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BIOLOGICAL THERMODYNAMIC DATA FOR THE CALIBRATION OF DIFFERENTIAL SCANNING CALORIMETERS: HEAT CAPACITY DATA ON THE UNFOLDING TRANSITION OF LYSOZYME IN SOLUTION

FREDERICK P. SCHWARZ

Chemical Thermodynamics Division, National Institute of Standards and Technology, Gaithersburg, MD 20899 (U.S.A.)

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ABSTRACT

Differential scanning calorimetry measurements of the unfolding transition of lysozyme in HCl–glycine buffer solutions were performed over a temperature range from 326 K at pH 2.3 to 349 K at pH 3.9. Van't Hoff transition enthalpies were calculated from the fit of a two-state transition model to the heat capacity measurements (ΔH_{vf}), from the van't Hoff plot of $\ln(1/K)$ vs. $1/T$ where K is the transition equilibrium constant, and from the ratio of the transition peak height to the area under the transition peak. The best linear fit of the van't Hoff enthalpies to the transition temperatures T_m was obtained with ΔH_{vf} and was ΔH_{vf} (kJ mol^{-1}) $= 432.7 \pm 1.7 + 5.81 \pm 0.24$ $(T_m - 337.2)$. Calorimetric transition enthalpies were determined from the transition peak area using an extrapolated sigmoidal baseline (ΔH_s) and an extrapolated straight linear baseline. The best linear dependence of the calorimetric enthalpy on T_m was obtained with ΔH_s (kJ mol^{-1}) $= 434.7 \pm 4.1 + 6.39 \pm 0.60$ $(T_m - 337.2)$. Linear least-squares fits of ΔH_{vf} and ΔH_s to T_m were independent of the DSC scan rate, the source of the lysozyme, the buffer concentration from 0.1 to 0.2 M and the concentration of lysozyme from 0.26 to 2.8 mM. The transition temperature exhibited a linear dependence on pH and concentration. Cooperativities of the transition ranged from 0.988 ± 0.007 at 326 K to 1.012 ± 0.007 at 349 K. The average heat capacity change of the solution accompanying the transition was 5.94 ± 3.10 kJ mol^{-1} K^{-1} over this temperature range.

INTRODUCTION

Recently, heat capacity data on the unfolding transition of ribonuclease a in HCl–glycine-buffered solutions have been reported for use in the calibration of differential scanning calorimeters (DSCs) [1]. Transition enthalpies, cooperativities and solution heat capacity changes were reported over a transition temperature range from 312 K at pH 2 to 335 K at pH 4. van't Hoff enthalpies were determined from a fit of a two-state transition model to the data and from a van't Hoff plot of $\ln K$ vs. $1/T$ where K is the transition equilibrium constant and T is the temperature. Calorimetric

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enthalpies were determined from the area under the transition profile both with a sigmoidal baseline and with a straight baseline. All the transition enthalpies exhibited a linear dependence on temperature, while the transition temperatures exhibited a linear dependence on pH and concentration over this temperature range [1]. The cooperativities were close to unity and exhibited a linear dependence on temperature [1]. The results were independent of the source of ribonuclease a, the differential scanning calorimetry (DSC) scan rate and the buffer concentration from 0.1 to 0.2 M [1]. To extend the temperature range of calibrations to 365 K, the unfolding transition of the more thermally stable enzyme, egg white lysozyme, was similarly investigated.

The unfolding transition of lysozyme, a globular protein consisting of 129 amino acid residues, in buffered aqueous solutions has been investigated over the past two decades by DSC [2–5]. Lysozyme catalyzes the breakdown of cell wall polysaccharides by hydrolysis of the glycosidic bond between the saccharides [6]. Privalov and Khechinashvili [2] have shown that the van't Hoff and calorimetric enthalpies of the unfolding transition of lysozyme at low concentration (0.05 mass%) in 0.05 M HCl–glycine-buffered solutions behaves very similarly to that of ribonuclease a. The transition enthalpies increase linearly with transition temperature and the cooperativity is 1.05 ± 0.03 over the temperature range 320.1–348.2 K [2]. In this study, the transition properties of lysozyme from three different sources were investigated as a function of concentration, pH, DSC scan rate and buffer concentration. Van't Hoff enthalpies similar to those calculated for ribonuclease a were determined and a third value for the van't Hoff enthalpy was calculated from the ratio of the peak height to the area of the transition profile. However, the van't Hoff enthalpies determined from fits of the two-state model to the data were computed without varying the number of moles of enzyme in contrast with the ribonuclease a transitions [1]; instead, the number of moles was held fixed at a known value determined from a spectroscopic analysis of the solution. Heat capacity changes of the solutions and cooperativities were also determined. This data was used to ascertain the suitability of the transition enthalpies of lysozyme in solution for the heat calibration of DSCs.

EXPERIMENTAL

Materials

Three samples of egg white lysozyme were obtained from two different commercial sources and are labeled a, b and c. Sample a was purchased in 1981 and had an activity of 49000 units (mg sample)$^{-1}$. A unit is the amount of enzyme which will cause a decrease of 0.001 min^{-1} in the

absorbancy of a suspension of *Micrococcus lysodeikticus* cells at pH 6.24 and 298.2 K [7]. Sample b had an activity of 49 000 units (mg sample)$^{-1}$ and was purchased in 1988 from the same commercial source as sample a. The third sample, labeled c, had an activity of 25 000 units mg^{-1} and was purchased in 1988 from a different commercial source. The hydrochloric acid, phosphoric acid, glycine, sodium phosphate and potassium hydroxide were of reagent quality.

Preparation and analysis of solutions

Solutions in the concentration range 0.2–3.0 mM were prepared by dissolving lysozyme (0.4 g) in 0.2 M HCl–glycine buffer solution (10 ml) at pH 3.5. The glycine buffer was prepared by dissolving glycine (0.2 M) in distilled water (1 l) and adding concentrated HCl dropwise with stirring until the pH reached 3.5 as monitored by an Orion 811 * pH meter operated with a Corning EX-L glass electrode. The solution was dialyzed using a membrane with a molecular weight cut-off of 3500 against a volume of about 400 ml of the buffer solution which was replaced twice during the course of the dialysis. Lysozyme solutions were stored in the dark in a refrigerator at 277 K for up to two weeks. Storage times greater than one month resulted in degradation of the lysozyme, especially at low pH, as indicated by the appearance of a low temperature shoulder in the transition peak. Prior to the DSC measurement, a 2 ml aliquot of solution at a specific concentration and pH was prepared by diluting the dialyzed solution with the buffer solution at a suitable pH. A portion of this solution was set aside for analysis by UV spectroscopy. The spectroscopic analysis consisted of diluting the sample to a concentration of about 0.07 mM with 0.1 M Na_2HPO_4–NaH_2PO_4 buffer at pH 7.0 and measuring the optical density at 280 nm with a Perkin–Elmer Lambda 4B spectrophotometer. An extinction coefficient of 3.85×10^4 mol l^{-1} cm^{-1} was calculated from an absorptivity of 26.35 dl g^{-1} cm^{-1} at 280 nm for a 1% solution at pH 7.0 [8] and a molar mass of 14.6×10^3 dalton for lysozyme [9]. The density of the 0.1 M sodium-phosphate-buffered solutions was 1.012 ± 0.001 g ml^{-1} and that of the 0.2 M HCl–glycine-buffered solutions was 0.994 ± 0.002 g ml^{-1} at room temperature.

To ascertain the purity of the lysozyme, aliquots of solutions prepared from each of the three sources were analyzed by gel filtration and an

* Certain commercial equipment, instruments and materials are identified in this paper in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material, instrument or equipment identified is necessarily the best available for the purpose.

enzymatic assay. Approximately 1 ml of 2 mM solutions were analyzed at pH 3.5 by gel filtration through a 1.25 cm × 75 cm column of Sephadex G-75-50 equilibrated with the 0.2 M HCl–glycine buffer at pH 3.5. The elution was maintained at a flow rate of 7 ml h^{-1} and the elutant was monitored by a UV absorption detector operated at 270 nm. The dependence of the rate of elution on molecular weight was determined from mixtures of ribonuclease a (13.7×10^3 dalton) and serum albumin (65×10^3 dalton). An enzymatic assay was performed on aliquots of solutions prepared by dissolving milligram quantities of lysozyme from each of the three sources in 0.066 M potassium phosphate buffer at pH 6.24 and dialyzing the solutions as described previously. Following the assay procedure described by Sigma Chemical Company, a 0.1 ml aliquot of the solution was added to 2.5 ml of *Micrococcus lysodeikticus* suspension in a quartz cuvette; these were then mixed by inversion. The suspension was prepared by dissolving 5.0 mg of dried lyophilized *Micrococcus lysodeikticus* cells from Sigma in the potassium phosphate buffer at pH 6.24. After mixing the enzyme solution with the suspension by inversion, the rate of decrease in absorption at 450 nm $d(A_{450\ nm})/dt$ was recorded. The enzymatic activity in terms of units per mole of protein was calculated using the equation

$$\text{Activity per mg of protein} = (d(A_{450\ nm})/dt)/(0.001 \times \text{mg of protein}) \quad (1)$$

where the mass of protein was determined by the spectroscopic analysis.

DSC measurements

DSC measurements were performed with a Hart 7707 differential heat conduction scanning microcalorimeter as described previously [1], but with one modification. The modification made it possible to run samples in three of the removable cells against one reference cell so that three samples could be run against a reference cell containing an equal mass of the buffer solution. The temperature and power calibrations of all three of the cells were performed in the same manner as described in detail previously [1,10]. The differential scanning calorimeter was normally operated at a scan rate of 20 K h^{-1} from 303.2 K to 363.2 K with sample masses of 0.801 g. An excess power vs. temperature scan for the lysozyme transition was obtained by subtracting the power input of a thermal scan of buffer vs. buffer from the power input of a scan of the solution vs. buffer. All the excess power thermal scans were corrected for the thermal lag of the differential scanning calorimeter by the Tian equation and were converted to excess heat capacity vs. temperature scans by dividing by the scan rate as described previously [1].

To determine the transitional baseline, temperature, van't Hoff transition enthalpy and change in the excess heat capacity of the baseline, the excess heat capacity vs. temperature profiles were fitted to the two-state transition

model described by Schwarz and Kirchhoff [1]. Instead of allowing the number of moles of protein to vary during the fitting procedure to determine the van't Hoff transition enthalpy, as was the case with the ribonuclease a results for the determination of ΔH_m in ref. 1, the number of moles of protein was fixed at a value N determined by the spectroscopic analysis. The sigmoidal transitional baseline was determined from extrapolations of the pre- and post-transitional baselines to the mid-point of the transition and the fractional area α under the transition profile at T was determined as described previously [1]. The temperature T_m of the transition was the temperature at 0.5 the area of the transition peak. The change in the excess heat capacity of the baselines ΔC_p was the difference between the pre- and post-transitional baselines extrapolated to T_m.

RESULTS AND DISCUSSION

Purity and activity analysis

Gel filtration analysis of the solutions of lysozyme from sources a, b and c revealed only one elutant at a mass of 15×10^3 dalton in agreement with the mass of 14.6×10^3 dalton for lysozyme. Under the operating conditions of the column, the baseline resolution was 3.6×10^3 dalton. The elutions were continued until two column volumes of the solution were eluted.

Enzymatic analysis showed that the activities measured within a few seconds after mixing were $102\,000 \pm 10\,000$ units (mg protein)$^{-1}$ from source c, $80\,000 \pm 8000$ units (mg protein)$^{-1}$ from source a and $78\,000 \pm 10\,000$ units (mg protein)$^{-1}$ from source b. The amount of enzyme in the solutions was determined by the spectroscopic analysis. Apparently, the enzymatic activity per milligram of enzyme of the dialyzed solutions of lysozyme was the same for the three different sources of lysozyme. On this basis and from the gel filtration results, the spectroscopic analysis of the dialyzed solutions yields the correct amount of lysozyme in the native state.

DSC measurements

A typical thermal scan of lysozyme (2 mM) in 0.2 M HCl–glycine buffer solution is shown in Fig. 1 together with the computer simulated excess heat capacity curve from the two-state model. The unfolding transition consists of a single symmetrical peak followed by an increase in the transitional baseline. An increase in the pH of the solution results in an increase in the transition temperature and the area under the transition profile. Both the pre-transitional and post-transitional baselines increase linearly with temperature which has been observed for the unfolding of other globular proteins such as ribonuclease a [1,2]. Extrapolation of the baselines to the

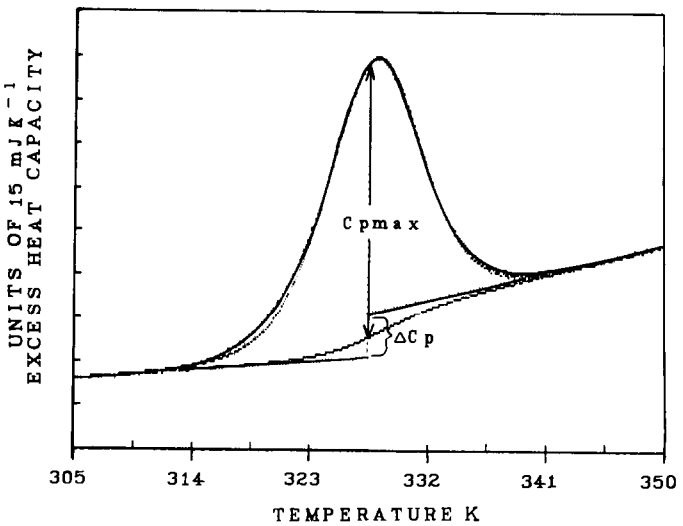

Fig. 1. DSC thermal scan 5 in Table 1 (2.63 mM lysozyme in 0.2 M HCl–glycine buffer at pH 2.4); scan rate = 20 K h^{-1}. Two-state model curve (— — —) and experimental data (· · · · · ·). The sigmoidal baseline was generated by the two-state model.

transition temperature shows a positive increase from the pre-transitional to the post-transitional baseline indicating an increase in the heat capacity ΔC_p of the solution on denaturation. This is also observed for ribonuclease a [1,2] and other globular proteins [2].

In repeated thermal scans of a sample, similar transition profiles are observed with a decrease in the transition enthalpy. The extent of the decrease depends on the pH of the solution and the temperature range and rate of the thermal scan. A repeated scan from 303 to 363 K of a transition occurring at 347 K results in a 20% decrease in the area under the transition profile, whereas a repeated scan of a transition occurring at 327 K results in a 50% decrease in the area. After prolonged heating at 363 K, a shoulder is observed at the low temperature side of the denaturation transition. Apparently there is some thermal degradation of the lysozyme at high temperature similar to that observed for ribonuclease a in solution [1]. Zale and Klibanov [11] have shown that denatured ribonuclease a undergoes irreversible hydrolysis of the peptide bonds at the aspartic acid residues and deamination of asparagine and/or glutamine residues at 363 K and pH 4. Despite a small amount of degradation of the enzyme when it is heated to temperatures just above the denaturation temperature, the unfolding transition of lysozyme can be considered to be essentially reversible. Only the results for the first thermal scan are reported for the samples studied.

The thermodynamic information on the unfolding transition of lysozyme in 0.2 M HCl–glycine buffer determined from DSC scans of 68 samples is summarized in Table 1. The lysozyme was obtained from the following three

sources: company 1 in 1982 (a), company 1 in 1988 (b) and company 2 in 1988 (c). Samples were scanned in three cells (1, 2 and 3) which have different calibration factors and at a scan rate of 20 K h^{-1}. The concentrations were obtained from spectroscopic analysis of the solutions and were used to determine the van't Hoff transition enthalpies from the fit (ΔH_{vf}) and the calorimetric transition enthalpies using the sigmoidal baseline (ΔH_s) and a linear baseline (ΔH_l). The van't Hoff enthalpy from the fit was corrected for the temperature variation of the van't Hoff enthalpy over the temperature range of the transition peak [1]. A value for the van't Hoff enthalpy ΔH_{ve} at the transition temperature was calculated from the equation

$$\Delta H_{ve} = 4.00\ R\ T_m^2 C_p^{max} / \Delta H_s N \tag{2}$$

where $R = 8.31451$ J mol^{-1} K^{-1} and C_p^{max} is the maximum height of the transition peak above the sigmoidal baseline. A van't Hoff enthalpy ΔH_{vs} without the temperature correction was calculated from a traditional van't Hoff plot described briefly as follows. The fraction of enzyme in the denatured state $\Theta(T)$ was taken as the ratio of the fractional area of the transition curve up to a temperature T over the total area of the curve above the sigmoidal baseline. The equilibrium constant is then $K(T) = \Theta(T)/(1 - \Theta(T))$ and ΔH_{vs} is obtained from the slope of ln K vs. $1/T$.

$$\mathrm{d} \ln K / \mathrm{d}T^{-1} = -\Delta H_{vs}/R \tag{3}$$

The cooperativity η of the transition was taken as the ratio of ΔH_s to the temperature-corrected van't Hoff enthalpy determined from the fit of the two-state model to the data (ΔH_{vf}). All five transition enthalpies were analyzed as linear functions of the transition temperature and the transition temperature was analyzed as a linear function of the pH.

Dependence of transition temperature on pH and concentration of lysozyme

A plot of the transition temperature as a function of pH is shown in Fig. 2 together with the linear least-squares fit of the data. Over the effective pH range of the HCl–glycine buffer (pH 2–4) the transition temperature increases with pH according to the following equation

$$T_m = 298.3 \pm 1.4 + (13.0 \pm 0.5)\mathrm{pH} \tag{4}$$

with a standard deviation of 1.93. A similar increase in the transition temperature with pH has also been observed with ribonuclease a [1] and other globular proteins [2]. This increase in the stability of the protein is brought about by a decrease in its net positive charge as the solution becomes less acidic [12]. According to the few DSC measurements of the denaturation transition of lysozyme in 0.2 M H_3PO_4–Na_3PO_4 buffer presented in Table 2, this equation also describes the dependence of T_m on pH

TABLE 1

Thermodynamic properties of the lysozyme transition in 0.2 M HCl–glycine-buffered solutions

Scan	Sample		pH	ΔC_p (kJ mol^{-1} K^{-1})	C_p^{max} (kJ mol^{-1} K^{-1})	T_m (K)	ΔH_{vf} (kJ mol^{-1})	ΔH_{ve} (kJ mol^{-1})	ΔH_{vs} (kJ mol^{-1})	ΔH_s (kJ mol^{-1})	ΔH_l (kJ mol^{-1})	η
	ID [a]	Concentration (mM)										
1	a3	1.10	2.3	1.60	36.3	325.8	353	387	380	331	415	0.938
2	a2	1.10	2.3	0.13	38.5	326.0	371	372	372	366	374	0.987
3	a1	1.26	2.4	5.39	33.8	326.3	344	373	373	321	336	0.933
4	c1	2.63	2.4	8.57	41.2	326.7	387	374	378	391	401	1.01
5	c3	2.63	2.4	5.87	43.6	326.8	399	371	372	418	381	1.05
6	c2	2.66	2.6	11.4	41.3	329.3	378	401	387	371	450	0.982
7	b2	2.40	2.6	5.03	40.1	329.5	378	409	404	354	373	0.936
8	b3	0.260	2.6	14.2	45.9	329.8	400	418	401	397	303	0.992
9	b1	1.19	2.6	8.21	41.9	329.8	395	367	374	413	403	1.04
10	b3	1.17	2.7	7.24	40.3	329.8	388	386	401	377	366	0.972
11	c2	2.72	2.6	10.0	43.0	329.8	400	382	381	408	418	1.02
12	b2	0.793	2.6	9.36	40.3	329.8	380	369	357	395	249	1.04
13	b3	0.618	2.6	11.7	38.2	330.1	377	405	433	341	349	0.906
14	a1	2.72	2.6	2.76	45.4	330.1	411	357	353	461	491	1.12
15	c3	1.46	2.6	7.56	41.6	330.6	379	441	424	343	411	0.905
16	b3	0.724	2.7	0.93	44.8	331.3	402	416	410	393	374	0.978
17	c1	1.46	2.6	2.60	49.5	331.3	431	402	400	450	571	1.04
18	c1	2.72	2.6	1.86	49.5	331.5	433	373	370	485	540	1.12
19	b2	1.20	2.7	3.18	47.9	332.1	411	429	407	409	361	0.996
20	c3	2.50	2.6	3.38	43.4	332.5	397	396	387	403	423	1.01
21	a2	2.50	2.6	2.91	41.9	332.8	389	404	394	382	419	0.982
22	a1	2.50	2.6	3.22	41.6	332.8	391	395	392	387	425	0.990
23	c2	2.55	2.8	1.40	51.3	333.3	436	394	381	481	515	1.10
24	a3	1.74	2.6	2.43	46.2	333.6	415	407	402	420	453	1.01
25	a1	1.74	2.6	3.54	44.0	333.7	407	410	415	397	458	0.976

26	a2	2.39	2.6	3.13	41.0	333.8	392	393	396	386	407	0.986	
27	a3	1.59	2.6	3.09	42.9	334.0	392	426	413	373	413	0.952	
28	a2	1.59	2.6	2.53	42.6	334.0	394	418	411	378	414	0.959	
29	a1	1.15	2.6	4.77	49.1	334.0	428	413	409	441	487	1.03	
30	a3	1.15	2.6	2.89	47.6	334.1	422	423	423	420	483	1.00	
31	a2	1.16	2.6	6.12	49.0	334.7	437	416	429	439	401	1.00	
32	a2	0.934	2.6	3.28	47.9	334.7	424	422	423	422	452	1.00	
33	a1	0.934	2.6	2.70	47.2	334.8	416	446	416	394	472	0.948	
34	a2	0.903	2.6	2.74	45.6	334.8	413	421	422	404	419	0.978	
35	a1	0.903	2.6	2.71	46.1	334.8	407	445	433	386	437	0.948	
36	a1	0.603	2.6	6.12	48.9	335.0	420	442	427	412	460	0.981	
37	a3	0.844	2.6	3.34	48.4	335.2	422	421	409	429	407	1.02	
38	a2	0.844	2.6	0	45.3	335.3	405	458	446	370	426	0.913	
39	a1	0.174	2.6	4.92	45.3	335.5	414	437	447	388	377	0.938	
40	a2	0.250	2.6	1.38	50.5	335.8	425	460	441	412	447	0.969	
41	c1	2.76	3.0	10.2	47.1	336.0	422	435	429	406	433	0.962	
42	c2	2.76	3.0	4.93	50.7	336.3	441	409	405	466	435	1.06	
43	c3	2.83	3.1	6.75	49.3	336.6	438	401	400	464	433	1.06	
44	c1	2.83	3.1	7.40	48.5	336.7	432	404	401	452	449	1.05	
45	a1	1.23	3.2	10.8	45.1	337.2	410	459	459	372	407	0.907	
46	b1	2.29	3.3	7.25	49.3	338.3	438	414	411	453	501	1.03	
47	b1	2.17	3.1	7.08	52.3	338.9	456	413	414	484	514	1.06	
48	a1	1.26	3.2	15.2	50.2	339.9	431	461	442	418	516	0.969	
49	a3	1.26	3.2	5.87	51.3	340.3	447	443	440	446	463	1.00	
50	a3	1.26	3.4	4.83	53.7	342.3	462	455	451	459	432	0.994	
51	c2	2.85	3.5	8.56	57.5	344.0	486	425	422	532	463	1.10	
52	c3	2.85	3.6	7.40	56.1	344.1	479	431	429	513	472	1.07	
53	c3	2.40	3.6	5.67	57.8	345.7	488	439	439	523	489	1.07	
54	c2	2.40	3.6	6.54	58.9	345.7	490	437	429	535	497	1.09	
55	a3	1.71	3.7	3.14	53.9	345.9	472	454	465	472	500	1.00	
56	a2	1.71	3.7	4.05	59.5	345.9	481	483	462	490	452	1.02	
57	a2	0.648	3.7	20.1	59.3	346.5	493	496	510	478	393	0.969	
58	a3	0.647	3.7	11.9	56.9	346.7	483	483	488	471	514	0.975	

TABLE 1 (continued)

Scan	Sample		pH	ΔC_p (kJ mol^{-1} K^{-1})	C_p^{max} (kJ mol^{-1} K^{-1})	T_m (K)	ΔH_{vf} (kJ mol^{-1})	ΔH_{ve} (kJ mol^{-1})	ΔH_{vs} (kJ mol^{-1})	ΔH_s (kJ mol^{-1})	ΔH_l (kJ mol^{-1})	η
	ID [a]	Concentration (mM)										
59	c1	2.78	3.7	7.43	60.1	346.8	496	455	451	528	528	1.07
60	c2	2.78	3.7	6.68	57.8	347.0	486	482	481	480	495	0.988
61	a3	0.780	3.8	11.7	64.3	347.0	506	498	477	517	456	1.02
62	a1	0.648	3.7	9.37	54.3	347.0	470	493	505	441	513	0.934
63	a1	0.647	3.7	4.62	63.9	347.2	507	470	462	544	560	1.07
64	b1	2.46	3.8	8.27	56.9	347.6	479	472	460	484	537	1.01
65	b2	2.46	3.8	8.75	60.9	347.6	501	463	456	528	456	1.05
66	a2	0.780	3.9	16.3	60.0	348.1	497	480	483	504	459	1.01
67	a2	0.846	3.9	8.82	61.8	348.7	500	513	511	487	482	0.973
68	a3	0.846	3.9	7.05	59.7	348.7	490	523	510	462	510	0.942

[a] The sample is described by a letter (a, b or c) which designates the source of the lysozyme and by a number (1, 2 or 3) which designates which of the three sample cells was used for the measurements. The sample size was 0.801 g of solution and the differential scanning calorimeter was run at a scan rate of 20 K h^{-1} from 303.2 K to 363.2 K.

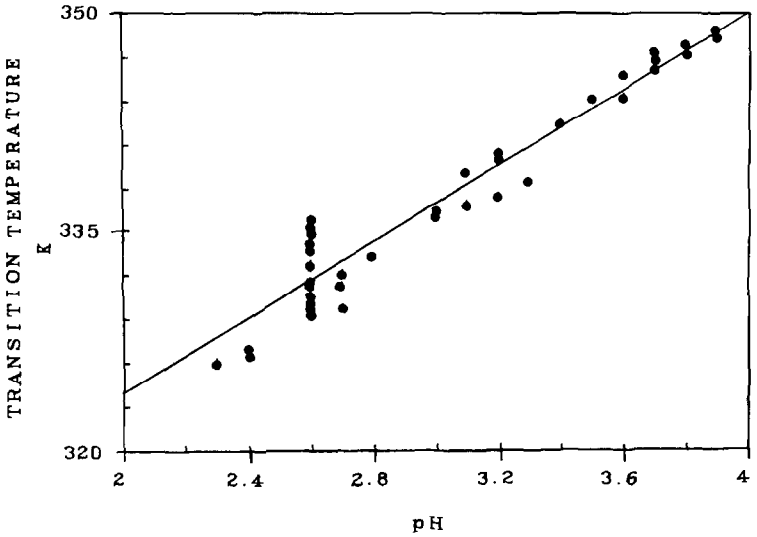

Fig. 2. Plot of the transition temperature vs. pH and the linear least-squares fit of T_m to pH; T_m (K) = 298.3 ± 1.4 + (13.0 ± 0.5) pH.

in phosphate buffers. This is in contrast with ribonuclease a where the dependence of T_m on pH depends on the buffer [13] since some anions such as PO_4^{3-} not only neutralize the positive charge on ribonuclease a but readily bind to the enzyme and enhance its stability.

The change in proton binding between the final, unfolded state (n_f) and the initial, folded state (n_i) of lysozyme in solution can be determined from the slope of eqn. (3) since [14]

$$n_f - n_i = \left(\Delta H_s / 2.303 R T_m^2\right) \partial(T_m)/\partial(\mathrm{pH}) \tag{5}$$

At the intermediate temperature of 337.2 K, $\Delta H_s = 434.7$ kJ mol^{-1}, $n_f - n_i = 2.6$ and thus 2.6 protons are absorbed per molecule of lysozyme as it unfolds at this temperature. This is close to the value of 2.2 protons per molecule of ribonuclease a [12]. Since the heat of ionization of glycine is about 2.51 kJ mol^{-1}, this will only contribute about 1% to the transition enthalpies, which is less than the experimental error in the transition enthalpies.

There is also a slight dependence of transition temperature on concentration of the enzyme as shown by the deviation of the points clustered at pH 2.6 and at pH 3.7 in Fig. 2. At pH 2.6 the concentration of the samples range from 0.11 to 2.74 mM and at pH 3.7 the concentrations range from 0.594 to 2.79 mM. At pH 2.6, the decrease in transition temperature with concentration is $-(1.09 \pm 0.43) \times 10^3$ K mol^{-1} l, while over the small concentration range at pH 3.7 it is $-(0.06 \pm 0.21) \times 10^3$ K mol^{-1} l. A similar decrease in T_m with increase in enzyme concentration was also observed with ribonuclease a and can be interpreted as a slight aggregation

TABLE 2

Thermodynamic properties of the lysozyme transition in solution under various experimental conditions

Scan	Sample		pH	ΔC_p (kJ mol^{-1} K^{-1})	C_p^{max} (kJ mol^{-1} K^{-1})	T_m (K)	ΔH_{vf} [b] (kJ mol^{-1})	ΔH_{ve} [b] (kJ mol^{-1})	ΔH_{vs} [b] (kJ mol^{-1})	ΔH_s [b] (kJ mol^{-1})	ΔH_l [b] (kJ mol^{-1})	η
	ID [a]	Concentration (mM)										
In 0.1 M HCl–glycine buffer												
1	b3	2.10	2.2	3.05	40.0	325.8	344	358	323	394	410	1.15
							367	376	372	362	387	
2	c3	2.10	2.2	4.90	40.5	326.8	385	364	380	395	360	1.03
							373	380	377	369	392	
3	c2	2.66	2.3	9.89	57.7	330.0	466	441	435	474	394	1.02
							391	396	392	389	408	
4	c1	3.02	3.8	8.29	55.8	348.8	481	474	477	476	541	0.990
							500	485	481	509	504	
5	c3	3.02	3.8	10.0	71.1	348.9	498	558	466	516	517	1.04
							501	486	481	510	505	
In 0.2 M HCl–glycine buffer at a scan rate of 5 K h^{-1}												
6	a2	2.41	3.3	5.82	48.1	334.3	424	413	403	433	409	1.02
							416	416	412	416	430	
7	a1	2.41	3.3	14.0	44.8	334.3	404	439	431	379	338	0.938
							416	416	412	416	430	
8	a3	0.624	3.3	24.4	56.0	336.2	451	478	456	440	270	0.976
							427	425	421	429	440	
In 0.2 M phosphoric acid–sodium phosphate buffer at a scan rate of 5 K h^{-1}												
9	a1	0.764	3.0	16.8	70.3	342.3	526	530	528	516	673	0.981
							463	454	450	468	471	
10	a2	1.05	3.6	17.8	59.6	345.5	484	485	468	487	510	1.01
							481	470	465	488	487	
11	a3	1.10	4.0	8.19	75.7	350.1	548	623	624	491	560	0.896
							508	491	487	517	511	

[a] The sample is described by a letter (a, b or c) which designates the source of the lysozyme and by a number (1, 2 or 3) which designates which of the three sample cells was used for the measurements. The sample masses were 0.801 g.

[b] Upper value, experimental; lower value, calculated. The equations for calculating the enthalpies are as follows: ΔH_{vf} (kJ mol^{-1}) = 432.7 + 5.81 (T_m − 337.2), ΔH_{ve} (kJ mol^{-1}) = 429.7 + 4.76 (T_m − 337.2), ΔH_{vs} (kJ mol^{-1}) = 425.5 + 4.74 (T_m − 337.2), ΔH_s (kJ mol^{-1}) = 434.7 + 6.39 (T_m − 337.2) and ΔH_l (kJ mol^{-1}) = 444.8 + 5.10 (T_m − 337.2).

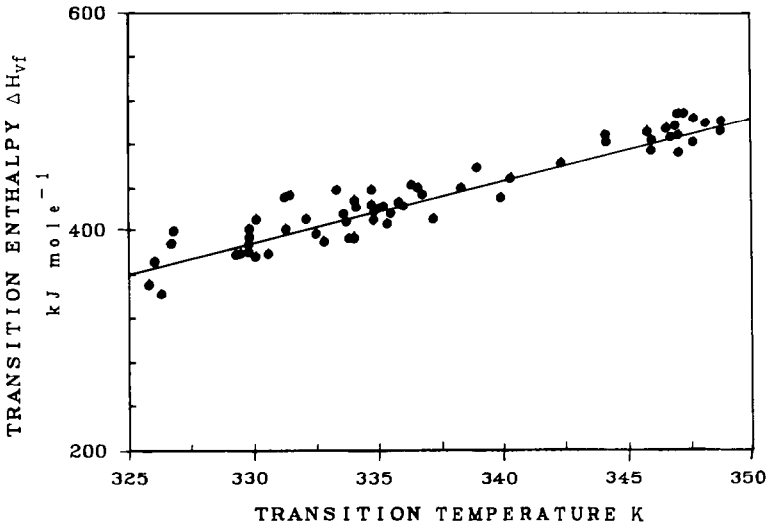

Fig. 3. Plot of ΔH_{vf} vs. the transition temperature and the linear least-squares fit of ΔH_{vf} to T_m; ΔH_{vf} (kJ mol^{-1}) = 432.7 ± 1.7 + (5.81 ± 0.24) (T_m − 337.2).

of the unfolded state of the enzyme. This may also account for the lack of 100% reversibility of the unfolding transition.

Dependence of transition enthalpy on temperature

The values of the enthalpies, ΔH_{vf}, ΔH_{ve}, ΔH_{vs}, ΔH_s and ΔH_l, are given in Table 1 and are plotted in Figs. 3–7 as a function of the transition temperature. The enthalpies were fitted by least squares to a linear dependence on T_m − 337.2 K; the fits are also shown in Figs. 3–7. A reference temperature of 337.2 K was adopted rather than the conventional 298.2 K since it falls at the mid-point of the temperature range of measurements where $d(\Delta H)/dT$ is linear. The results of these fits to all the data are presented in Table 3. The van't Hoff enthalpies, ΔH_{vf}, ΔH_{ve} and ΔH_{vs}, provide a much better linear fit of the enthalpy to the temperature with standard deviations from 13.7 to 19.7 kJ mol^{-1} than the calorimetric enthalpies, ΔH_s and ΔH_l, with standard deviations of 33.8 kJ mol^{-1} and 51.4 kJ mol^{-1}, respectively. In comparing the linear dependences of the van't Hoff enthalpies on temperature, the best fit is with the temperature-corrected van't Hoff enthalpy from the fit of the two-state transition model to the data (ΔH_{vf}). The linear dependence of ΔH_{ve} on T_m is within a standard deviation of the linear dependence of ΔH_{vs} on T_m as expected since ΔH_{ve} from eqn. (2) is the value of $R\ d(\ln K)/dT^{-1}$ from eqn. (3) at the midpoint of the transition. In addition, the linear dependence of ΔH_{vf} on T_m is within the standard deviation of the linear dependence of ΔH_s on T_m.

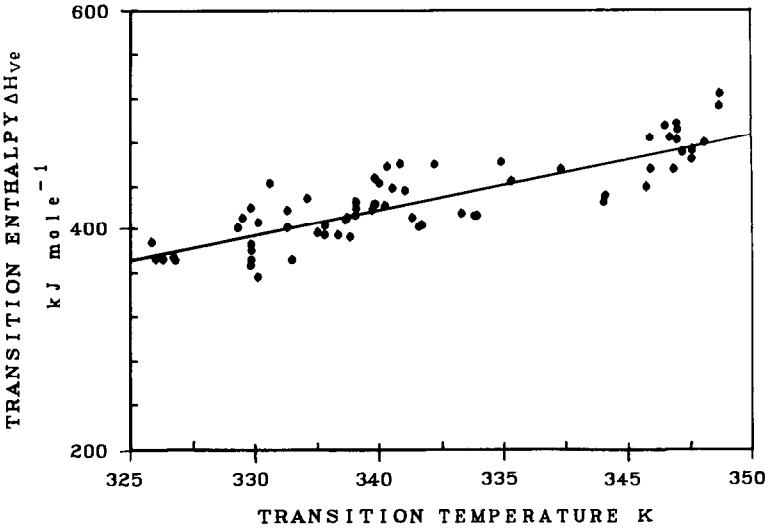

Fig. 4. Plot of ΔH_{ve} vs. the transition temperature and the linear least-squares fit of ΔH_{ve} to T_m; ΔH_{ve} (kJ mol^{-1}) = 429.7 ± 2.5 + (4.76 ± 0.36) (T_m − 337.2).

DSC measurements were also performed at a slower scan rate of 5 K h^{-1} on solutions at a lower buffer concentration of 0.1 M HCl–glycine, and on solutions containing a phosphoric acid–sodium phosphate buffer instead of the 0.2 M HCl–glycine buffer. The enthalpies determined from these measurements and the calculated enthalpies from the linear fits are presented in Table 2. At the slower scan rate of 5 K h^{-1}, 75% of the experimentally

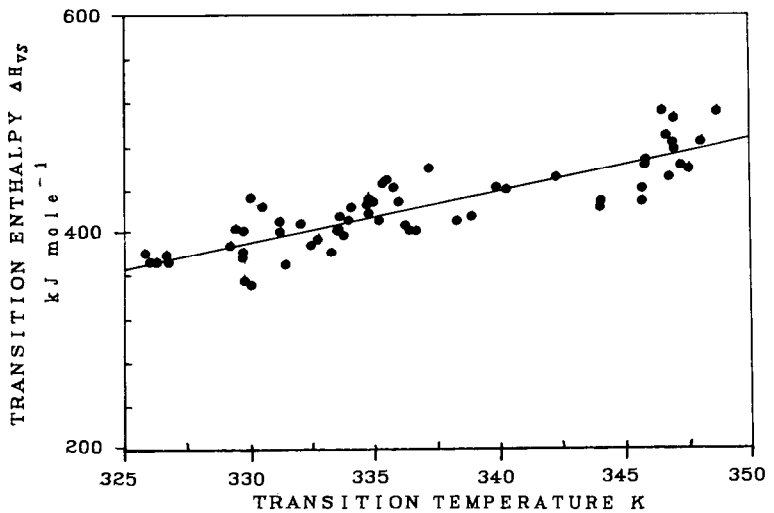

Fig. 5. Plot of ΔH_{vs} vs. the transition temperature and the linear least-squares fit of ΔH_{vs} to T_m; ΔH_{vs} (kJ mol^{-1}) = 425.5 ± 2.4 + (4.74 ± 0.35) (T_m − 337.2).

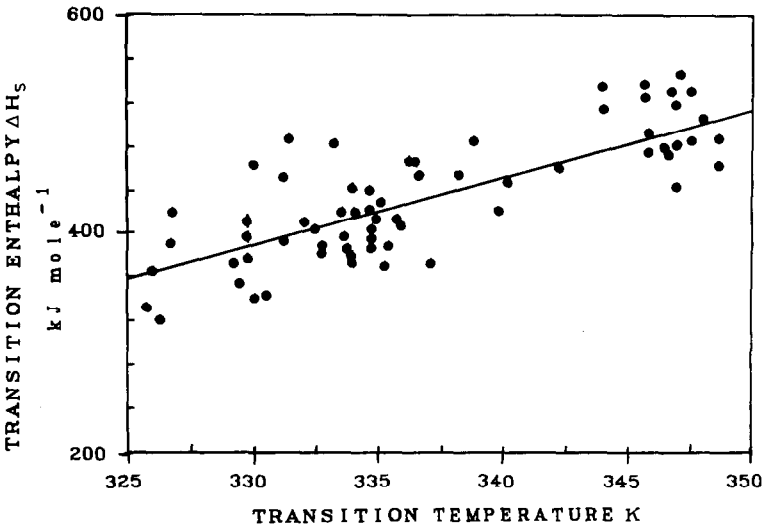

Fig. 6. Plot of ΔH_s vs. the transition temperature and the linear least-squares fit of ΔH_s to T_m; ΔH_s (kJ mol^{-1}) = 434.7 ± 4.1 + (6.39 ± 0.60) (T_m − 337.2).

determined enthalpies obtained with a sigmoidal baseline, ΔH_{vf}, ΔH_{ve}, ΔH_{vs} and ΔH_s, are within 10% of the calculated values. Similarly, 88% of all the experimentally determined enthalpies are within 10% of their calculated values at the lower buffer concentration of 0.1 M. Apparently the linear dependence of the enthalpies on temperature is unaffected by scan rate and by a change in buffer concentration from 0.1 M to 0.2 M. This also appears

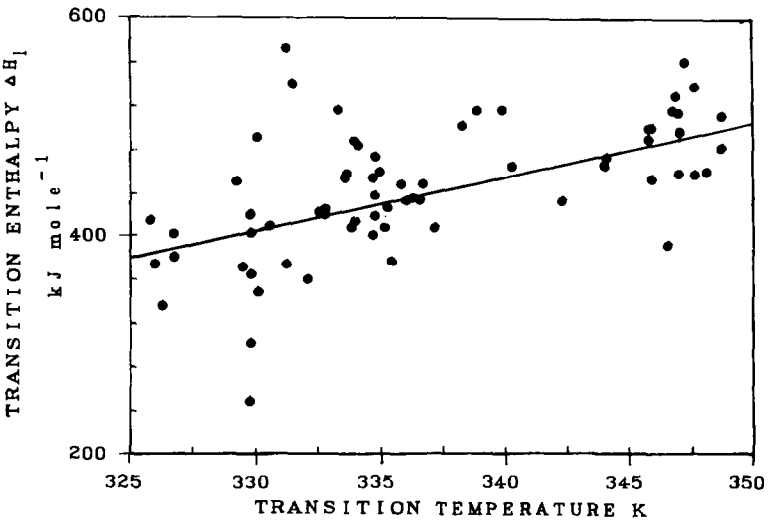

Fig. 7. Plot of ΔH_I vs. the transition temperature and the linear least-squares fit of ΔH_I to T_m; ΔH_I (kJ mol^{-1}) = 444.8 ± 6.2 + (5.10 ± 0.92) (T_m − 337.2).

TABLE 3

Transition enthalpies of lysozyme in 0.2 M HCl–glycine-buffered solutions as a function of transition temperature

Transition enthalpy	Result of linear fit to T_m $\Delta H = \Delta H_0 + \Delta C_p' (T_m - 337.2)$		
	ΔH_0 (kJ mol^{-1})	$\Delta C_p'$ (kJ mol^{-1} K^{-1})	σ (kJ mol^{-1})
ΔH_{vf}	432.7 ± 1.7	5.81 ± 0.24	13.7
ΔH_{ve}	429.7 + 2.5	4.76 ± 0.36	20.4
ΔH_{vs}	425.5 ± 2.4	4.74 ± 0.35	19.7
ΔH_s	434.7 ± 4.1	6.39 ± 0.60	33.8
ΔH_l	444.8 ± 6.2	5.10 ± 0.92	51.4

to be true when substituting a phosphate buffer for the glycine buffer as shown by the agreement (within 10%) of 60% of the experimentally determined and calculated enthalpies in Table 3. More measurements are needed to substantiate this. However, the unfolding transition of lysozyme is totally irreversible in the phosphate-buffered solutions and the DSC scan is noisier at the higher scan rate of 20 K h^{-1} in phosphate-buffered solutions than in HCl–glycine-buffered solutions. At pH > 5.0 where the phosphate buffer performance is at an optimum, the lysozyme exhibits a small amount of self-association in the native state [15] which will introduce a concentration dependence in the transition enthalpies and temperatures. The agreement between the transition enthalpies of the glycine and phosphate solutions does substantiate the conclusion that the heat ionization of the buffer contributes an amount to the transition enthalpies well within experimental error of the enthalpy determinations.

The heat capacity change of the solution

In Table 1, the change in the heat capacity ΔC_p of the solutions accompanying denaturation is the same within experimental error at all the transition temperatures, which is also true for the denaturation of ribonuclease a [1]. Excluding the scans with the three highest and with the lowest values of ΔC_p, the average value is 5.94 ± 3.10 kJ mol^{-1} K^{-1} which is in agreement with the value of 6.72 kJ mol^{-1} K^{-1} obtained by Privalov and Khechinashvili [2] for 0.5 mass% lysozyme in 0.05 M HCl–glycine solutions. The average value of ΔC_p is also within the average values of ΔC_p for the 0.1 M buffered solutions, the 0.2 M buffered solutions scanned at 5 K h^{-1} and the 0.2 M phosphate-buffered solutions scanned at 5 K h^{-1}.

Privalov and Khechinashvili [2] showed that since $\Delta C_p = \mathrm{d}(\Delta H)/\mathrm{d}T$ for lysozyme and other globular proteins, the dependence of the transition enthalpy on temperature results from the difference in the heat capacities of

the folded and unfolded states of the protein. All the $d(\Delta H)/dT_m$ ($\Delta C_p'$) values in Table 3, e.g. $d(\Delta H_s)/dT_m = 6.39 \pm 0.60$ kJ mol^{-1} K^{-1} and $d(\Delta H_{vf})/dT_m = 5.81 \pm 0.24$ kJ mol^{-1} K^{-1}, are within experimental error of the average value of 5.94 ± 3.10 kJ mol^{-1} K^{-1} for ΔC_p in Table 1. Therefore, as concluded by Privalov and Khechinashvili [2], the temperature dependence of the transition enthalpy results from the increase in the heat capacity of the unfolded state relative to that of the folded (native) state of the protein.

Cooperativity of the transition

The two-state model is based on the assumption that the transition is fully cooperative, i.e. that the total protein unfolds as a single entity. Thus the observed calorimetric heat for the unfolding transition should be the same as the heat determined by the temperature dependence of ln K, the van't Hoff enthalpy. Actually Privalov and Khechinashvili [2] observed that the ratio of the calorimetric enthalpy to the van't Hoff enthalpy, the cooperativity, was 1.05 ± 0.03 for the globular proteins, indicating that the unfolding was not exactly two state but involved the existence of intermediate states in the denaturation. Since ΔH_{vf} includes a temperature dependence in its evaluation, whereas ΔH_{ve} and ΔH_{vs} do not, ΔH_{vf} was chosen for comparison with the calorimetric enthalpy ΔH_s in determining the cooperativity η of the unfolding transition of lysozyme in solution. Although the standard deviation of the linear dependence of ΔH_l is within the standard deviation of the linear dependence of ΔH_s on T_m, ΔH_l is not thermodynamically accurate since it was determined with a straight baseline instead of the more correct sigmoidal baseline.

Values of the cooperativity are given in Table 1 and show the following temperature dependence,

$$\eta = 1.0022 \pm 0.0063 + (0.0013 \pm 0.0009)(T_m - 337.2) \quad (6)$$

which is small but statistically significant. The increase in the cooperativity with the transition temperature is more pronounced for the unfolding transition of ribonuclease a in solution [1] where

$$\eta = 1.057 \pm 0.014 + (0.007 \pm 0.001)(T_m - 333.15) \quad (7)$$

Apparently, the unfolding transition of lysozyme is more cooperative over a 23 K (326–349 K) temperature range than that of ribonuclease a in solution. With the exception of scans 1, 2 and 7 in Table 3, all the cooperativities of the 0.1 M buffered solutions, the slower scanned solutions (5 K h^{-1}) and the phosphate-buffered solutions are within 1% of the dependence shown in eqn. (6).

Error analysis of the enthalpy data

To determine the possible sources of error in the temperature dependence of the enthalpy data, the T_m dependence of the temperature-corrected van't Hoff enthalpies (ΔH_{vf}) and the calorimetric enthalpies with the sigmoidal baseline (ΔH_s) were examined in more detail. The transition temperature dependences of ΔH_{vf} and ΔH_s were determined for subsets of the data, consisting of two different concentration levels, the three different sources of lysozyme and the three different DSC cells used in the measurements. The results of the least-squares fits of the data in the subsets are presented in Table 4.

In Table 4, the constants describing the linear dependence of ΔH_{vf} on T_m for the two concentration subsets ($\geqslant 2.0$ mM and $\leqslant 1.0$ mM) are within the standard deviations of the constants describing the dependence of all the

TABLE 4

Transition enthalpies of lysozyme in 0.2 M HCl–glycine-buffered solutions as a function of transition temperature under various experimental conditions

Data set	ΔH_{vf0} (kJ mol^{-1})	$\Delta C'_{pvf}$ (kJ mol^{-1} K^{-1})	σ (kJ mol^{-1})	No. of data points
Results of the linear fit $\Delta H_{vf} = \Delta H_{vf0} + \Delta C'_{pvf}(T_m - 337.2)$				
All data	432.7 ± 1.7	5.81 ± 0.24	13.7	68
High concentration $\geqslant 2.0$ mM	437.0 ± 2.9	5.58 ± 0.42	14.5	26
Low concentration $\leqslant 1.0$ mM	430.2 ± 2.2	6.08 ± 0.31	10.0	21
Cell 1, all sources	432.6 ± 3.5	5.26 ± 0.57	16.7	23
Cell 2, all sources	433.0 ± 2.5	6.15 ± 0.36	12.5	24
Cell 3, all sources	432.0 ± 2.6	5.85 ± 0.37	12.0	21
Sample from source a	428.1 ± 2.1	6.13 ± 0.31	12.5	37
Sample from source b	432.7 ± 3.2	5.88 ± 0.45	10.3	12
Sample from source c	441.3 ± 3.2	5.40 ± 0.46	13.9	19
Data set	ΔH_{s0} (kJ mol^{-1})	$\Delta C'_{ps}$ (kJ mol^{-1} K^{-1}	σ (kJ mol^{-1})	No. of data points
Results of the linear fit $\Delta H_s = \Delta H_{s0} + \Delta C'_{ps}(T_m - 337.2)$				
All data	434.7 ± 4.1	6.39 ± 0.60	12.5	68
High concentration $\geqslant 2.0$ mM	454.6 ± 6.7	6.51 ± 0.99	34.4	26
Low concentration $\leqslant 1.0$ mM	419.9 ± 6.0	6.44 ± 0.83	26.5	21
Cell 1, all sources	436.3 ± 8.8	5.3 ± 1.4	41.4	23
Cell 2, all sources	435.8 ± 6.3	6.92 ± 0.89	30.7	24
Cell 3, all sources	430.5 ± 6.5	6.65 ± 0.91	29.8	21
Sample from source a	420.9 ± 4.7	6.32 ± 0.70	28.4	37
Sample from source b	438.7 ± 7.8	7.4 ± 1.1	25.1	12
Sample from source c	460.8 ± 8.1	6.7 ± 1.2	34.8	19

ΔH_{vf} values on T_m. The linear fit of ΔH_{vf} to T_m is thus independent of the concentration range from 0.26 mM to 2.8 mM. For the ΔH_s concentration subsets, however, only the slopes $\Delta C'_{ps}$ are within the standard deviation of $\Delta C'_{ps}$ for the linear fit of all the ΔH_s values to T_m. From the perspective of the agreement of the slopes of the fits, the linear fit of ΔH_s to T_m is also independent of concentration over this temperature range.

The constants describing the linear dependence of ΔH_{vf} on T_m, ΔH_{vf0} and $\Delta C'_{pvf}$, for each of the cell subsets in Table 4 are within the standard deviations of the constants describing the linear dependence for all the cells. This is also true for the results of the least-squares fits of the ΔH_s data. In addition, the fits are worst for the cell 1 subset in both cases which may reflect poorer instrumental performance with cell 1. Despite the separate calibration of each of the three cells, it appears that the DSC measurements with the three different cells yield the same enthalpy data.

A comparison of the results of the linear fits with the different source subsets in Table 4 shows that the slopes of the fits $\Delta C'_p$ are within a standard deviation of the slopes for the ΔH_{vf} and ΔH_s fits with all the data. Only the intercept of the source b subset is within a standard deviation of the intercepts for the ΔH_{vf} and ΔH_s fits with all the data. The intercept of the fit with the source c data is higher while the intercept of the fit with the source a data is lower than the intercept of the fit with all the data. From the standpoint of the slopes of the fits, it appears that the enthalpy data are independent of the source of lysozyme.

The values of ΔH_{vf} and ΔH_s are in good agreement with the transition enthalpies from the literature presented in Table 5. The literature values from 325.2 to 348.7 K are within 5% of the ΔH_{vf} and ΔH_s values, while the literature values at 320.2 and 350.2 K are within 10% of the ΔH_{vf} and ΔH_s values. At 351.7 K, however, the enthalpy values from Fujita et al. [5] are within 13% of the ΔH_{vf} and ΔH_s values. Standard deviations of the linear

TABLE 5

Comparison of transition enthalpies with literature values

Temperature (K)	ΔH_{vf} [a] (kJ mol^{-1})	ΔH_s [b] (kJ mol^{-1})	Literature values (kJ mol^{-1})
320.2	334	326	356 [2]
325.2	363	358	377 [2]
326.2	369	364	382 ± 8 [3]
328.2	380	377	398 [2]
339.2	444	447	481 [2]
348.7	500	505	509 [4]
350.2	508	518	560 [2]
351.7	517	527	585 ± 6 [5]

[a] Calculated from ΔH_{vf} (kJ mol^{-1}) = 432.7 + 5.81 (T_m − 337.2).

[b] Calculated from ΔH_s (kJ mol^{-1}) = 434.7 + 6.39 (T_m − 337.2).

fits of ΔH_{vf} and ΔH_s given in Table 2 correspond to a maximum error of 4% which is close to the 5% deviation between these values and most of the literature values reported in Table 5.

The probable sources of error in the ribonuclease a measurements have been attributed to analytical errors from incomplete balancing of the sample and reference cells leading to non-linear baseline distortions of the transition profile and from scatter in the transition profile integration limits [1]. The analytical errors were estimated to be about 1%.

Another source of error in the ribonuclease a measurements has been attributed to variations in cell loading which may affect the cell calibration [1]. Probable sources of the 4% error in the lysozyme measurements may also be attributed to the same analytical errors as for ribonuclease a. However, the error in the cell loading is negligible for the lysozyme measurements since the cells were loaded with the same amount of solution for all the measurements. An additional source of error is in the concentration determinations and this is expected to be at least 1%.

CONCLUSIONS

The transition enthalpies for lysozyme in 0.2 M HCl–glycine-buffered solutions can be determined with sufficient accuracy and reproducibility for the evaluation of DSC performance in the 326–349 K temperature range. DSC scans of dialyzed solutions of lysozyme in HCl–glycine buffer yield transition enthalpies which are independent of the source of the lysozyme, the buffer concentration from 0.1 to 0.2 M and the scan rate. Since the ΔH_{vf} enthalpies were corrected for temperature, it is recommended that they be used for reporting the van't Hoff enthalpies. Since the ΔH_s enthalpies were determined with the sigmoidal baseline, it is recommended that they be used for reporting the calorimetric enthalpies.

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