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Differential scanning calorimetric studies of *E. coli* aspartate transcarbamylase

III. The denaturational thermodynamics of the holoenzyme with single-site mutations in the catalytic chain

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Aspartate transcarbamylase (EC 2.1.3.2) from *E. coli* is a multimeric enzyme consisting of two catalytic subunits and three regulatory subunits whose activity is regulated by subunit interactions. Differential scanning calorimetric (DSC) scans of the wild-type enzyme consist of two peaks, each comprised of at least two components, corresponding to denaturation of the catalytic and regulatory subunits within the intact holoenzyme (Vickers et al., J. Biol. Chem. 253 (1978) 8493; Edge et al., Biochemistry 27 (1988) 8081). We have examined the effects of nine single-site mutations in the catalytic chains. Three of the mutations (Asp-100-Gly, Glu-86-Gln, and Arg-269-Gly) are at sites at the C1 : C2 interface between c chains within the catalytic subunit. These mutations disrupt salt linkages present in both the T and R states of the molecule (Honzatko et al., J. Mol. Biol. 160 (1982) 219; Krause et al., J. Mol. Biol. 193 (1987) 527). The remainder (Lys-164-Ile, Tyr-165-Phe, Glu-239-Gln, Glu-239-Ala, Tyr-240-Phe and Asp-271-Ser) are at the C1 : C4 interface between catalytic subunits and are involved in interactions which stabilize either the T or R state. DSC scans of all of the mutants except Asp-100-Gly and Arg-269-Gly consisted of two peaks. At intermediate concentrations, Asp-100-Gly and Arg-269-Gly had only a single peak near the T_m of the regulatory subunit transition in the holoenzyme, although their denaturational profiles were more complex at high and low protein concentrations. The catalytic subunits of Glu-86-Gln, Lys-164-Ile and Asp-271-Ser appear to be significantly destabilized relative to wild-type protein while Tyr-165-Phe and Tyr-240-Phe appear to be stabilized. Values of $\Delta\Delta G^{\circ}_{tr}$, the difference between the subunit interaction energy of wild-type and mutant proteins, evaluated as suggested by Brandts et al. (Biochemistry 28 (1989) 8588) range from -3.7 kcal mol^{-1} for Glu-86-Gln to 2.4 kcal mol^{-1} for Tyr-165-Phe.

1. Introduction

Aspartate transcarbamylase (c_6r_6; carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) from *E. coli* is a complex multimeric protein comprised of six c chains and six r chains, organized as two catalytic trimers (c_3) and three regulatory dimers (r_2), respectively (for recent reviews, see refs 1–5). This enzyme catalyzes the first committed step in pyrimidine biosynthesis, transfer of a carbamoyl group from carbamoyl phosphate to the α-amino group of L-aspartate. Both substrates bind cooperatively [6–8] and enzymatic activity is further regulated by ATP, an

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Abbreviations: c_6r_6, aspartate transcarbamylase; c_3, catalytic subunit; PALA, *N*-(phosphonacetyl)-L-aspartic acid; r_2, regulatory subunit.

activator [6], and CTP and UTP, which inhibit cooperatively [6,9]. High-resolution crystal structures of the unliganded enzyme [10–12] as well as its complexes with CTP [13], PALA [14–16] and carbamoyl phosphate and succinate [17] have been determined. The crystal structures confirm the conclusion drawn earlier from solution studies (cf. refs 18 and 19), that binding of substrates and substrate analogs is linked to a major change in molecular architecture. The CTP- and PALA-liganded crystal structures are designated the T and R states, respectively. The structure of the unliganded protein is similar to that of the T state.

In order to explore the roles of specific residues in enzyme function, site-directed mutagenesis has been used to alter many residues (cf. refs 2 and 20–26). Although the functional properties of these mutants have been studied in detail, their effects on the physical properties of the protein remain largely unexplored. In the present study, we report the results of a DSC study of the effects on thermal stability of mutations at the interface between c chains either within a catalytic subunit or between catalytic subunits. As shown previously [27–29], DSC has the advantage that the effects of mutations on catalytic and regulatory subunits can be resolved, since each subunit melts at a characteristic temperature. Further, as shown by Brandts et al. [30], the effects of mutations on ΔG°_{cr}, the free energy of intersubunit c-r interactions, can be calculated from the difference in the temperatures of denaturation of isolated regulatory subunits and regulatory subunits within the intact enzyme.

2. Materials and methods

2.1. Materials

Plasmids obtained from either E.R. Kantrowitz (Boston College, Chestnut Hill, MA) or J.R. Wild (Texas A & M University, College Station, TX) containing mutations in the *pyr*B gene were transformed into *E. coli* strain EK1104 for overexpression. Purified protein was obtained by the same procedures used to prepare wild-type protein [31] and was characterized by polyacrylamide gel electrophoresis, generally under non-denaturing conditions [32] but occasionally under denaturing conditions [33], by pH-stat activity assays at pH 7.0 or 8.3 and by analytical gel chromatography [31]. The homogeneity of the mutant proteins was comparable to that of the wild type. Kinetic parameters were very similar to those reported by Kantrowitz, Wild and their collaborators (refs 23–26; and E.R. Kantrowitz, personal communication; M.E. Wales and J.R. Wild, personal communication). Proteins were stored as $(NH_4)_2SO_4$ precipitates at 4°C and used within 1 month of purification. In preparation for DSC experiments, precipitated protein was dissolved in and dialyzed against 40 mM sodium phosphate, 0.2 mM dithiothreitol, 0.2 mM EDTA (pH 7). Any insoluble material was removed by centrifugation at 15 000 rpm for 10 min. Concentrations were determined spectrophotometrically, assuming an extinction coefficient of 0.59 cm ml mg^{-1}.

2.2. Calorimetry

The DSC experiments reported here were performed in two different instruments, the MC-2 (Microcal, Inc., Northampton, MA) and the DASM-4 (Vneshbio, Moscow, U.S.S.R.). It was ascertained that these instruments gave closely agreeing results. A scan rate of 0.33 K min^{-1} (MC-2) or 0.28 K min^{-1} (DASM-4) was employed. As noted earlier (ref. 28, footnote 2), a slow scan rate is needed to avoid exothermic disturbances in cells having pill-box geometry, as in the MC-2, and was employed also in the DASM-4 to ensure comparable results.

2.3. Analysis of data

The DSC curves, after deduction of instrumental baselines, were resolved into component curves by a least-squares procedure (ref. 28, appendix; and ref. 34). Calculations were made on the basis of two alternative models, one involving a strictly sequential series of unfolding steps and the other the independent unfolding of domains [34]. As in the case of the wild-type holoenzyme, it was found in most cases that five steps were necessary to obtain an adequate fit of calculated to observed

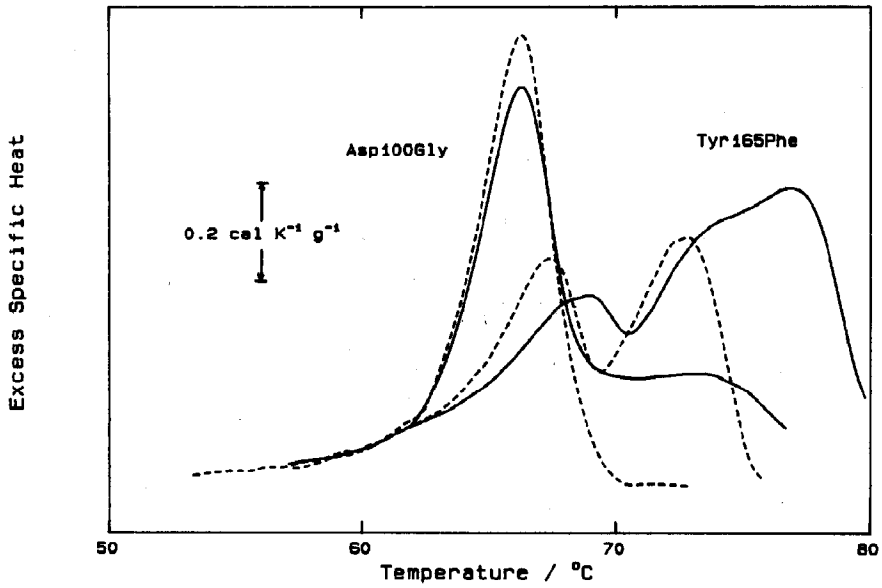

Fig. 1. DSC curves observed with Asp-100-Gly (———, 13.1 mg ml^{-1}; - - - - - -, 6.55 mg ml^{-1}); and Tyr-165-Phe (———, 12.3 mg ml^{-1}; - - - - - -, 5.0 mg ml^{-1}).

data. In all cases where both models were employed, the parameters obtained on the basis of the two models showed no significant differences.

In the curve-resolution procedure which we employ there are three adjustable parameters for each component of the overall DSC curve, the temperature of half-completion, $t_{1/2}$ (°C), the specific enthalpy at that temperature, $\Delta h_{1/2}$ (cal g^{-1}), and a quantity β defined as the ratio of the van't Hoff enthalpy, ΔH_{vH} (kcal mol^{-1}), to the calorimetric enthalpy, $\Delta h_{1/2}$. In general, β was required to have the same value for all components. For a strictly two-state process, i.e., a reversible process following the van't Hoff equation,

$$\mathrm{d} \ln K/\mathrm{d}t = \Delta H_{vH}/Rt^2 \quad (1)$$

the parameter β should equal the molecular weight. It has frequently been found in other cases (cf. ref. 35) that β may differ significantly from the molecular weight, in most cases for unknown reasons. However, it can be shown by computer simulation that two possible causes for such behavior are some degree of aggregation in either the initial or the final state or both, and variations in domain interactions [30] during the unfolding of multidomain proteins. It is thus of interest that in the present complex situation the value $\beta = 308\,000 \pm 10\,000$, close to the molecular weight of the holoenzyme, was obtained in most instances. As will be discussed later, one mutant, Asp-100-Gly,* gave $\beta = 161\,000 \pm 4000$, close to half the molecular weight.

All the denaturations studied here appeared to be irreversible in that a second scan of material cooled in the calorimeter after the first scan gave no significant endotherm. The admittedly unorthodox procedure of employing equilibrium thermodynamics in the analysis of the present data is discussed elsewhere [28,34].

3. Results

As was the case with the wild-type protein [29], the DSC curves obtained with all except two of the mutant proteins showed two well-resolved peaks, but with temperatures and areas more or

* For each mutant protein, the number indicates the position of the mutation in the c chain sequence; the amino acid to the left of the number is the amino acid that occurs in the wild-type protein; the number to the right is its replacement in the mutant.

Table 1

Parameters obtained by curve fitting the data for mutants showing two DSC peaks [a]

Component no.	Protein (no. of expts): Concn range (mg ml^{-1})	Wild type (5): 3.15–10.5		Glu-86-Gln (5): 3.05–13.25		Lys-164-Ile (5): 3.27–13.07		Tyr-165-Phe (4): 2.50–12.3	
	β/1000:	305 ± 3		304 ± 3		323 ± 4		293 ± 5	
	S.D. (%C_{max}):	2.4 ± 0.3		2.4 ± 0.4		2.8 ± 0.6		2.6 ± 0.4	
		$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})	$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})	$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})	$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})
1		64.14 ± 0.14	0.60 ± 0.01	62.57 ± 0.11	0.55 ± 0.06	63.66 ± 0.19	0.47 ± 0.04	65.84 ± 0.33	0.62 ± 0.07
2		66.54 ± 0.11	1.00 ± 0.04	64.89 ± 0.09	1.04 ± 0.07	66.04 ± 0.06	0.92 ± 0.02	67.72 ± 0.57	0.97 ± 0.10
3		70.86 ± 0.12	0.80 ± 0.01	68.29 ± 0.23	0.69 ± 0.11	69.84 ± 0.25	0.79 ± 0.08	71.92 ± 0.52	1.02 ± 0.05
4		72.75 ± 0.14	0.96 ± 0.05	70.00 ± 0.25	0.78 ± 0.03	70.95 ± 0.15	0.94 ± 0.12	73.93 ± 0.66	1.19 ± 0.10
5		74.10 ± 0.21	1.07 ± 0.03	71.70 ± 0.13	0.83 ± 0.02	71.57 ± 0.49	1.00 ± 0.09	77.58 [b]	1.15 [b]
			Sum 4.42 ± 0.05		Sum 3.88 ± 0.04		Sum 3.93 ± 0.05		

[a] Indicated uncertainties are standard errors of the mean.
[b] Fifth component needed only at highest concentration.

less altered from those for the wild-type protein. Some of the more pronounced effects resulting from the mutations studied here are illustrated in fig. 1. Replacement of aspartate at position 100 by glycine (Asp-100-Gly) leads to a DSC curve showing a single peak at low concentrations but with a shoulder on the high-temperature side appearing at higher concentrations. The mutant Tyr-165-Phe shows two normal appearing peaks at low concentration while at high concentration the upper peak is markedly broadened and increased in area. A similar effect, albeit to a less marked degree,

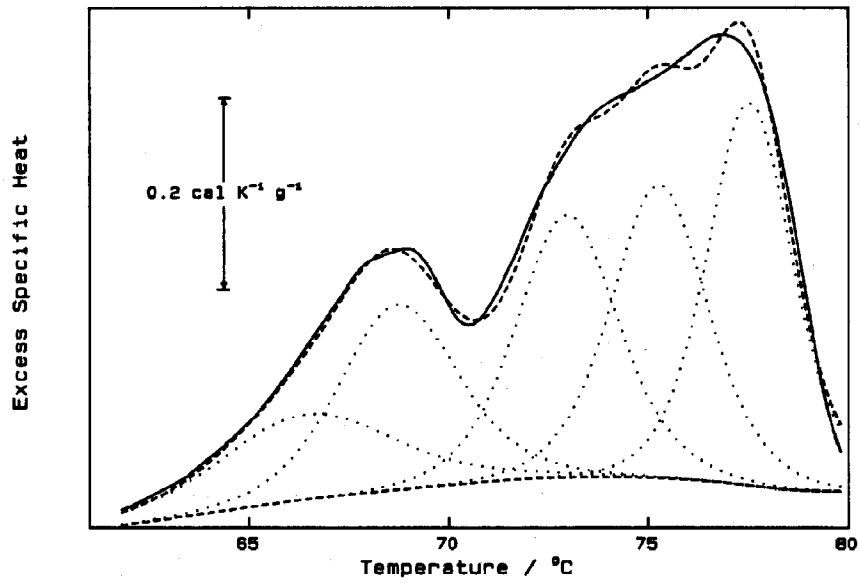

Fig. 2. Resolution for the DSC curve for Tyr-165-Phe at a concentration of 12.3 mg ml^{-1}. Observed data, ———; calculated data and baseline, - - - - - -; calculated components, ·····. The standard deviation of 46 calculated points from the observed points was 0.010 cal K^{-1} g^{-1}, and the value of β (see text) was 298 000.

Table 1 (continued)

Glu-239-Gln (5)		Glu-239-Ala (5)		Tyr-240-Phe (5)		Asp-271-Ser (5)	
3.46–10.8		3.00–8.91		4.95–13.05		2.03–6.78	
3.08 ± 5		310 ± 2		301 ± 1		318 ± 3	
2.6 ± 0.3		2.6 ± 0.4		1.8 ± 0.2		4.1 ± 0.5	
$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})	$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})	$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})	$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})
63.79 ± 0.36	0.52 ± 0.05	64.22 ± 0.11	0.59 ± 0.08	64.90 ± 0.18	0.55 ± 0.08	63.06 ± 0.44	0.38 ± 0.08
65.70 ± 0.09	0.85 ± 0.02	66.11 ± 0.16	0.90 ± 0.03	66.72 ± 0.08	1.09 ± 0.02	66.39 ± 0.06	0.98 ± 0.03
70.66 ± 0.09	0.89 ± 0.06	70.88 ± 0.10	0.83 ± 0.04	71.25 ± 0.19	0.95 ± 0.06	69.68 ± 0.21	0.98 ± 0.07
72.64 ± 0.12	0.98 ± 0.06	72.69 ± 0.14	0.97 ± 0.06	72.96 ± 0.24	1.17 ± 0.08	71.10 ± 0.17	0.98 ± 0.02
73.80 ± 0.24	1.04 ± 0.04	73.79 ± 0.06	1.08 ± 0.04	74.29 ± 0.37	1.21 ± 0.04		
	Sum 4.28 ± 0.17		Sum 4.37 ± 0.15		Sum 4.83 ± 0.13		Sum 3.04 ± 0.19

was seen with the wild-type protein at very high concentrations [29].

The thermodynamic parameters obtained by curve fitting to the data for the mutants showing two DSC peaks are summarized in table 1, together with the protein concentration range, the value of β obtained in the calculation employed, and the mean standard deviation of the calculated points from the observed curve, expressed as percent of the maximal value of the excess specific heat. A representative fit is shown in fig. 2. For some mutants at low concentrations fewer than five components gave satisfactory fits, as indicated. In these cases, when a calculation was started using five components, a component was eventually discarded by the computer by being

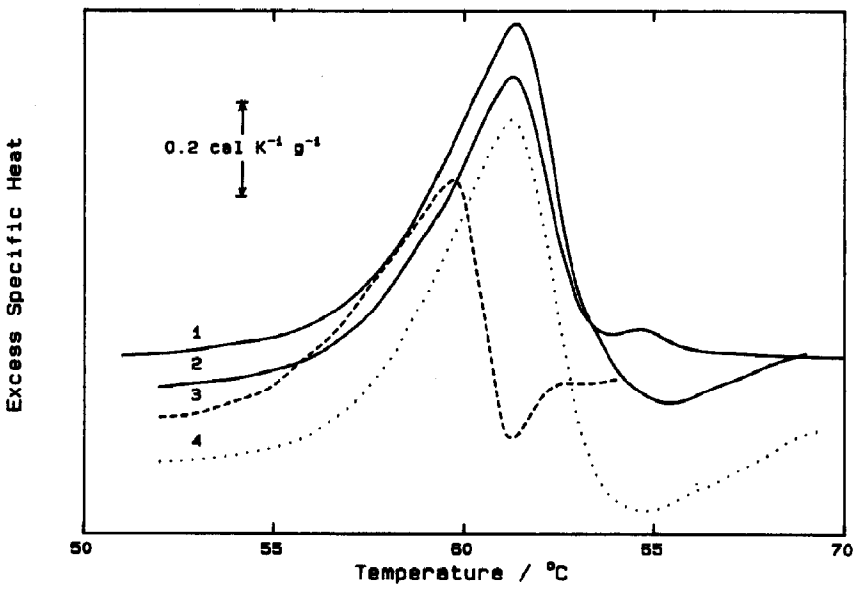

Fig. 3. DSC curves observed with Arg-269-Gly. Scan rates: 0.28 K min^{-1} (curves 1, 2 and 4); 0.14 K min^{-1} (curve 3). Curve 1, 8.04 mg ml^{-1}; curve 2, 6.99 mg ml^{-1}; curve 3, 6.83 mg ml^{-1}; curve 4, 5.47 mg ml^{-1}.

assigned a negligible enthalpy. The uncertainties given throughout table 1 are standard errors of the mean.

In addition to Asp-100-Gly, Arg-269-Gly also showed a single DSC peak. The behaviors of these two mutants were very different from each other. Whereas Asp-100-Gly showed an apparently simple single peak at low concentrations, becoming complicated by a shoulder on the high-temperature side at higher concentrations, Arg-269-Gly showed a single peak at high concentrations which became complicated at lower concentrations by an *exothermic* peak which became more pronounced as the concentration was decreased. This peak was decreased but not removed at a concentration of 6.83 mg ml^{-1} by going to a scan rate of 0.13 K min^{-1} (fig. 3).

The results of curve fitting to the data for Asp-100-Gly and Arg-269-Gly are summarized in table 2. Up to a concentration of about 7 mg ml^{-1} the data for Asp-100-Gly were well fitted with three components. At higher concentrations the increasingly evident high-temperature shoulder necessitated a fourth component with $t_{1/2}$ equal to 73.0 °C and enthalpy increasing with increasing concentration. The total enthalpy is similar to the values found for the mutants giving two DSC peaks.

For Arg-269-Gly a satisfactory curve-fitting calculation was possible only at the highest concentration, yielding the parameters given in table 2, but curve fitting to the data which included exotherms could not be carried out unless the exotherms were removed by some highly arbitrary revision of the experimental data which we did not undertake. The total denaturational enthalpy change was smallest for this mutant.

4. Discussion

4.1. Wild-type aspartate transcarbamylase and the mutants showing two DSC peaks

In the discussion of our results which follows we shall employ a simplified characterization of the DSC peaks in terms of weighted mean transition temperatures, t_L and t_H, and total peak enthalpies, Δh_L and Δh_H.

$$t_L = (t_1\Delta h_1 + t_2\Delta h_2)/\Delta h_L;\ \Delta h_L = \Delta h_1 + \Delta h_2 \quad (2)$$

$$t_H = (t_3\Delta h_3 + t_4\Delta h_4 + t_5\Delta h_5)/\Delta h_H;\ \Delta h_H = \Delta h_3 + \Delta h_4 + \Delta h_5 \quad (3)$$

where the subscripts L and H refer to the peaks at lower and higher temperatures, respectively. Table 3 lists the values of t_L, ΔH_L, t_H and ΔH_H for wild-type enzyme and seven mutants.

We found in our DSC study of the isolated subunits of the holoenzyme [28] that the data for r_2 required two two-state components for an ade-

Table 2

Parameters obtained by curve fitting the data for mutants showing a single DSC peak

Component no.	Protein (no. of expts): Asp-100-Gly (6)		Arg-269-Gly (4) [a]	
	Concn range (mg ml^{-1}): 3.95–13.1		5.47–8.04	
	β/1000: 161 ± 4		250	
	S.D. (%C_{max}): 1.7 ± 0.3		2.3	
	$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})	$t_{1/2}$ (°C)	$\Delta h_{1/2}$ (cal g^{-1})
1	62.15 ± 0.59	0.35 ± 0.04	58.91	0.93
2	65.15 ± 0.15	1.32 ± 0.04	61.20	1.40
3	66.48 ± 0.08	2.01 ± 0.08	64.50	0.17
4	72.96 [b]	0.50 ± 0.98 [b]		Sum 2.50
		Sum 4.05 ± 0.14		

[a] Only the run at highest concentration was analyzed by curve fitting. See text.
[b] At the three highest concentrations only (8.73–13.1 mg ml^{-1}).

Table 3

Values at 5 mg ml^{-1} for the weighted mean temperatures and total enthalpies of the DSC peaks at lower temperature (L) and higher temperature (H)

Protein	t_L (°C)	ΔH_L (kcal mol^{-1})	t_H (°C)	ΔH_H (kcal mol^{-1})
Wild type	65.78	488	72.67	863
Mutations at C1 : C2 interface				
Glu-86-Gln	64.27	483	70.11	699
Asp-100-Gly [a]	65.59	592	–	–
Arg-269-Gly [a]	60.57	625	–	–
Mutations at C1 : C4 interface				
Lys-164-Ile	65.18	449	70.61	882
Tyr-165-Phe	66.77	466	72.75	984
Glu-239-Gln	64.96	422	72.69	896
Glu-239-Ala	65.42	462	72.58	893
Tyr-240-Phe	65.97	494	72.96	1002
Asp-271-Ser	65.57	432	70.52	623
Isolated subunits [b]	50.60	890 [c]	71.40	1440 [c]

[a] Calculated from the parameters for components 1–3 in table 2. The values given for the mutants showing a single DSC peak undoubtedly include contributions from the catalytic chains.

[b] Ref. 28.

[c] Calculated using $\beta = 310\,000$.

quate fit of calculated to experimental data, and that three two-state components were needed in the case of c_3. This is true whether the model of sequential steps or that of independent domains is employed. Furthermore, the fact that the values of $t_{1/2}$ for the components were essentially independent of concentration indicated that neither r_2 nor c_3 dissociated on denaturation. The fact that wild-type c_6r_6 and all the mutants listed in table 1, with the exception of Asp-271-Ser, require two components in the peak at lower temperature and three in the peak at higher temperature, and that β automatically takes on a value in the curve-fitting calculation closely equal to the molecular weight of c_6r_6, suggests that the melting of c_6r_6 consists first in the fully cooperative melting of the three r_2 subunits followed by the fully cooperative melting of the two c_3 subunits. On the basis of this view of the melting process, since all the mutations considered here are located in the catalytic chains, we may, at least as a first approximation, interpret the thermodynamic effects of the mutations in the following manner: changes in the values of t_H reflect apparent stabilization or destabilization of the catalytic subunits, and changes in the values of t_L reflect alterations in the interactions between catalytic subunits and regulatory subunits. This interpretation involves the reasonable assumption [30] that all c-r interactions are lost when the regulatory chains are denatured.

Taking the standard free energies of denaturation of the wild-type and mutant catalytic subunits at the respective t_H values to be zero, application of the Gibbs-Helmholtz equation leads to the values of $\Delta\Delta G_d^\circ = \Delta G_d^\circ$ (WT) $- \Delta G_d^\circ$ (mutant) at t_H of wild-type protein listed in table 4. Positive values for $\Delta\Delta G_d^\circ$ indicate apparent destabilization relative to the wild-type protein. The qualification 'apparent' is necessary here, and will be assumed throughout the following discussion, since the variation in $\Delta\Delta G_d^\circ$ could be due, at least in part, to stabilization of the denatured state. In this calculation ΔC_p, the permanent change in heat capacity accompanying denaturation, has been set

Table 4

Changes in free energy and enthalpy of denaturation of catalytic subunits and in free energy of catalytic-regulatory subunit interactions caused by mutations

Protein	$\Delta\Delta G_d^\circ$ (kcal mol^{-1})	$\Delta\Delta H_d$ (kcal mol^{-1}) [a]	$\Delta\Delta G_{cr}^\circ$ (kcal mol^{-1}) [b]
Mutations at C1 : C2 interface [c]			
Glu-86-Gln	5.2	161	−3.7
Mutations at C1 : C4 interface			
Lys-164-Ile	5.3	32	−1.5
Tyr-165-Phe	−0.2	−155	2.4
Glu-239-Gln	−0.05	−25	−2.0
Glu-239-Ala	0.2	−16	−0.9
Tyr-240-Phe	−0.8	−150	0.5
Asp-271-Ser	3.9	277	−0.5

[a] In calculating $\Delta\Delta H_d$, the value of ΔH_H for wild type in table 3, obtained with $\beta = 305\,000$, has been scaled for each mutant.

[b] In calculating $\Delta\Delta G_{cr}^\circ$ the value for ΔH_L^* (eq. 4) is 890 (β/310 000) where β is the value obtained for wild-type protein (table 1). ΔG_{cr}° (wild type) = −39.22 kcal mol^{-1}.

[c] Asp-100-Gly and Arg-269-Gly are not included here, since it was not possible to separate the contribution from the regulatory chains from those of the catalytic chains.

equal to zero although small values for ΔC_p, some positive and some negative, were observed in some of the experiments. However, in no case were values observed which were large enough to be of significance; for example, the mean ΔC_p for Glu-86-Gln was 9.1 ± 4.9 kcal K^{-1} mol^{-1}, and inclusion of this value gave $\Delta\Delta G_d^\circ = 5.2$ kcal K^{-1} mol^{-1} instead of the value 5.3 listed in table 4. The values for $\Delta\Delta H_d$ are also given in the table. As has previously been found for mutant forms of T_4 lysozyme and staphylococcal nuclease (unpublished results), there is absolutely no correlation between $\Delta\Delta G_d^\circ$ and $\Delta\Delta H_d$, and the enthalpy values are frequently of much larger magnitude than the free energy values, showing that large compensating entropy values are also present. These effects are particularly evident in the present work because of the very large molecular weight of c_6r_6.

A simple and reasonable model for the analysis of domain interactions in proteins has been introduced and discussed by Brandts et al. [30]. As discussed above, it would appear that, to a first approximation, we can consider the manifold domain interactions in the holoenzyme as being lumped together into a single interaction between catalytic and regulatory units. On this basis, we can apply the simplest formulation proposed by Brandts et al., and evaluate the free energy of c-r interactions by means of an equation analogous to their eq. 2:

$$\Delta G_{cr}^\circ = -\Delta H_L^*(1 - t_L^*/t_L) \qquad (4)$$

where t_L and t_L^* are, respectively, the weighted mean temperatures for the denaturation of the regulatory subunit in the holoenzyme and in the reference state devoid of subunit interactions, and ΔH_L^* denotes the enthalpy of denaturation of the regulatory subunit in the reference state. We can take as the reference state the isolated subunits, with the parameters listed in the final row of table 3. Application of eq. 4 then gives the values for $\Delta\Delta G_{cr}^\circ = \Delta G_{cr}^\circ$ (WT) $- \Delta G_{cr}^\circ$ (mutant) listed in column 4 of table 4. These values are of the same general magnitude as those for $\Delta\Delta G_d^\circ$ though not at all correlated with them.

A perplexing situation arises in connection with the ratio of enthalpy values, $\Delta H_H/\Delta H_L$. In three cases, as seen in table 5, this ratio increases significantly with increasing concentration. Although such behavior would appear to be a clear indication of intermolecular interaction, we have no other indication of this, such as a significant variation of either t_L or t_H or β with concentration.

Table 5

Least-squared coefficients for the variation of $\Delta H_H/\Delta H_L$ with concentration for mutants showing two DSC peaks

Protein	A [a]	B (ml mg^{-1}) [a]	r^2 [b]	Comment
Wild type	1.513	0.0381	0.68	barely significant
Mutations at C1 : C2 interface				
Glu-86-Gln	0.897	0.0717	0.92	significant
Mutations at C1 : C4 interface				
Lys-164-Ile	1.494	0.0422	0.72	barely significant
Tyr-165-Phe	0.815	0.1181	0.96	significant
Glu-239-Gln	2.069	0.0078	0.03	not significant
Glu-239-Ala	1.762	0.0295	0.37	not significant
Tyr-240-Phe	1.529	0.0591	0.96	significant
Asp-271-Ser	1.108	0.0288	0.23	not significant

[a] $\Delta H_H/\Delta H_L = A + Bc$; c = protein concentration in mg ml^{-1}.
[b] r^2 = coefficient of determination.

4.2. *Mutants showing a single DSC peak*

As mentioned earlier, Asp-100-Gly and Arg-269-Gly show a single DSC peak in contrast to the usual two peaks. The behavior of Asp-100-Gly at two concentrations is shown in fig. 1 and curve-fitting parameters are listed in table 2. The outstanding feature of the results obtained with this mutant is the value for β, $161\,000 \pm 4000$, close to one half the molecular weight of c_6r_6. In addition, a second endotherm with $t_{1/2} = 73.0\,^\circ$C appeared at high concentrations. The simplest interpretation of these unexpected results is that in the temperature range of thermal transitions the cooperative unit for the denaturation of this mutant is one half of the full molecule, with association to the holoenzyme occurring at higher concentration. It is of course difficult to understand how $2c_3 \cdot 3r_2$ can be divided into two equal parts unless the structure of this mutant is fundamentally different from that of wild-type c_6r_6. There is no independent evidence for an aberrant structure.

Arg-269-Gly proved to be unique among the proteins studied here in its proclivity to show exotherms. As discussed earlier [28], very sharp exotherms were observed with wild-type c_6r_6 and also *lac* repressor and λ repressor of *E. coli*, when scanned in the DASM-1M calorimeter at 1 K min^{-1}, but were substantially reduced by going to a scan rate of 0.22 K min^{-1}, and restricting concentrations to 5 mg ml^{-1} or higher. No exotherms were observed when these proteins were scanned even at 1 K min^{-1} in the DASM-4, and it was concluded that the exotherms were instrumental artifacts of completely unknown origin seen in the pill-box-shaped cells of DASM-1M but not in the cells of the DASM-4 which are horizontal capillary helices. The experiments showing exotherms with Arg-269-Gly were all performed in the DASM-4 calorimeter, and were much broader than those previously observed in the DASM-1M. It therefore seems likely that the exotherms observed with this mutant are an integral part of the denaturation process, perhaps an aggregation.

4.3. Relations between thermodynamic, structural and functional properties

Three of the mutants examined here, Glu-86-Gln, Asp-100-Gly and Arg-269-Gly, are involved in salt linkages at the C1 : C2 interface between c chains within the catalytic subunit in both the T and R states [11,16]. All three mutations substantially reduce the stability of the catalytic subunit; melting of the catalytic subunits of Asp-100-Gly (at low concentrations) and Arg-269-Gly can no longer be resolved from melting of the regulatory subunit. Although the effects of the mutation Glu-86-Gln are less striking, values of t_H and ΔH_H for the catalytic subunit are still reduced substantially relative to the wild-type protein. Both Glu-86-Gln and Arg-269-Gly also have very low affinity for aspartate and very low activity (E.R. Kantrowitz, personal communication).

All of the remaining mutations are located at the C1 : C4 interface between catalytic subunits [11]. One of the critical differences between the T and R states is the position of the 240s loop of the c chain at this interface which is involved in *intersubunit* interactions in the T state and *intrachain* interactions in the R state [15,16]. All of the mutated residues at this interface are part of the 240s loop or interact with it. Lys-164, Tyr-165, and Glu-239 are involved in an *intersubunit* interaction in the T state and an *intrachain* interaction in the R state [11,15,16]. Tyr-240 and Asp-271 form an *intrachain* interaction in the T state [11]. Activity data suggest that Lys-164-Ile, Tyr-165-Phe and Glu-239-Ala are shifted towards the T state (ref. 23; and M.E. Wales and J.R. Wild, personal communication), while activity [26], low angle X-ray scattering [36] and analytical gel chromatography (Burz et al., in preparation) data suggest that the conformation of Glu-239-Gln is shifted towards the R state. The activities of Tyr-240-Phe and Asp-271-Ser are very similar (ref. 24; and E.R. Kantrowitz, personal communication) and suggest a shift towards an R state structure; however, Asp-271-Ser is activated by low concentrations of PALA (E.R. Kantrowitz, personal communication), and crystalline Tyr-240-Phe is T-like, except in the vicinity of the mutation [37].

Of the C1 : C4 mutants, Lys-164-Ile and Asp-271-Ser appear to be the least stable. As shown in tables 3 and 4, the free energies of denaturation of their catalytic subunits are significantly more negative than that of wild-type protein. The denaturational enthalpy of the catalytic subunit of Asp-271-Ser is also substantially reduced. In contrast, Tyr-165-Phe and Tyr-240-Phe appear to be exceptionally stable proteins, with higher values of the denaturational enthalpies of the catalytic subunits and stronger c-r interactions than wild-type protein. Both Glu-239-Gln and Glu-239-Ala have similar denaturational thermodynamics and subunit interaction energies that are reduced relative to wild-type protein (tables 3 and 4).

There appears to be no general correlation between the functional properties of a mutant and its thermal stability. This in turn suggests that classifying mutants as T or R state is likely to be an oversimplification that at best holds true only over a restricted range of conditions. For example, the energetics of denaturation of Lys-164-Ile and Tyr-165-Phe are quite different, although both Lys-164 and Tyr-165 are involved in an interaction with Glu-239 and both mutants appear in functional assays to be shifted towards the T

state; similarly, Asp-271-Ser and Tyr-240-Phe have quite different thermodynamic properties despite their nearly identical activities. Finally, the mutations at position 239 yield virtually identical denaturational parameters but opposite functional properties.

As a final note, there is a weak unexpected correlation between $\Delta\Delta H_d$ and $\Delta\Delta G_{cr}^{\circ}$, with more positive values of the former being correlated with apparently weaker subunit interactions. There is also a positive correlation between $\Delta\Delta H_d$ and the partition coefficient evaluated by analytical gel chromatography, with more positive values of the former being correlated with smaller Stokes radii (Burz et al., in preparation). These correlations suggest that there are as yet unexplored relationships between the structure of the catalytic subunit and the subunit interactions and quaternary structure of the native enzyme.

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