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Differential Scanning Calorimetric Study of the Thermal Denaturation of Aspartate Transcarbamoylase of *Escherichia coli*†

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ABSTRACT: The thermal denaturation of *Escherichia coli* aspartate transcarbamoylase (c_6r_6) in the absence and presence of various ligands has been studied by means of high-sensitivity differential scanning calorimetry (DSC). As previously reported [Vickers, K. P., Donovan, J. W., & Schachman, H. K. (1978) *J. Biol. Chem. 253*, 8493–8498], the denaturational endotherm consists of two peaks, the lower of which is due to denaturation of the three regulatory, r_2, subunits while the upper involves the two catalytic, c_3, subunits. The temperature of maximal excess apparent specific heat, t_m, of the lower peak is raised from the value of 51.4 °C for the isolated subunit to 66.8 °C as a result of subunit interactions, whereas t_m for the c_3 peak is essentially the same in the isolated subunit and in the holoenzyme, indicating that the denatured r_2 subunits do not interact with the c_3 subunits. The total specific denaturational enthalpy for c_6r_6, 4.83 ± 0.16 cal g^{-1}, is significantly larger than the weighted mean, 4.08 cal g^{-1}, of the enthalpies for c_3 and r_2. The fact that no endotherm is observed when previously scanned protein is rescanned indicates that the denaturation is irreversible, as is also the case with the r_2 and c_3 subunits. Empirical justification for analyzing the data in terms of equilibrium thermodynamics is cited. The observed DSC curves can be expressed within experimental uncertainty as the sum of five sequential two-state steps. The value of $t_{1/2}$, the temperature of half-completion, for each step increases with increasing protein concentration, indicating that some dissociation of the protein takes place during denaturation. Since earlier work [Edge, V., Allewell, N. M., & Sturtevant, J. M. (1985) *Biochemistry 24*, 5899–5906] indicated that neither the regulatory nor the catalytic subunits dissociate on denaturation, the dissociation of c_6r_6 is presumably either to $2c_3 + 3r_2$ or to $2c_3 + r_6$. The effects of ligand concentration on the five steps in the denaturation are complex. The changes in overall denaturational enthalpies produced by the ligands lead to enthalpies of dissociation from the native enzyme as follows: for *N*-(phosphonoacetyl)-L-aspartate, 45 ± 3 kcal mol^{-1}; for ATP, 2.1 ± 0.1 kcal mol^{-1}; and for CTP, −36 ± 4 kcal mol^{-1}.

Aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase; carbamoyl-phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) has been extensively used as a model system for investigating how linkages between ligand binding, conformational transitions, and protein–protein interactions regulate protein function [for recent reviews, see Kantrowitz et al. (1980a,b) and Allewell (1987)]. The enzyme catalyzes the first committed step in pyrimidine biosynthesis, transfer of the carbamoyl moiety of carbamoyl phosphate to the α-amino group of L-aspartate. Both substrates bind cooperatively, and at nonsaturating concentrations, ATP is an

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activator while CTP is an inhibitor (Gerhart & Pardee, 1962). *N*-(Phosphonoacetyl)-L-aspartate (PALA), a bisubstrate analogue (Collins & Stark, 1971), has been widely used in mechanistic studies.

The native enzyme is a dodecamer, consisting of six c chains, organized as two catalytic trimers (c_3), and six r chains, organized as three regulatory dimers (r_2) (Gerhart & Schachman, 1965; Weber, 1968; Wiley & Lipscomb, 1968). Each r chain contains a zinc ion coordinated by four sulfhydryl groups near the c–r interface (Rosenbusch & Weber, 1971; Hunt et al., 1984). The structures of the unliganded enzyme and its complexes with PALA and CTP have been solved to approximately 2.5-Å resolution (Honzatko et al., 1982; Krause et al., 1987; Kim et al., 1987). Both c and r chains consist of two domains; the c chain is comprised of polar and equatorial domains, while the r chain is made up of allosteric and zinc domains. The active site lies between c chains (Honzatko et al., 1982; Wente & Schachman, 1987) while the primary nucleotide binding site lies in the allosteric domain of the r chain. Nucleotides also bind competitively at the active site (Suter & Rosenbusch, 1977; Burz & Allewell, 1982).

Substrates and substrate analogues produce a major change in the three-dimensional structure of the protein, both in solution [cf. Gerhart and Schachman (1968), Griffin et al. (1972), and Moody et al. (1979)] and in the crystal (Krause et al., 1987). Two loops in the c chain swing into the active site, and the contacts between catalytic subunits and between catalytic and regulatory subunits are reorganized (Krause et al., 1987). Conformational changes accompanying binding of nucleotides are primarily at the level of tertiary structure (Kim et al., 1987).

Differential scanning calorimetry is a powerful method for investigating how ligand binding alters the thermodynamic properties of proteins. This method was first applied to ATCase by Vickers et al. (1978). In a previous paper, we extended their experimental studies of the isolated subunits and quantitatively modeled the results (Edge et al., 1985). This paper reports the results of an analogous study of c_6r_6. Primary attention is given to the effect of protein concentration in the absence of ligands, and to the effects of PALA, CTP, and ATP, on the thermal denaturation of ATCase.

Materials and Methods

Chemicals. PALA, assayed as >80% pure, was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. ATP and CTP (Na salts) were purchased from Sigma. Concentrations were determined by weight, correcting for impurities present in PALA. All other chemicals were reagent grade and were used without further purification.

Protein. ATCase was purified from the derepressed diploid *Escherichia coli* strain developed by Gerhart and Holoubek (1967) as described previously (Holoubek, 1967; Allewell et al., 1975). Cells were grown at Oak Ridge National Laboratories, Oak Ridge, TN. Purity, as verified by polyacrylamide gel electrophesis, by using the procedures of Weber and Osborn (1969) and Davis (1964) and by a pH stat assay of enzymatic activity, was similar to that of previous preparations, which have been examined by equilibrium dialysis (Allewell et al., 1975), reaction microcalorimetry (Allewell et al., 1975; Knier & Allewell, 1977; Burz & Allewell, 1982; McCarthy & Allewell, 1983), pH stat (Allewell et al., 1979), hydrogen exchange methods (Lennick & Allewell, 1981), and fluorescence spectroscopy (Maliwal et al., 1984). The protein was stored as a precipitate in 3.6 M $(NH_4)_2SO_4$, 0.1 M Tris-HCl, 2 mM β-mercaptoethanol, and 0.2 mM NaEDTA, pH 8.3 at 0 °C. All DSC experiments were completed within 2 months of preparing the protein. Concentrations of protein solutions were determined spectrophotometrically, assuming an absorbance of 0.59 $(mg/mL)^{-1}$ at 280 nm (Blackburn & Schachman, 1976).

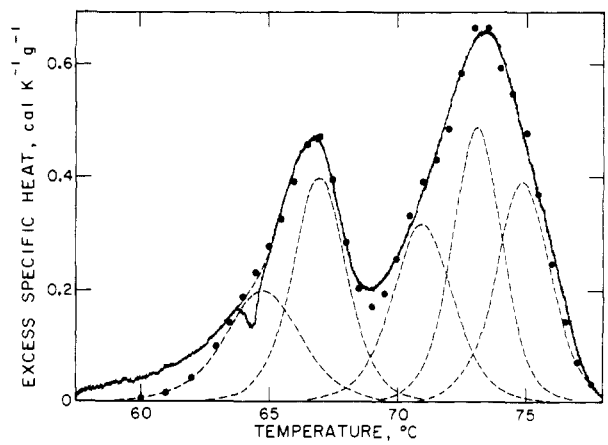

FIGURE 1: Tracing of a DSC scan observed with c_6r_6 at 9.75 mg mL^{-1}. Scan rate, 0.22 K min^{-1}. The noise level shown is typical for all the DSC experiments reported here. See text for discussion of the small exotherm at 64 °C. The dashed curves are for the five sequential steps obtained by curve resolution, and the closed circles are calculated points obtained by summing the component curves, with a standard deviation from the observed amounting to 3.2% of the maximal value (at 73 °C) of the excess specific heat.

Calorimetry. Scanning calorimetric experiments were performed with a DASM-1M instrument (Privalov et al., 1975) purchased from Mashpriborintorg, Smolenskaya Pl. Moscow, USSR. As explained in our previous publication (Edge et al., 1985), a scan rate of 0.22 K min^{-1} was employed in order to minimize the interference due to exothermic peaks presumably associated with protein aggregation. Under these conditions, data obtained with the DASM-1M are fully comparable with those obtained with the DASM-4. Although scanning at a faster rate results in a large exotherm, the increase in T_m values at a scan rate of 1 K min^{-1} is only 2 K. All calorimetric experiments were performed with protein dissolved in 40 mM sodium phosphate buffer, pH 7.0, containing 0.2 mM dithiothreitol and 0.2 mM EDTA. Any precipitate was removed by centrifugation at 4 °C for 2 h at 15000*g*. The DSC curves were resolved into component curves according to the assumed model of strictly sequential two-state steps, as outlined in the Appendix of our preceding paper (Edge et al., 1985) and in a recent review (Sturtevant, 1987).

Results

Thermal Denaturation of c_6r_6 in the Absence of Ligands. A typical DSC curve for c_6r_6 in the absence of ligands is shown in Figure 1. The small exothermic peak, which seems to be essentially an instrumental artifact associated with aggregation of the protein [see footnote 2 of Edge et al. (1985)], appears not to deduct significantly from the denaturational endotherm. Comparison of the values of t_m for the peaks, 66.8 and 73.4 °C, with those for the isolated subunits, 51.4 °C for r_2 and 72.8 °C for c_3, suggests that the lower peak is due to denaturation of the regulatory units present in c_6r_6 and the upper peak to that of the catalytic subunits (Vickers et al., 1978; Edge et al., 1985). The difference in t_m for the catalytic and regulatory subunits appears to be due to the presence of phosphate, which binds at the active site, since a single broad DSC peak is observed when the buffer is Hepes instead of phosphate (Bromberg, Allewell, and Sturtevant, unpublished observations).

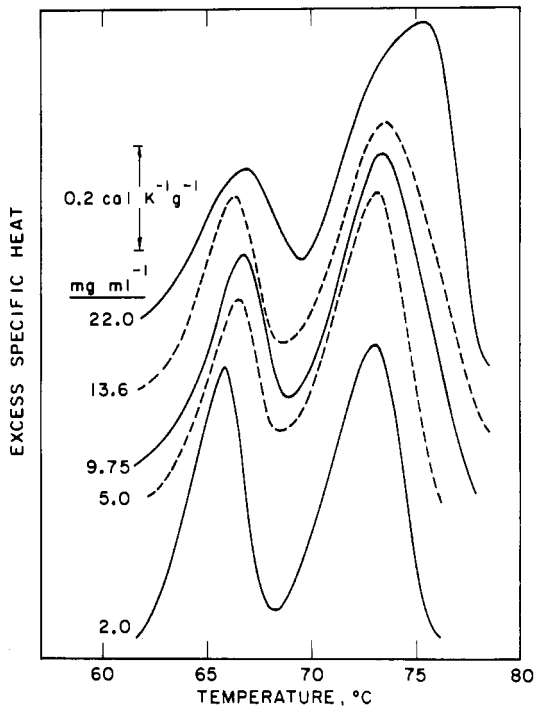

FIGURE 2: Tracings of DSC curves observed at various protein concentrations, normalized to constant sensitivity.

Figure 2 illustrates the variation of the DSC curves with protein concentration. There is little or no change in the temperature, t_m, at which the excess apparent specific heat is maximal for the lower peak in the concentration range 5–22 mg mL^{-1}, and an increase of 2.25 °C in t_m for the upper peak.

As observed with the separated subunits, the denaturation of c_6r_6 was, in terms of DSC, operationally irreversible. The propriety of applying equilibrium thermodynamics to apparently irreversible processes has been discussed by Manly et al. (1985) in connection with the denaturation of the core protein of *lac* repressor, and by Edge et al. (1985) for the ATCase subunits. In both cases, support for the application of equilibrium thermodynamics was based in part on the results of computer simulations of models of the type

$$N_4 \rightleftarrows 4D; \qquad D \xrightarrow[E_a]{k} I$$

for the tetrameric core protein of *lac* repressor, or

$$N \rightleftarrows D; \qquad D \xrightarrow[E_a]{k} I$$

for the nondissociating (see below) subunits of ATCase, with the rate constant, k, and the activation energy, E_a, for the irreversible step selected to result in as much as 90% of the protein being in the irreversibly denatured form, I, at 95% completion of the denaturation. It was found that even in such cases the parameters obtained by analyses, by our procedure based on the van't Hoff equation, of simulated DSC curves gave parameters in reasonable agreement with those used to generate the curves.

Resolution of the DSC curves for isolated r_2 and c_3 subunits on the basis of sequential two-state steps showed that two steps sufficed in the case of r_2 but that three steps were required to give an adequate representation of the curves for c_3 (Edge et al., 1985). Correspondingly, it appears that a total of five sequential steps are required for c_6r_6, as indicated in Figure 1. This conclusion is based on the fact that curve resolutions using five steps generally gave significantly lower standard deviations of calculated from observed specific heats than obtained with four steps. In the curve resolutions reported in this paper, the ratio $\beta = \Delta H_{vH}/\Delta h$, where ΔH_{vH} is the van't Hoff enthalpy in kilocalories per mole, and Δh is the calorimetric, or true, enthalpy in calories per gram, has been held equal to the molecular weight of c_6r_6, 310 000. In the curve resolutions for c_3 and r_2 (Edge et al., 1985), β was taken as an adjustable parameter. It turned out to be approximately equal to 2.5 times the molecular weight in the case of r_2 both in the absence and in the presence of ligands, suggesting that r_2 is aggregated under all the experimental conditions employed and, since t_m is independent of protein concentration, remains aggregated to the same extent after denaturation. The fact that good fits for c_6r_6 both in the absence and in the presence of ligands were obtained with β taken equal to the molecular weight shows that the native protein is not aggregated under our experimental conditions.

It should be mentioned here that the information content (Weber & Anderson, 1965) of our DSC curves probably is such that the evaluation of 10 parameters, $t_{1/2}$ (the temperature of half-completion), and Δh for each component curve by curve resolution cannot be expected to yield uniquely correct results. Nevertheless, the fact that the best fits of the data for the subunits and for the holoenzyme are obtained with models involving the same total number of components lends credence to these models.

The average values for the parameters obtained in the curve resolutions for c_6r_6 are listed in Table I, together with those for c_3 and r_2 given in our previous paper (Edge et al., 1985). As indicated in the footnotes to Table I, curve resolutions were carried out for numerous experiments over wide ranges of protein concentrations. The values given for $t_{1/2}$ for c_6r_6 indicate the range of variation with protein concentration, in each case the lower value corresponding to the lowest protein concentration and the higher value to the highest concentration. An experiment with protein at 9.75 mg mL^{-1} is the one shown in Figure 1. The range of values of $t_{1/2}$ found for each step is listed in column 2 of Table I, and mean values for the specific enthalpies in column 3. In columns 4, 5, and 6 are given values for ΔH_{vH} calculated in three different ways: (1) the products $\beta\Delta h$; (2) by means of the expression

$$\Delta H_{vH} = ART_{1/2}{}^2 c_{ex,1/2}/\Delta h \qquad (1)$$

where $T_{1/2} = t_{1/2} + 273.15$ and $c_{ex,1/2}$ is the value of c_{ex} at $t_{1/2}$ (for a two-state process not involving dissociation or association, $A = 4.00$; this is the value used in calculating the quantities in Table I); (3) the products of −1.987 times the slopes of van't Hoff plots of ln (protein concentration) vs $1/T_{1/2}$. Column 7 gives the standard deviations of the experimental points from the van't Hoff plots expressed in degrees centrigrade. The facts that $\sum\Delta h$, the sum of the five specific enthalpies, agrees well with the total enthalpy, Δh_{obsd}, as evaluated by planimeter integration of the DSC curves and that the curve-fitting process gave calculated values for the excess apparent specific heat, c_{ex}, agreeing with the experimental values within a mean standard deviation of only 3.5 ± 0.2% of the maximal value of c_{ex} lend support to the model on which the curve resolution was based. It should be noted that with as closely spaced values of $t_{1/2}$ as found here essentially the same results would be obtained with a model assuming five independently denaturing domains instead of five strictly sequential steps.

Thermal Denaturation of c_6r_6 in the Presence of PALA. Figure 3 shows the effects of PALA at concentrations up to 50 mM on the denaturation of c_6r_6. The values of t_m for both peaks are increased by the ligand, the increase at 50 mM PALA amounting to 5.3 °C for the lower peak and 7.1 °C

Table I: Parameters for Fitting the DSC Data for c_6r_6[a] to the Sum of Five Sequential Two-State Steps, for c_3[b] to the Sum of Three Steps, and for r_2[c] to the Sum of Two Steps

step	$t_{1/2}$ (°C)	Δh (cal g^{-1})	ΔH_{vH} (kcal mol^{-1}) $\beta\Delta h$	by eq 1	vH plot[d]	SD of van't Hoff plot (°C)	β (daltons)
			c_6r_6				
1	64.1–65.6	0.73 ± 0.05	226 ± 16	244 ± 12	290	±0.14	
2	65.9–68.0	0.99 ± 0.03	307 ± 9	336 ± 11	227	±0.16	
3	70.65–72.2	0.96 ± 0.04	289 ± 12	308 ± 9	234	±0.17	310000
4	72.05–74.45	1.12 ± 0.05	347 ± 16	377 ± 8	215	±0.12	
5	73.65–76.5	1.19 ± 0.06	369 ± 19	383 ± 8	180	±0.13	
			c_3 (Edge et al., 1985)				
1	68.6 ± 0.1	0.94 ± 0.08	98 ± 9	135 ± 4			106 000 ± 4000
2	70.4 ± 0.1	1.36 ± 0.05	145 ± 7	197 ± 9			
3	73.1 ± 0.1	2.35 ± 0.08	249 ± 9	275 ± 5			
			r_2 (Edge et al., 1985)				
1	48.4 ± 0.4	1.01 ± 0.08	86 ± 9	88 ± 11			85 100 ± 5600
2	51.8 ± 0.3	1.86 ± 0.17	158 ± 15	153 ± 7			

[a] Protein concentration 2–23.3 mg mL^{-1}; 14 experiments. $\sum\Delta h$ = 4.99 ± 0.15 cal g^{-1}; Δh_{obsd} = 4.83 ± 0.16 cal g^{-1}. Mean standard deviation in curve fitting = 3.5 ± 0.2% of c_{max}. [b] Protein concentration 1–15 mg mL^{-1}; 8 experiments. $\sum\Delta h$ = 4.65 ± 0.16 cal g^{-1}; Δh_{obsd} = 4.54 ± 0.22 cal g^{-1}. Mean standard deviation in curve fitting = 4.5 ± 0.3% of c_{max}. [c] Protein concentration 1–11.6 mg mL^{-1}; 9 experiments. $\sum\Delta h$ = 2.87 ± 0.12 cal g^{-1}; Δh_{obsd} = 2.77 ± 0.11 cal g^{-1}. Mean standard deviation in curve fitting = 3.1 ± 0.4% of c_{max}. [d] 1.987 times slope of van't Hoff plots of ln protein concentration vs $1/T_{1/2}$. Uncertainties are standard errors of the mean value unless otherwise specified.

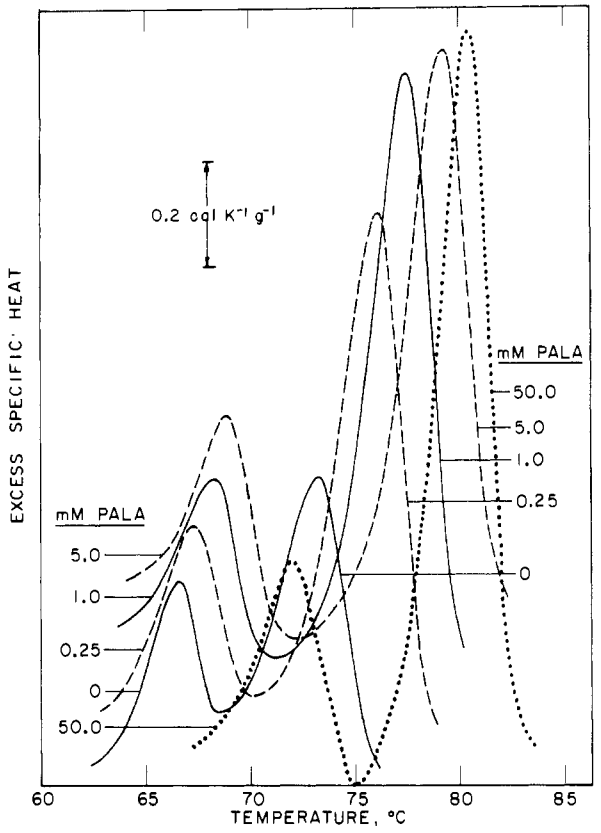

FIGURE 3: Tracings of DSC curves showing the effects of various concentrations of PALA. Protein concentration, 5 mg mL^{-1}.

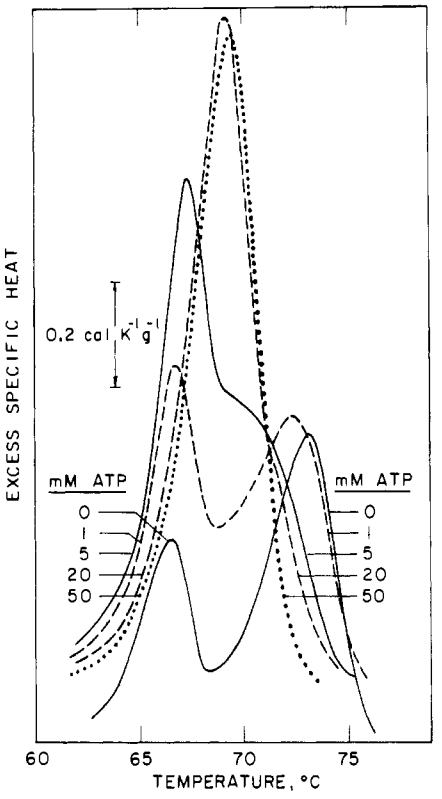

FIGURE 4: Tracings of DSC curves showing the effects of various concentrations of ATP. Protein concentration, 5 mg mL^{-1}.

for the upper peak. The increase in t_m for the lower peak is in sharp contrast to the lowering produced by PALA in the case of isolated regulatory subunit, indicating pronounced interactions between the subunits. On the other hand, the effects of PALA on the denaturational enthalpies are qualitatively the same as observed for the isolated subunits (Edge et al., 1985), with an increase in that of the catalytic subunit and with only a slight effect on that of the regulatory subunit.

The parameters resulting from resolution of the DSC curves observed in the presence of PALA are listed in Table II, along with the corresponding values for c_3 + PALA (Edge et al., 1985) for comparison. The arrangement of the data is the same as in Table I.

Thermal Denaturation of c_6r_6 in the Presence of ATP. The effects of the allosteric effector ATP on the denaturation of c_6r_6, which are shown in Figure 4, are qualitatively similar to those observed with the separated subunits. The value of t_m for the lower peak steadily increases as the concentration of the ligand is increased while that of the upper peak is decreased, and the enthalpies are not greatly affected by the ligand. The apparent increase in the size of the lower peak appears to be due to the coalescence of the two peaks.

The parameters resulting from resolution of the DSC curves in the presence of ATP are listed in Table III, along with the corresponding values for r_2 + ATP (Edge et al., 1985) for comparison. The organization of the data is the same as in the previous tables.

Table II: Parameters for Fitting the DSC Data for c_6r_6[a] in the Presence of PALA[b] to the Sum of Five Sequential Two-State Steps and for c_3[c] in the Presence of PALA[b] to the Sum of Three Steps

step	$t_{1/2}$ (°C)	Δh (cal g^{-1})	ΔH_{vH} (kcal mol^{-1}) $\beta\Delta h$	ΔH_{vH} (kcal mol^{-1}) by eq 1	ΔH_{vH} (kcal mol^{-1}) vH plot[d]	SD of van't Hoff plot (°C)	β (daltons)
			c_6r_6 + PALA				
1	64.3–69.6	0.64 ± 0.05	198 ± 16	219 ± 16	188	±0.46	
2	66.6–72.0	0.97 ± 0.06	301 ± 19	325 ± 12	241	±0.29	
3	73.5–78.6	1.06 ± 0.06	329 ± 19	358 ± 18	240	±0.14	310000
4	75.3–79.9	1.44 ± 0.05	446 ± 16	517 ± 18	255	±0.14	
5	76.7–81.1	1.53 ± 0.06	474 ± 19	506 ± 19	267	±0.16	
			c_3 + PALA (Edge et al., 1985)				
1	71.9–75.35	1.13 ± 0.09	127 ± 12	137 ± 14	281	±0.23	112500 ± 6700
2	75.0–78.5	2.23 ± 0.10	249 ± 13	280 ± 16	290	±0.20	
3	77.1–81.2	3.04 ± 0.21	338 ± 26	362 ± 14	273	±0.15	

[a] Protein concentration 5 mg mL^{-1}; 8 experiments. For c_6r_6 + PALA, $\sum\Delta h$ = 5.63 ± 0.19 cal g^{-1}; Δh_{obsd} = 5.71 ± 0.30 cal g^{-1}. Mean standard deviation in curve fitting = 2.2 ± 0.3% of c_{max}. [b] Range of PALA concentration 0.25–50 mM. [c] Protein concentration 5 mg mL^{-1}; 6 experiments. For c_3 + PALA, $\sum\Delta h$ = 6.39 ± 0.31 cal g^{-1}; Δh_{obsd} = 6.29 ± 0.43 cal g^{-1}. Mean standard deviation in curve fitting = 2.7 ± 0.2% of c_{max}. [d] 1.987 times slopes of van't Hoff plots of ln protein concentration vs $1/T_{1/2}$. Uncertainties are standard errors of the mean values unless otherwise specified.

Table III: Parameters for Fitting the DSC Data for c_6r_6[a] in the Presence of ATP[b] to the Sum of Five Sequential Two-State Steps and for r_2[c] in the Presence of ATP[b] to the Sum of Two Steps

step	$t_{1/2}$ (°C)	Δh (cal g^{-1})	ΔH_{vH} (kcal mol^{-1}) $\beta\Delta h$	ΔH_{vH} (kcal mol^{-1}) by eq 1	ΔH_{vH} (kcal mol^{-1}) vH plot[d]	SD of van't Hoff plot (°C)	β (daltons)
			c_6r_6 + CTP				
1	65.14 ± 0.19	0.63 ± 0.05	196 ± 16	228 ± 7			
2	67.00 ± 0.14	1.05 ± 0.05	326 ± 16	398 ± 12			
3	68.34 ± 0.12	1.12 ± 0.12	347 ± 37	424 ± 41			310000
4	70.02 ± 0.24	1.11 ± 0.07	344 ± 22	409 ± 33			
5	73.0–70.05	0.99 ± 0.05	307 ± 16	340 ± 16	307	±0.13	
			r_2 + ATP (Edge et al., 1985)				
1	47.8–59.2	1.32 ± 0.10	113 ± 15	128 ± 11	83	±0.43	83 ± 3700
2	52.4–62.35	2.48 ± 0.06	212 ± 9	229 ± 11	101	±0.19	

[a] Protein concentration 5 mg mL^{-1}; 8 experiments. $\sum\Delta h$ = 4.85 ± 0.18 cal g^{-1}; Δh_{obsd} = 4.87 ± 0.20 cal g^{-1}. Mean standard deviation in curve fitting = 1.6 ± 0.3% of c_{max}. [b] Range of ATP concentration 1–75 mM. [c] Protein concentration 5 mg mL^{-1}; 12 experiments. $\sum\Delta h$ = 3.79 ± 0.13 cal g^{-1}; Δh_{obsd} = 3.77 ± 0.18 cal g^{-1}. Mean standard deviation in curve fitting = 1.9 ± 0.2% of c_{max}. [d] 1.987 times slopes of van't Hoff plots of ln protein concentration vs $1/T_{1/2}$. Uncertainties are standard errors of the mean values unless otherwise stated.

Table IV: Parameters for Fitting the DSC Data for c_6r_6[a] in the Presence of CTP[b] to the Sum of Five Sequential Two-State Steps and for r_2[c] in the Presence of CTP[b] to the Sum of Two Steps

step	$t_{1/2}$ (°C)	Δh (cal g^{-1})	ΔH_{vH} (kcal mol^{-1}) $\beta\Delta h$	ΔH_{vH} (kcal mol^{-1}) by eq 1	ΔH_{vH} (kcal mol^{-1}) vH plot[d]	SD of van't Hoff plot (°C)	β (daltons)
			c_6r_6 + CTP				
1	65.9–70.7	0.41 ± 0.04	127 ± 12	222 ± 13	209	±0.22	
2	67.6–70.9	0.67 ± 0.07	208 ± 22	346 ± 13	280	±0.18	
3	68.25–71.8	0.94 ± 0.09	291 ± 28	417 ± 28	283	±0.15	310000
4	71.31 ± 0.35	1.17 ± 0.08	363 ± 25	440 ± 40			
5	72.88 ± 0.30	1.18 ± 0.09	366 ± 28	339 ± 32			
			r_2 + CTP				
1	51.65–61.9	1.66 ± 0.09	139 ± 14	144 ± 12	81	±0.65	82800 ± 8200
2	54.25–64.55	2.77 ± 0.11	229 ± 4	233 ± 18	104	±0.31	

[a] Protein concentration 5 mg mL^{-1}; 12 experiments. $\sum\Delta h$ = 4.37 ± 0.17 cal g^{-1}; Δh_{obsd} = 4.13 ± 0.45 cal g^{-1}. Mean standard deviation in curve fitting = 1.6 ± 0.3% of c_{max}. [b] Range of CTP concentration 1–50 mM. [c] Protein concentration 5 mg mL^{-1}; 10 experiments. $\sum\Delta h$ = 4.43 ± 0.26 cal g^{-1}; Δh_{obsd} = 4.31 ± 0.27 cal g^{-1}. Mean standard deviation in curve fitting = 2.4 ± 0.4% of c_{max}. [d] 1.987 times slopes of van't Hoff plots of ln protein concentration vs $1/T_{1/2}$. Uncertainties are standard errors of the mean values unless otherwise specified.

Thermal Denaturation of c_6r_6 in the Presence of CTP. The effects of CTP on the thermal denaturation of c_6r_6 are very similar to those of ATP as shown in Figure 4. The t_m of the lower, or regulatory, peak is markedly increased by CTP, and that of the upper, or catalytic, peak is slightly lowered, with the result that in the presence of 50 mM CTP the two peaks appear to have merged. Here again, as with c_6r_6 alone and in the presence of PALA and of ATP, it was found that curve resolution was most satisfactory on the assumption of five sequential two-state steps, even at high concentrations of CTP where only one peak is seen. The results of curve resolution are summarized in Table IV, including resolution of the curves for r_2 in the presence of CTP as reported in our earlier paper. It is interesting that the enthalpies for the successive steps are quite accurately independent of CTP concentration despite the changes suggested by Figure 4.

Discussion

Allosteric regulation of the enzymatic activity of ATCase depends upon interactions between its subunits, and a wide variety of methods have been used to demonstrate and probe these interactions in solution. These include functional assays (Gerhart & Pardee, 1962), the use of reporter groups (Hensley & Schachman, 1979; Johnson & Schachman, 1980, 1983;

Wang et al., 1981; Lahue & Chachman, 1986), thermodynamic (Knier & Allewell, 1978; Allewell et al., 1979; Burz & Allewell, 1982) and kinetic (Kihara et al., 1984) methods, hydrogen exchange (Lennick & Allewell, 1981), and the use of mutant [cf. Gibbons et al. (1975)] and hybrid (Foltermann et al., 1984) enzymes.

This study provides further evidence for strong subunit interactions. The mean value of the total observed denaturational enthalpy, 4.83 ± 0.16 cal g^{-1} [incorrectly stated in our earlier paper (Edge et al., 1985) to be 7.72 cal g^{-1}], is significantly higher than the weighted mean, 4.08 cal g^{-1}, of the enthalpies of the isolated subunits. The value of t_m for the r_2 peak in c_6r_6 is 67 °C compared with 51 °C for the isolated subunit, and that for the c_3 peak in c_6r_6 is 73 °C compared with 73 °C for c_3 alone. Thus, subunit interactions appear to lead to pronounced stabilization of the regulatory subunit but little or none of the catalytic subunit by the denatured r_2 subunits. These results differ qualitatively from those of hydrogen-exchange studies which sample the intact molecule and indicate that assembly stabilizes not only r_2 but also c_3 (Lennick & Allewell, 1981). They also differ quantitatively from the results of Vickers et al. (1978), who reported higher t_m values under identical solution conditions. This difference is probably due to differences in the scan rates and protein concentrations used in the two studies, although subtle differences in the protein preparations cannot be ruled out.

The effects of PALA on the values of t_m give further indication of subunit interactions. This ligand increases t_m for both peaks of c_6r_6 whereas it increases that for c_3 and decreases that for r_2. In contrast, the effects of ATP and CTP on the value of t_m for c_6r_6 are qualitatively similar to those observed with the isolated subunits; i.e., both increase the t_m of r_2 and decrease that of c_3. Variations in t_m with ligand concentration reflect differences in moles of ligand bound by the folded and unfolded species (Schellman, 1976; Fukada et al., 1983), with preferential binding to the folded species resulting in an increase in t_m.

Variations of Temperatures of Half-Denaturation. The values of $t_{1/2}$ for each step in the denaturation of c_6r_6 in the absence of ligands increase with increasing protein concentration, indicating that some dissociation of the protein takes place during denaturation. It can be shown by means of computer simulation that in cases of domain interactions, $t_{1/2}$ for the denaturation of a domain not involved in dissociation or in ligand binding can nevertheless change with protein or ligand concentration. For example, in a two-domain case where domain 1 binds ligand and domain 2 does not, if $t_{1/2}$ and Δh of domain 1 are significantly affected by unfolding of domain 2, it is found that $t_{1/2}$ for domain 2 increases with increasing ligand concentration. It is therefore impossible to conclude from the data in Table I at which step or steps dissociation takes place. In view of the fact that both r_2 and c_3 remain undissociated on denaturation (Edge et al., 1985), the dissociation of c_6r_6 would be expected to be of the overall form $c_6r_6 \rightleftarrows 2c_3 + 3r_2$, or if, as indicated by the earlier data, r_2 actually is primarily oligomerized to r_6 under the present experimental conditions, $c_6r_6 \rightarrow 2c_3 + r_6$. If the reasonable assumption is made that dissociation takes place only in the last step, then the enthalpy derived from the van't Hoff plot for this step is $4 \times 180 = 720$ kcal mol^{-1} if the product includes $3r_2$ and $2 \times 180 = 360$ if it includes r_6 (Sturtevant, 1987). The agreement of this latter figure with the values for ΔH_{vH} for the fifth step calculated analytically (columns 4 and 5, Table I) supports this assumption.

Inspection of Table II shows that the values for $t_{1/2}$ for all five steps in the denaturation of c_6r_6 in the presence of PALA increase with increasing PALA concentration. Simulations similar to those mentioned in the preceding paragraph show that $t_{1/2}$ can depend on ligand concentration even for steps not directly involved in ligand dissociation. It is thus impossible to decide from the data in Table II at which step or steps PALA is dissociated.

The data shown in Tables III and IV indicate that over the range 1–75 mM the concentration of ATP affects $t_{1/2}$ for only the final step, while that of CTP affects the values of $t_{1/2}$ for the first three steps. This difference in the effects of ATP and CTP, and the others observed in this study, is consistent with a substantial body of evidence which indicates that ATP and CTP regulate the activity of the enzyme by different mechanisms. Support for this hypothesis, summarized by Thiry and Hervé (1978), has been derived from NMR (Moore & Browne, 1980), equilibrium isotope (Wedler & Gasser, 1974), and equilibrium dialysis experiments (Winlund-Gray et al., 1973), fluorescence spectroscopy (Wong, 1971), enzyme kinetics (Heyde et al., 1973a,b), microcalorimetry (Burz & Allewell, 1982), and studies with mutants (Silver et al., 1983; Kantrowitz et al., 1981). On the other hand, measurements of activity (Howlett et al., 1977), sedimentation velocity (Howlett & Schachman, 1977), and sulfhydryl reactivity (Markus et al., 1971; Blackburn & Schachman, 1977) suggest that both ligands exert their effect by acting on the same conformational equilibrium.

Enthalpies of Dissociation of Ligands. According to Hess's Law, the enthalpy of denaturation in the presence of an approximately saturating ligand which completely dissociates during denaturation must differ from the enthalpy of denaturation in the absence of ligand by the enthalpy of dissociation of the ligand from the native protein. Of course, in this comparison, the enthalpies must all be calculated to the same temperature. In the present case, since no consistent value for ΔC_p was observed, no corrections were made. The differences in enthalpies in the presence and the absence of PALA is 273 kcal mol^{-1}, giving 45 ± 3 kcal mol^{-1} for the average enthalpy of dissociation of PALA assuming all six molecules are dissociated. Similarly, we can calculate 2.1 ± 0.1 kcal mol^{-1} as the average enthalpy of dissociation of ATP and −36 ± 4 kcal mol^{-1} for CTP. The difference between the enthalpies of binding of ATP and CTP calculated here and those determined by Burz and Allewell (1982) at 25 °C indicates that a large change in heat capacity is associated with nucleotide binding.

Registry No. ATCase, 9012-49-1; ATP, 56-65-5; CTP, 65-47-4; PALA, 51321-79-0.

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