Differential Scanning Calorimetry of the Irreversible Denaturation of *Escherichia coli* Glucosamine-6-phosphate Deaminase†

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ABSTRACT: The thermal denaturation of *Escherichia coli* glucosamine-6-phosphate deaminase (G6PD) at neutral pH was studied by means of differential scanning calorimetry (DSC). In the concentration range 0.6–7.3 mg mL^{-1}, the denaturation of this hexameric enzyme was completely irreversible as judged by the absence of any endotherm on rescanning of previously scanned samples. However, the study of the effect of scanning rate on DSC curves indicated that the denaturation of G6PD is, most likely, a complex process which includes transitions in equilibrium as well as an irreversible step; in addition, it was found that application of the equilibrium formalism to the analysis of calorimetric data seems to be justified in this case, provided that scanning rates used are above 0.75 K min^{-1}. The calorimetric and van't Hoff enthalpies for G6PD were 1260 ± 118 and 160 ± 27 kcal mol^{-1}, respectively, indicating the presence of intermediates in the process. Accordingly, the DSC curves were adequately fitted to a model including six two-state sequential transitions. The observed protein-concentration dependence of the temperature at the maximum heat capacity, T_m, for each of the individual transitions suggests that G6PD dissociates to dimers in two consecutive steps. Using a model that includes dissociation explicitly, we calculated the thermodynamic parameters for each step. From this data, the enthalpy and free energy for the disruption of one dimer–dimer contact were roughly estimated, at pH 7.1 and 51 °C, as 57 and 2.1 kcal mol^{-1}, respectively.

Glucosamine-6-phosphate deaminase (EC 5.3.1.10) catalyzes the isomerization–deamination of D-glucosamine 6-phosphate into D-fructose 6-phosphate and ammonia. It has been mostly studied in microorganisms, mainly *Escherichia coli* (Comb & Roseman, 1958; Midelfort & Rose, 1977; Calcagno et al., 1984). *E. coli* deaminase (G6PD)[1] is a hexamer made up of six identical subunits of 29.7 kDa each (Calcagno et al., 1984), whose sequence is known from DNA (Rogers et al., 1988). *N*-Acetyl-D-glucosamine 6-phosphate activates G6PD, which is a typical allosteric enzyme of the K type (Calcagno et al., 1984; Altamirano et al., 1989). Prediction of secondary structure from amino acid sequence and circular dichroism spectroscopy suggests that G6PD subunits follow an α/β folding pattern (Altamirano et al., 1991). Chemical and crystallographic evidences point out that the deaminase quaternary structure consists of a trimer of dimers, the subunits being linked by interchain disulfide bonds. The hexamer subunits are arranged as dimers with local 2-fold axes and further associated by the crystallographic 3-fold axis to form a hexagonal array. (Altamirano et al., 1992; Horjales et al., 1992)

These structural characteristics, as well as its known catalytic and regulatory properties, make this enzyme an interesting model for studying the influence of subunit interactions on the stability of the oligomer. In this work we studied the thermal denaturation of G6PD by means of differential scanning calorimetry (DSC). In spite of the irreversible nature of the denaturation process, it was found that the calorimetric data could be analyzed in terms of equilibrium thermodynamics if high scanning rates were used. The effect of protein concentration on the excess heat capacity curves indicated that, on denaturation, hexameric G6PD dissociates to dimers in two consecutive steps. Additionally, thermodynamic parameters obtained from curve fitting of experimental data were used to estimate the free energy associated with dimer–dimer contacts.

MATERIALS AND METHODS

Enzyme. Glucosamine-6-phosphate deaminase from *E. coli* K12 was prepared by allosteric site affinity chromatography from an overproducing strain (Altamirano et al., 1991). The concentration of the enzyme was calculated from its absorbance at 278 nm, pH 7.7, and its known molar absorptivity, $\epsilon_{278} = 2.005 \times 10^5$ M^{-1} cm^{-1} (Altamirano et al., 1987).

Calorimetry. Scanning calorimetric measurements were made with a MicroCal MC-2 instrument (MicroCal Inc., Northampton, MA) using the DA-2 system for data acquisition and analysis. Protein samples were extensively dialyzed against 0.01 M sodium phosphate buffer, pH 7.1, for 20 h at 4 °C. After dialysis, the protein concentration was determined spectrophotometrically. All solutions were degassed under vacuum before being loaded into the calorimeter cells. Experiments were conducted under a nitrogen atmosphere at a total pressure of 2.2 kg cm^{-2}. Six different scanning rates were used (0.17–1.50 K min^{-1}), choosing in each case the

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[1] Abbreviations: G6PD, glucosamine-6-phosphate deaminase from *Escherichia coli*; DSC, differential scanning calorimetry.

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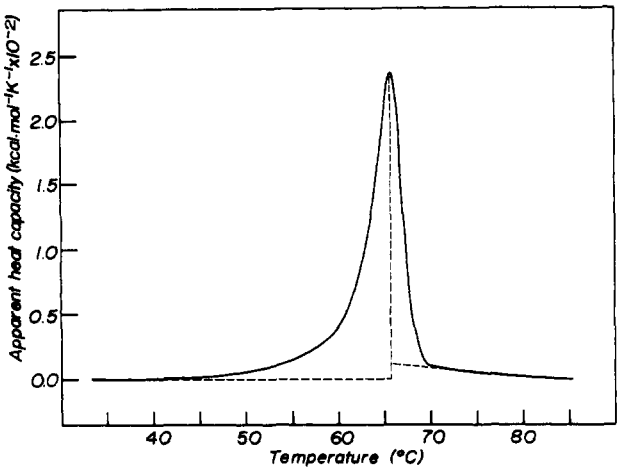

FIGURE 1: DSC curve of glucosamine-6-phosphate deaminase after subtraction of buffer–buffer base line. Protein concentration, 7.3 mg mL^{-1}; pH, 7.1; scanning rate, 1.0 K min^{-1}. Pre- and postdenaturational dashed lines are presented to show the permanent heat capacity change observed in this particular experiment (see text).

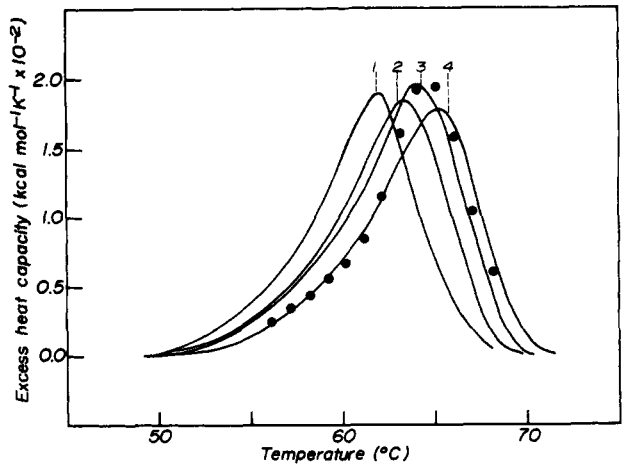

FIGURE 2: Effect of scanning rate on the excess heat capacity of glucosamine-6-phosphate deaminase: (1) 0.17, (2) 0.50, (3) 0.75, and (4) 1.50 K min^{-1}. Solid circles (●) represent calculated values that correspond to infinite scanning rate (see text for details of the extrapolation procedure). Protein concentration was the same (approximately 1.0 mg mL^{-1}) in all experiments.

appropriate response time of the instrument according to the manufacturer's recommendations. Buffer–buffer base lines were obtained at the same scanning rates and subtracted from sample curves. All thermodynamic data are given per mole of hexameric protein (M_r = 178.2), unless otherwise stated.

RESULTS AND DISCUSSION

Figure 1 shows a DSC curve, corrected by subtraction of the buffer–buffer tracing, for G6PD (7.3 mg mL^{-1}) at a scan rate of 1.0 K min^{-1}. As can be seen, in this particular curve the difference between final and initial heat capacity is approximately 12.0 kcal mol^{-1} K^{-1}; however, the values observed in several experiments were rather variable. Therefore, we restricted our analysis of data to the excess heat capacity function, C_p^{ex}, which was routinely obtained by using straight base lines that were as nearly tangent as possible to both the pre- and postdenaturational regions of the DSC tracings. In the concentration range studied (0.6–7.3 mg mL^{-1}), the thermal denaturation of this protein was completely irreversible, since no endotherm was observed by rescanning the sample after it was cooled from the first run. Moreover, after the sample was cooled from the first scan, the protein had precipitated appreciably. Similar irreversible behavior has been found with other oligomeric proteins (Manly et al., 1985; Edge et al., 1985, 1988), and in those cases equilibrium thermodynamics has been applied to the analysis of calorimetric curves. Justification for this procedure has relied on the finding that simulated curves for model systems, in which a protein undergoes a reversible denaturation followed by an irreversible step, can be analyzed in terms of the equilibrium formalism to yield parameters in agreement with those used to generate the curves. Nevertheless, when an irreversible process is present, the kind of information that can be obtained from calorimetric experiments depends on each particular situation (Freire et al., 1990). In any case, a study of the dependence of DSC curves on scanning rate is essential to performing an adequate analysis of data (Sánchez-Ruíz et al., 1988; Freire et al., 1990).

Effect of Scanning Rate on Calorimetric Curves. Denaturation endotherms of G6PD (ca. 1.0 mg mL^{-1}) were obtained at six different scanning rates in the range 0.17–1.5 K min^{-1} (Figure 2). It can be observed that the temperature at the maximum heat capacity, T_m, is dependent on the scanning rate, indicating that denaturation occurs as a kinetically controlled process (Freire et al., 1990). To determine which type of thermodynamic information could be extracted from the calorimetric curves, we first confronted our data with a simple two-state irreversible transition:

$$N \xrightarrow{k} D \qquad (I)$$

where N and D represent the native and denatured states, respectively; k, the first-order rate constant for the reaction, can be calculated at any given temperature according to Sánchez-Ruíz et al. (1988) and Freire et al. (1990),

$$k = rC_p^{ex}/(\Delta H - \langle \Delta H \rangle) \qquad (1)$$

where r is the scanning rate, ΔH is the total enthalpy of the process, and $\langle \Delta H \rangle$ is the cumulative enthalpy at a given temperature. Since the rate constant is only a function of temperature, eq 1 should give the same value of k when applied to calorimetric data obtained at different scanning rates. In our study the rate constant calculated at a fixed temperature from curves in Figure 2 varied by factors of 3 to 5 (depending on the temperature at which the calculations were made). This result suggests that the denaturation of the deaminase does not follow the two-state mechanism mentioned above.

Recently, Freire et al. (1990) developed equations to account for the effect of irreversibility on calorimetric data according to the more complex model

$$N \rightleftarrows I_1 \rightleftarrows I_2 \rightleftarrows \ldots \rightleftarrows I_n \xrightarrow{k} D \qquad (II)$$

which consists of a series of sequential equilibrium transitions connecting the native state to a number of intermediate species, I_i, followed by a final irreversible step. Unfortunately, straightforward application of those equations to the case of G6PD is not possible since there is evidence indicating that this protein dissociates during denaturation (see below). However, if it is assumed that equilibrium between the intermediate states is always established, extrapolation of experimental data, such as $\langle \Delta H \rangle$, to infinite scanning rate should permit isolation of the part pertaining to the species in thermodynamic equilibrium. In order to reduce the experimental uncertainties arising from the different total enthalpies of the curves in Figure 2 (ΔH = 1300 ± 120 kcal mol^{-1}), we decided to use the fractional cumulative enthalpy ($\langle \Delta H \rangle / \Delta H$). Figure 3 shows that the quotient $\langle \Delta H \rangle / \Delta H$

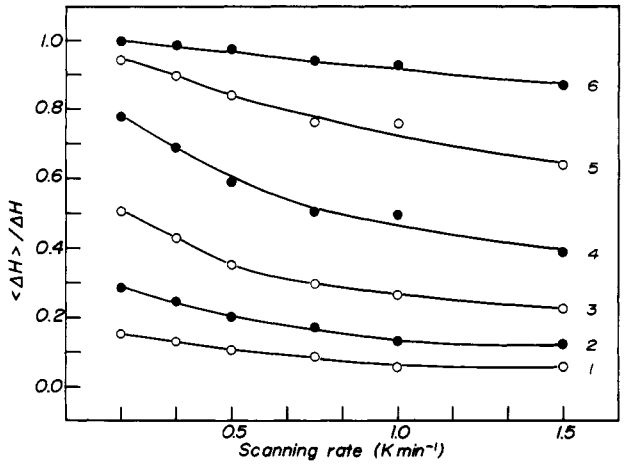

FIGURE 3: Plots of the fractional cumulative enthalpy of denaturation ($\langle\Delta H\rangle/\Delta H$) vs scanning rate, for the following temperatures: (1) 57, (2) 59, (3) 61, (4) 63, (5) 65, and (6) 67 °C.

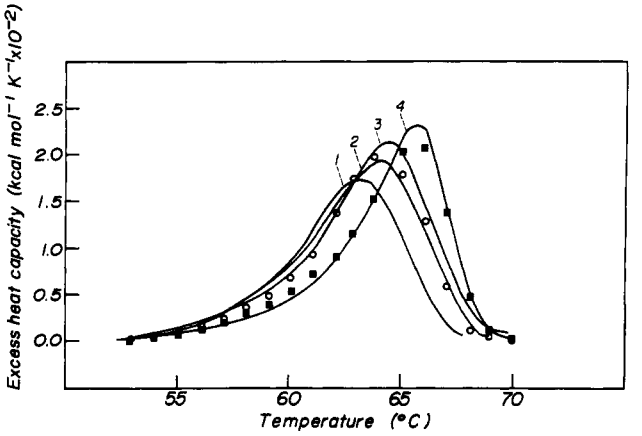

FIGURE 4: Curves of the excess heat capacity of glucosamine-6-phosphate deaminase at different protein concentrations: (1) 0.61, (2) 0.97, (3) 1.99, and (4) 7.30 mg mL^{-1}. In all cases, the scanning rate was 1.0 K min^{-1}. Calculated values of C_p^{ex} from curve fitting to mechanism III are shown with circles (○) for curve 2 and squares (■) for curve 4.

seems to approach a limiting value at high scanning rates. Furthermore, good linear relationships (correlation coefficients between −0.86 and −0.99) were obtained from plots of $\ln[1 - (\langle\Delta H\rangle/\Delta H)]$ vs the reciprocal of the scanning rate ($1/r$), thus making possible an appropriate extrapolation to a zero value of $1/r$. In this way, we constructed a curve of excess enthalpy and, from its derivative with respect to temperature, a curve of excess heat capacity, which is shown with solid circles in Figure 2. This curve, which according to the assumption stated above should correspond to the transitions in equilibrium, does not deviate too much from experimental curves obtained at high scanning rates. Therefore, it appears that it would be correct to apply the formalism of equilibrium thermodynamics to the analysis of our calorimetric data, provided that the scanning rate employed is above 0.75 K min^{-1}.

Effect of Protein Concentration on Excess Heat Capacity Curves. Denaturation experiments were done at enzyme concentrations ranging from 0.6 to 7.3 mg mL^{-1}, using a scanning rate of 1.0 K min^{-1} in all cases. The C_p^{ex} curves obtained showed that T_m increases with protein concentration (Figure 4), indicating that some dissociation of the oligomeric molecule takes place during denaturation (Takahashi & Sturtevant, 1981; Sturtevant, 1987). The total enthalpy of the process displayed no dependence on protein concentration, its average value being 1260 ± 118 kcal mol^{-1}, or 7.07 cal g^{-1}. This value of specific enthalpy lies in the range observed for small compact proteins (7–9 cal g^{-1} at 70 °C; Privalov, 1979). The van't Hoff enthalpy, ΔH_{vH}, amounted to only 160 ± 27 kcal mol^{-1}. Since the cooperative ratio (CR = $\Delta H/\Delta H_{vH}$) is much larger than unity, it can safely be concluded that one or more intermediate states are present in the overall process (Tanford, 1968; Biltonen & Freire, 1978; Privalov 1979), as would be expected from the molecular complexity of this enzyme. In agreement with this result, we found that each of the curves in Figure 4 could be resolved into six two-state sequential transitions with a standard deviation of fitting that was below 1.5% of the maximum value of C_p^{ex}. The use of only five transitions gave, in most cases, significantly higher standard deviations and some important discrepancies with experimental curves. The results obtained in the curve resolutions for G6PD are summarized in Table I. In the row corresponding to T_m, the values given indicate the range of variation that resulted from experiments performed at the different protein concentrations.

The increase of T_m with the total protein concentration, $[P]_0$ can be stated in a quantitative way by the relationship (Sturtevant, 1987)

$$\ln[P]_0 = [-\Delta H_{vH}/(n-1)RT_m] + \text{constant} \qquad (2)$$

which applies to a two-state transition that involves complete dissociation of an oligomeric molecule: $N_n \rightleftarrows nD$. In the case of partial dissociation, i.e., $N_n \rightleftarrows mD + N_l$, it can be shown that the equation relating $[P]_0$ to T_m is

$$\ln[P]_0 = [-\Delta H_{vH}/mRT_m] + \text{constant} \qquad (3)$$

In our case, plots of $\ln[P]_0$ vs $1/T_m$ adjusted reasonably well to straight lines only for the fourth and fifth two-state transitions of Table I (Figure 5). From the slopes of these plots, which are nearly identical to each other, it was found that $\Delta H_{vH} = 280\ (n-1)$ kcal mol^{-1} (according to eq 2), or $\Delta H_{vH} = 280m$ kcal mol^{-1} (according to eq 3). Since the average enthalpies of the fourth and fifth transitions are 251 ± 56 and 252 ± 40 kcal mol^{-1} (Table I), the above results suggest that either $n = 2$ or $m = 1$. Thus, it seems reasonable to propose that dissociation of the oligomeric enzyme occurs in two of the latest stages of the denaturation process.

In those cases where protein denaturation is accompanied by dissociation of subunits, the use of a sequential model with two-state transitions in the deconvolution of DSC curves is

Table I: Parameters Obtained in the Resolution of Calorimetric Curves for Glucosamine-6-phosphate Deaminase Based on Six Two-State Sequential Transitions[a]

	transition					
parameter	1	2	3	4	5	6
T_m (°C)[b]	57.0–58.3	58.6–61.3	61.4–63.4	62.8–64.9	64.0–66.1	65.3–66.9
ΔH (kcal mol^{-1})[c]	141 ± 29	190 ± 32	218 ± 44	251 ± 56	252 ± 40	238 ± 26

[a] From six experiments in the concentration range 0.6–7.3 mg mL^{-1}. [b] Lowest and highest values found. [c] Enthalpies expressed as mean ± standard deviation; $\Sigma\Delta H$ = 1290 ● 96 kcal mol^{-1}; average calorimetric enthalpy = 1260 ± 118 kcal mol^{-1}.

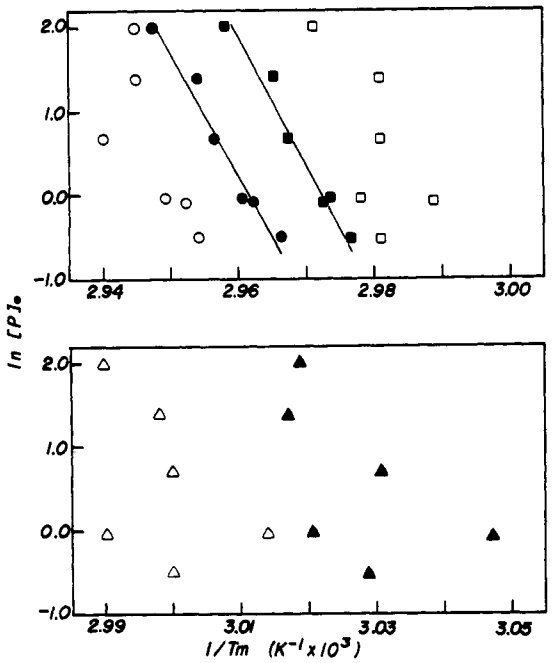

FIGURE 5: Plots of the logarithm of the total protein concentration, $[P]_0$, vs $1/T_m$ for each of the sequential transitions obtained by resolution of the calorimetric curves for glucosamine-6-phosphate deaminase. Data are for transitions 1 (▲), 2 (△), 3 (□), 4 (■), 5 (●), and 6 (○). Straight lines shown are the best fit for transitions 4 and 5, which gave correlation coefficients of –0.99 and –0.98. In all other cases, the correlation coefficients were between –0.44 and –0.73.

only a rough approximation. Therefore, we analyzed our data in terms of a model that explicitly considers two dissociation steps. For simplicity, dissociation was considered to occur at the end of the pathway:

$$I_0 \overset{K_1}{\rightleftharpoons} I_1 \overset{K_2}{\rightleftharpoons} I_2 \overset{K_3}{\rightleftharpoons} I_3 \overset{K_4}{\rightleftharpoons} I_4 \overset{K_5}{\rightleftharpoons} I_5 + I_6 \quad \text{(III)}$$

$$2I_6 \overset{K_6}{\rightleftharpoons}$$

In this mechanism, it is assumed that the subunits of the enzyme remain associated as dimers even in the final state. I_0 represents the native state, and I_6 the final state in equilibrium; species I_0–I_4 are hexameric, I_5 is tetrameric, and I_6 is dimeric. Thus, the first four steps would correspond to changes in protein regions that do not participate significantly in dimer–dimer contacts, while the last two steps would include disruption of such contacts.

In model III, the equilibrium constants are defined as

$$K_i = [I_i]/[I_{i-1}] \quad \text{for} \quad i = 1\text{–}4 \tag{4}$$

$$K_5 = [I_5][I_6]/[I_4] \tag{5}$$

$$K_6 = [I_6]^2/[I_5] \tag{6}$$

The values of the equilibrium constants can be calculated from the known thermodynamic relationship

$$K_i = \exp(-\Delta Gi/RT) = \exp[-\Delta H_i[1 - (T/T_{m,i})]] \tag{7}$$

where ΔH_i is the enthalpy change of the ith step and $T_{m,i}$ is the particular temperature at which $K_i = 0$. In eq 7, permanent heat capacity changes are neglected. The total concentration of molecules (expressed as moles of hexamer per liter) is given by

$$C = \sum_{i=0}^{4}[I_i] + \frac{2}{3}[I_5] + \frac{1}{3}[I_6] \tag{8}$$

which can be written in terms of $[I_0]$ by using eqs 4–6,

$$C = (1 + K_1 + K_1K_2 + K_1K_2K_3 + K_1K_2K_3K_4)[I_0] + \{(2/3)(K_1K_2K_3K_4K_5)^{2/3}/K_6^{1/3}\}[I_0]^{2/3} + (1/3)(K_1K_2K_3K_4K_5K_6)^{1/3}[I_0]^{1/3} \tag{9}$$

Equation 9 can be solved numerically to obtain $[I_0]$, which in turn allows calculation of the concentrations of all other species. At any temperature, the heat absorbed during denaturation of the enzyme should be equal to

$$Q = \sum_{i=1}^{6} H_i[I_i]V \tag{10}$$

where H_i is the enthalpy of I_i with respect to that of I_0 and V is the sample volume in the experiment. Since throughout this work enthalpy changes are expressed per mole of hexamer, eq 10 must be divided by the number of moles of hexamer, $(C)(V)$, to give the cumulative enthalpy change:

$$\langle \Delta H \rangle = \sum_{i=1}^{6} H_i[I_i]/C \tag{11}$$

It must be noted that all the H_i values are determined by the set of ΔH_i, since

$$\Delta H_i = H_i - H_{i-1} \quad \text{for} \quad 1 \le i \le 4$$

$$\Delta H_5 = H_5 + H_6 - H_4$$

and

$$\Delta H_6 = 2H_6 - H_5$$

Finally, the derivative of $\langle \Delta H \rangle$ with respect to temperature gives C_p^{ex}. Thus, to perform the above calculations, the parameters needed are the six values of ΔH_i and their corresponding $T_{m,i}$ values.

Data of curves 2 and 4 in Figure 4 were simultaneously fitted to the equations that describe mechanism III, as outlined above. The nonlinear least squares procedure employed was based on the GRIDLS program of Bevington (1969). Results are summarized in Table II, and values of C_p^{ex} calculated from the regression analysis are also shown in Figure 4. The standard deviation of fitting (5.2% of the maximum C_p^{ex}) was

Table II: Parameters Obtained from Curve Fitting of Calorimetric Data for Glucosamine-6-phosphate Deaminase, Based on Mechanism III[a]

	transition					
parameter	1	2	3	4	5	6
T_m (°C)[b]	58.1	61.9	62.4	68.1	69.1	71.2
ΔH (kcal mol^{-1})[c]	140 ± 20	182 ± 50	10 ± 20	199 ± 35	284 ± 30	396 ± 20

[a] From curves 2 and 4 of Figure 4. [b] The estimated uncertainty of these values is ±0.6 °C. [c] Enthalpies expressed as mean ± standard deviation; $\Sigma\Delta H$ = 1211 ± 76 kcal mol^{-1}; average calorimetric enthalpy = 1260 ± 118 kcal mol^{-1}.

certainly higher than in the previous deconvolution procedure; it must be recalled, however, that in this case two different calorimetric curves were fitted at the same time, thus increasing the amount of experimental data that should be explained with the set of parameters.

The parameters listed in Table II allowed us to estimate the contribution of each dimer–dimer contact to the stability of the whole molecule. That is, if the hexagonal array of subunits is conserved during the first four sequential steps, then ΔH_5 would include the enthalpy change for the disruption of two dimer–dimer contacts, ΔH^C, plus the change associated with the final denaturation of one dimeric unit, ΔH^D. In the sixth step, two dimers would denature, but only one dimer–dimer interaction would remain to be disrupted. Therefore,

$$\Delta H_5 = 2\Delta H^C + \Delta H^D \quad (12)$$

$$\Delta H_6 = \Delta H^C + 2\Delta H^D \quad (13)$$

By solving these equations we found that $\Delta H^D = 170$ kcal and $\Delta H^C = 57$ kcal. This latter number is comparable to 37 kcal, which is a rough estimation for the enthalpy change corresponding to the rupture of one catalytic–regulatory subunit interaction in aspartate transcarbamoylase (Brandts et al., 1989). ΔS^C was calculated as 0.17 eu by following a similar procedure. Furthermore, neglecting heat capacity effects, the free energy to break one dimer–dimer contact was found to be 2.1 kcal at 50 °C, which is close to the 4.2 kcal of interaction for each catalytic and regulatory subunit in aspartate transcarbamoylase at 51 °C, as estimated by Brandts et al. (1989) from the analysis of the calorimetric data of Edge et al. (1985, 1988).

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