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High-Resolution Differential Scanning Calorimetric Analysis of the Subunits of *Escherichia coli* Aspartate Transcarbamoylase[†]

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ABSTRACT: The thermal denaturation of the catalytic (c_3) and regulatory (r_2) subunits of *Escherichia coli* aspartate transcarbamoylase (c_6r_6) in the absence and presence of various ligands has been studied by means of highly sensitive differential scanning calorimetry. The denaturation of both types of subunit is irreversible as judged by the facts that the proteins coagulate when heated and that no endotherm is observed when previously scanned protein is rescanned. Despite this apparent irreversibility, there is empirical justification for analyzing the calorimetric data in terms of equilibrium thermodynamics as embodied in the van't Hoff equation. The observed curves of excess apparent specific heat vs. temperature are asymmetric and can be expressed within experimental uncertainty as the sums of sequential two-state steps, a minimum of two steps being required for r_2 and three for c_3. As previously reported [Vickers, K. P., Donovan, J. W., & Schachman, H. K. (1978) *J. Biol. Chem. 253*, 8493–8498], the addition of the effectors ATP and CTP raises the denaturation temperature of r_2 and lowers that of c_3 while the addition of the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate raises the denaturation temperature of c_3 and lowers that of r_2. These effects vary with ligand concentration in the manner expected from the van't Hoff equation, indicating that they are simply manifestations of Le Chatelier's principle rather than being due to "stabilization" or "destabilization" of the proteins. The denaturational enthalpy is increased in those cases of ligand binding in which the denaturation temperature is increased, because of the contribution from the enthalpy of dissociation of the ligand.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2; L-aspartate carbamoyltransferase) has been used extensively as a model system for investigating the interplay between ligand binding, tertiary and quaternary structure, and biological activity [reviewed by Kantrowitz et al. (1980a,b) and Cohen et al., (1985)]. The native enzyme is composed of 12 polypeptide chains consisting of two catalytic trimers (c_3)[1] of molecular weight 1.0×10^5 and three regulatory dimers (r_2) of molecular weight 3.3×10^4 (Gerhart & Schachman, 1965; Weber, 1968; Wiley & Lipscomb, 1968). Each subunit consists of two domains (Honzatko et al., 1982). c_3 and r_2 can be obtained in pure form by treating c_6r_6 with mercurials (Gerhart & Holoubek, 1967; Yang et al., 1978). The interactions of the subunits in the holoenzyme and of the separated subunits and the holoenzyme with various ligands were studied by Vickers et al. (1978) by means of differential scanning calorimetry. In view of the importance of this enzyme as a model system for studying allosteric interactions, it seemed worthwhile to extend the work of Vickers et al. using a highly sensitive scanning calorimeter. The present paper is primarily concerned with the isolated subunits and their interactions with the allosteric effectors ATP and CTP and the bisubstrate analogue PALA. A later paper will focus on the DSC results obtained with c_6r_6 in the absence and presence of the ligands mentioned above.

Materials and Methods

Proteins. The native enzyme was prepared from the derepressed diploid *E. coli* strain developed by Gerhart and Holoubek 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 according to the procedures of Weber & Osborn (1969) and Davis (1964) and by enzymatic activity, was similar to that of previous preparations. c_3 and r_2 were prepared according to Yang et al. (1978) with the modifications introduced by Burz & Allewell (1982). The Zn content of r_2 was determined by colorimetric analysis (Kägi & Vallee, 1958), and its degradation was ruled out by N-terminal analysis and analytical gel chromatography. The ligand binding properties of both c_3 and r_2 have been examined by hydrogen exchange (Lennick & Allewell, 1981), reaction calorimetry (Burz & Allewell, 1982), and fluorescence spectroscopy (Maliwal et al., 1984). Assembly has been examined by both hydrogen exchange (Lennick & Allewell, 1981) and reaction calorimetry (McCarthy & Allewell, 1983). Both analytical gel chromatography and microzone electrophoresis were used to verify that the assembly reaction was stoichiometric.

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[1] Abbreviations: ATCase (c_6r_6), *E. coli* aspartate transcarbamoylase; c_3, catalytic subunit of ATCase; DSC, differential scanning calorimet(er)(ry); DTT, dithiothreitol; PALA, *N*-(phosphonoacetyl)-L-aspartate; r_2, regulatory subunit of ATCase; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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All DSC experiments were performed within a few days of preparing the subunits. In the interim, the proteins were stored as $(NH_4)_2SO_4$ precipitates at 4 °C under 3.6 M $(NH_4)_2SO_4$ containing 0.1 M Tris-HCl and 0.1 mM dithiothreitol at pH 8.3. In addition, the precipitation buffer for c_3 contained 0.2 mM EDTA; that for r_2 contained 2 mM $Zn(OAc)_2$, 180 mM benzamidine hydrochloride, and 50 mM streptomycin (to inhibit proteolysis and bacterial growth). The proteins were equilibrated with the buffer used in the DSC experiments by extensive dialysis in the 24 h preceding the experiments. Any precipitate was removed by centrifugation at 4 °C for 2 h at 15000*g*. Protein and nucleotide concentrations were determined spectrophotometrically as described previously (Blackburn & Schachman, 1976; Burz & Allewell, 1982).

Chemicals. ATP and CTP were purchased from Sigma and used without further purification. PALA was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Its purity was determined as previously described (Burz & Allewell, 1982).

Calorimetry. The DASM-1M microcalorimeter (Privalov et al., 1975) was used in the present study. The sensitivity of this instrument is such that the upper limit of the protein concentrations used, about 15 mg mL^{-1}, coincided with the lower limit used in the work of Vickers et al. (1978). All the results presented here were obtained at a scan rate of 0.22 K min^{-1}. This is $^1/_5$th of the rate usually employed in studies of protein denaturation with the DASM-1M and $^1/_{45}$th of the rate used by Vickers et al. We observed troublesome exotherms, presumably associated with protein aggregation, at a scan rate of 1 K min^{-1}, particularly at protein concentrations below 5 mg mL^{-1}. For reasons which we do not understand, these exotherms disappeared nearly completely at a scan rate of 0.22 K min^{-1} and protein concentrations of 5 mg mL^{-1} and higher.[2] All calorimetric experiments were performed with protein dissolved in 40 mM sodium phosphate buffer, pH 7.0, containing 0.2 mM DTT and, in the case of c_3 and c_6r_6, 0.2 mM EDTA.

Resolution of DSC Curves. The curves of excess specific heat vs. temperature were resolved into component two-state curves as described in the Appendix.

van't Hoff Analysis of Data for Irreversible Denaturation. As pointed out by Vickers et al., the thermal denaturation of c_6r_6 and its separated subunits is irreversible overall; at the end of each scan, the protein has precipitated, and it does not redissolve on being cooled. Furthermore, when a sample which has been heated through its denaturational endotherm is rescanned after being cooled, little or no endotherm is observed. We shall, nevertheless, analyze our DSC data in terms of equilibrium thermodynamics, primarily as expressed in the van't Hoff equation

$$d \ln K/dT = \Delta H_{vH}/RT^2 \qquad (1)$$

where K is the "equilibrium constant" for the denaturation and ΔH_{vH} is the (van't Hoff) enthalpy which determines the variation of K with the absolute temperature T. We offer two justifications for this unorthodox procedure. In the first place, we have observed not only with the proteins studied here but also with other proteins such as the *lac* repressor of *E. coli* (Manly et al., 1985) that the effects of variations of protein and ligand concentration on denaturational parameters can be accurately and consistently expressed in terms of the van't Hoff equation. Second, calculations for the model system

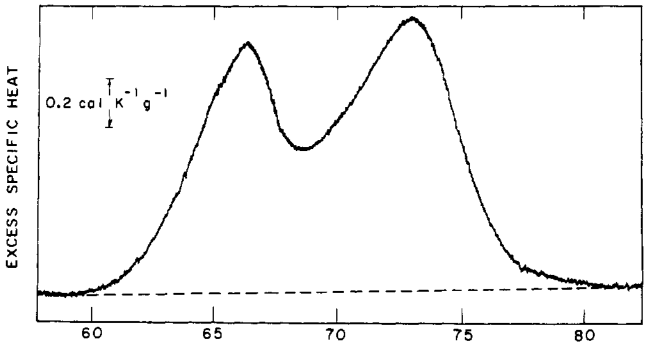

FIGURE 1: Tracing of a DSC span of aspartate transcarbamoylase, showing the noise level encountered in this work. In this case, the initial and final apparent specific heats were nearly equal. Protein concentration, 5.94 mg mL^{-1}; scan rate, 0.22 K min^{-1}.

$$P \rightleftharpoons D \qquad D \rightarrow A \qquad (2)$$

in which a protein undergoes a reversible denaturation followed by an irreversible step show that data for such a system can be analyzed according to the van't Hoff equation to give parameters close to those originally assigned to the reversible system. This is true even when the rate of the irreversible step is such as to cause 90% of the protein to be in the irreversible form at a temperature at which the reversible reaction is 95% completed (unpublished results).

It can be shown [Fukada et al., 1983; see also Schellman (1976)] that for the process

$$P_mL_n \rightleftharpoons mD + nL \qquad (3)$$

in which the oligomeric protein P_m with n molecules of bound ligand undergoes dissociation and loss of bound ligand on denaturation:

$$\Delta H_{vH}/RT_m = (m-1) \ln [P]_0 - n \ln [L]_0 + \text{constant} \qquad (4)$$

where $[P]_0$ is the total protein concentration and $[L]_0 \gg [P]_0$ is the total ligand concentration. Thus, a plot of ln $[P]_0$ vs. $1/T_m$ at constant or zero $[L]_0$ should be a straight line with slope $-\Delta H_{vH}/[(m-1)R]$, or a plot of ln $[L]_0$ vs. $1/T_m$ at constant $[P]_0$ should be a straight line with slope $-\Delta H_{vH}/nR$. It is important to note that no assumption concerning the degree of saturation of the protein by the ligand is involved in this treatment.

RESULTS

Thermal Denaturation of c_6r_6. A tracing of the DSC output curve for an experiment with c_6r_6 at a concentration of 5.94 mg mL^{-1} is shown in Figure 1. The noise level amounted to 14 mcal K^{-1} g^{-1}. As is frequently the case in DSC studies of protein denaturation, the base lines were rather variable, even more so with c_6r_6 and its subunits than with other proteins we have previously studied. In some experiments, the final apparent specific heat of the protein was larger than the initial value while in others it was smaller. There was also a lack of consistency in the initial and final variation of the specific heat with temperature. This variability is definitely not instrumental and must be due to subtle irreproducibilities in the preparation of the protein. We have used as base lines straight

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[2] Since completion of the work reported here, we have obtained the new model of the Privalov calorimeter, designated DASM-4. We have found that ATCase and its subunits show no exotherms when scanned in this instrument even at 1 K min^{-1}. This has also been found to be the case with the *lac* repressor and λ repressor of *E. coli*, both of which gave large exotherms when scanned at 1 K min^{-1} in the DASM-1M instrument. It appears that these exotherms are not of chemical origin but result in some unknown way from the pillbox geometry of the cells of the DASM-1M. The cells of the DASM-4 are horizontal helices of small-bore platinum tubing.

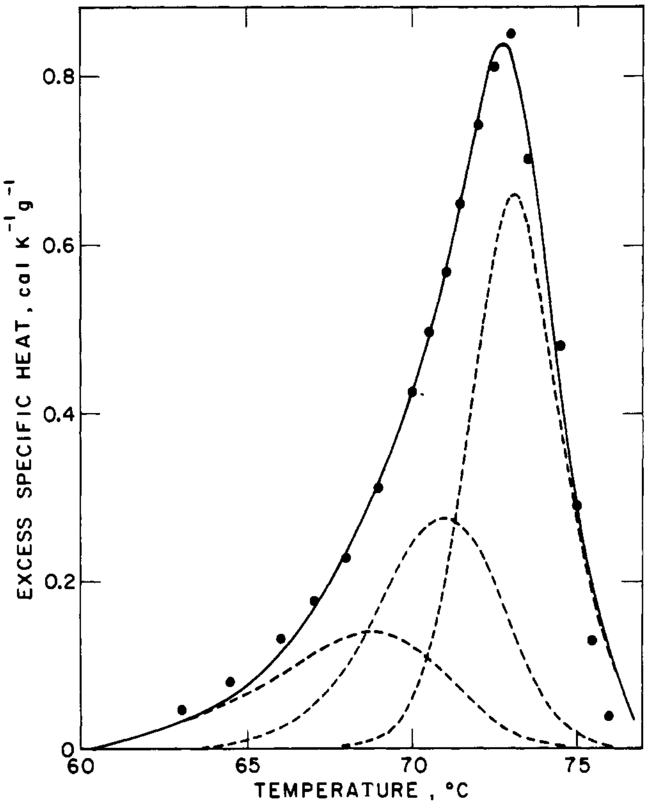


FIGURE 2: Resolution of a DSC curve for c_3 into three sequential two-state steps. (---) Component contributions; (—) sum of component contributions; (●) observed values. Protein concentration, 5.0 mg mL^{-1}; scan rate, 0.22 K min^{-1}. See Appendix for comment concerning the asymmetry of the component contributions.

lines which are as nearly as possible tangent to both the pre- and postdenaturational base lines, as illustrated in the figure. As reported by Vickers et al. (1978), the curve has two peaks; in our experience, the one at lower temperature is considerably larger in relation to the peak at higher temperature than they observed. We obtain peak temperatures of 66.4 and 73.3 °C, while they found values of 72 and 82 °C at a scan rate 45 times larger than we used. We derive a total enthalpy of 7.72 cal g^{-1} compared to their value of 5.05 cal g^{-1}.

A denaturation curve with more than one peak is usually taken to indicate the presence in the protein molecule of more than one domain, with the various domains undergoing denaturation more or less independently of each other. Vickers et al. concluded, primarily on the basis of comparisons with the denaturational curves for the separated subunits, that the lower peak is due to the denaturation of the regulatory subunits and the higher peak to that of the catalytic subunits. If the process were as simple as the independent denaturation of two domains, the overall curve should be resolvable into the sum of two nearly symmetrical van't Hoff (two-state) curves. The pronounced asymmetry of both peaks shows the situation to be more complex, as a result of domain interactions or other causes.

Thermal Denaturation of c_3. The thermal denaturation of c_3 was investigated over the concentration range of 1.0–15.0 mg mL^{-1}. The t_m observed in 23 experiments was 73.0 ± 0.1 °C, and the enthalpy (ΔH_{cal}) was 4.00 ± 0.15 cal g^{-1}. The constancy of t_m indicates that there is no change in oligomerization of the protein on denaturation and thus that the trimer either has completely dissociated well below the denaturation temperature or remains intact during the denaturation. The DSC curves for c_3 are markedly asymmetric (Figure 2), and it therefore seems likely that the second alternative is the correct one and that the asymmetry is due to the existence of two or more steps in the denaturation of the protein.

Table I: Parameters for Fitting the DSC Data for c_3 to the Sum of Three Sequential Two-State Steps[a]

step	$t_{1/2}$ (°C)	Δh (cal g^{-1})	ΔH_{vH} (kcal mol^{-1})[b]	β (daltons)
1	68.6 ± 0.1	0.94 ± 0.08	98 ± 8	
2	70.4 ± 0.1	1.36 ± 0.05	145 ± 8	106000 ± 4000
3	73.1 ± 0.1	2.35 ± 0.08	249 ± 4	

[a] Range of protein concentrations 1–15 mg mL^{-1}; six experiments. Uncertainties are standard errors of the mean. [b] $\Delta H_{vH} = \beta \Delta h$.

It was found that within experimental uncertainty the DSC curves could be represented as the sum of three sequential two-state steps. Analysis into three steps gave significantly better fits to the experimental data than obtained with only two steps. The mean standard deviation between 18–20 observed and calculated points per experiment for 6 DSC experiments was 2.8% of the maximal value of the excess heat capacity. Similar accuracy was obtained in the analysis of experiments with c_3 in the presence of PALA, and with r_2 alone and in the presence of ATP and CTP. It should be noted that essentially as good fits for c_3 can be obtained with the assumption of three *independent* two-state components; however, models of this type are not consistent with evidence in the literature indicating extensive interdomain, interchain, and intersubunit interactions [reviewed by Kantrowitz et al. (1980a,b) and Cohen et al. (1985)]. The analysis into sequential steps is illustrated in Figure 2, and the mean values of the derived parameters are listed in Table I. The individual values of $t_{1/2}$, ΔH_{cal}, and β are independent of the protein concentration, and at each concentration, the sum of the values of ΔH_{cal} is closely equal to ΔH_{obsd}, the value for the enthalpy obtained by integration of the DSC curve. It is interesting that β, a freely varying parameter, assumed a value close to the molecular weight of c_3, thus indicating that the sequential steps are indeed closely two state in character.

Thermal Denaturation of c_3 in the Presence of Ligands. The effects of the nucleotides ATP and CTP on the denaturation of c_3 consist of a pronounced lowering of t_m and no significant change in enthalpy. We may therefore conclude that these ligands are more tightly bound to the denatured than to the native form of c_3 and that the enthalpy of binding is small. On the other hand, the bisubstrate analogue PALA increases t_m and the enthalpy of denaturation, the enthalpy increase being fully developed at the lowest PALA concentration employed, 0.5 mM, indicating effective saturation of the PALA binding sites in all our experiments. The value of t_m at a constant concentration of c_3, 5 mg mL^{-1}, increases with increasing PALA concentration in accordance with the van't Hoff equation.

As was the case with c_3 in the absence of ligands, the DSC curves observed in the presence of PALA are asymmetric and can be resolved into sequential steps. This is illustrated in Figure 3 for an experiment in the presence of 20 mM PALA. The mean values of the enthalpies obtained at various concentrations of PALA and protein at 5 mg mL^{-1} are listed in Table II. The value of β obtained in the curve resolution is again approximately equal to the molecular weight of c_3.

The values of $t_{1/2}$ for each step obtained by curve resolution give reasonably satisfactory van't Hoff plots, as shown in Figure 4. If one molecule of ligand is released at each step, then values for ΔH_{vH} are obtained by multiplying the slopes of the least-squares lines in the figure by −1.987 and are listed

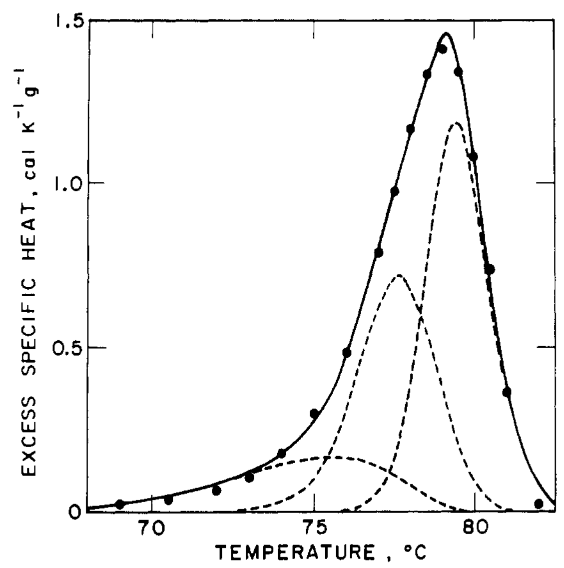


FIGURE 3: Resolution of the DSC curve for an experiment with c_3 (5.0 mg mL^{-1}) plus PALA (5 mM) into three sequential two-state steps. (---) Component contributions; (—) sum of component contributions; (●) observed values.

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

		ΔH_{vH} (kcal mol^{-1})		
step	Δh (cal g^{-1})	curve resolution[c]	van't Hoff plot[d]	β (daltons)
1	1.13 ± 0.09[e]	127 ± 14[e]	281	
2	2.23 ± 0.10	249 ± 12	290	112500 ± 6700[e]
3	3.04 ± 0.21	338 ± 13	273	

[a] Protein concentration 5 mg mL^{-1}; six experiments. [b] Range of PALA concentrations 0.5–50 mM. [c] $\Delta H_{vH} = \beta \Delta h$. [d] −1.987 times the slopes of the van't Hoff plots of ln $[\mathrm{PALA}]_0$ vs. $1/T_{1/2}$ (Figure 4). [e] Uncertainties are standard errors of the mean.

in the fourth column of Table II. A direct simulation for the model

$$c_3L_3 \rightleftarrows c_2dL_2 + L \rightleftarrows cd_2L + L \rightleftarrows d_3 + L \qquad (5)$$

where c and d represent respectively native and denatured monomer chains, verifies that, as expected for two-state steps, the enthalpies in columns three and four in Table II should agree for each step. More elaborate models than are justified by the information now available would be required to account for the apparent constancy of the enthalpies in column 4.

Thermal Denaturation of r_2. Nine experiments with r_2 at concentrations in the range 1–11.6 mg mL^{-1} gave a value for t_m of 51.7 ± 0.3 °C and for ΔH_{cal} of 2.77 ± 0.11 cal g^{-1}. The constancy of t_m indicates either that r_2 is already dissociated by 45–50 °C, which seems unlikely, or that the protein remains dimeric after denaturation. Vickers et al. (1978) reported a value for t_m of 55 °C at a 40-fold higher scan rate than we employed. Their value for the enthalpy of denaturation is 73% of the mean value given above. This difference may result from differences in protein preparations or in scan rates.

The DSC curves for r_2 were somewhat asymmetric, and it was found that they could be represented within experimental uncertainty as resulting from two sequential two-state steps. The fit is illustrated in Figure 5, and the mean values of the parameters obtained by curve fitting are listed in Table III. The value for β is 2.5 times larger than the molecular weight of r_2, strongly suggesting that what is being observed is denaturation of an aggregate. As in the case of c_3, the large difference in ΔH for the two steps indicates strong interchain or intersubunit interactions.

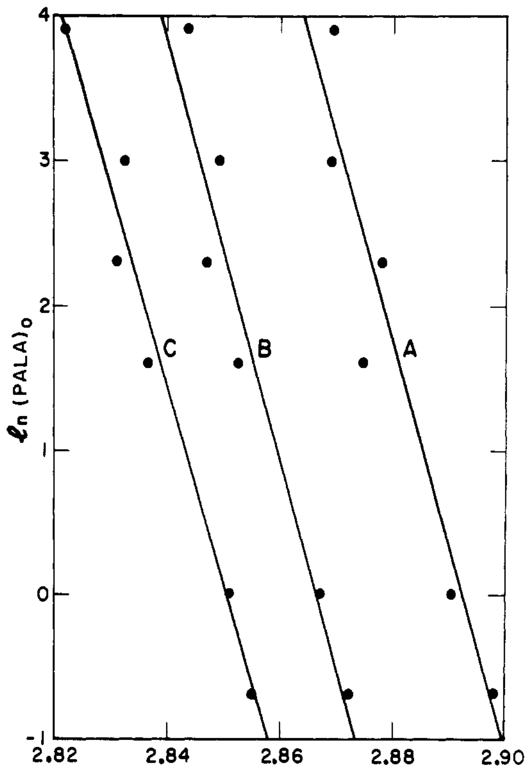


FIGURE 4: van't Hoff plots of the logarithm of the total concentration of PALA vs. $1000/T_{1/2}$ for sequential steps 1, 2, and 3 (plots A, B, and C, respectively) obtained by resolution of the DSC curves for c_3 (5.0 mg mL^{-1}) in the presence of concentrations of PALA of 0.50–50.0 mM.

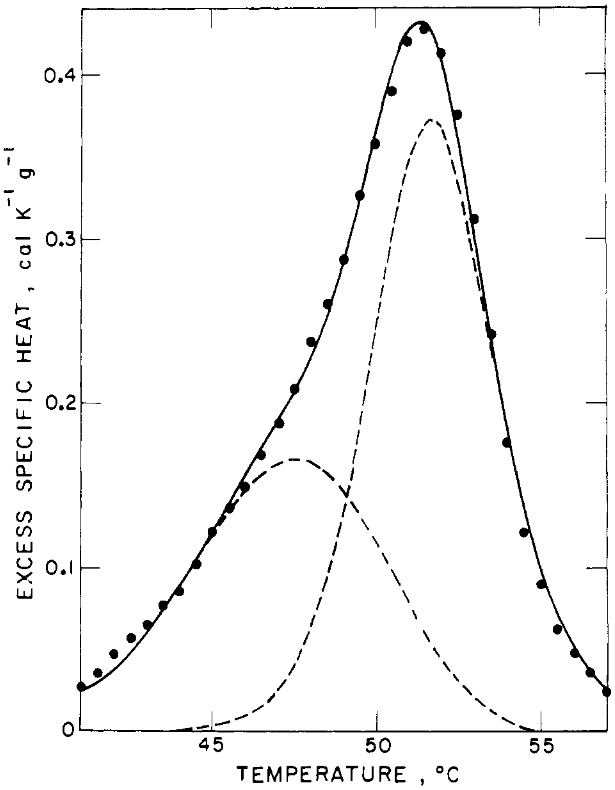


FIGURE 5: Resolution of the DSC curve for an experiment with r_2 into two sequential two-state steps. (---) Component contributions; (—) sum of component contributions; (●) observed values. Protein concentration, 5.0 mg mL^{-1}; scan rate, 0.22 K min^{-1}.

Thermal Denaturation of r_2 in the Presence of Ligands. In contrast to the case of c_3, PALA lowers the t_m of r_2 with little

Table III: Parameters for Fitting the DSC Data for r_2 to the Sum of Two Sequential Two-State Steps[a]

step	$t_{1/2}$ (°C)	Δh (cal g^{-1})	Δh [kcal (mol of r_2)$^{-1}$]	ΔH_{vH} (kcal mol^{-1})[b]	β (daltons)
1	48.4 ± 0.4	1.01 ± 0.08	34.3 ± 2.7	86 ± 9	85100 ± 5600
2	51.8 ± 0.3	1.86 ± 0.17	63.2 ± 3.1	158 ± 15	

[a] Range of protein concentrations 1–11.6 mg mL^{-1}. Uncertainties are standard errors of the mean. [b] $\Delta H_{vH} = \beta\Delta h$.

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

				ΔH_{vH} (kcal mol^{-1})		
ligand	step	Δh (cal g^{-1})	ΔH [kcal (mol of r_2)$^{-1}$]	curve resolution[b]	van't Hoff plot[c]	β (daltons)
ATP	1	1.32 ± 0.10[d]	44.9 ± 3.4[d]	113 ± 15[d]	83 ± 17[d]	85400 ± 3700[d]
	2	2.48 ± 0.06	84.3 ± 2.0	212 ± 9	101 ± 10	
CTP	1	1.66 ± 0.09	56.4 ± 3.1	137 ± 14	81 ± 16	82800 ± 8200
	2	2.77 ± 0.11	94.2 ± 3.7	229 ± 3.7	104 ± 10	

[a] Protein concentration 5.0 mg mL^{-1}. [b] $\Delta H_{vH} = \beta\Delta h$. [c] −1.987 times the slopes of the van't Hoff plots of ln $[ATP]_0$ or ln $[CTP]_0$ vs. $1/T_{1/2}$ (cf. Figure 5). [d] Uncertainties are standard errors of the mean.

or no effect on its denaturational enthalpy, while ATP and CTP increase both the t_m and the enthalpy of denaturation. At a concentration of 15 mM, PALA lowers the t_m for r_2 by 3.5 °C. The data for PALA as ligand give a van't Hoff plot with slope = +12 800 K^{-1} and coefficient of determination r^2 = 0.80. Comparison of this slope with the molar enthalpy of denaturation suggests that as many as six more molecules of PALA may be bound to the denatured than to the native protein. If this suggestion is correct, then it may be concluded that the binding of PALA to r_2 is nearly athermal.

A total of 13 experiments were run in the presence of ATP at ligand concentrations of 1–75 mM and 9 in the presence of CTP at concentrations of 1–50 mM. The results of expressing the DSC curves for these experiments in terms of two sequential two-state steps are summarized in Table IV. The value for β obtained by curve resolution was almost the same as that obtained from the experiments run in the absence of ligand.

The values for $t_{1/2}$ for each step with ATP are shown in the form of van't Hoff plots in Figure 6. Similar plots were obtained for CTP. The value of the coefficient of determination for the first step in each case (r^2 = 0.88 and 0.73 for ATP and CTP, respectively) is considerably lower than that for the second step (r^2 = 0.97 and 0.95). If we assume two molecules of ligand to be bound to r_2 one of which is dissociated at each step, the least-squared slopes of the plots in Figure 6 lead to the values for ΔH_{vH} given in column 5 of Table IV. As was the case for c_3 + PALA, the discrepancies between the enthalpies in column 4 and column 5 of the table cannot be accounted for with the information now available.

The enthalpies in column 3 of Table IV exceed those in column 3 of Table III by 5.3 and 10.5 kcal (mole of binding site)$^{-1}$ for ATP as ligand and by 11.0 and 15.5 kcal mol^{-1} for CTP. These quantities presumably reflect the heats of dissociation of the nucleotides at the first and second steps of the denaturation.

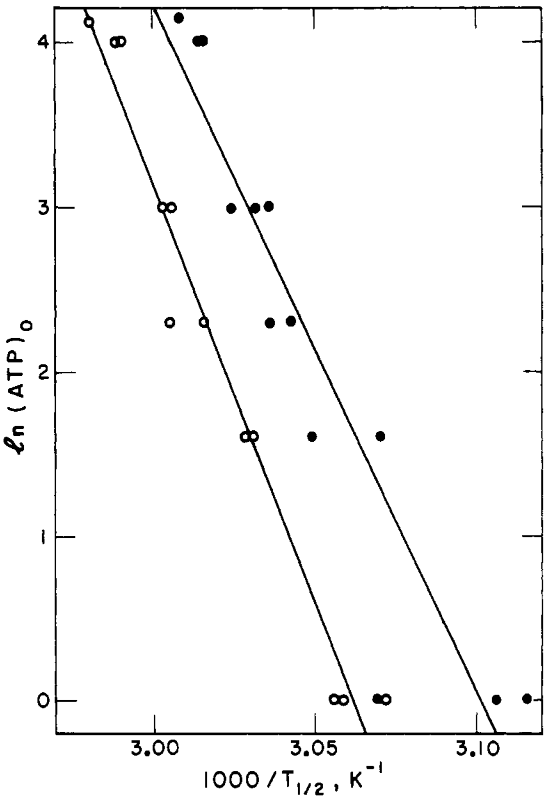


FIGURE 6: van't Hoff plots of the logarithm of the total concentration of ATP vs. $1000/T_{1/2}$ for sequential steps 1 (●) and 2 (○) obtained by resolution of the DSC curves for r_2 (5.0 mg mL^{-1}) in the presence of concentrations of ATP of 1.00–75.0 mM.

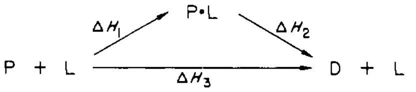
DISCUSSION

The results of this study are in qualitative agreement with those of Vickers et al. (1978); i.e., we also find that PALA increases t_m and the enthalpy of denaturation of c_3 while both CTP and ATP increase t_m and the enthalpy of denaturation of r_2. Our results are not, however, in quantitative agreement. In general, our t_m values are lower than those of Vickers et al., while our enthalpy values are larger. For example, our values for t_m and the enthalpy of denaturation for c_3 in the absence of PALA are 73 °C and 4.65 cal g^{-1}, respectively, compared to the values of Vickers et al. of 80 °C and 3.94 cal g^{-1}. Similarly, our values for unliganded r_2 are 51.7 °C (for t_m) and 2.77 cal g^{-1} relative to values from Vickers et al. of 55 °C and 1.91 cal g^{-1}. The very rapid scan rates (10 °C/min) and very high protein concentrations (~18 mg mL^{-1} for c_3 and ~45 mg mL^{-1} for r_2) used by Vickers et al. may contribute to these differences. There may also be some differences in the proteins used in the two studies although our preparative procedures are generally very similar to those described by Schachman and his collaborators.

The high sensitivity of the Privalov calorimeter has made it possible to make measurements over a wide range of protein and ligand concentrations and to resolve the components of the two major transitions. In the case of c_3, the data were best fit to a sequential model involving two intermediates in addition to the native and fully denatured species. The lack of dependence of t_m on protein concentration and the relatively good agreement between β, the ratio of the van't Hoff and calorimetric enthalpies, and the molecular weight of c_3 suggest that the protein does not dissociate upon denaturation and that the apparently irreversible aggregation which occurs at high temperatures does not contribute to the observed thermodynamic effects. Continued association of c chains, even at high temperatures, is consistent with the conclusions of Schachman and his collaborators about the strength of interchain bonds in c_3 [cf. Subramani et al. (1977)] and also the observation that these bonds become stronger as the temperature increases (Burns & Schachman, 1982). Although each step in the denaturation pathway could correspond to the denaturation of one c chain, this is not necessarily the case; as Privalov (1982) and others have pointed out, cooperative units detected by differential scanning calorimetry need not correspond to structural domains. Interpretation of the data is further complicated by the existence of domains within each chain (Honzatko et al., 1982). Hence, the increases in the values of both the calorimetric and van't Hoff enthalpies at each stage of the reaction could reflect differences in the structural elements denaturing at each stage. Alternatively, if each step corresponds to the melting of one chain, the differences must be attributed to changes in chain conformation and interchain interactions as denaturation progresses.

As reported by Vickers et al. (1978), binding of the bisubstrate analogue PALA increases the total enthalpy of denaturation by approximately 40%; $1/t_m$ also increases linearly with ln [PALA], indicating, as might be expected, that PALA is bound more tightly by native c_3 than by denatured protein. van't Hoff enthalpies calculated from the dependence of t_m on PALA concentration are approximately equal for each step in the denaturation process, although those calculated by curve fitting show the same trends observed in the absence of PALA.

The difference in the enthalpies observed in the presence and absence of PALA is to be attributed to the dissociation of PALA. This is made clear by the general isothermal scheme:



Since $\Delta H_1 + \Delta H_2 = \Delta H_3$, it follows that $\Delta H_{\text{dissoc}} = -\Delta H_1 = \Delta H_2 - \Delta H_3$. The observed differences correspond to 0.19, 0.87, and 0.69 cal g^{-1} for steps 1, 2, and 3, respectively, giving a mean value of 0.58 cal g^{-1} or 58 kcal (mol of PALA)$^{-1}$ at a t_m of 79 °C. Shrake et al. (1981) reported a value of −40.3 kcal (mol of c_3)$^{-1}$ for the binding of PALA in the same buffer at 30 °C. Hence, the apparent value of the change in heat capacity on dissociation of PALA is roughly 910 cal K^{-1} (mol of PALA)$^{-1}$.

There is ample evidence indicating that these effects reflect not only local interactions between PALA and the protein at the active site but also long-range changes in tertiary structure and interchain interactions induced by binding of the ligand. The crystal structure of c_6r_6 ligated with PALA indicates that each PALA binding site involves two c chains and that binding results in large motions of two loops (Krause et al., 1985). Although analogous crystallographic studies have not been carried out with c_3, the results of complementation experiments provide independent evidence that two chains contribute to each active site (Robey & Schachman, 1985). Evidence that PALA also produces a conformational change in c_3 has come from sedimentation velocity [cf. Howlett & Schachman (1977)], c chain interchange (Burns & Schachman, 1982), and hydrogen exchange (Lennick & Allewell, 1981) experiments and from several thermodynamic studies (Knier & Allewell, 1977; Vickers et al., 1978; Shrake et al., 1982). The contribution to the observed changes in excess heat capacity due to uptake by the buffer of the protons released when PALA is dissociated (Knier & Allewell, 1977; Allewell et al., 1979; Shrake et al., 1981) is small because of the small enthalpy of protonation of the HPO_4^{2-} ion.

ATP and CTP have been shown to bind competitively at the active site (Honzatko et al., 1979) and to increase rates of hydrogen exchange from c_3 (Lennick & Allewell, 1981). We find that they lower the t_m, indicating that they are bound more tightly by the denatured species than by native c_3, but that there is no significant change in the enthalpy of denaturation, implying negligible differences in the enthalpy of binding to the two forms of the protein. Burz & Allewell (1982) have reported enthalpy changes of −6.2 and −8.9 kcal (mol of c_3)$^{-1}$ for the binding of CTP and ATP, respectively, to native c_3 at pH 7 and 25 °C.

In considering the results for r_2, the first point to be noted is that the β values obtained for r_2 alone or in the presence of ligands strongly suggest that what is being observed is denaturation of an aggregated species. At 25 °C, r_2 ligated with Zn(II) exists primarily as a dimer with a molecular weight of 34 000 (Colberg et al., 1972); however, the β values which we obtained were consistently in the range of 85 000 daltons. Since r_2 forms visible aggregates as the temperature is raised, it is not surprising that the data reflect their presence. What is perhaps more surprising is the fact that the aggregates which also form as c_3 is heated apparently did not affect the data.

The data for both liganded and unliganded r_2 were best fit to a model requiring one intermediate between the undenatured and fully denatured species. The enthalpy change associated with the second step is almost twice that associated with the first. While the two steps in the denaturation reaction might correspond to melting of the two monomeric units, this need not be the case. If so, the difference in ΔH_{cal} for the two steps provides evidence for strong interchain interactions. This would be consistent with the evidence indicating that the two nucleotide binding sites of both r_2 and the regulatory subunits of native c_6r_6 are not equivalent [Suter & Rosenbuch, 1977; see also Klotz (1982)].

The effects on t_m of varying protein and ligand concentrations indicate that there is no significant change in the state of aggregation of the protein during denaturation and that ATP and CTP bind preferentially to the native species while PALA binds preferentially to the denatured form. PALA has no significant effect on the apparent enthalpy of denaturation, indicating that there is no significant difference in its enthalpy of binding to the two protein species. Both CTP and ATP increase the enthalpy change associated with each step by at least 30%, with CTP having a slightly greater effect than ATP. As was the case for PALA and c_3, the differences in the enthalpy changes observed in the presence and absence of ligands are the result of dissociation of the ligand. A large body of evidence indicates that the binding of nucleotides to r_2 alters both tertiary structure and subunit interactions [cf. McClintock & Markus (1968), Cohlberg et al. (1972), Hensley & Schachman, (1979), Lennick & Allewell (1981), and Honzatko et al. (1982)], with CTP, which binds more tightly than ATP, generally having a larger effect. The dissociation heats necessarily include any contributions from structural changes in the protein.

In view of the interest which exists in whether hydrogen exchange proceeds via a "local unfolding" mechanism [cf. Woodward et al. (1982) and Englander & Kallenbach (1984)], it is of interest to compare the effects of ligands on the kinetics of hydrogen exchange and thermal denaturation of c_3 and r_2. Results for other proteins have been reviewed by Privalov (1979), who noted correlations between rates of exchange and the enthalpy, entropy, and heat capacity changes associated with denaturation. We find similar correlations; the binding of PALA to c_3, and that of either ATP or CTP to r_2, is associated with decreases in rates of exchange and increases in t_m and the enthalpy of denaturation. Conversely, binding of either CTP or ATP to c_3 results in a slight increase in the rate of exchange and a decrease in t_m although there is no significant change in the enthalpy of denaturation. These correlations are consistent with hydrogen exchange proceeding by a local unfolding mechanism but do not rule out other possibilities.

Nevertheless, because both subunits are clearly aggregated at temperatures at which denaturation occurs, attempts to draw conclusions from DSC data about the behavior of either protein at room temperature are fraught with danger. The same caveat will apply to the native enzyme, since its denaturational behavior is analogous to that of the subunits (Vickers et al., 1978).

We have reported here that ATP and CTP raise the denaturation temperature of r_2 and lower that of c_3 and that PALA raises the denaturation temperature of c_3 and lowers that of r_2. It should be noted that continuing changes in t_m with increases in ligand concentration are observed even at ligand concentrations at which the proteins are very nearly fully saturated with ligand. This situation, which has been previously reported for the binding of arabinose to the arabinose binding protein of *E. coli* [Fukada et al., 1983; see also Schellman (1976)], shows that the effect of the ligands on t_m can be viewed as a manifestation of Le Chatelier's principle and need not be interpreted as a mysterious "stabilization" (or "destabilization" when t_m is lowered) of the protein.

Appendix

Resolution of DSC Curves. None of the DSC curves of excess specific heat vs. temperature observed with ATCase or its catalytic or regulatory subunits was of the nearly symmetrical form expected for a two-state process not involving dissociation. We have assumed that the complex processes represented by the observed curves can be expressed as the resultants of two or more two-state processes all of which are at equilibrium throughout the overall process. In the case of ATCase and its subunits, there is much evidence for strong interpeptide chain and intersubunit interactions [cf. Subramani et al. (1977) and Burns & Schachman (1982)] and for the simultaneous interaction of ligand molecules with more than one peptide chain (Burns & Schachman, 1982; Krause et al., 1985; Robey & Schachman, 1985). It is therefore inappropriate in this case to apply a model based on the more or less independent unfolding of separate molecular domains despite the demonstrated adequacy of this model for several other proteins (Novokhatny et al., 1984; Hecht et al., 1984). We have instead restricted our treatment to models involving strictly sequential two-state steps.

The procedure outlined here is a "brute force" type of treatment in which the adjustable parameters for each step are varied in succession until a minimal value for the standard deviation of the calculated from the observed data is obtained. This calculation can also be carried out, with some saving in computer time, by a nonlinear least-squares procedure (B. G. Barisas, personal communication). In all cases where both methods were employed, they gave closely agreeing results.

Our procedure will be illustrated for the case of three sequential steps:

$$N_1 \rightleftarrows N_2 \rightleftarrows N_3 \rightleftarrows N_4 \qquad \text{(A1)}$$

with equilibrium constants

$$K_1 = [N_2]/[N_1]; \ldots; K_3 = [N_4]/[N_3] \qquad \text{(A2)}$$

where the quantities in brackets represent concentrations. This formulation applies with only minor changes to a system in which bound ligand molecules are dissociated at one or more steps, provided the total ligand concentration, $[L]_0$, is large compared to the total concentration of binding sites. If n ligand molecules are dissociated in step i, then the equilibrium constant for that step is taken as

$$K_i' = K_i[L]_0^{-n}$$

The equilibria for a model involving independent domains would be

$$N_1 \rightleftarrows D_1 \qquad N_2 \rightleftarrows D_2 \qquad N_3 \rightleftarrows D_3$$

with no equilibrium, for example, between N_1 and D_2.

Setting

$$[N]_0 = [N_1] + [N_2] + [N_3]$$
$$D = 1 + K_1 + K_1K_2 + K_1K_2K_3 \qquad \text{(A3)}$$

we find

$$[N_1]/[N]_0 = 1/D; \ldots; [N_4]/[N]_0 = K_1K_2K_3/D \qquad \text{(A4)}$$

The two-state assumption means that each K obeys the van't Hoff equation, which in integrated form is

$$\ln K_i = \frac{\Delta H_{vH,i}}{R}\left(\frac{1}{T_{1/2,i}} - \frac{1}{t}\right) \qquad \text{(A5)}$$

where $T_{1/2,i}$ is the absolute temperature at which $K_i = 1$. If we assume specific values for the parameters $T_{1/2,i}$ and $\Delta H_{vH,i}$, we can calculate $[N_i]/[N]_0$ at $T + \delta T$ and $T - \delta T$ and then evaluate $\delta([N_i]/[N]_0)$ for the temperature interval $2\delta T$, and the increment in enthalpy for the interval due to the ith step:

$$\delta q_i = \delta([N_i]/[N]_0)(\Delta h_1 + \ldots + \Delta h_i) \qquad \text{(A6)}$$

In this expression, Δh_i is the change in specific enthalpy (calories per gram) at the ith step, the third adjustable parameter for that step. Finally, the excess specific heat (calories per degrees kelvin per gram) at the temperature T is given by

$$c_{ex} = \sum \delta q_i/2\delta T \qquad \text{(A7)}$$

The component curves shown in Figures 2 and 3 in the text are actually the total accrued contribution of each step

$$c_i = \frac{\Delta h_i}{2\delta T}\,\frac{\delta[P_{i+1}] + \ldots + \delta[P_{n+1}]}{[P]_0} \qquad \text{(A8)}$$

where n is the number of steps, and in some cases have shapes which obviously deviate from that expected for an isolated two-state process.

In the analysis of a DSC curve, preliminary estimates are made of the number of steps involved and the parameters for each step. Adequate estimates can usually be obtained from a rough graphical calculation. Each parameter is then adjusted in sequence by a specific increment until the standard deviation of the calculated from the observed data is minimized. The number of incrementations of each parameter at each pass is limited to five or six, and in order to reduce the change of settling in a local minimum removed from the global minimum, the initial incrementations are specified to be several times larger than the final ones.

In the absence of intermolecular cooperation at one or more steps, it would appear that the ratio $\beta = \Delta H_{vH}/\Delta h$ should be the same for each step and should equal the molecular weight of the whole molecule. In this case, there are two adjustable parameters for each step.

The information content of a DSC curve with a modest level of accuracy is by any measure quite limited [cf. Weber & Anderson (1965)]. It must therefore be realized that an analysis of such a curve to evaluate six or more adjustable parameters cannot lead to a truly unique result, despite the significant restriction of possible solutions imposed by the requirement that all components follow the van't Hoff equation.

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

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