Differential Scanning Calorimetric Study of the Thermal Unfolding of Taka-amylase A from *Aspergillus oryzae*†

Harumi Fukada and Katsutada Takahashi

Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan

Julian M. Sturtevant*

Department of Chemistry, Yale University, New Haven, Connecticut 06511

Received December 19, 1986; Revised Manuscript Received March 3, 1987

ABSTRACT: The thermally induced unfolding of Taka-amylase A, isolated from *Aspergillus oryzae*, was studied by differential scanning calorimetry. The experimental curves of excess apparent specific heat vs. temperature showed a single asymmetric peak. Curve resolution indicated that this asymmetry is due to the two-state unfolding of three domains in the molecule, with dissociation of the single tightly bound Ca^{2+} ion occurring during the unfolding of the last domain. Further indication of the dissociation of the specifically bound Ca^{2+} during denaturation is afforded by the fact that the temperature of maximal excess specific heat, t_m, increases with increasing protein concentration in the absence of added excess Ca^{2+} and with increasing Ca^{2+} concentration in the presence of added Ca^{2+}. Experiments in a variety of buffers with different enthalpies of ionization indicated that 11.8 ± 1.5 protons are lost from the protein during unfolding at pH 7.0. In apparent contradiction of this result, the value of t_m was found to be essentially independent of pH in the range pH 7–8. No explanation of this anomaly is available. The enthalpy of unfolding at pH 7 and 62 °C in the absence of added Ca^{2+}, corrected for the change in buffer protonation, is 2250 ± 40 kJ mol^{-1} (42.5 J g^{-1}), and the permanent change in apparent heat capacity is 36.4 ± 4.1 kJ K^{-1} mol^{-1} (0.687 J g^{-1}). Both of these quantities are unusually large for a globular protein.

Taka-amylase A (TAA)[1] (Akabori et al., 1954) is an α-amylase isolated from *Aspergillus oryzae* having a molecular weight of 53 000. At ordinary temperatures in solution the enzyme is a monomer that consists of a single polypeptide chain of 478 amino acid residues with four intrachain disulfide bonds (Toda et al., 1982). The denaturation of TAA has been studied quantitatively and shown to be reversible both in acid solution (Oikawa, 1957; Takagi & Toda, 1962) and in urea solution (Fujita-Ikeda & Isemura, 1960; Takagi & Isemura, 1962). It has also been shown, as was first observed with ribonuclease A (Anfinsen & Haber, 1961), that reduced and unfolded TAA is renatured upon being returned to neutral pH in aqueous solution with reoxidation of the sulfhydryls (Isemura et al., 1961; Isemura et al., 1963; Takagi & Isemura, 1965). An important feature of this enzyme is that it contains one tightly bound Ca^{2+} ion per molecule, which is essential for enzymic activity (Oikawa & Maeda, 1957; Vallee et al., 1959). Its three-dimensional structure has been determined by X-ray crystallography at a resolution of 2.3 Å (Matsuura et al., 1979, 1980, 1984). The molecule appears to be composed of a main domain, containing a cleft in which the active site of the enzyme is located, and a smaller C-terminal domain, the two domains being joined by single polypeptide chain. In this paper, we report the thermodynamic parameters for the thermal unfolding of TAA as measured by high-sensitivity differential scanning calorimetry.

Materials and Methods

Taka-amylase A was prepared from Taka-diastase Sankyo of Sankyo Co. Ltd., Tokyo, by the method of Akabori et al. (1954) with modification to include DEAE column chromatography. The enzyme preparation crystallized from acetone solution was dissolved in water at pH 7.0 and dialyzed against the appropriate buffer, the dialyzate being used for further dilution of the protein for calorimetric measurements and for filling the reference cell of the calorimeter. The TAA concentration was determined spectrophotometrically by using a value of specific absorptivity at 280 nm of 2.21 cm^2 mg^{-1} at pH 6.0 (Toda & Akabori, 1963). The solutions employed from measurements had a protein concentration of 0.5–2 mg mL^{-1}.

The buffers employed were cacodylate, PIPES, MES, HEPES, MOPS, BES, ACES, triethanolamine, Tris, and acetate at a concentration of 0.05 M and containing 0.1 M NaCl. All the chemicals used were commercial products of reagent grade. Solutions were prepared with doubly deionized water.

Differential Scanning Calorimetry. The DASM-1M differential heat capacity microcalorimeter designed by Privalov et al. (1975) was used with some minor electronic modifications. The scan rate was usually 1 K min^{-1}. Calorimetric enthalpy and heat capacity changes associated with the unfolding of the protein were evaluated from the DSC curves of excess heat capacity according to the methods described previously (Velicelebi & Sturtevant, 1979; Takahashi &

† This research was aided by grants from the National Institutes of Health (GM-04725) and the National Science Foundation (PCM81-17341). Travel costs for this research program were supported in part by the Ministry of Education, Japan (K.T.).

[1] Abbreviations: TAA, Taka-amylase A from *Aspergillus oryzae*; DSC, differential scanning calorimetric (calorimetry); ACES, *N*-(2-acetimido)-2-aminoethanesulfonic acid; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; HEPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TEA, triethanolamine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/87/0426-4063$01.50/0

Table I: Thermal Denaturation of TAA in 0.05 M Buffers, 0.1 M NaCl, with No Added $CaCl_2$

buffer	prot concn (mg mL^{-1})	t_m (°C)	ΔH_d (kJ mol^{-1})	ΔH_d(62 °C) (kJ mol^{-1})	ΔC_d (J K^{-1} g^{-1})
HEPES, pH 7.0	0.53	61.9	2040	2045	0.87
	1.06	62.75	1990	1970	0.53
	2.13	63.25	2020	1980	0.57
	3.19	63.75	1875	1830	0.50
				mean 1960	0.62
				2207 ± 60[a]	±0.13
HEPPS, pH 7.5	0.53	62.5	2035	2015	0.77
	1.05	63.2	2070	2040	0.48
	2.10	63.7	2130	2070	0.65
	3.15	63.9	2190	2140	0.50
				mean 2070	0.60
				2395 ± 40[a]	±0.10
cacodylate, pH 6.8	0.50	62.1	2070	2030	0.93
	1.01	62.9	2190	2160	0.79
	2.01	62.95	2240	2230	0.77
	3.02	63.75	2290	2220	0.87
				mean 2160	0.84
				2135 ± 65[a]	±0.06

[a] Values corrected for buffer ionization heat as described below.

Sturtevant, 1981; Fukada et al., 1983).

Results and Discussion

Denaturation of TAA at pH 7.0 in the Absence of Added Calcium. A tracing of a typical DSC curve for the thermal denaturation of TAA in the absence of any added Ca^{2+} is shown in Figure 1. The results of 12 experiments in three different buffers at varying protein concentrations are summarized in Table I. The enthalpies listed in column 5 are values calculated to 62 °C from the observed values in column 4 and the specific heat changes in column 6. The means of these values have been corrected for buffer ionization heats as described in a later section. The mean denaturational enthalpy at 62 °C, 2246 kJ mol^{-1}, corresponds to 42.4 J g^{-1} (10.13 cal g^{-1}), which is an unusually large value for a globular protein (Privalov & Khechinashvili, 1974). The average increase in heat capacity, 0.69 J K^{-1} g^{-1} (0.16 cal K^{-1} g^{-1}) is also usually large.

The pronounced asymmetry of the curve in Figure 1 indicates that the process is more complicated than simple two state. An additional indication of this follows from the fact that the van't Hoff enthalpy, calculated by means of the equation (eq 1) appropriate for a two-state process involving

$$\Delta H_{vH} = 4RT_m^2(c_{max}/\Delta h_{cal}) \quad (1)$$

neither dissociation nor association, where T_m is the absolute temperature at which the excess specific heat reaches its maximal value, c_{max}, and Δh_{cal} is the specific enthalpy obtained by evaluating the area of the DSC curve, has the value 339 kJ mol^{-1}, whereas the calorimetric enthalpy is 2020 kJ mol^{-1}. Application of the procedure for DSC curve resolution previously described (Edge et al., 1985) showed that the DSC data could be adequately represented as the resultant of the two-state unfolding of three domains with dissociation of the specifically bound Ca^{2+} ion during the last step:

$$N_1N_2N_3Ca^{2+} \rightleftarrows D_1N_2N_3Ca^{2+} \rightleftarrows D_1D_2N_3Ca^{2+} \rightleftarrows D_1D_2D_3 + Ca^{2+} \quad (2)$$

The observed data were read relative to the base line shown in Figure 1, which was calculated by the procedure of Takahashi and Sturtevant (1981). Although this procedure for obtaining a base line is strictly valid only for a simple two-state process, it can be shown by simulations that its use in the present case is not likely to introduce serious errors. The component curves obtained by the resolution procedure are shown in Figure 1. The standard deviation of calculated from observed excess specific heats (35–40 values for each DSC curve) averaged 1.4% of c_{max} for the 22 curves that were resolved.

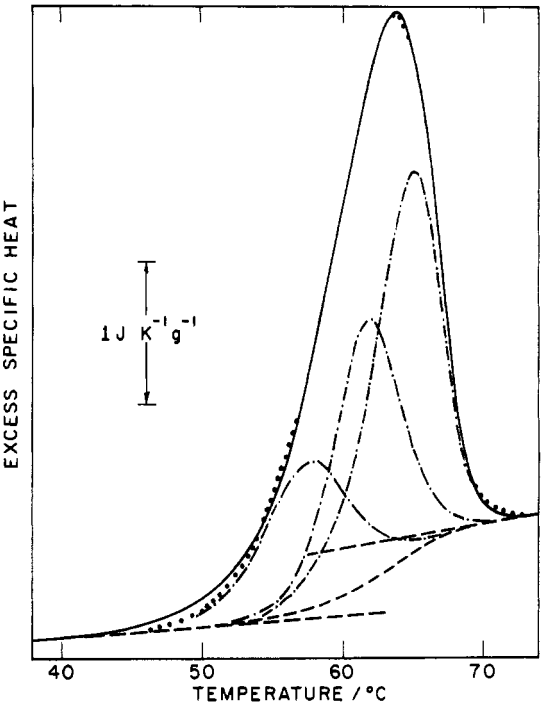

FIGURE 1: Tracing of a typical DSC curve for the thermal denaturation of TAA in the absence of added Ca^{2+}. The instrumental base line (nearly flat) has been deducted. The noise level was approximately 0.02 J K^{-1} g^{-1} peak to peak. Protein at 3.19 mg mL^{-1} was dissolved in 0.05 M HEPES buffer containing 0.1 M NaCl at pH 7.0. Scan rate 1 K min^{-1}. The dashed lines are the initial and final base lines, and the dashed curve is the calculated base line, which goes from the initial to the final base line in proportion to the extent of denaturation. The three dot-dash curves are the component transitions arrived at by curve fitting (see text), and the filled circles show the deviation of the calculated sum curve from the observed curve.

This model for the denaturation process is consistent with the results of the X-ray crystallographic determination of the structure of TAA (Matsuura et al., 1984). The molecule of 478 amino acid residues is composed of two domains, the A domain made up of the first 380 residues from the N-terminus and the B domain containing the remaining residues. The two domains are linked by a single polypeptide chain. The A domain contains the Ca^{2+} binding site and a large cleft which is the catalytically active site of the enzyme. It seems likely

Table II: Result of Resolution of the DSC Curves for the Experiments Listed in Table I

buffer	prot concn (mg mL^{-1})	$t_{1/2,1}$ (°C)	Δh_1 (J g^{-1})	$t_{1/2,2}$ (°C)	Δh_2 (J g^{-1})	$t_{1/2,3}$ (°C)	Δh_3 (J g^{-1})	$\sum\Delta h$ (J g^{-1})	ΔC_d[a] (J K^{-1} g^{-1})	Δh_{diss}[b] (J g^{-1})
HEPES, pH 7.0	0.53	55.8	9.02	60.1	12.24	62.4	17.80	39.07	0.75	3.83
	1.06	57.0	8.61	61.0	11.57	63.3	17.05	37.23	0.74	3.72
	2.13	57.3	8.84	61.4	11.77	64.05	17.15	37.77	0.72	3.50
	3.19	57.8	8.75	62.0	11.20	64.3	17.05	37.00	0.59	4.50
			mean 9.51		11.70		17.26	37.77[c]	0.70	3.89
			SE ±0.25		±0.31		±0.26	±0.66	±0.05	±0.30
HEPPS, pH 7.5	0.53	57.8	9.51	61.35	11.85	63.1	17.14	38.50	0.66	4.14
	1.05	57.75	9.13	61.6	12.15	63.85	17.78	39.06	0.78	3.88
	2.10	57.75	9.20	61.8	12.18	64.4	18.08	39.46	0.73	4.00
	3.15	56.8	9.55	61.6	12.33	64.65	17.93	39.81	0.58	3.82
			mean 9.35		12.13		17.73	39.21[d]	0.69	3.96
			SE ±0.15		±0.14		±0.29	±0.40	±0.06	±0.10
cacodylate, pH 6.8	0.50	57.0	11.07	60.8	14.72	63.2	19.78	45.57	0.97	2.73
	1.01	57.55	9.46	61.35	12.99	63.75	18.32	40.77	0.92	3.13
	2.01	57.4	10.27	61.25	13.09	63.70	17.95	41.31	0.73	3.06
	3.02	58.2	10.75	61.95	13.38	64.50	18.12	42.25	0.70	2.95
			mean 10.39		13.55		18.54	42.48[e]	0.83	2.97
			SE ±0.50		±0.57		±0.59	±1.52	±0.10	±0.12

[a] $\Delta C_d = (\Delta h_2 - \Delta h_1)/(t_{1/2,2} - t_{1/2,1})$. [b] $\Delta h_{diss} = \Delta h_3 - [\Delta C_d(t_{1/2,3} - t_{1/2,2}) + \Delta h_2]$. [c] Mean value of Δh_{obsd} = 37.40 J g^{-1}. [d] Mean value of Δh_{obsd} = 39.73 J g^{-1}. [e] Mean value of Δh_{obsd} = 43.39 J g^{-1}.

that the three domains suggested by our curve resolution are the B domain and the roughly equal parts of the A domain on either side of the cleft.

The results of the curve resolutions are listed in Table II. The adjustable parameters for each component are $t_{1/2,i}$, the temperature of half-completion, and Δh_i, the specific enthalpy change. In each step the van't Hoff enthalpy was set equal to Δh_i times the molecular weight, 53 000 daltons. The sum of the Δh_i given in column 9 of the table differs on average by only 1.6% from the value corresponding to ΔH_d in column 4, Table I.

It appears that the difference between the values of Δh_i for each experiment can be accounted for largely on the basis of an apparent ΔC_d as listed in column 6, Table I, plus the heat of dissociation of Ca^{2+} from the denaturated protein. The values of ΔC_d calculated from the values for $t_{1/2,1}$, $t_{1/2,2}$, Δh_1, and Δh_2 as indicated in Table II are listed in column 10 of Table II and agree reasonably well with those given in Table I. If these values are utilized with $t_{1/2,2}$, $t_{1/2,3}$, and Δh_2 to predict values for Δh_3, it is found that the predicted values are less than the values obtained by curve resolution by the amounts given in the last column of the table. We assign these values to the enthalpy of dissociation of the Ca^{2+} ion during the last step of the deviation in obtaining a mean value of 190 ± 30 kJ mol^{-1}. This seems large for the dissociation of a single ion, suggesting that the unfolding of the Ca^{2+} binding domain is much more extensive than that which takes place in the other two domains. This analysis of the individual enthalpies of denaturation of the domains also suggests that the domains N_1 and N_2 (eq 2) are energetically quite similar.

It is evident in Table II that, with the exception of $t_{1/2,1}$ in EPPS buffer, the values for $t_{1/2}$ increase with increasing protein concentration. If these increases are examined in the form of van't Hoff plots (Takahashi & Sturtevant, 1981; Fukada et al., 1983; Edge et al., 1985; Manly et al., 1985) of ln $(TAA)_0$ vs. $1/T_{1/2}$, where $(TAA)_0$ is the total protein concentration and $T_{1/2} = t_{1/2} + 273.15$, it is found that only in the case of $t_{1/2,3}$ are reasonably constant slopes obtained. This dependence of $t_{1/2,3}$ on the protein concentration is further indication that the tightly bound Ca^{2+} is dissociated during the last unfolding step. The roughly similar behavior of $t_{1/2,1}$ and $t_{1/2,2}$ may result from domain interactions. The mean slope of the van't Hoff plots for $t_{1/2,3}$ is $(-125 \pm 12) \times 10^3$, which when multiplied by $-R$ gives ΔH_{vH} = 1040 ± 100 kcal mol^{-1}. Comparison of this figure with the mean value of ΔH_3, 945 ± 30 kcal mol^{-1}, lends further support to the analysis of the DSC curves presented here.

The expected small increase of each Δh_i with protein concentration resulting from the increase of $t_{1/2,i}$ is obscured by the scatter of the data.

The dissociation of Ca^{2+} ion during the thermal denaturation of TAA is further evidenced by the fact that the value of t_m for solutions containing a constant concentration of TAA in phosphate buffer is lowered from 61.0 to 58.5 °C with increase in the buffer concentration from 0.01 to 0.1 M, presumably due to lowering of the Ca^{2+} activity by binding to phosphate. A similar effect is produced by the addition of EDTA to TAA in cacodylate buffer.

The curve resolution employed here is based on the van't Hoff equation and is thus in principle only applicable to reversible processes, whereas the thermal denaturation of TAA is, at least by the DSC criterion of repeatable scans, irreversible. However, it may be argued that the observed dependence of $t_{1/2}$ on protein concentration would not be observed if the process were truly irreversible. Furthermore, as will be seen later, the value of $t_{1/2}$ is affected by the concentration of added Ca^{2+}, again as expected on the basis of the van't Hoff equation, and it is thus evident that there is reversible communication between the protein and the Ca^{2+} ions in solution. Similar effects have been observed with other apparently irreversible denaturations studied by DSC (Manly et al., 1985; Edge et al., 1985).

Denaturation of TAA at pH 7.0 in a Variety of Buffers. The thermal denaturation of TAA was run in nine different buffers with enthalpies of ionization ranging from −5 to +45 kJ mol^{-1}, with the results summarized in Figure 2 and Table III. The buffer heats of ionization calculated to 62 °C are given in column 2. Column 5 lists the denaturational enthalpies calculated to 62 °C from the observed enthalpies by using the values for ΔC_d given in column 6. The latter values fit the equation

$$\Delta H_d(62\ °C) = 2251 - 11.41\Delta H_i(62\ °C) \qquad (3)$$

with a standard deviation of ±63 kJ mol^{-1}. The second term in this equation corresponds to a release of 11.4 ± 1.5 protons from the protein during unfolding. This result suggests that several ionizable groups, probably mostly carboxyl groups which have high pK values when immersed in a medium of

Table III: Thermal Denaturation of TAA at pH 7 in Various Buffers

buffer	ΔH_i(62 °C) (kJ mol^{-1})	t_m (°C)	ΔH_d (kJ mol^{-1})	ΔH_d(62 °C) (kJ mol^{-1})	ΔC_d (kJ K^{-1} mol^{-1})
cacodylate	−5.1[a]	64.4	2321	2216	43.9
PIPES	12.2[a]	62.8	2205	2163	52.9
MES	15.6[a]	63.5	2200	2120	53.1
MOPS	22.3[a]	64.1	2195	2089	50.6
HEPES	22.7[a]	64.3	2063	1968	41.2
BES	25.2[a]	64.6	2058	1966	35.4
ACES	30.4[a]	63.6	1939	1863	47.2
TEA	35.5[a]	64.0	1898	1841	28.3
Tris	45.6[b]	66.0	1871	1752	29.8
					mean 42.5
					SE 3.5

[a] Calculated by extrapolation from values of ΔH_i determined over the temperature range 5–40 °C (K. Takahashi and H. Fukada, unpublished results). [b] Calculated by eq 7 in Grenthe et al. (1970).

Table IV: Thermal Denaturation of TAA at pH 7 in 0.05 M HEPES Buffer, 0.1 M NaCl, in the Presence and Absence of Added Calcium

prot concn (mg mL^{-1})	$CaCl_2$ concn (mM)	t_m (°C)	ΔH_d (kJ mol^{-1})	ΔH_d(62 °C) (kJ mol^{-1})	ΔC_d (J K^{-1} g^{-1})
		Constant Calcium Concentration			
0.57	0.50	68.2	2021	1817	0.62
1.14	0.50	68.1	1989	1861	0.40
2.28	0.50	68.2	2056	1938	0.36
2.28	0.50	68.2	1979	1896	0.25
4.55	0.50	68.2	2030	1909	0.39
		mean 68.2		1884	0.40
		SE ±0.0		±27	±0.08
		Varying Calcium Concentration			
2.28	0.12	67.5	2046	1936	0.38
2.28	0.50	68.2	2056	1938	0.36
0.57–4.55	0.50	68.2	1979	1896	0.25
2.27	0.60	68.2	1933	1856	0.23
2.27	1.19	69.9	2032	1865	0.40
2.27	2.38	71.7	2042	1821	0.46
				mean 1885	0.35
				SE ±23	±0.04

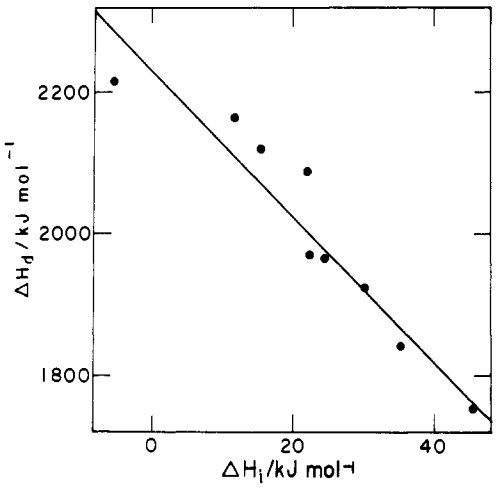

FIGURE 2: Enthalpy of denaturation, ΔH_d, in various buffers calculated to 62 °C plotted against the enthalpies of ionization at 62 °C of the buffers (cf. Table III). The line in the figure was determined by linear least squaring.

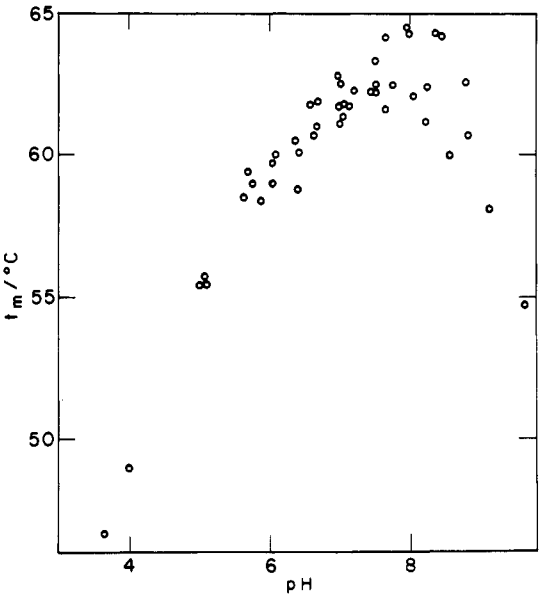

FIGURE 3: Effect of pH on the denaturation of TAA. The observed denaturational t_m (°C) is plotted against the pH of denaturation.

low dielectric constant, become exposed to the solvent during denaturation and undergo ionization with liberation of protons. The value of the denaturational enthalpy at zero buffer ionization heat is seen to be 2251 ± 40 kJ mol^{-1} (pH 7, 62 °C).

Effect of pH on the Thermal Unfolding of TAA. Experiments were performed to investigate the effect of pH on the denaturation of TAA. In these experiments, the buffer pH was adjusted to the desired pH at 50 °C to reduce errors due to the different temperature coefficients of pH for the various buffers used. The effect of pH on t_m is shown in Figure 3. There is significant variation in t_m at high and low pH's, but only minor changes in the range 6.5–8.5. This latter fact leads to the conclusion that there is little or no change in the protonation of the protein during denaturation at neutral pH, in sharp disagreement with the unusually large variation of ΔH_d with buffer ionization heat discussed above. We can offer no resolution of this discrepancy. The enthalpy of denaturation was observed to decrease at low pH by an amount larger than could be accounted for on the basis of the change in t_m and the value of ΔC_d. The enthalpies are not listed here because the uncertainty in the change of protonation precludes the possibility of applying corrections for the buffer ionization heat.

Table V: Results of the Resolution of the DSC Curves for the Experiments Listed in Table IV

prot concn (mg mL^{-1})	$CaCl_2$ concn (mM)	$t_{1/2,1}$ (°C)	Δh_1 (J g^{-1})	$t_{1/2,2}$ (°C)	Δh_2 (J g^{-1})	$t_{1/2,3}$ (°C)	Δh_3 (J g^{-1})	$\sum \Delta h$ (J g^{-1})
			Constant Calcium Concentration					
0.57	0.50	63.4	8.58	66.65	12.08	68.75	19.55	40.21
1.14	0.50	64.0	7.20	66.2	10.85	68.4	19.67	37.72
2.28	0.50	61.6	5.30	65.45	10.75	68.2	19.65	37.19
2.28	0.50	62.2	7.67	66.3	12.05	68.55	19.68	39.40
4.55	0.50	62.85	7.28	66.35	11.51	68.75	19.49	38.28
		mean 62.8	7.21	66.2	11.45	68.5	19.61	38.56[a]
		SE ±0.5	±0.69	±0.3	±0.37	±0.2	±0.05	±0.71
			Varying Calcium Concentration					
2.28	0.12	61.9	7.74	65.65	11.93	67.8	19.01	38.68
2.28	0.50	61.6	5.30	65.45	10.75	68.2	19.65	37.19
0.57–4.55	0.50	63.0	7.39	66.3	11.42	68.55	19.45	38.26
2.27	0.60	62.8	6.42	66.1	11.34	68.5	19.87	37.63
2.27	1.19	64.2	6.84	67.7	11.54	70.1	20.00	38.38
2.27	2.38	65.6	7.14	68.9	11.43	71.2	20.57	39.14
			mean 6.81		11.40		19.76	38.21[b]
			SE ±0.43		±0.19		±0.26	±0.35

[a] Mean value of Δh_{obsd} = 38.02 J g^{-1}. [b] Mean value of Δh_{obsd} = 37.89 J g^{-1}.

Denaturation of TAA at pH 7.0 in the Presence of Added Calcium Ion. As seen in the upper part of Table IV, in the presence of a constant concentration of added Ca^{2+}, t_m for the thermal unfolding of TAA is independent of protein concentration. This interesting observation is precisely what is to be expected on the basis of the model of eq 2; dissociation of the specifically bound Ca^{2+} from the enzyme at a maximum concentration of 86 μM does not significantly alter the concentration of free Ca^{2+} present, and thus the tendency toward reversal of the dissociation remains constant.

The lower part of Table IV shows that, as expected at constant protein concentration, t_m increases with increasing Ca^{2+} concentration. It is important to note that the continuing increase in t_m, which would continue up to high concentrations of Ca^{2+} as long as no complications such as nonspecific binding or effective change in the medium entered the picture, has nothing to do with "stabilizing" the protein and is simply a result of Le Chatelier's principle. Certainly the first equimolar addition of Ca^{2+} to the apoenzyme has a dramatic effect in stabilizing the molecule and in rendering it enzymically active, but further additions have no such effects.

The DSC curves for the runs included in Table IV have been analyzed according to the model of eq 1, and the resulting best-fit parameters are listed in Table V. As in the case of the parameters listed in Table II, here we ignore the variation of $t_{1/2,1}$ and $t_{1/2,2}$ with Ca^{2+} concentration in the lower part of Table V and focus on the variation of $t_{1/2,3}$. These data give a moderately good van't Hoff plot yielding an apparent value for ΔH_{vH} of 680 kJ mol^{-1} with a coefficient of determination of 0.82. ΔH_{cal} obtained from the mean value of Δh_3 is 1047 kJ mol^{-1}. These two enthalpy values are brought into agreement if ΔH_{vH} is evaluated by multiplying the slope of the van't Hoff plot by $-1.5R$ instead of R, suggesting that on average 1.5 Ca^{2+} ions are dissociated on denaturation, which seems reasonable in view of reports of as many as nine additional Ca^{2+} ions being bound per molecule in the presence of excess Ca^{2+} (Oikawa & Maeda, 1957). The increase in the values for ΔH_i with increasing $t_{1/2,i}$ can be largely rationalized in terms of a constant value for ΔC_d as were those in Table II, although the values for Δh_2 and Δh_3 in Table V are larger than would be expected on the basis of the value for ΔC_d found in Table II, presumably because of the binding of more than one Ca^{2+} per molecule.

The most important results emerging from the present work are that the protein, so far as its thermal unfolding is concerned, appears to be composed of three more or less independent domains; two of these domains are probably located in the A domain recognized by X-ray crystallography, and the third one is the C-terminal domain. The specifically bound Ca^{2+} ion is dissociated during the unfolding of the highest melting of these domains. The domains N_1 and N_2 (eq 2) are energetically similar. The overall specific enthalpy at pH 7 and 62 °C, 42.6 J g^{-1}, and the permanent change in apparent specific heat, 0.68 J K^{-1} g^{-1}, are both unusually large for a globular protein (Privalor & Khechinashvili, 1974).

References

Akabori, S., Ikenaka, T., & Hagihara, B. (1954) *J. Biochem. (Tokyo) 41*, 577–582.

Anfinsen, C. B., & Haber, E. (1961) *J. Biol. Chem. 236*, 1361–1363.

Edge, V., Allewell, N. M., & Sturtevant, J. M. (1985) *Biochemistry 24*, 5899–5906.

Fujita-Ikeda, S., & Isemura, T. (1960) *J. Biochem. (Tokyo) 47*, 537–547.

Fukada, H., Sturtevant, J. M., & Quiocho, F. A. (1983) *J. Biol. Chem. 258*, 13193–13198.

Grenthe, I., Ots, H., & Ginstrup, O. (1970) *Acta Chem. Scand. 24*, 1067–1080.

Isemura, T., Takagi, T., Maeda, Y., & Imai, K. (1961) *Biochem. Biophys. Res. Commun. 5*, 373–377.

Isemura, T., Takagi, T., Maeda, Y., & Yutani, K. (1963) *J. Biochem. (Tokyo) 53*, 155–161.

Manly, S. P., Matthews, K. S., & Sturtevant, J. M. (1985) *Biochemistry 24*, 3842–3846.

Matsuura, Y., Kusunoki, M., Date, W., Harada, W., Bando, S., Tanaka, N., & Kakudo, M. (1979) *J. Biochem. (Tokyo) 86*, 1773–1783.

Matsuura, Y., Kusunoki, M., Harada, W., Tanaka, N., Iga, Y., Yasuoka, N., Toda, H., Narita, K., & Kakudo, M. (1980) *J. Biochem. (Tokyo) 87*, 1555–1558.

Matsuura, Y., Kusunoki, M., Harada, W., & Kakudo, M. (1984) *J. Biochem. (Tokyo) 95*, 697–702.

Oikawa, A. (1957) *J. Biochem. (Tokyo) 44*, 623–630.

Oikawa, A., & Maeda, A. (1957) *J. Biochem. (Tokyo) 44*, 745–752.

Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665–684.

Privalov, P. L., Plotnikov, V. V., & Filimonov, V. V. (1975) *J. Chem. Thermodyn. 7*, 41–47.

Takagi, T., & Isemura, T. (1962) *J. Biochem. (Tokyo) 52*, 314-323.
Takagi, T., & Toda, H. (1962) *J. Biochem. (Tokyo) 52*, 16-27.
Takagi, T., & Isemura, T. (1965) *J. Biochem. (Tokyo) 57*, 89-95.
Takahashi, K., & Sturtevant, J. M. (1981) *Biochemistry 20*, 6185-6190.
Toda, H., & Akabori, S. (1963) *J. Biochem. (Tokyo) 53*, 102-110.
Toda, H., Kondo, K., & Narita, K. (1982) *Proc. Jpn. Acad., Ser. B, 58B*, 208-212.
Vallee, B. L., Stein, E. A., Summerwell, W. N., & Fischer, E. H. (1959) *J. Biol. Chem. 234*, 2901-2905.
Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180-1186.

Structure, Dynamics, and Thermodynamics of Mismatched DNA Oligonucleotide Duplexes $d(CCCAGGG)_2$ and $d(CCCTGGG)_2$†

Frances H. Arnold,‡ Steven Wolk, Phillip Cruz,§ and Ignacio Tinoco, Jr.*

Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, Berkeley, California 94720

Received October 23, 1986; Revised Manuscript Received February 20, 1987

ABSTRACT: The structures and hydrogen exchange properties of the mismatched DNA oligonucleotide duplexes $d(CCCAGGG)_2$ and $d(CCCTGGG)_2$ have been studied by high-resolution nuclear magnetic resonance. Both the adenine–adenine and thymine–thymine mismatches are intercalated in the duplexes. The structures of these self-complementary duplexes are symmetric, with the two strands in equivalent positions. The evidence indicates that these mismatches are not stably hydrogen bonded. The mismatched bases in both duplexes are in the anti conformation. The mismatched thymine nucleotide in $d(CCCTGGG)_2$ is intercalated in the duplex with very little distortion of the bases or sugar–phosphate backbone. In contrast, the bases of the adenine–adenine mismatch in $d(CCCAGGG)_2$ must tilt and push apart to reduce the overlap of the amino groups. The thermodynamic data show that the T·T mismatch is less destabilizing than the A·A mismatch when flanked by C·G base pairs in this sequence, in contrast to their approximately equal stabilities when flanked by A·T base pairs in the sequence d(CAAAXAAAG·CTTTYTTTG) where X and Y = A, C, G, and T [Aboul-ela, F., Koh, D., & Tinoco, I., Jr. (1985) *Nucleic Acids Res. 13*, 4811]. Although the mechanism cannot be determined conclusively from the limited data obtained, exchange of the imino protons with solvent in these destabilized heteroduplexes appears to occur by a cooperative mechanism in which half the helix dissociates.

When an incorrect base is incorporated during DNA synthesis, in the great majority of cases it will be recognized and excised. Failure to repair a misincorporated base will result in a mutation when the newly synthesized strand is replicated. Thus, DNA that contains non-Watson–Crick base pairs is an intermediate in mutagenesis.

The probability that a mismatched base will be recognized and repaired does not correlate with the thermodynamic stability of the mismatch (Tinoco et al., 1987). The proofreading or repair enzymes are apparently capable of recognizing some aspect of the mismatch structure or motions. Several oligonucleotides containing non-Watson–Crick base pairs and extra nucleotides have been studied by NMR in order to determine the approximate orientation of the bases and the hydrogen exchange behavior in the destabilized molecules. By nuclear Overhauser effect (NOE) experiments, it was found that the extra cytosine in $d(CA_3CA_3G{\cdot}CT_6G)$ is outside the helix (Morden et al., 1983), whereas the extra A in self-complementary d(CGCAGAATTCGCG) appears to be incorporated inside the duplex structure (Patel et al., 1982). Patel and co-workers (Patel et al., 1984a) have studied the mismatches G·T, G·A, C·T, and A·C in the duplexes $d(CGTGAATTCGCG)_2$, $d(CGAGAATTCGCG)_2$, $d(CGCGAATTCTCG)_2$, and $d(CGCGAATTCACG)_2$. In all cases, the mismatches were incorporated inside the helix. Imino hydrogens at and adjacent to the mismatch site were found to exchange with solvent by a high activation energy mechanism in which more than one base pair became accessible to the solvent at one time.

The structures of thymine–thymine and adenine–adenine mismatches have not been determined, although there has been some evidence that T·T is also incorporated in the helix. Haasnoot and co-workers (Cornelis et al., 1979) studied the self-complementary duplex $d(ATCCTATTAGGAT)_2$, in which a T·T mismatch occurs at base pair 7. They compared the chemical shift of the mismatched thymine imino proton at 10.4 ppm to that of d(TTTT) and thymidine 5′-(*O*-methylphosphate) at 11.2 ppm, from which they inferred that the mismatched T was shielded in the duplex and therefore intercalated in the helix. They also observed that the imino protons near the T·T mismatch melted at lower temperatures

†This work was supported by National Institutes of Health Grant GM 10840 and by the U.S. Department of Energy Office of Energy Research, under Contract AT03-82-ER60090.
‡Present address: Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125.
§Present address: Department of Biological Chemistry, Wright State University, Dayton, OH 45435.

0006-2960/87/0426-4068$01.50/0