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Thermal Denaturation of Staphylococcal Nuclease†

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ABSTRACT: The fully reversible thermal denaturation of staphylococcal nuclease in the absence and presence of Ca^{2+} and/or thymidine 3′,5′-diphosphate (pdTp) from pH 4 to 8 has been studied by high-sensitivity differential scanning calorimetry. In the absence of ligands, the denaturation is accompanied by an enthalpy change of 4.25 cal g^{-1} and an increase in specific heat of 0.134 cal K^{-1} g^{-1}, both of which are usual values for small globular proteins. The temperature (t_m) of maximal excess specific heat is 53.4 °C. Each of the ligands, Ca^{2+} and pdTp, by itself has important effects on the unfolding of the protein which are enhanced when both ligands are present. Addition of saturating concentrations of these ligands raises the denaturational enthalpy to 5.74 cal g^{-1} in the case of Ca^{2+} and to 6.72 cal g^{-1} in the case of pdTp. The ligands raise the t_m by as much as 11 °C depending on ligand concentration. From the variation of the denaturational enthalpies with ligand concentrations, binding constants at 53 °C equal to 950 M^{-1} and 1.4×10^4 M^{-1} are estimated for Ca^{2+} and pdTp, respectively, and from the enthalpies at ligand saturation, binding enthalpies at 53 °C of −15.0 and −19.3 kcal mol^{-1}.

Staphylococcal nuclease A (deoxyribonucleate 3′-nucleotidohydrolase, EC 3.1.4.4) is a well-characterized globular protein containing 149 amino acid residues in a single peptide chain of molecular weight 16 807. It contains no sulfhydryl groups or disulfide bonds. Because of its relatively simple structure, ease of isolation and purification, and good thermal stability, it has been much studied (Anfinsen et al., 1971; Tucker et al., 1978, 1979a,b,c) with the aim of understanding the interrelations between the amino acid sequence and three-dimensional structure of the enzyme and its biological and physical properties. In this paper, we report the results of a high-sensitivity differential scanning calorimetric (DSC) study of the reversible thermal unfolding of staphylococcal nuclease (Nase). This study was undertaken primarily as the first step in a program of investigations of the effects of single amino acid replacements on the thermodynamics of unfolding of the protein.

The enzymic activity and many of the other properties of Nase are strongly affected by Ca^{2+}. Included in these properties is the binding of the strongest known inhibitor of the enzyme, thymidine 3′,5′-diphosphate (pdTp). We have therefore included in our study the thermal unfolding of Nase in the presence of either or both of these ligands.

Materials and Methods

All chemicals were of analytical reagent grade. pdTp (tetralithium salt, dihydrate) was obtained from Calbiochem-Boehring Corp. as A grade. Nase was isolated from homogenates of *Escherichia coli* cells that had been transformed with a recombinant plasmid containing the gene for the enzyme and inducers for production of the enzyme. This plasmid, obtained from Dr. Robert Fox of Yale University, was constructed by insertion of a *Sau*3A restriction fragment containing the structural gene for nuclease A in the unique *Bam*HI restriction site in the expression vector pAS1 (Rosenberg et al., 1983). This construction results in the synthesis of a modified nuclease in which the heptapeptide Met-Asp-Pro-Thr-Val-Tyr-Ser is appended to the amino-terminal alanine of nuclease A. By analogy with staphylococcal nuclease B (Davis et al., 1977), it is assumed that these extra residues

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have little or no effect on the properties of the protein. The nuclease gene is under the transcriptional control of the bacteriophage λ P_L promoter, which requires that the plasmid be propagated in an *E. coli* host strain whose chromosome contains the gene for a temperature-sensitive repressor; induction of the gene is performed by raising the temperature of medium containing exponentially growing cells from 30 to 42 °C. Under these conditions, approximately 40% of the total cellular protein of the *E. coli* host accumulates as Nase. The bacterial cells were suspended in two volumes of 0.05 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), pH 7.0, and lysed by passage through a French pressure cell. Following centrifugation at 30000*g*, the pellet containing unbroken cells and debris was discarded, and the supernatant was applied to a 2.2 × 20 cm column of the cation-exchange resin Bio-Rex 70 (Bio-Rad, dry mesh 100–200). Elution was carried out with a 1.2-L linear gradient of NaCl from 0 to 0.6 M in 0.05 M Pipes buffer, pH 7.0. The effluent was collected in fractions of 10 mL, the course of the elution being followed by optical absorption at 280 nm and enzymic activity. A considerable fraction of the contaminating protein was eluted in the first 400 mL. The fractions with maximal specific activity were loaded on a second column for affinity chromatography. The competitive inhibitor 3′-[(4-aminophenyl)phosphoryl]deoxythymidine 5′-phosphate was coupled to Sepharose 4B by the cyanogen bromide method (Cuatrecasas et al., 1968). The matrix (2.2 × 20 cm) was equilibrated with 0.1 M ammonium acetate and 5 mM $CaCl_2$, pH 8.8, and the elution was started with the same mixture. The effluent containing the remaining contaminating proteins and without affinity for the inhibitor was discarded. The nuclease was then eluted with 0.5 M guanidinium chloride in fractions of 7 mL. The fractions with maximal specific activity were pooled and dialyzed against deionized water for 24–48 h with at least two changes of water. These fractions showed a single band in sodium dodecyl sulfate (SDS) electrophoresis on slab gels. The dialyzed protein was lyophilized and stored at −10 °C. Typically, 200 mg of Nase could be isolated from 10 g of bacterial cells. Before use, the dry protein was dissolved in 0.05 M Pipes, pH 7.0, and dialyzed against the same buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) in order to remove traces of bivalent cations and then against the same buffer without EDTA. Protein concentrations were determined spectrophotometrically by using $E^{1\%}_{280nm} = 9.3$. When denaturation was studied at other values of the pH, the enzyme was dissolved in an appropriate 0.05 M buffer [acetate or citrate at low pH, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) or Pipes at higher pH] at the desired pH and containing 0.1 M NaCl.

The enzyme activity was determined by the method of Cuatrecasas et al. (1967) which is based on the hypochromicity caused by the hydrolysis of singled-stranded DNA.

DSC measurements were performed with a Model DASM-1M scanning microcalorimeter (Privalov et al., 1975) at a scan rate of 1 K min^{-1} with protein concentrations of 1.0-1.6 mg mL^{-1}. It was ascertained that the DSC results were unchanged at a scan rate of 0.5 K min^{-1}. The signal to noise ratio is illustrated by the curves of excess apparent specific heat vs. temperature given in Figure 1. The denaturation was observed to be 90–100% reversible as judged by the DSC curves observed on rescanning samples which were cooled in the calorimeter after a first scan. The thermodynamic parameters were obtained from the DSC curves according to the procedures outlined by Privalov & Khechinashvili (1974), with base lines drawn as described by Takahashi & Sturtevant (1981).

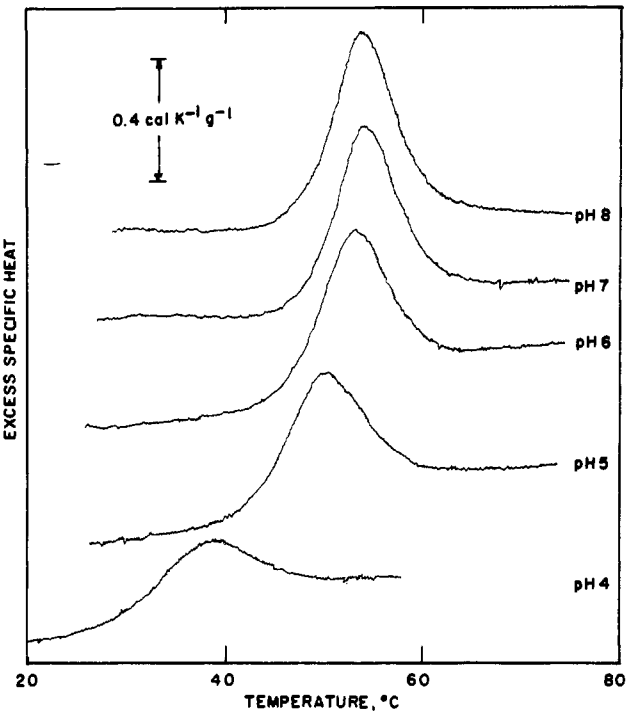


FIGURE 1: Tracings of DSC curves for staphylococcal nuclease run at the indicate values of pH (cf. Table V). The tracings illustrate the noise level generally observed in this work. Protein concentration, 1.05 mg mL^{-1}; scan rate, 1.0 K min^{-1}.

Table I: Thermodynamics of the Thermal Unfolding of Staphylococcal Nuclease in 0.05 M Pipes Buffer at pH 7.0

t_m (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$ ratio
After Dialysis against EDTA				
53.40 ± 0.10	71.5 ± 0.7	2.25 ± 0.09	103.6 ± 1.5	1.45 ± 0.04
Before Dialysis against EDTA				
53.05 ± 0.25	83.5 ± 1.3	1.74 ± 0.09	110.5 ± 1.2	1.32 ± 0.02

Results and Discussion

Thermal Unfolding of Nase in the Absence of Ligands. The DSC data obtained at pH 7.0 with Nase are summarized in Table I. Since none of the observed quantities varied significantly with protein concentration over the range 0.65–3.62 mg mL^{-1}, only mean values, with standard errors of the mean, are listed in Table I. A total of 13 experiments with protein which had been dialyzed against EDTA and 11 experiments with undialyzed protein were run. The temperatures of maximal excess specific heat (t_m), which did not differ significantly from the temperatures of half-completion (t_d), are listed in column 1 of Table I. The calorimetric enthalpies obtained by planimeter integration of the DSC curves are given in column 2 and the permanent changes in heat capacity due to denaturation in column 3. The values for the van't Hoff enthalpy listed in column 4 were calculated according to

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

where $T_m = t_m + 273.15$ and C_{ex}^{max} is the maximal value of the excess apparent heat capacity.

The mean value of ΔH_{cal} for enzyme dialyzed against EDTA corresponds to 4.25 cal g^{-1}, a value not unusual for a globular protein. In contrast, the value for bovine pancreatic ribonuclease A (RNase A) at pH 7 is 12.3 cal g^{-1} (Tsong et al., 1970). The large value for ΔC_p^d, which corresponds to 0.134 cal K^{-1} g^{-1} (0.150 cal K^{-1} g^{-1} for RNase A), is also not unusual

Table II: Thermodynamics of the Thermal Denaturation of Staphylococcal Nuclease in the Presence of either Ca^{2+} or pdTp at pH 7.0

protein concn (mg mL^{-1})	Ca^{2+} concn (mM)	pdTp concn (mM)	t_m (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$ ratio
1.22	0	0	53.50	72.8	2.28	115.7	1.58
1.22	1.88	0	54.75	79.6	1.77	112.7	1.41
1.22	3.75	0	55.50	81.9	1.99	110.1	1.34
1.22	7.50	0	56.50	86.5	2.10	112.0	1.29
1.22	11.25	0	57.25	90.1	1.99	114.7	1.27
1.22	15.00	0	57.75	94.2	1.99	118.0	1.25
1.22	20.00	0	58.00	92.8	1.71	114.6	1.23
1.27	40.00	0	58.00	92.2	2.30	111.2	1.20
1.27	80.00	0	58.50	94.9	2.30	111.0	1.17
1.27	160.0	0	57.50	97.0	1.92	111.5	1.15
1.27	320.0	0	56.00	97.9	1.92	108.3	1.11
					mean 2.02	112.7	
					SE ±0.07	±0.8	
1.65	0	0	53.00	73.2	2.32	110.8	1.51
1.65	0	0.10	53.75	77.3	1.99	109.6	1.41
1.65	0	0.20	54.50	80.5	1.79	108.9	1.35
1.65	0	0.30	54.75	85.5	2.21	117.3	1.37
1.65	0	0.40	55.25	82.8	2.11	110.7	1.33
1.65	0	0.60	55.75	88.7	1.95	106.0	1.19
1.5	0	1.10	56.25	91.9	2.13	120.2	1.30
1.65	0	2.20	58.00	100.7	2.11	107.6	1.06
1.5	0	3.30	57.75	101.4	2.17	119.8	1.18
1.5	0	4.40	58.50	106.1	2.01	118.9	1.12
1.27	0	6.00	59.00	107.5	2.33	116.4	1.08
1.27	0	12.0	60.75	108.9	1.92	118.1	1.09
1.27	0	24.0	62.00	111.5	1.78	126.7	1.14
1.46	0	50.00	63.00	113.3	1.67	115.1	1.02
1.46	0	60.0	63.00	113.3	2.14	124.6	1.10
1.46	0	80.0	64.00	112.4	1.43	118.5	1.05
					mean 2.00	115.6	
					SE ±0.06	±1.6	

for globular proteins and is presumably largely attributable to increased exposure to the solvent of hydrophobic groups resulting from the unfolding (Sturtevant, 1977). In the case of a reversible process such as the unfolding of Nase, the only cause for a value for the ratio $\Delta H_{vH}/\Delta H_{cal}$ to be greater than unity is intermolecular cooperation, an extreme example of this being a phase transition. We therfore conclude on the basis of the value of 1.45 for this ratio (Table I) that Nase is partially dimerized under the experimental conditions employed (Taniuchi & Bohnert, 1975). The fact that t_m is independent of protein concentration shows that the extent of dimerization of the unfolded protein is the same as that of the native protein (Takahashi & Sturtevant, 1981). It thus seems likely that the denaturational enthalpy is not significantly affected by the dimerization.

The data in Table I show that the major effects of EDTA dialysis were to lower the value of ΔH_{cal} by 15% and to raise that of ΔC_p^d by 30%. Statistically significant changes were also produced in ΔH_{vH} and the ratio $\Delta H_{vH}/\Delta H_{cal}$. It is interesting that the t_m of the unfolding process was very little affected by EDTA dialysis. It will be shown below that addition of Ca^{2+} to both dialyzed and nondialyzed Nase produces significant increases in both t_m and ΔH_{cal} but has little effect on ΔC_p^d or ΔH_{vH}. We may therefore conclude that the effects of EDTA dialysis are not due to removal of Ca^{2+} from the enzyme. Presumably, other metal ions are present in the enzyme as originally isolated. Unless otherwise indicated, all the data reported below were obtained with dialyzed enzyme.

Thermal Unfolding of Nase in the Presence of either Ca^{2+} or pdTp. The DSC data obtained for the denaturation of Nase with added Ca^{2+} in the absence of pdTp and of added nucleotide in the absence of Ca^{2+} are listed in Table II. There has been some question as to whether these ligands bind when the other ligand is absent (Cuatrecasas et al., 1967a,b). It is evident that for both Ca^{2+} and pdTp, t_m and ΔH_{cal} increase markedly with increasing ligand concentration, which clearly shows that both these ligands do in fact bind and remain bound until the temperature becomes so high that denaturation takes place with accompanying dissociation of the ligand. NMR evidence for the binding of Ca^{2+} alone and of pdTp alone has been published (Markley et al., 1968, 1970; Markley & Jardetzky, 1970; Meadows et al., 1967). The plots in Figure 2 show that the protein becomes saturated with Ca^{2+} at approximately 100 mM and with pdTp at approximately 20 mM at denaturation temperatures. In contrast, ΔH_{vH} and ΔC_p^d

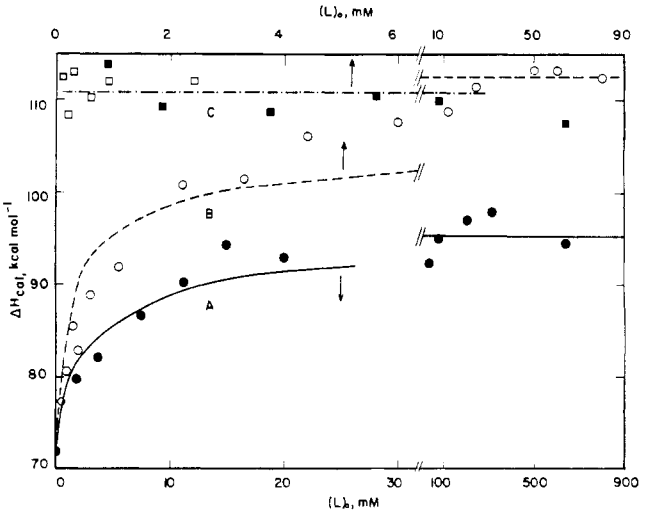


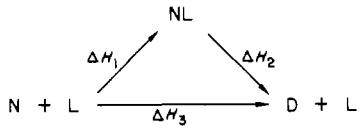
FIGURE 2: Variation of the enthalpy of denaturation of staphylococcal nuclease with ligand concentration. (○) pdTp in the absence of Ca^{2+}; (●) Ca^{2+} in the absence of pdTp; (□) pdTp in the presence of 20 mM Ca^{2+}; (■) Ca^{2+} in the presence of 0.30 mM pdTp. Curve A, calculated for Ca^{2+} binding with K_b(53 °C) = 950 M^{-1}; curve B, calculated for pdTp binding with K_b(53 °C) = 1.4 × 10^4 M^{-1}. Protein concentration, 1.22–1.65 mg mL^{-1}, pH 7.0; scan rate, 1.0 K min^{-1}.

Table III: Thermodynamic Parameters for the Binding of Ligands to Staphylococcal Nuclease at 53 °C

ligand	K_b (M^{-1})	$\Delta G°$ (kcal mol^{-1})	ΔH (kcal mol^{-1})	$\Delta S°$ (cal K^{-1} mol^{-1})
Ca^{2+}	950	−18.6 ± 1.0	−15.0 ± 1.0	11 ± 3
pdTp	1.4×10^4	−25.9 ± 1.0	−19.3 ± 1.0	20 ± 4

appear to be unchanged by the addition of ligands.

If we consider the isothermal scheme



where N and D represent native and denatured protein, respectively, it is evident that the enthalpy of denaturation of the protein in the presence of a saturating concentration of ligand L minus that in the absence of L, $\Delta H_2 - \Delta H_3$, is equal to the enthalpy of dissociation of ligand from the native protein, $\Delta H_d = -\Delta H_1$. Taking 96.5 kcal mol^{-1} as the value at 57.3 °C for ΔH_2, which when corrected to 53 °C by using $\Delta C_p^d = 2.02$ kcal K^{-1} mol^{-1} is 87.8 kcal mol^{-1}, and 72.8 kcal mol^{-1} for ΔH_3, we obtain $\Delta H_d = 15.0$ kcal mol^{-1} at 53 °C. Similarly for pdTp, the heat of dissociation at 53 °C is calculated to be 19.3 kcal mol^{-1}.

The variation of enthalpy with ligand concentration for both ligands can be reasonably well fitted to theoretical titration curves as illustrated by the solid and dashed curves in Figure 2. In calculating these fits to minimize the respective standard deviations, it is, of course, necessary to include the variation of both the equilibrium constant and the enthalpy with temperature. The thermodynamic parameters for the binding of Ca^{2+} and of pdTp obtained by the calculations outlined in these last two paragraphs are listed in Table III. In each case, both the enthalpy and entropy changes are favorable to binding.

Dunn & Chaiken (1975) reported a value for K_b for pdTp in the absence of Ca^{2+} at pH 7.5 and 25 °C of 2.6×10^4 M^{-1}. If the value for ΔH_b is taken to be temperature independent, K_b is calculated to be 1.6×10^3 M^{-1} at 53 °C. The reason for this discrepancy, which cannot be removed by any reasonable value for ΔC_b, the heat capacity change on binding the nucleotide, is unknown.

If we are correct in interpreting the curves in Figure 2 in terms of binding equilibria, then the data in Table II for the variation of t_m with ligand concentration should be interpretable in terms of the van't Hoff equation (Fukada et al., 1983). van't Hoff plots of these data are given in Figure 3. The slopes of lines A and B give values for ΔH_{vH} of $147n$ kcal mol^{-1} and $138n$ kcal mol^{-1}, respectively, where n is the number of ligand molecules bound per protein molecule. Comparison of these quantities with those in column 7 of Table II indicates that $n = 1$. The reason for the van't Hoff plots leading to enthalpies which are 20–30% larger than ΔH_{vH} deduced directly from the DSC curves is unknown.

It may be noted that we have here further examples [cf. Fukada et al. (1983)] of increases in t_m with increasing ligand concentrations resulting from simple displacements of binding equilibria in accordance with Le Chatelier's principle.

Thermal Unfolding of Nase in the Presence of both Ca^{2+} and pdTp. The DSC data obtained in the presence of both ligands are given in Table IV. Here it is seen that as long as both ligands are present in excess, ΔH_{cal}, ΔH_{vH}, and ΔC_p^d are independent of ligand concentrations, with only t_m remaining variable. It is thus evident that each ligand greatly increases the tightness of binding of the other ligand, as has been previously observed at lower temperatures (Markley & Jardetzky, 1970). This point is further illustrated by line C in Figure 2.

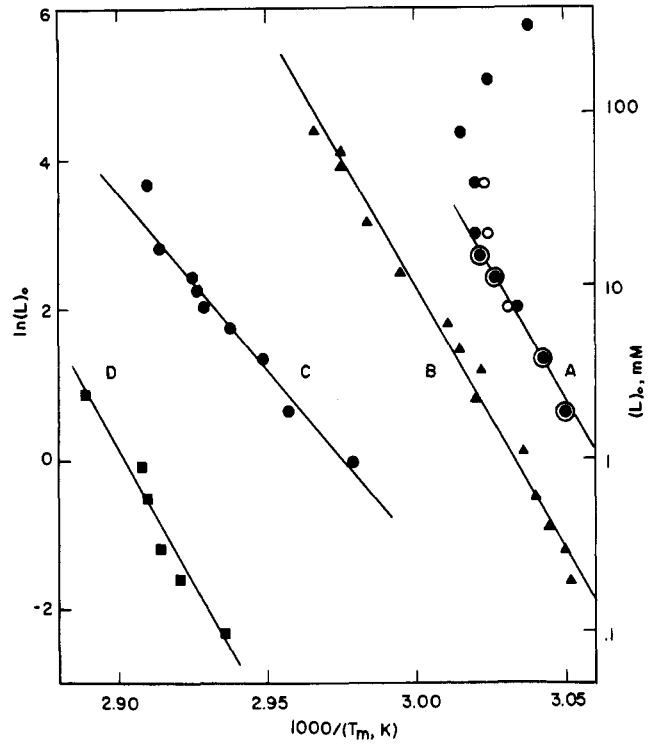


FIGURE 3: Effects of ligand concentration on the temperature of maximal excess specific heat presented in the form of van't Hoff plots. (A) Ca^{2+} binding in the absence of pdTp (two series of experiments); (B) pdTp binding in the absence of Ca^{2+}; (C) Ca^{2+} binding in the presence of 0.30 mM pdTp; (D) pdTp binding in the presence of 20 mM Ca^{2+}. Protein concentration, 1.22–1.65 mg mL^{-1}, pH 7.0; scan rate, 1 K min^{-1}.

The variation of t_m with ligand concentration is presented as van't Hoff plots C and D in Figure 3. In the case of pdTp, the slope is similar to that of lines A and B, indicating that one molecule of nucleotide is bound per molecule of Nase. On the other hand, the data for Ca^{2+} have a lower slope which indicates the binding of approximately 1.5 atoms per molecule of protein. Cuatrecasas et al. (1967a) and Markley & Jardetzky (1970) concluded that in the presence of pdTp there are two binding sites for Ca^{2+}, one strong and one weak. Our data suggest either that the weaker binding site is not saturated at the denaturational temperature or that some Ca^{2+} remains bound to the denatured protein in the presence of pdTp.

It is interesting that the ratio $\Delta H_{vH}/\Delta H_{cal}$ is very close to unity when both ligands are present. We may thus infer that the protein is not oligomerized under these conditions and that no intermediate states of enthalpy significantly different from that of native or denatured protein are present to a detectable degree at equilibrium. Kinetic experiments have been reported [see, for example, Schechter et al. (1970)] which indicate important intermediates in both the unfolding and refolding of Nase. These intermediates must be present at low concentrations under equilibrium conditions.

Effect of pH on the Thermal Unfolding of Nase. The effects of pH on the denaturational properties of Nase in the absence and presence of ligands are illustrated in Table V and Figure 4. At low pH, t_m, ΔH_{cal}, and ΔH_{vH} are much lower than at neutral pH, while ΔC_p^d and the ratio $\Delta H_{vH}/\Delta H_{cal}$ remain essentially unchanged.[1] This is true in both the ab-

[1] Only minor fractions of the changes in ΔH_{cal} are due to buffer heats of ionization.

Table IV: Thermodynamics of the Thermal Denaturation of Staphylococcal Nuclease in the Presence of both Ca^{2+} and pdTp at pH 7.0

protein concn (mg mL^{-1})	Ca^{2+} concn (mM)	pdTp concn (mM)	t_m (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$ ratio
2.50	20	0	57.75	(90.5)	1.88	105.3	(1.16)
1.23	20	0.10	67.50	112.4	2.26	92.8	0.83
1.23	20	0.20	69.25	108.3	1.70	102.3	0.94
1.23	20	0.30	70.00	112.9	1.70	105.4	0.93
1.23	20	0.60	70.50	110.1	2.54	118.0	1.07
1.23	20	0.90	71.75	111.9	2.26	121.8	1.09
1.23	20	2.40	73.00	111.5	1.98	123.3	1.11
				mean 111.2	2.05	109.8	1.00
				SE ±0.14	±0.14	±5.1	±0.06
1.65	0	0.30	54.75	(85.5)	2.21	117.3	(1.37)
1.56	0.94	0.30	62.50	113.9	1.78	102.5	0.90
1.56	1.88	0.30	65.00	109.2	2.00	111.3	1.01
1.56	3.75	0.30	66.00	108.6	2.23	123.3	1.14
1.56	5.63	0.30	67.75	110.3	2.00	120.4	1.09
1.40	7.50	0.30	68.25	109.8	1.56	116.5	1.06
1.40	9.38	0.30	68.50	110.2	1.53	122.6	1.11
1.40	11.25	0.30	68.75	109.1	2.23	122.1	1.12
1.23	20.00	0.30	70.00	110.6	2.26	113.8	1.03
				mean 110.2	1.98	116.6	1.06
				SE ±0.7	±0.11	±2.5	±0.03

Table V: Effect of pH on the Thermal Unfolding of Staphylococcal Nuclease in 0.05 M Buffer and 0.1 M NaCl

protein concn (mg mL^{-1})	buffer	pH	t_m (°C)	ΔH_{cal}[a] (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$ ratio
				No Ligand			
1.05	acetate	4.00	38.75	29.5	(0)	62.8	(2.20)
1.05	acetate	5.00	50.00	52.8	2.65	78.8	1.50
1.05	Pipes	6.00	53.00	67.9	2.32	93.7	1.36
1.05	Pipes	7.00	54.00	74.0	2.32	95.1	1.29
1.05	Hepes	8.00	54.50	78.8	2.15	96.8	1.23
					mean 2.36		1.34
					SE ±0.15		±0.08
				20 mM Ca^{2+}			
2.50	acetate	3.60	28.25	31.2	2.03	52.6	(1.80)
2.50	citrate	4.65	47.00	63.1	1.53	84.2	1.35
2.50	acetate	4.70	46.00	63.2	1.74	75.2	1.20
2.50	citrate	5.50	52.25	67.8	2.02	90.5	1.34
2.50	Pipes	7.00	57.75	89.7	1.88	105.3	1.16
2.50	Pipes	7.50	57.50	91.5	2.08	107.0	1.16
2.50	Hepes	7.50	56.75	92.5	1.88	112.4	1.21
					mean 1.88		1.24
					SE ±0.09		±0.04
				20 mM Ca^{2+} + 2 mM pdTp			
2.80	acetate	4.10	34.75	37.2	1.40	64.2	(1.73)
2.80	acetate	4.85	51.00	67.3	1.60	87.4	1.30
2.80	Pipes	5.25	54.50	82.9	1.40	81.9	0.99
2.80	Pipes	6.65	70.25	109.2	1.80	128.9	1.18
2.80	Pipes	7.10	71.75	120.3	1.45	133.3	1.11
					mean 1.53		mean 1.15
					SE ±0.10		SE ±0.09

[a] These values have been corrected for the contribution due to buffer deprotonation. See text for details.

sence and presence of ligands. The increase of t_m with increasing pH indicates that protons are taken up by the protein from the buffer during unfolding. van't Hoff plots of pH vs. $1000/T_m$ are given in Figure 4. The slopes of these plots are equal to $\Delta H_{vH}/2.303nR$ (Fukada et al., 1983)[2] where n is the number of protons per protein molecule taken up by the protein during denaturation. Rough estimates of the value of n can be obtained by selecting n to make ΔH_{vH} equal to ΔH_{cal}, with results which are shown in the inset to Figure 4. The fact that the change in protonation is larger at low than at neutral pH suggests that carboxyl groups are involved in the change.

It has been found in the case of several proteins that the change in the enthalpy of denaturation with pH correlates with the change in the temperature of denaturation with pH in terms of the heat capacity due to denaturation, ΔC_p^d, as observed in individual DSC experiments (Privalov & Khechinashvili, 1974). This appears to be approximately the case with Nase. The data in Table V with no Ca^{2+} present give a value of 2.9 kcal K^{-1} mol^{-1} for ΔC_p, those with Ca^{2+} present give 2.1 kcal K^{-1} mol^{-1}, and those with both Ca^{2+} and pdTp present give 2.2 kcal K^{-1} mol^{-1}.

In conclusion, an additional comparison between RNase A and Nase is of interest. Despite the facts that the molecular weight of RNase A is 20% lower than that of Nase and that its peptide chain is seriously restricted in its conformational possibilities by four disulfide bonds, nevertheless the thermal

[2] In the derivation given by Fukada et al. (1983), it was assumed that the total ligand concentration was essentially unaffected by changes in the extent of ligation of the protein present at relatively low concentration. This assumption holds in the present case since the hydrogen ion concentration is buffered.

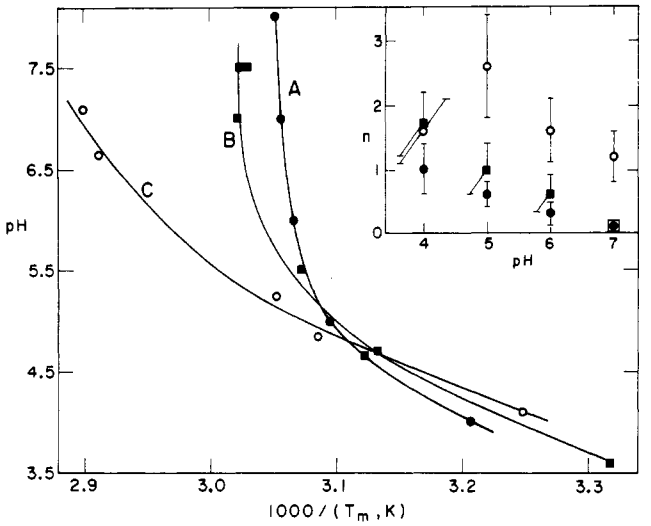


FIGURE 4: Effects of pH on the temperature of maximal excess specific heat. (A) No ligand present; (B) 20 mM Ca^{2+} present; (C) 20 mM Ca^{2+} and 2 mM pdTp present. Inset: Estimates of the number of protons taken up per molecule of staphylococcal nuclease during denaturation, based on the slopes of curves A, B, and C, as detailed in the text. Protein concentration, 1.05–2.80 mg mL^{-1}; scan rate, 1 K min^{-1}.

denaturation of RNase A is readily reversible only at low pH (pH 2–4) while that of Nase is fully reversible at least over the range pH 4–8. It appears that according to this criterion intramolecular cross-links do not necessarily facilitate the refolding of a protein from the unfolded state.

Registry No. pdTp, 2863-04-9; Ca, 7440-70-2; staphylococcal nuclease, 9013-53-0.

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