binding of the bisubstrate analogue *N*-phosphonoacetyl-L-aspartate (PALA)[1] does not alter the binding constant for ATP. If ATP does not increase the affinity of the enzyme for substrates, as this result implies, what then is the mechanism of ATP activation?

The marked pH dependence of the effects of ATP and CTP on enzymatic activity (Kerbiriou & Hervé, 1973; Thiry & Hervé, 1978) implicates ionizable groups in the allosteric mechanism. In an earlier paper (Allewell et al., 1975), we compared the energetics of binding CTP and ATP to the native enzyme under a restricted set of conditions. We have now made extensive potentiometric and calorimetric measurements of the binding of adenosine and cytidine mono-, di-, and triphosphates to the native enzyme and its subunits over a broad range of pH values. Free energies, enthalpies, and entropies have been derived from calorimetric titration curves, while the linkage between proton and nucleotide binding has been elucidated in potentiometric experiments. With these data, the energetics of binding to the isolated subunits and native enzyme can be compared and the relative magnitudes of contributions from sites on the catalytic and regulatory subunits and changes in subunit interactions upon binding assessed. Similarly, if each phosphate residue of the nucleoside triphosphates is assumed to bind independently, the contribution of each to the overall energetics can be evaluated by comparing nucleoside mono-, di-, and triphosphates. Our results indicate that several ionizable groups are differentially perturbed by CTP and ATP and that ATP perturbs these groups to different degrees in the native enzyme and isolated subunits. Furthermore, there is a strict correlation between the numbers of protons bound and the magnitudes of the enthalpy and entropy changes associated with binding. Our interpretation of this result is that proton binding and structural changes in the protein are tightly coupled.

Materials and Methods

Chemicals. PALA was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Purity was determined

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[1] Abbreviations: β-ME, β-mercaptoethanol; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PALA, *N*-phosphonoacetyl-L-aspartate; c_6r_6, aspartate transcarbamylase; c_3, catalytic subunit; r_2, regulatory subunit; EDTA, ethylenediaminetetraacetic acid.

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by ultraviolet difference spectroscopy (Collins & Stark, 1971) and verified by ^{31}P NMR, using an inorganic phosphate standard. All concentrations determined by weight were corrected for the impurities present. Neohydrin [1-[3-(chloromercuri)-2-methoxypropyl]urea] was obtained from ICN-K & K Laboratories and twice recrystallized from 95% ethanol. All other chemicals were reagent grade and used without further purification.

Proteins. The native enzyme was prepared from the derepressed, diploid *E. coli* strain developed by Gerhart & Holoubek (1967) as described previously (Gerhart & Holoubek, 1967; Allewell et al., 1975). Cells were grown either at the New England Enzyme Center, Boston, MA, or at Oak Ridge National Laboratories, Oak Ridge, TN. Purity, as verified by polyacrylamide gel electrophoresis, by using the procedures of Weber & Osborn (1969) and Davis (1964), and by enzymatic activity, was similar to that of previous preparations. c_6r_6 was stored as a precipitate in 3.6 M $(NH_4)_2SO_4$, 0.1 M Tris-HCl, 2 mM β-ME, and 0.2 mM NaEDTA, pH 8.3 at 4 °C.

c_3 and r_2 were prepared according to Yang et al. (1978) with the following modifications: (1) The dissociation reaction was carried out at 4 °C. (2) The final concentration of Zn(II) added to fractions containing r_2 from the DEAE-cellulose column was 0.2 mM. (3) Fractions containing r_2 were extensively dialyzed [4 × 12 h, 20:1 (v/v)] against 25 mM Tris-HCl and 1 mM dithiothreitol. c_3 was stored under the same conditions as the native enzyme. r_2 was stored at 4 °C in 3.6 M $(NH_4)_2SO_4$, 0.1 M Tris-HCl, 2 mM $Zn(OAc)_2$, 2 mM β-ME, 1.8×10^{-4} M benzamidine hydrochloride, and 50 μg/mL streptomycin sulfate. While the presence of protease inhibitors and antimicrobial agents may not be necessary, their presence appears desirable since aged solutions of r_2 show N-terminal heterogeneity (N. M. Allewell and H. Steinman, unpublished experiments), and they have been shown not to affect nucleoside triphosphate binding to c_6r_6 (N. M. Allewell and A. Zaug, unpublished experiments).

Concentrations of protein and nucleotide solutions were determined spectrophotometrically as described previously (Allewell et al., 1975). Extinction coefficients and values of λ_{max} for nucleotides were obtained from Bock et al. (1956).

Calorimetry. Calorimetric experiments were performed with a modified (Knier & Allewell, 1978) LKB 10700-1 flow microcalorimeter submerged in a Tronac CTB-1005 water bath thermostated with a Tronac PTC-40 precision temperature controller to 25.0 ± 0.1 °C. Except where noted, measurements were made in either 0.10 M Hepes (pH 7.0–8.2) or 0.10 M Ches (pH 9.0–10.0). In addition, solutions of c_6r_6 or c_3 contained 2 mM β-ME and 0.2 mM NaEDTA while solutions of r_2 contained 2 mM β-ME, 1.8×10^{-4} M benzamidine hydrochloride, and 50 μg/mL streptomycin sulfate. Protein concentrations ranged from 8 to 18 mg/mL for c_6r_6 and c_3 and from 2.5 to 6.0 mg/mL for r_2.

Free ligand concentrations were approximated by initially fitting the calorimetric titration curves to the equation

$$\Delta H_{app} = \frac{B - \sqrt{B^2 - C}}{2E_{tot}} \Delta H_{max} \quad (1)$$

where $B = K_d + N_{tot} + E_{tot}$ and $C = 4E_{tot}N_{tot}$. K_d is the dissociation constant, N_{tot} the total concentration of nucleotide (free + bound), and E_{tot} the total site concentration. Free nucleotide concentrations were calculated by subtracting the amount bound from the total. This equation is rigorously correct only when there is only one binding site per molecule; however, since the amount bound rarely amounted to more than a few percent of the total ligand present, it was deemed adequate. Furthermore, the equilibrium constant obtained will be only an apparent value, since ΔH_{app} is a composite of enthalpies of binding, conformational changes, and other unspecified reactions coupled with the reaction being studied, which calorimetry cannot resolve.

Potentiometry. Changes in proton equilibria accompanying ligand binding were determined by reestablishing the initial pH of an unbuffered solution, perturbed upon the addition of ligand, by back-titration with HCl on a Brinkmann Metrohm E412 Dosimat fitted with a Brinkmann EA-147 microcombination glass electrode.

Two sets of data were collected. The first set was obtained over the pH range 6.6–9.0 at constant ionic strength (I = 0.020 M) and the second at ionic strengths corresponding to the calorimetric experiments at the same pH. These ionic strengths were calculated by assuming pK values of 7.5 and 9.3 (Sigma Chemical Co.) for Hepes and Ches, respectively, and complete cancellation of charges on the zwitterion. Ionic strength was maintained with NaOAc. β-ME, NaEDTA, benzamidine hydrochloride, and streptomycin sulfate were added as required.

In a typical experiment, the volumes of the protein solution and the aliquots of nucleotide and titrant added were 4.5 mL, 50–75 μL, and 100–600 μL, respectively. Successive aliquots of nucleotide were added until a saturating concentration was reached. Addition of nucleotide solutions to buffer alone consistently produced substantial increases in pH (0.08–0.3 unit), which decreased in magnitude as nucleotide concentration and pH were increased. This effect was approximately 3 times greater for ATP than for CTP and may be attributable to dissociation of stacked nucleotide bases upon dilution (Heyn & Bretz, 1975).

$\Delta\bar{\nu}_{H^+}$, the change in moles of protons bound per mole of nucleotide, was calculated from

$$\Delta\bar{\nu}_{H^+} = \frac{(V_{exp} - V_{con})M_{titrant}}{m_{bs}} \quad (2)$$

where V_{exp} and V_{con} are the experimental and control volumes of titrant required to back-titrate nucleotide diluted into the protein solution and buffer, respectively, $M_{titrant}$ is the molarity of the titrant, and m_{bs} is the moles of binding sites in the reaction vessel. The resulting values of $\Delta\bar{\nu}_{H^+}$, when plotted against ligand concentration, produce a titration curve comparable to those obtained by calorimetric titration. The values reported are the result of 3–10 determinations.

Protein concentrations ranged from 0.8 to 2.1 mg/mL for c_6r_6 and c_3 and from 0.3 to 0.5 mg/mL for r_2. Concentrations of nucleotide stock solutions were such that addition of a 50- or 75-μL aliquot changed the pH by at least 0.03 unit and the concentration of ligand was still saturating ($5 \times K_d$) after addition of four to six aliquots and back-titration. Stock concentrations of 60 mM for adenine nucleotides and 25 mM for cytosine nucleotides were found suitable. Titrant concentrations ranged from 0.01×10^{-3} to 1.00×10^{-3} M.

Between pH 7.8 and 8.2, the pH of unbuffered protein solutions increased significantly (0.02–0.06 unit) during preliminary experiments. Purging all solutions and the reaction chamber with dry nitrogen eliminated this problem.

$\Delta\bar{\nu}_{H^+}$, the change in moles of protons bound per mole of nucleotide, as a function of pH was fit to the equation

$$\Delta\bar{\nu}_{H^+} = m\sum_i n_i \left(\frac{[H^+]^{n_i}}{[H^+]^{n_i} + K_d'^{n_i}} - \frac{[H^+]^{n_i}}{[H^+]^{n_i} + K_d^{n_i}} \right) \quad (3)$$

where the summation is over i classes of ionizable groups, K_d'

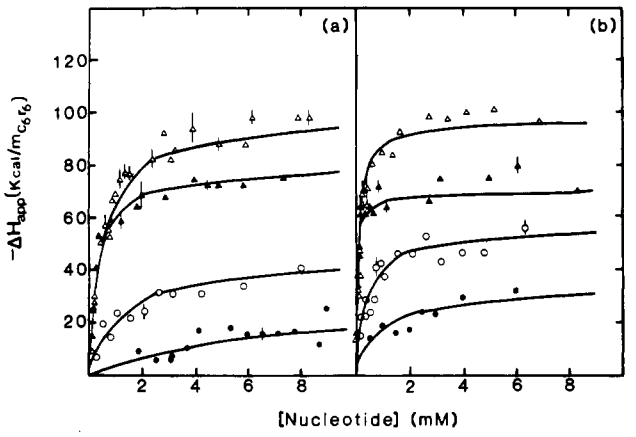

FIGURE 1: Calorimetric titrations of c_6r_6 with (a) adenine nucleotides and (b) cytosine nucleotides in 0.1 M Hepes, 2 mM β-ME, and 0.2 mM NaEDTA, pH 7 at 25 °C. (△) XTP in the absence of PALA; (▲) XTP in the presence of saturating concentrations of PALA; (○) XDP (saturating PALA concentrations present); (●) XMP (saturating PALA concentrations present). The curves correspond to the best fit to eq 1.

and K_d are acid dissociation constants for ionizable groups in class i in the protein–ligand complex and unliganded protein, respectively, n_i is the number of protons in this class, and m is equal to 3, 2, and 6 for c_3, r_2, and c_6r_6, respectively. Raising $[H^+]$ to the n_ith power implies that the ionizations of the n_i groups in class i are infinitely cooperative. It was necessary to make this assumption to fit the data.

Numerical Analysis. All analysis of data was done by using a nonlinear least-squares program written by Dr. Michael Johnson (University of Virginia, Charlottesville, VA) which provides reliable confidence limits and correlation coefficients for all variables, plots residuals as functions of both independent and dependent variables, and has interactive graphics capabilities (Johnson et al., 1976).

Results

Models. Calorimetric titrations of the native enzyme with cytidine and adenosine mono-, di-, and triphosphates at pH 7, uncorrected for proton effects, are shown in Figure 1. The upper curve in each panel pertains to measurements made in the absence of PALA; all other experiments with the native enzyme were carried out in the presence of saturating PALA (concentrations ~20% greater than the concentration of active sites, corresponding to free concentrations of $\sim 10^{-5}$ M). Under these conditions, PALA would be expected both to block binding of nucleotides to the active site (Suter & Rosenbusch, 1977) and to maintain the quaternary structure in the R (relaxed) state (Howlett et al., 1977).[2] The difference between the data obtained in the presence and absence of PALA suggests that binding at the active site may contribute ~−19 kcal/mol of c_6r_6 to ΔH_{app} for ATP and ~−27 kcal/mol of c_6r_6 for CTP.

The first question to be considered is whether multiple classes of binding sites can be resolved. In a previous paper (Allewell et al., 1975), we fit calorimetric and equilibrium dialysis data on nucleoside triphosphate binding to c_6r_6 to models which assumed six strong binding sites on the regulatory subunits (divided into two classes of three sites each in the case of CTP) and six weaker sites on the catalytic subunits. Since that time, the question of stoichiometry has been raised anew by Suter & Rosenbusch (1977), who found, with a filter binding assay, one tight binding site on each of the catalytic and regulatory subunits. While a purely calorimetric study provides no information on the total number of sites, the existence of a large body of highly precise data provides an opportunity to ask how many types of sites can be distinguished and possibly to determine enthalpies of binding for individual classes of sites.

A total of 22 stoichiometric models were formulated and tested against the data in Figure 1, as well as comparable data for c_3 and r_2 at pH 7. Following Suter & Rosenbusch (1977), all of the models assumed one tight binding site on each catalytic and regulatory subunit, with binding to tight sites on the catalytic subunits blocked by PALA. Within this framework, both sequential and independent binding to lower affinity sites on both catalytic and regulatory subunits were considered. The approach taken in fitting the data was to use the best fit to an appropriate simpler case as initial guesses and to float the enthalpies first, then the binding constants, and finally all the parameters simultaneously. With this approach, convergence to physically reasonable values could be achieved with several models. Correlation coefficients were generally no greater than 0.95, and often much less. Representative results are given in Table I. These results are consistent with the existence of multiple classes of sites; however, only in the case of ATP binding to r_2 is the fit significantly better than that obtained with eq 1.

Competition between Nucleoside Triphosphates. Clearer resolution of catalytic and regulatory sites on c_6r_6 was achieved in competition experiments. In each experiment, a solution of c_6r_6 (or c_6r_6·6 PALA) containing a given concentration of ATP was mixed in the calorimeter with a second solution containing both the same concentration of ATP and a known concentration of CTP. For a single class of sites

$$\Delta H_{app} = f_2^{CTP}\Delta H_{max}^{CTP} + (f_2^{ATP} - f_1^{ATP})\Delta H_{max}^{ATP} \quad (4)$$

where f_2^{CTP} and f_2^{ATP}, the final fractional occupancy by CTP and ATP, respectively, have values of

$$f_2^{CTP} = \frac{[CTP]N}{K_d^{CTP} + [CTP]N} \qquad N = \frac{K_d^{ATP}}{K_d^{ATP} + [ATP]}$$

and

$$f_2^{ATP} = \frac{[ATP]M}{K_d^{ATP} + [ATP]M} \qquad M = \frac{K_d^{CTP}}{K_d^{CTP} + [CTP]}$$

while f_1^{ATP}, the initial fractional occupancy of ATP sites, is given by

$$f_1^{ATP} = \frac{[ATP]}{K_d^{ATP} + [ATP]}$$

The data shown in Figure 2 were obtained at three concentrations of ATP. As expected, if ATP and CTP compete for the same sites, the heat effects decrease as the concentration of ATP increases. More surprisingly, in view of the differences between c_6r_6 and c_6r_6·6PALA in Figure 1, the results for c_6r_6 and c_6r_6·6PALA in Figure 2 are very similar. Furthermore, the curves calculated with eq 4 and the results of the best fit of the calorimetric titrations of c_6r_6 in Figure 1 to a single class of sites (Table II) do not fit the data from the competition experiments. They can, however, be fit with values of ΔH_{max} similar to those derived by fitting the data in Figure 1 for c_6r_6·6PALA to a single class of sites.

Taken in combination, these results suggest that even the lowest concentration of ATP used in the competition exper-

[2] Note, however, that in contrast to the results of Gray et al. (1973) and predictions of the two-state model both ATP and CTP appear to bind more tightly (by factors of 1.5 and 2, respectively) in the presence of PALA than in its absence.

Table I: Representative Fits of Calorimetric Titration Data at pH 7 to Models Involving Two Classes of Sites[a]

	model	nucleoside triphosphate	k_1, $-\Delta H_1$	k_2, $-\Delta H_2$	σ[b]
r_2:	sequential binding to two nonequivalent sites $\Delta H = \frac{2k_1\Delta H_1[L] + k_1k_2(\Delta H_1 + \Delta H_2)[L]^2}{1 + 2k_1[L] + k_1k_2[L]^2}$	ATP	6.0 ± 1.3[c] 17.7 ± 2.6	1.7 ± 0.8 12 ± 1	0.48
		CTP	6.3 ± 2.1 34 ± 3	1.8 ± 0.1 3 ± 13	1.6
c_3:	sequential binding to two classes of sites, with one and two members, respectively $\Delta H = \frac{3k_1\Delta H_1[L] + 3k_1k_2[L]^2(\Delta H_1 + \Delta H_2) + k_1k_2^2[L]^3(\Delta H_1 + \Delta H_2)}{1 + 3k_1[L] + 3k_1k_2[L]^2 + k_1k_2^2[L]^3}$	ATP	7 ± 6 6.6 ± 1.3	0.4 ± 0.3 4.3 ± 2.3	1.25
		CTP[d]			
c_6r_6·6PALA:	sequential binding to two classes of sites on r_2 subunits, with one and two members, respectively (three sites identical on unliganded enzyme, but binding at first site alters affinity of remaining two) ΔH: same as above	ATP	3.4 ± 1.7 13 ± 8	6.9 ± 1.5 32 ± 5	8.9
		CTP	5.4 ± 1.8 56 ± 29	5 ± 10 7 ± 4	6.2
c_6r_6:	three sites on r_2 subunits with identical intrinsic affinities and enthalpies; two identical sites on c_3 subunits; no interactions between c and r sites $\Delta H = \frac{(3k_1\Delta H_1[L])(1 + 2k_1[L] + k_1^2[L]^2)}{1 + 3k_1[L] + 3k_1^2[L]^2 + k_1^3[L]^3} + \frac{(2k_2\Delta H_2[L])(1 + k_2[L])}{1 + 2k_2[L] + k_2^2[L]^2}$	ATP	2.3 ± 1.2 28.6 ± 2.8	34 ± 22 0.03 ± 0.02	10.3
		CTP	9.6 ± 1.0 28.5 ± 1.3	1.1 ± 0.9 1.2 ± 0.9	9.1

[a] Units for microscopic association constants and enthalpies are mM^{-1} and kcal mol^{-1}, respectively. [b] Square root of variance. [c] Error corresponding to outer confidence limit. [d] Data at saturating concentrations not available.

Table II: Values of the Parameters Used To Calculate the Curves in Figure 2 from Equation 4

fit	data set	K_d^{ATP} (mM)	K_d^{CTP} (mM)	$-\Delta H_{max}^{ATP}$[a] (kcal/mol)	$-\Delta H_{max}^{CTP}$[a] (kcal/mol)
1	Figure 1, −PALA	0.47 ± 0.09	0.12 ± 0.02	97 ± 5	97 ± 7
2	Figure 1, +PALA	0.30 ± 0.04	0.06 ± 0.01	78 ± 2	70 ± 2
3	Figure 2, −PALA	1.2 ± 0.4	0.03 ± 0.025	62 ± 6	62 ± 7
4	Figure 2, +PALA	0.62 ± 0.09	0.01 ± 0.008	68 ± 2	73 ± 1

[a] Uncorrected for proton effects.

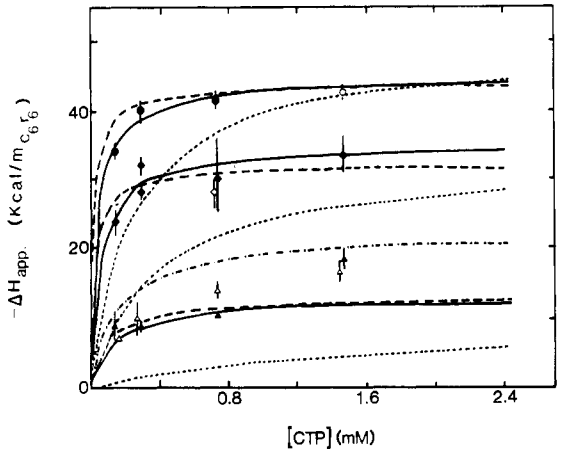

FIGURE 2: Calorimetric titration of c_6r_6 or c_6r_6·6PALA at pH with CTP in the presence of various concentrations of ATP. Closed symbols, c_6r_6; open symbols, c_6r_6·6PALA. (● or ○) 0.47 mM ATP; (♦ or ◇) 0.93 mM ATP; (▲ or △) 4.67 mM ATP. The parameters used to calculate the various curves are listed in Table II. (···) Fit 1; (-·-·) fit 2; (—) fit 3; (- - -) fit 4.

iments was sufficient to saturate the active sites and that ATP and CTP compete only for sites on the regulatory subunits. The K_d values for c_3 in Table V are consistent with this possibility. It is of interest that two classes of sites which could not be unambiguously distinguished with calorimetric titration data alone could be resolved in a competition experiment. It should be kept in mind, however, that the need to resolve catalytic and regulatory sites analytically in the experiments described below is eliminated by the presence of sufficient PALA to block binding at the active site.

Comparisons of Nucleoside Mono-, Di-, and Triphosphates. Energy changes associated with binding nucleoside mono-, di-, and triphosphates to c_6r_6·6PALA at pH 7 and 10 derived by fitting the data in Figure 1 to a single class of three sites are shown in Figure 3.[3] The contributions of individual groups to the energetics of binding, assuming that each group binds independently, are tabulated in Table III. The values for $\Delta H_{binding}$ were corrected for the heat effects associated with removal of protons from the buffer by using the following equation:

$$\Delta H_{binding} = \Delta H_{app} - \Delta\bar{\nu}_{H^+}\Delta H_{ion} \quad (5)$$

where ΔH_{ion} is the enthalpy of ionization of the buffer. Values of $\Delta G°_{binding}$ and $\Delta S°_{binding}$ are unitary values, obtained by adding −2.4 kcal/mol and 8 cal K^{-1} mol^{-1} to the values calculated from the standard thermodynamic expressions (Knier & Allewell, 1978).

As the number of phosphate residues increases, $\Delta\bar{\nu}_{H^+}$ for both nucleotides becomes larger, and all three thermodynamic parameters become more negative. This correlation between more positive values of $\Delta\bar{\nu}_{H^+}$ and more negative values of ΔH and $\Delta S°$ was encountered frequently. In each series, the β and γ residues make approximately equal contributions at pH 7 to the energetics of binding; however, the variation in $\Delta H_{binding}$ and $\Delta S°_{binding}$ is significantly greater for adenine nucleotides than for cytosine nucleotides. Interestingly, this difference is less pronounced at pH 10, where the enzyme lacks

[3] Note that the assumption that there are only three tight binding sites in c_6r_6·6PALA, rather than six, results in the calculated values of $\Delta H_{binding}$ being larger by a factor of 2 than those given by Allewell et al. (1975).

Table III: Contributions of Individual Groups in Nucleoside Triphosphates to the Overall Energetics of Binding[a]

	pH 7				pH 10			
group	ΔG°_{unit} (kcal/mol)	ΔH (kcal/mol)	ΔS°_{unit} (cal K^{-1} mol^{-1})	$\Delta\bar{\nu}_{H^+}$	ΔG°_{unit} (kcal/mol)	ΔH (kcal/mol)	ΔS°_{unit} (cal K^{-1} mol^{-1})	$\Delta\bar{\nu}_{H^+}$
AMP	−5.3	−10.9	−19	0	−4.9	−9.9	−17	0
βP	−1.1	−9.5	−28	1.1	−0.3	−2.9	−8	0
γP	−0.8	−19.5	−63	1.6	−0.9	−10.7	−33	0.2
CMP	−6.7	−12.6	−20	0.4	−5.2	−9.3	−14	0
βP	−0.3	−10.8	−35	0.5	−1.1	−3.3	−7	0.3
γP	−1.1	−7.3	−21	0.5	−0.2	−14.5	−48	0.3

[a] This analysis assumes additivity of the contributions of various groups. As Jencks (1981) has pointed out, this is not necessarily the case.

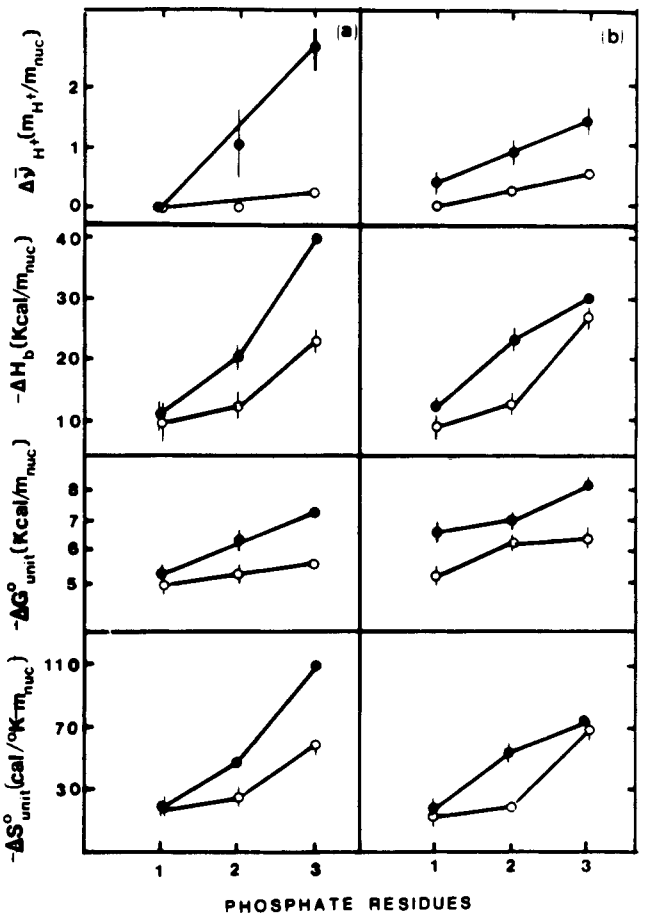

FIGURE 3: Dependence of the thermodynamic parameters for nucleotide binding to c_6r_6·6PALA on the number of phosphate residues. (a) Adenine nucleotides and (b) cytosine nucleotides at (●) pH 7 and (○) pH 10. Experimental conditions were those described in Figure 1, except for the potentiometric experiments which were carried out in NaOAc at the same ionic strength as the comparable calorimetric experiments. Calculations were performed as described in the text.

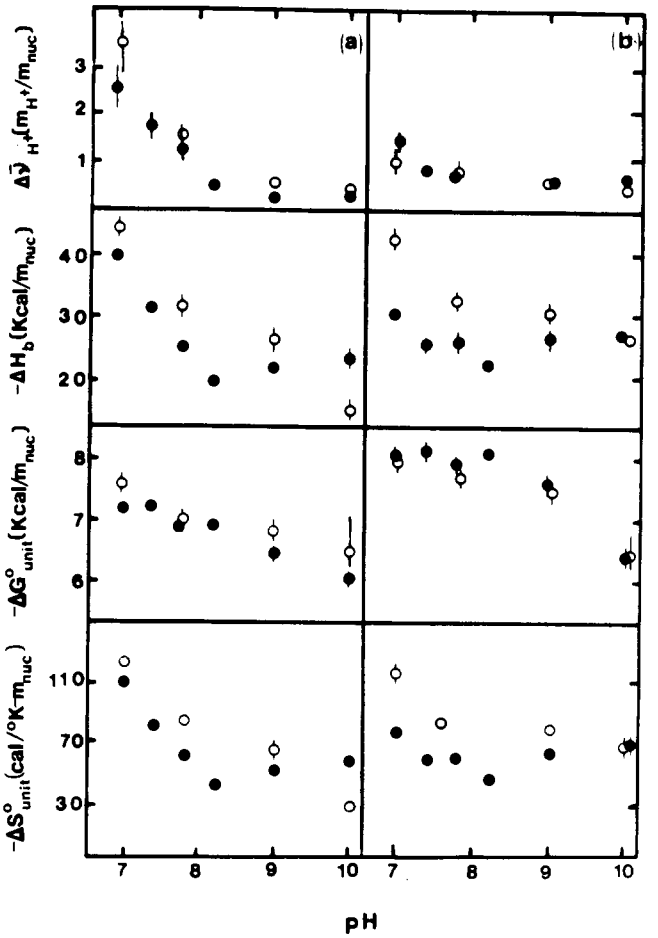

FIGURE 4: pH dependence of the thermodynamic parameters for nucleoside triphosphate binding to c_6r_6·6PALA (●) and r_2 (○). (a) ATP; (b) CTP. Experimental conditions were those described in Figure 3. Calculations were performed as described in the text.

both cooperativity and sensitivity to nucleoside triphosphates (Weitzman & Wilson, 1966; Kerbiriou & Hervé, 1973). Deviations from linearity are also greater at pH 10, suggesting that a group titrating between pH 7 and 10 is involved in interactions between the β- and γ-phosphate residues and the protein.

Energetics of Binding ATP and CTP to c_6r_6·6PALA and r_2. The stoichiometry of proton binding and changes in thermodynamic parameters which accompany binding of ATP and CTP to c_6r_6·6PALA and r_2 are shown as a function of pH in Figure 4. A single class of binding sites and a stoichiometry of one site per regulatory dimer have been assumed. The quality of the data was comparable to that of Figure 1, and the calculations were performed as described previously. For both nucleoside triphosphates, $\Delta H_{binding}$ and $\Delta S^\circ_{binding}$ are negative over the entire pH range, $\Delta\bar{\nu}_{H^+}$ is consistently positive, $\Delta G^\circ_{binding}$ increases while $\Delta\bar{\nu}_{H^+}$ decreases as the pH increases, and larger values of $\Delta\bar{\nu}_{H^+}$ are generally correlated with more negative values of ΔH and ΔS°. On the other hand, superimposed upon these qualitative similarities are well-defined quantitative differences. Of particular interest is the fact that variations in $\Delta\bar{\nu}_{H^+}$, ΔH, and ΔS° with pH are smaller for CTP than for ATP. Similarly, while the energetics of binding of both nucleoside triphosphates to c_6r_6·6PALA and r_2 are similar, differences outside experimental error clearly exist. Again, more positive values of $\Delta\bar{\nu}_{H^+}$ (for r_2) are by and large correlated with more negative values of ΔH and ΔS°.

Calorimetric Determination of $\Delta\bar{\nu}_{H^+}$. Since proton effects evaluated by potentiometry are susceptible to a variety of systematic errors, values of $\Delta\bar{\nu}_{H^+}$ for both ATP and CTP

Table IV: Changes in Subunit Interaction Energies[a] in c_6r_6·6PALA Produced by Binding of CTP and ATP

pH	$\Delta\Delta G^\circ_{unit}$ (kcal/mol)	$\Delta\Delta H$ (kcal/mol)	$\Delta\Delta S^\circ_{unit}$ (cal K^{-1} mol^{-1})	$\Delta\Delta\bar{\nu}_{H^+}$
		ATP		
7.0	1.1 ± 0.3[b]	14 ± 2	42 ± 5	−2.8 ± 1.8
7.8	0.3 ± 0.3	20 ± 2	66 ± 5	−1.1 ± 0.4
9.0	1.0 ± 0.5	13 ± 3	39 ± 9	−0.8 ± 0.1
10.0	1.2 ± 0.9	−25 ± 4	−87 ± 9	−0.5 ± 0.1
		CTP		
7.0	−0.5 ± 0.4	35 ± 3	120 ± 6	1.1 ± 0.7
7.8	−0.7 ± 0.5	20 ± 4	66 ± 9	−0.1 ± 0.3
9.0	−0.5 ± 0.4	14 ± 3	48 ± 6	−0.2 ± 0.1
10.0	0.1 ± 0.7	−1 ± 7	−3 ± 17	0.5 ± 0.1

[a] Calculated by multiplying the difference in the binding parameters for c_6r_6·6PALA and r_2 by a factor of 3. For example, $\Delta\Delta G^\circ_{unit} = 3[\Delta G^\circ_{binding}(c_6r_6\text{·6PALA}) - \Delta G^\circ_{binding}(r_2)]$. [b] Root mean square errors.

binding to r_2 were verified at one pH (pH 7.8) by calorimetry. ΔH_{app} was determined in a series of buffers with a range of ΔH_{ion}, and $\Delta\bar{\nu}_{H^+}$ was calculated according to eq 5. Values of 1.4 ± 0.1 mol of H^+/mol of nucleotide were obtained for both ATP and CTP. This result is in good agreement with the potentiometrically determined value for ATP but somewhat larger than the corresponding value for CTP.

Changes in Subunit Interaction Energies. Differences in the thermodynamic parameters for binding both nucleoside triphosphates to c_6r_6·6PALA and r_2 are given as a function of pH in Table IV. These differences are formally a measure of the changes in subunit interaction energies produced by binding of ligands (Ackers & Halvorson, 1974; Weber, 1975). Note that while both nucleoside triphosphates have only small effects on the free energies of subunit interactions, each produces substantial changes in the subunit interaction enthalpies and entropies. Also of interest is the monotonic variation in ΔH and ΔS° for CTP and the familiar correlation between larger values of $\Delta\bar{\nu}_{H^+}$ and more negative values of $\Delta H_{binding}$ and $\Delta S^\circ_{binding}$.

Energetics of Binding CTP and ATP to c_3 and c_3·3PALA. Binding of either CTP or ATP to c_3 in the absence of PALA is accompanied by extremely small heat and proton effects which decrease in magnitude as the pH increases. In the presence of PALA, no effects larger than the uncertainty of the measurements could be detected. Values for K_d, $\Delta H_{binding}$, and $\Delta\bar{\nu}_{H^+}$ under the restricted set of conditions where measurements could be made are given in Table V. One binding site per c_3 has been assumed. The affinity of c_3 for nucleoside triphosphates appears comparable to r_2, as Suter & Rosenbusch (1977) reported. Both the similarity in the K_d values of r_2 and c_3 and the small heat effects associated with binding to c_3 would be expected to make resolution of individual classes in the thermal titration data difficult. At pH 7, the values of $\Delta S^\circ_{binding}$ (unitary) derived from the calorimetric measurements are 0.5 ± 4 and −24 ± 3 cal K^{-1} mol^{-1} for CTP and ATP, respectively. These values indicate that the energetics of binding to these sites do not depend solely upon electrostatic interactions. K_d values derived from the potentiometric data are considerably larger and would necessitate substantial

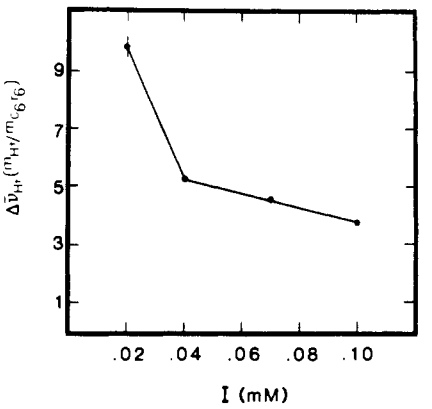

FIGURE 5: Dependence of $\Delta\bar{\nu}_{H^+}$ for binding ATP to c_6r_6 at pH 7.0 on I, the ionic strength of NaOAc.

negative entropy changes in both cases; however, these data are less reliable, because of the large correction required for effects associated with nucleoside triphosphate dilution.

Potentiometric Determination of $\Delta\bar{\nu}_{H^+}$. While the pH dependence of the results shown in Figure 4 implicates ionizable groups, these data do not allow these groups to be characterized in detail. In particular, eq 3 cannot be used to calculate pK values since $\Delta\bar{\nu}_{H^+}$ decreases as the ionic strength increases (Figure 5) and the molarity of the buffer, rather than its ionic strength, was held constant in the calorimetric experiments. Potentiometric experiments carried out at constant ionic strength (0.02 M) proved, however, quite informative.

The results for c_3, c_3·3PALA, and r_2 are shown in Figure 6a,c. The fine structure indicates that in all cases several ionizable groups are perturbed when nucleoside triphosphates bind, and the sharpness of the peaks suggests that several groups titrate cooperatively. In all three cases, the curves for the two nucleoside triphosphates are qualitatively similar; however, the magnitudes of the effects are consistently greater for ATP. Binding of ATP to c_3·3PALA was readily detectable in these experiments, at concentrations less than 1 mM, in spite of the fact that the heat effects are too small to be measured.

Comparable results for c_6r_6, in the presence and absence of PALA, are shown in panels b and d. Also shown are curves calculated by summing, with appropriate stoichiometries, the effects for individual subunits. The differences between the observed and calculated curves are minor in the case of CTP but dramatic for ATP.

The results of nonlinear least-squares analysis of these data in terms of eq 3 are given in Table VI. The pK values for the unliganded proteins are generally close, suggesting that both nucleoside triphosphates interact with a common set of ionizable groups; however, ATP appears to interact with a larger number of groups and to produce larger shifts in pK values.

Using the method of Saroff & Minton (1972), we can estimate the contribution of linked protonation reactions to the total free energy of nucleoside triphosphate binding. The appropriate equation is

$$\Delta G(\mathrm{H}) = -RT \ln \frac{\Pi_i(1 + k_i^{\mathrm{L}}[\mathrm{H}^+])n_i^{\mathrm{L}}}{\Pi_i(1 + k_i^{\mathrm{U}}[\mathrm{H}^+])n_i^{\mathrm{U}}} \qquad (6)$$

Table V: Thermodynamic Parameters for CTP and ATP Binding to c_3 in the Absence of PALA

nucleotide	pH	K_d (mM) (calorimetric)	K_d (mM) (potentiometric)	ΔH_{max} (kcal/c_3)	$\Delta H_{binding}$ (kcal/c_3)	$\Delta\bar{\nu}_{H^+}$
CTP	7	0.01 ± 0.01	0.22 ± 0.03	−6.2 ± 0.4	−9.4 ± 0.4	0.74 ± 0.02
ATP	7	0.1 ± 0.1	2 ± 0.7	−8.9 ± 1.3	−15.1 ± 0.9	1.7 ± 0.4
ATP	8		0.9 ± 0.2			1.1 ± 0.1

Table VI: Results of Nonlinear Least-Squares Analysis of Data in Figure 6 to Equation 3

protein	nucleoside triphosphate	$n_1:n_2:n_3$	$pK_1 - pK_1'$	$pK_2 - pK_2'$	$pK_3 - pK_3'$
c_3	CTP	1:1:1	6.97-7.43	7.60-7.68	9.23-9.48
c_3	ATP	1:2:1	7.00-7.30	7.33-7.47	8.21-8.66
c_3·3PALA	CTP	2:2:1	7.45-7.57	7.70-7.58	8.50-8.30
c_3·3PALA	ATP	2:4:1	7.08-7.30	8.11-8.04	8.50-8.30
r_2	CTP	2:2:1	6.45-6.85	7.65-7.85	8.70-9.08
r_2	ATP	4:3:1	6.45-6.98	7.53-7.97	8.70-12.5
c_6r_6	CTP	2:2:1	6.50-7.10	7.44-7.68	8.17-9.34
c_6r_6	ATP	3:2:1	6.44-7.03	7.45-7.70	7.96-8.66
c_6r_6·6PALA	CTP	2:2:1	6.65-7.05	7.75-7.90	8.70-9.23
c_6r_6·6PALA	ATP	3:2:1	6.70-7.00	7.50-7.65	7.77-8.25

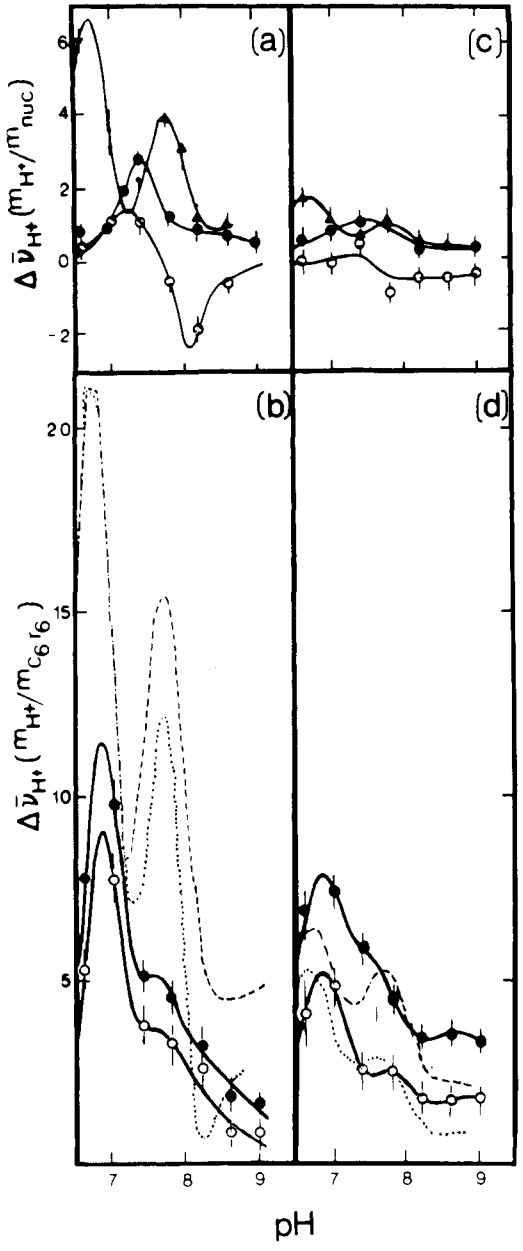

FIGURE 6: pH dependence of proton uptake effects associated with binding ATP (panels a and b) and CTP (panels c and d) to r_2, c_3, c_3·3PALA, c_6r_6, and c_6r_6·6PALA in 0.02 M NaOAc at 25 °C. Results for the subunits are shown in the upper pair of panels [(▲) r_2; (●) c_3; (○) c_3·3PALA]; results for the native enzyme in the presence (○) and absence (●) of PALA are in the lower panels. The solid curves are calculated with eq 3 and the parameter values listed in Table VI. The dotted curves in the lower panels indicate the results expected for the native enzyme by assuming the same contributions to $\Delta\bar{\nu}_{H^+}$ from the various classes of proton binding sites in the native enzyme as in the subunits. (---) c_6r_6; (···) c_6r_6·6PALA.

where n_i^L and n_i^U are the numbers of linked proton binding sites of class i in the liganded and unliganded protein, respectively, and k_i^L and k_i^U are the corresponding microscopic proton association constants (Minton, 1980). Except for binding to r_2, these values are not significantly different for the two nucleoside triphosphates. For c_6r_6 and c_6r_6·6PALA, the values are −3.6 and −2.1 kcal/mol of nucleotide, respectively, at pH 7 and −1.3 and −0.7 kcal/mol of nucleotide at pH 9. Protonation reactions contribute −4.2 and −1.9 kcal/mol of nucleotide to binding of ATP and CTP, respectively, to r_2 at pH 7, and −0.75 kcal/mol of nucleotide for both nucleoside triphosphates at pH 9. They make their greatest contribution (~55% of the total free energy change) in binding ATP to r_2 at pH 7. For comparison, the corresponding values for PALA and carbamoyl phosphate binding to c_6r_6, calculated from the data of Allewell et al. (1979), are −6.4 and −4.2 kcal/mol, respectively.

Discussion

Although it has been known for some time that the phosphate residues of nucleoside triphosphates are involved both in binding and in the allosteric mechanism (Gerhart & Pardee, 1963; Tondre & Hammes, 1974; Thiry & Hervé, 1978), interactions between nucleoside triphosphates and ionizable groups on the protein have not previously been systematically studied. While the use of calorimetry to acquire binding data is more time consuming than most other methods and requires large amounts of material, it has the advantages of allowing measurements to be made over a wide range of ligand concentrations and pH and of allowing the enthalpic and entropic components of the free energy to be resolved.

Our results indicate that each phosphate residue makes a substantial contribution not only to the enthalpy and entropy of binding but also to linked proton binding. At pH 7, the magnitudes of all of these effects are consistently greater for adenine nucleotides than for cytosine nucleotides. At pH 10, where the enzyme is not regulated, the magnitudes of all parameters are reduced, and the energetics of binding are very similar for both families of nucleotides.

We have also obtained independent evidence for high-affinity nucleoside triphosphate binding sites on c_3, and, at least in the case of ATP, two classes of sites can be distinguished. One class, presumably at the active site, since binding can be blocked by PALA, is characterized by a small negative enthalpy of binding and concomitant binding of protons between pH 7 and 8. Binding to these sites is sufficient to account for the entire difference in the values of ΔH_{app} for ATP in the presence and absence of PALA at pH 7, but for only about 50% of the difference for CTP. A second class has an enthalpy of binding close to zero but can be detected by the release of protons which accompanies binding near pH 8. Independent evidence of two classes of nucleotide binding sites on c_3 has

been presented recently by Issaly et al. (1982).

This study also represents the first detailed comparison of the binding of nucleoside triphosphates to r_2 and c_6r_6. We find that, for both CTP and ATP, free energies of binding generally differ by less than 1 kcal for c_6r_6·6PALA and r_2. On the other hand, there are substantial differences in the enthalpies and entropies of binding.

These differences, which provide a particularly striking example of enthalpy–entropy compensation, are a measure of the changes in the c–r subunit interaction energies produced when nucleoside triphosphates bind to c_6r_6·6PALA. They indicate that while nucleoside triphosphate binding does not significantly alter the strength of the c–r interactions, as reflected in the free energy, there are changes in specific interactions. The effects of these changes on the thermodynamic parameters are larger than those produced by PALA (Knier & Allewell, 1978); however, they appear similar for both nucleoside triphosphates.

In spite of these similarities, there are substantial quantitative differences between CTP and ATP which indicate that the two nucleoside triphosphates interact differently with ionizable groups on the protein. Binding of ATP invariably results in the binding of more protons, either because more groups are perturbed or because the perturbations are larger. The variation with pH in the thermodynamic parameters for binding is also greater for ATP, as predicted by linkage theory. Similarly, the contribution of each phosphate group to the energetics of binding is greater at pH 7 for ATP, although not at pH 10. Finally, and most interestingly, the stoichiometries of proton binding to the assembled enzyme and its isolated subunits are rather similar for CTP but radically different for ATP. The maximum differences are at pH 6.8 and 7.7, where approximately three additional protons are bound by r_2. It is of interest that ATP is the only effector for which the stoichiometry of linked proton binding is substantially different for the subunits and the native enzyme; the proton effects which accompany binding of PALA to c_6r_6 and c_3 are very similar (Allewell et al., 1979). This difference between ATP, on the one hand, and CTP and PALA, on the other hand, may reflect a fundamental difference in the mechanism of ATP regulation. An analogous difference in the binding of Cibacron Blue to c_3 and c_6r_6 has recently been reported by Issaly et al. (1982).

The only reasonable candidates for the residues perturbed by bound nucleotides are His, Cys, and Lys. Cys-47, Lys-84, and His-106 on c_3 are close to the active site, while His-117 and His-146 are located in the nucleotide binding domain of r_2 (Monaco, 1978). Furthermore, His-106 on c_3, His-117 on r_2, and the r_2 sulfhydryls form part of the c–r contact region and hence could be differentially perturbed in the isolated subunits and native enzyme. Moore & Browne (1980) have also shown recently that a histidine is perturbed when nucleoside triphosphates bind to r_2.

The strict correlation between $\Delta\bar{\nu}_{H^+}$, $\Delta H_{binding}$, and $\Delta S°_{binding}$ is particularly noteworthy in view of the fact that the corresponding parameters for both substrate analogue binding (Allewell et al., 1979) and subunit association (McCarthy & Allewell, 1982) follow the same pattern. This pattern also characterizes oxygenation-linked proton release in human hemoglobin (Ackers, 1980).

The correlation between $\Delta\bar{\nu}_{H^+}$ and $\Delta H_{binding}$ appears largely a consequence of the linkage between proton and nucleotide binding; the average values of $\Delta\Delta H_{binding}/\Delta\Delta\bar{\nu}_{H^+}$ (10.2 and 12.7 kcal/mol for adenine and cytosine nucleotides, respectively) are comparable to those for side-chain ionizations. The contribution of protonation reactions to $\Delta S°_{binding}$ would, however, be expected to be positive rather than negative.[4] Instead, it seems likely that $\Delta S°_{binding}$ reflects primarily the tightening of the structure which hydrogen exchange results indicate accompanies not only nucleotide binding but also subunit association (Lennick & Allewell, 1981). As Sturtevant (1979) and Ross & Subramanian (1981) have pointed out, any accompanying decrease in vibrational degrees of freedom, or increase in hydrophobic bonding, hydrogen bonding, or van der Waals interactions, would make a negative contribution to $\Delta S°_{binding}$. If this interpretation of the thermodynamic effects is correct, the correlation between $\Delta H_{binding}$ and $\Delta S°_{binding}$ in turn requires that proton binding and structural changes in the protein be tightly coupled. A model system which may be analogous is the C peptide of ribonuclease, where formation of an ionic bond is coupled to formation of a helix (Bierzynski et al., 1982).

A substantial body of information which bears on the role of protons in the allosteric mechanism now exists. Evidence for their involvement includes not only the pH dependence of both homotropic (Pastra-Landis et al., 1978) and heterotropic effects (Kerbiriou & Hervé, 1973; Thiry & Hervé, 1978) and sulfhydryl reactivity (Lauritzen & Lipscomb, 1980) but also the demonstration of salt bridges at both c–c and c–r interfaces (Honzatko et al., 1982). This paper adds new information on the thermodynamic linkage between proton binding, nucleotide binding, and structural changes in the protein to that already available on linkages between proton and substrate analogue binding (Allewell et al., 1979) and subunit association (McCarthy & Allewell, 1982). We have also shown that there are significant differences in the interactions of ATP and CTP with ionizable groups at the c–r interface. With the crystal structure of c_6r_6·6PALA available (Ladner et al., 1982), it should now be possible to develop more detailed models of the allosteric mechanism.

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[4] Ross & Subramanian (1981), following Shiao & Sturtevant (1976), state that both ΔH and $\Delta S°$ for protonation reactions are negative. This is the case, however, only when the standard state for protons is defined as unit activity at pH 7. Throughout this paper, the standard state is considered to be 1 M.

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