pool for interaction with enzymes and carrier proteins than core-located TG (Hamilton & Small, 1981). If so, the structural organization of both the surface and core components of emulsions would be important. As their physical properties become better defined, triglyceride-rich emulsions with a simple composition should become increasingly useful model systems for the complex emulsions of biological systems (Miller & Small, 1987).

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Registry No. DPPC, 2644-64-6; 1-oleyl-2-palmitylphosphatidylcholine, 81624-29-5; triolein, 122-32-7; tripalmitin, 11140-06-0.

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A New Method for Determining the Heat Capacity Change for Protein Folding†

C. Nick Pace* and Douglas V. Laurents
Biochemistry Department, Texas A&M University, College Station, Texas 77843
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ABSTRACT: In order to use results from calorimetry or thermal unfolding curves to estimate the free energy change for protein unfolding at 25 °C, it is necessary to know the change in heat capacity for unfolding, ΔC_p. We describe a new method for measuring ΔC_p which is based on results from urea and thermal unfolding curves but does not require a calorimeter. We find that ΔC_p = 1650 ± 200 cal/(deg·mol) for the unfolding of ribonuclease T1 and that ΔC_p = 2200 ± 300 cal/(deg·mol) for the unfolding of ribonuclease A.

There is currently considerable interest in measuring the conformational stability of globular proteins, i.e., how much more stable the folded, biologically active conformation is than unfolded conformations under physiological conditions (Goldenberg, 1988; Matthews, 1987). Most often, estimates of the conformational stability are based on an analysis of urea or thermal unfolding curves (Pace et al., 1989) or on calorimetric studies (Sturtevant, 1987; Privalov & Potekhin, 1986). With thermal denaturation curves or calorimetry, this requires extrapolating measurements made in the narrow temperature range where unfolding occurs to an ambient temperature, such as 25 °C. This is generally done with a form of the Gibbs–Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (1)$$

where $\Delta G(T)$ is ΔG at a temperature T, T_m is the midpoint of the thermal unfolding curve, ΔH_m is the enthalpy change for unfolding measured at T_m, and ΔC_p is the difference in heat capacity between the folded and unfolded conformations. Thus, in order to calculate $\Delta G(T)$, T_m, ΔH_m, and ΔC_p are required. Becktel and Schellman (1987) have recently discussed both theoretical and experimental aspects of this equation in detail. T_m and ΔH_m can be readily determined from thermal unfolding curves with an uncertainty of about ±0.5 °C for T_m and ±5 kcal/mol for ΔH_m. The determination

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of a reliable value of ΔC_p is more difficult. Three methods have been used to determine ΔC_p. The simplest is a van't Hoff analysis of a thermal unfolding curve. This method has been successful in some cases (Brandts & Hunt, 1967; Pace & Tanford, 1968; Alexander & Pace, 1971; Shiao et al., 1971; Jackson & Brandts, 1970; Nojima et al., 1977) but not with T4 lysozyme (Becktel & Schellman, 1987) or RNase T1, as discussed here. A second method involves measuring T_m and ΔH_m as a function of pH; if proper precautions are taken and ΔH_m and ΔC_p do not depend on pH, an estimate of ΔC_p can be obtained (Shortle et al., 1988; Becktel & Schellman, 1987; Privalov, 1979). The method of choice is to determine ΔC_p with a calorimeter (Sturtevant, 1987). Becktel and Schellman (1987) state that the minimum error in determining ΔC_p with a calorimeter is ±4%; however, the actual error is about ±10% in favorable cases, and it is clear from data such as those shown in Figure 6 of Privalov's review (Privalov, 1979) that the error in typical cases may be considerably greater.

This paper describes a method for measuring ΔC_p with reasonable accuracy that does not require a calorimeter.

Experimental Procedures

Ribonuclease T1 was purified from a crude *Aspergillus oryzae* extract (product P 4755) obtained from Sigma as described previously (Pace et al., 1987). RNase T1 concentrations were determined by using an absorbance of 1.9 for a 1 mg/mL solution (Takahashi et al., 1970). Ribonuclease A (type XII-A) was purchased from Sigma. "Ultrapure" urea was purchased from Schwarz/Mann Biotech, and stock solutions were prepared as previously described (Pace, 1986). Urea unfolding curves were determined by measuring the intrinsic fluorescence of RNase T1 (278-nm excitation, 320-nm emission) or RNase A (278-nm excitation, 305-nm emission) solutions in thermostated 1 cm^2 cuvettes with a Perkin-Elmer MPF 44B spectrofluorometer. Thermal unfolding curves were determined by measuring the optical rotation at 295 nm of ≈1 mg/mL solutions of RNase T1 in a Cary 60 spectropolarimeter. For all experiments, the temperature was controlled to ±0.05 °C, and the pH was measured to ±0.01 after a double buffer adjustment on a Radiometer Model 26 pH meter. The determination and analysis of urea and thermal unfolding curves have been discussed in detail elsewhere (Pace et al., 1989).

Results

Typical urea unfolding curves for RNase T1 and RNase A are shown in Figure 1. The single tryptophan residue in RNase T1 is completely buried near the center of the molecule, and several of the nine tyrosine residues transfer excitation energy to the tryptophan (Pongs, 1970). Thus, it is not surprising that the intrinsic fluorescence decreases substantially when the protein unfolds. RNase A contains two buried and four partially buried tyrosine residues. The intrinsic fluorescence increases when the protein unfolds for reasons which are not clear (Schmid, 1989). The fluorescence and ORD spectra of folded and unfolded RNase T1, and thermal unfolding curves typical of those reported here, have been published previously (Pace et al., 1989).

The free energy of unfolding, ΔG, was calculated as a function of urea concentration and temperature by assuming a two-state mechanism and using the equation:

$$\Delta G = -RT \ln K = -RT \ln [(y_f - y)/(y - y_u)] \quad (2)$$

where K is the equilibrium constant, y is the observed value of the parameter used to follow unfolding in the transition region, and y_f and y_u are the values of y characteristic of the

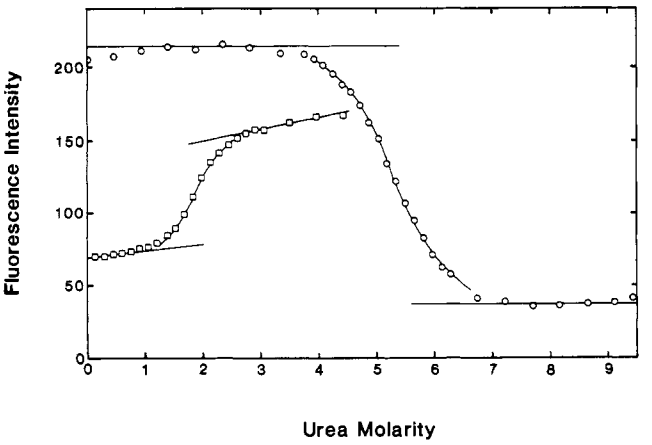

FIGURE 1: Urea unfolding curves for RNase T1 (○) at pH 7.0 (30 mM MOPS), 19.2 °C, and RNase A (□) at pH 2.80 (30 mM glycine), 21.1 °C. The fluorescence intensity after excitation at 278 nm was measured at 320 nm for RNase T1 and at 305 nm for RNase A. The solid curves in the transition region are theoretical curves based on the parameters given in Table I.

folded and unfolded conformations of the proteins, respectively (Pace et al., 1989). The unfolding of both proteins has been shown to closely approach a two-state mechanism (Freire & Biltonen, 1978; Pace et al., unpublished observations).

ΔG was found to vary linearly with urea concentration, and a least-squares analysis was used to fit the data to the equation:

$$\Delta G = \Delta G(H_2O) - m(\text{urea}) \quad (3)$$

where $\Delta G(H_2O)$ is the value of ΔG in the absence of urea and m is a measure of the dependence of ΔG on urea concentration. Values of m and $(\text{urea})_{1/2}$, the midpoint of the urea unfolding curve, are given in Table I. For the analysis described below, it was necessary to have $\Delta G(H_2O)$ values from the urea unfolding experiments at the same pH as the thermal unfolding experiments. We have determined that $\Delta(\text{urea})_{1/2}/\Delta(\text{pH}) = 1.28 \pm 0.02$ M/pH unit for RNase T1 in the temperature range and buffers used here. This was used to correct the $(\text{urea})_{1/2}$ values to a common pH, since the pH generally differs slightly among individual experiments. Over the narrow temperature (19–29 °C) and pH (6–8) ranges investigated, the m values do not vary significantly, and the average values are 1195 ± 35 cal mol^{-1} M^{-1} for RNase T1 and 2405 ± 40 cal mol^{-1} M^{-1} for RNase A. These average values were used to calculate the values of $\Delta G(H_2O)$ in Table I and those used in Figures 2 and 3, below. The estimates of ΔC_p are not changed significantly when the actual m values are used rather than the average m values (see Table II).

For analyzing thermal unfolding curves, a least-squares analysis of plots of ΔG as a function of temperature was used to determine the midpoint, $T_m = T$ at $\Delta G = 0$, and the enthalpy change for unfolding at T_m, $\Delta H_m = (T_m \text{ in K})(\text{slope at } T_m)$. The values are given in Table I.

The experiment at pH 7 (30 mM MOPS) in the presence of 4.31 M urea was a thermal unfolding experiment, but the results were used to calculate $\Delta G(H_2O)$ values at the same temperatures as the six urea denaturation curves determined at pH 7. This was done by adding 5150 cal/mol [(1195 cal mol^{-1} M^{-1})(4.31 M)] to the ΔG values calculated directly from the thermal unfolding curve at each of the temperatures. The average difference between the two sets of $\Delta G(H_2O)$ values is ±0.12 kcal/mol. This excellent agreement between the $\Delta G(H_2O)$ values from the urea unfolding curves determined by using fluorescence measurements and the values from the thermal unfolding curve determined by using optical rotation measurements is reassuring.

Table I: Parameters Characterizing the Urea and Thermal Unfolding of RNase T1 and RNase A

	T (°C)	m^a (cal mol^{-1} M^{-1})	$(U)_{1/2}{}^b$ (M)	$\Delta G(H_2O)^c$ (kcal/mol)	$\Delta C_p{}^d$ [cal/(deg·mol)]
		RNase T1			
pH 5.95 (30 mM MES)	19.05	1190	6.64	7.94	1580
	22.00	1190	6.32	7.55	1535
T_m = 52.8 °C	25.05	1150	5.92	7.07	1490
ΔH_m = 104.3 kcal/mol	29.10	1180	5.45	6.51	1215
pH 7.0 (30 mM MOPS)	19.20	1210	5.26	6.29	1710
	21.10	1100	4.98	5.95	1770
T_m = 48.25 °C	23.05	1200	4.86	5.81	1630
	25.00	1200	4.58	5.47	1645
ΔH_m = 95.2 kcal/mol	27.00	1200	4.14	4.95	1870
	29.00	1265	3.93	4.70	1705
pH 7.9 (30 mM HEPPSO)	19.05	1110	4.19	5.01	1745
	22.00	1250	3.91	4.67	1625
T_m = 43.3 °C	25.00	1280	3.44	4.11	1715
ΔH_m = 87.1 kcal/mol	28.95	1190	2.95	3.52	1300
pH 7.0 (30 mM MOPS)^e	19.20			6.31	1695
[urea] = 4.31 M	21.10			6.07	1670
	23.05			5.83	1610
T_m = 26.95 °C	25.00			5.59	1505
	27.00			5.16	1580
ΔH_m = 46.4 kcal/mol	29.00			4.90	1365
		RNase A			
pH 2.80 (30 mM Gly)	17.10	2435	2.25	5.41	2135
	21.10	2425	2.02	4.85	2160
T_m = 42.6 °C^f	24.90	2325	1.80	4.33	1970
ΔH_m = 95 kcal/mol^f	27.75	2445	1.44	3.48	2785

^{a} Slope of plots of ΔG as a function of urea concentration (eq 3). ^{b} Midpoint of the urea unfolding curve, $(urea)_{1/2}$. ^{c} ΔG at 0 M urea = $(urea)_{1/2}$ (1195 mol^{-1} M^{-1}) for RNase T1 and =$(urea)^{1/2}$ (2407 cal mol^{-1} M^{-1}) for RNase A. These are the average values of m for both proteins (see Results). ^{d} Calculated with eq 1 using T_m, ΔH_m, and $\Delta G(H_2O)$ for $\Delta G(T)$. ^{e} These results are from a thermal unfolding curve determined in the presence of 4.31 M urea. The $\Delta G(H_2O)$ values were calculated by adding 5150 cal/mol [=(1195 cal mol^{-1} M^{-1} (4.31 M)] to the values of ΔG calculated from the thermal unfolding curve at the temperatures indicated. ^{f} Freire and Biltonen (1978).

Discussion

We begin by explaining why a straightforward approach will not yield a reliable estimate of ΔC_p for RNase T1. By measuring the equilibrium constant, K, as a function of temperature, the enthalpy change, ΔH, can be calculated by using the van't Hoff equation:

$$d(-R \ln K)/d(1/T) = \Delta H \quad (4)$$

For protein unfolding, ΔH is generally found to increase markedly with temperature because the heat capacity of the unfolded protein, $C_p(U)$, is greater than that of the folded protein, $C_p(F)$, so there is a difference in heat capacity, ΔC_p, for the reaction folded ↔ unfolded:

$$\Delta C_p = C_p(U) - C_p(F) \quad (5)$$

If ΔH can be measured as a function of temperature, ΔC_p can be calculated by using the Kirchoff equation:

$$d(\Delta H)/d(T) = \Delta C_p \quad (6)$$

Throughout this discussion, we will assume that ΔC_p is independent of the temperature. ΔC_p must vary with temperature (Becktel & Schellmann, 1987), but the dependence is too small to measure given the accuracy of the techniques available for measuring ΔC_p (Jackson & Brandts, 1970; Shiao et al., 1971; Privalov, 1979).

In previous studies of the β-lactoglobulins, we were able to obtain reasonable estimates of ΔC_p using this method (Pace & Tanford, 1968; Alexander & Pace, 1971). When the same approach was used with RNase T1, the calculated values of ΔC_p ranged from negative to as high as 18 000 cal/(deg·mol). For example, the values of ΔC_p calculated from the four thermal denaturation curves given in Table I were the following (in calories per degree per mole): −730 (pH 5.95), −90 and 750 (pH 7.0, the results in Table I are the average of two thermal denaturation curves), 2250 (pH 7.9), and 2650 (pH 7.0, 4.31 M urea). With the β-lactoglobulins, the van't Hoff approach was successful mainly because these proteins are most stable at 35 °C so that ΔH varied from −40 to 40 kcal/mol in the temperature range where measurements could be made. This is the optimum situation for determining ΔC_p. First, the magnitude of the ΔH values is as low as possible so that K varies less with temperature and this improves the accuracy of the measurement of ΔH, and, second, measurements can be made over a wide temperature range (10–50 °C). In contrast, RNase T1 is most stable near −5 °C, K can be accurately measured over only a narrow temperature range, about 7 °C, and the magnitude of ΔH is large, 95 kcal/mol at pH 7. Assuming that ΔC_p = 2000 cal/(deg·mol), which is in the range expected for small globular proteins (Edelhoch & Osborne, 1976), ΔH would change by only 14 kcal/mol over this temperature range. Since we estimate an error of about ±5 kcal/mol inmeasuring ΔH, it is clear why this approach has not been successful with RNase T1. In other cases where ΔC_p was measured by this approach, the thermal unfolding curves were determined at low pH where T_m and the ΔH values are lower so that ΔH could be measured over a wider temperature range [see, for example, Shiao et al. (1971)]. Under any conditions, this approach requires great care in maintaining and measuring the temperature and in making careful measurements because the determination of ΔC_p requires taking a second derivative of the experimental data.

For protein folding, ΔG can be measured much more accurately than ΔH. A consideration of eq 1 shows that at temperatures near T_m, ΔG depends on ΔH_m but not significantly on ΔC_p. However, if ΔG could be measured at temperatures considerably below T_m, it should be possible to use

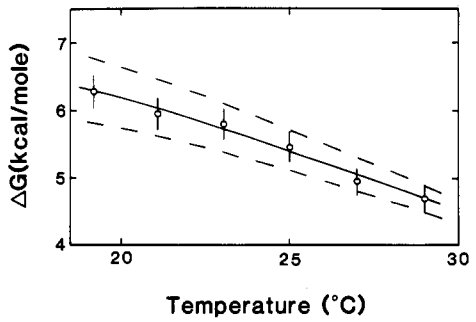

FIGURE 2: $\Delta G(H_2O)$ as a function of temperature for the unfolding of RNase T1 at pH 7.0 (30 mM MOPS). The $\Delta G(H_2O)$ values are from Table I. The error bars show the error expected in the $\Delta G(H_2O)$ values based on errors of ±0.05 in the $(\text{urea})_{1/2}$ values and of ±3% in the m values. The solid line was calculated with eq 1 using ΔC_p = 1715 cal/(deg·mol) and the T_m and ΔH_m values given in Table I. The dashed lines were calculated in the same way using ΔC_p values 20% higher and lower than 1715 cal/(deg·mol).

the results to estimate ΔC_p. This is the basis of the approach described here. We have used urea to decrease the stability of the protein. As discussed above, the analysis of urea unfolding curves leads to an estimate of $\Delta G(H_2O)$, ΔG for unfolding in the absence of urea. Hence, determining urea unfolding curves as a function of temperature will yield $\Delta G(T)$ values which can be used with T_m and ΔH_m in the Gibbs–Helmholtz equation (eq 1) to calculate ΔC_p. The ΔC_p values in Table I were calculated in this way. The average of the 20 ΔC_p values given for RNase T1 is 1600 ± 120 (average deviation) cal/(deg·mol). Another approach that can be used to calculate ΔC_p is illustrated in Figure 2.

The $\Delta G(H_2O)$ values from Table I for the experiments at pH 7 are plotted as a function of temperature in Figure 2. Note that the $\Delta G(H_2O)$ values vary from 4.7 to 6.3 kcal/mol over this 10 °C temperature range. The solid line shows the variation in ΔG calculated with Eq 1 using ΔC_p = 1715 cal/(deg·mol) and the values of T_m and ΔH_m given in Table I. This value of ΔC_p minimizes the deviation of the experimental values of $\Delta G(H_2O)$ from those calculated with eq 1. The dashed lines in Figure 2 show the effect of increasing or decreasing the ΔC_p value by 20%. Thus, the value of ΔC_p is defined to within about ±10–15% by the $\Delta G(H_2O)$ values. Note from the dashed lines that the dependence of the ΔG values on ΔC_p becomes greater the further the measurements are made from T_m. This suggests that the $\Delta G(H_2O)$ values should be measured at the lowest possible temperatures to get the best estimate of ΔC_p. This will become even clearer in the discussion of errors below. With RNase T1, it is not convenient to work at lower temperatures where the unfolding reaction takes days to reach equilibrium.

In favorable cases, it will be possible to measure $\Delta G(T)$ values such as those shown in Figure 2 with a single experiment. This is illustrated by the results in Table I from the thermal unfolding curve in the presence of 4.31 M urea. Note that the $\Delta G(H_2O)$ and ΔC_p values from this experiment are in good agreement with those from the individual urea unfolding curves at pH 7. Thus, if the m value is known and does not vary with temperature, the $\Delta G(T)$ values needed to estimate ΔC_p can be determined with a single thermal unfolding curve in the presence of urea.

One obvious concern with this method is whether ΔC_p values based in part on experiments in urea will be equivalent to ΔC_p values determined in water. To investigate this, we also measured ΔC_p at pH 5.95 and 7.9 where RNase T1 is more and less stable than at pH 7. This extends the measurements over a 2–7 M range of urea concentrations. The ΔC_p values

Table II: ΔC_p for the Unfolding of RNase T1 and RNase A

conditions	ΔC_p[a] [cal/(deg·mol)]
RNase T1	
pH 5.95 (30 mM MES)	1490 (1565)
pH 7.0 (30 mM MOPS)	1715 (1720)
pH 7.9 (30 mM HEPPSO)	1650 (1600)
pH 7.0 (30 mM MOPS), 4.31 M urea	1680
RNase A	
pH 2.80 (30 mM glycine)	2200 (2200)

[a] ΔC_p was estimated by using the data in Table I as described in Figure 2 and under Discussion. The ΔC_p values given in parentheses were calculated by using the m values given for each experiment in Table I rather than the average of all of the m values.

at the three pHs (Table II) do not differ significantly from the average value of 1600 cal/(deg·mol). This also suggests that ΔC_p does not vary significantly with pH, as has been shown with several other proteins (Privalov, 1979). Similar results have been obtained with ribonuclease A where ΔC_p does not vary significantly between 0 and 4 M urea (Brandts & Hunt, 1967), with lysozyme where ΔC_p does not vary significantly between 0 and 2 M Gdn-HCl (Pfeil & Privalov, 1976), and with β-lactoglobulin where identical ΔC_p values are observed between 4.4 and 5.5 M urea, and in 3.4 M Gdn-HCl (Pace & Tanford, 1968).

The ΔC_p observed on protein unfolding is thought to result mainly from the exposure of buried nonpolar side chains to solvent (Baldwin, 1986). Wetlaufer et al. (1964) measured the temperature dependence of the solubility of hydrocarbons such as propane in water, urea, and guanidine hydrochloride solutions. Edelhoch and Osborne (1976) used these data to show that ΔC_p for the transfer of hydrocarbons to water is somewhat greater than for the transfer to 7 M urea. Thus, model compound data suggest that ΔC_p should be reduced somewhat in the presence of urea.

A related concern is the use of linear extrapolation (eq 3) to estimate $\Delta G(H_2O)$. For RNase T1, the denaturant binding model and Tanford's model yield $\Delta G(H_2O)$ values which are 0.5 and 1.0 kcal/mol greater, respectively, than the value obtained by linear extrapolation (Pace, 1986). Much larger differences are observed when Gdn-HCl is used as a denaturant. Recent studies by Bolen and Santoro (1988) provide the best experimental support to date for the use of linear extrapolation to estimate $\Delta G(H_2O)$. Theoretical arguments also support the use of linear extrapolation (Schellman, 1978; Dill, 1985).

How do errors in the measured values of $\Delta G(H_2O)$, ΔH_m, and T_m affect the ΔC_p values calculated with eq 1? Our estimates of the upper limits of the probable errors are ±0.3 kcal/mol for $\Delta G(H_2O)$, ±5 kcal/mol for ΔH_m, and ±0.5 °C for T_m. These errors would lead, respectively, to errors in ΔC_p of ±30, 30, and 9% at 29 °C, of ±13, 19, and 2.5% at 19 °C, and of ±7.5, 15, and 1% at 9 °C. This points out again the importance of measuring $\Delta G(H_2O)$ at the lowest possible temperatures and that an accurate value of ΔH_m is essential for using this method to estimate ΔC_p.

As noted in the introduction, by measuring ΔH_m and T_m as a function of pH, ΔC_p can be calculated by using eq 6. This approach assumes that ΔH and ΔC_p do not depend on pH and has been shown to give good agreement with calorimetric values of ΔC_p for several proteins (Privalov, 1979). This approach yields ΔC_p = 1810 cal/(deg·mol) using the ΔH_m and T_m values from the thermal unfolding experiments at pH 5.95, 7.0, and 7.9. This is in reasonable agreement with the values of ΔC_p in Table II. This method is not as well-suited for RNase T1 as it is for most other proteins. RNase T1 is most

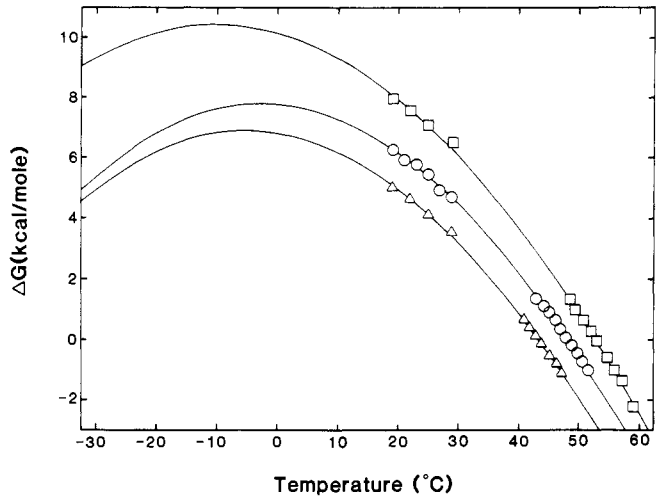

FIGURE 3: ΔG as a function of temperature for the unfolding of RNase T1 at pH 5.95 (30 mM MES) (□), at pH 7.0 (30 mM MOPS) (○), and at pH 7.9 (30 mM HEPPSO) (△). The points in the temperature range 19–30 °C are the $\Delta G(H_2O)$ values from urea unfolding curves (Table I), and those above 40 °C are from the thermal unfolding curves used to determine the values of T_m and ΔH_m given in Table I. The solid curves were calculated with eq 1 using the T_m and ΔH_m values from Table I, and the ΔC_p values from Table II.

stable near pH 5, and it is difficult to vary T_m by more than 15 °C by either raising or lowering the pH.

On the basis of our results, we estimate that ΔC_p = 1650 ± 200 cal/(deg·mol) for RNase T1 unfolding. This estimate gives more weight to the data at lower temperatures and the data at pH 7.0 where the thermal unfolding was studied most carefully. Calorimetric studies of RNase T1 unfolding have been hampered by a lack of sufficient protein. The availability in the near future of large amounts of RNase T1 prepared from the gene cloned in *Escherichia coli* (Quaas et al., 1988) will allow us to determine ΔC_p with a calorimeter for comparison with ΔC_p determined by the method described here.

The thermodynamics of unfolding of RNase A have been studied in more detail than for any other protein (Tsong et al., 1970; Tiktopulo & Privalov, 1974; Freire & Biltonen, 1978). The conditions used most often were pH 2.75–2.80 in glycine buffers. Consequently, this protein and these conditions were chosen to test the method of determining ΔC_p proposed here. The reported values of T_m range from 39 to 43 °C, and the values of ΔH_m range from near 50 to 95 kcal/mol, with the calorimetric values generally between 80 and 95 kcal/mol. The values of T_m and ΔH_m given in Table I are the most recent calorimetric values (Freire & Biltonen, 1978). Using these values and ΔC_p = 2200 cal/(deg·mol) minimized the deviations of the values of ΔG calculated with eq 1 from the experimental values of $\Delta G(H_2O)$ given in Table I. Decreasing ΔH_m to 90 kcal/mol leads to a value of ΔC_p = 1730 cal/(deg·mol), and decreasing T_m by 0.5 °C leads to a value of ΔC_p = 2090 cal/(deg·mol). The values of ΔC_p previously reported were 2.4 (at 40 °C) (Brandts & Hunt, 1967) and 2.0 ± 0.2 (Shiao et al., 1971) kcal/(deg·mol) based on the van't Hoff method and 2.1 (Tsong et al., 1970), 1.2 (Tiktopulo & Privalov, 1974; Privalov & Khechinashvili, 1974), and 1.8 ± 0.5 (Freire & Biltonen, 1978) kcal/(deg·mol) based on calorimetry. Thus, the ΔC_p value determined by the method reported here is in good agreement with the values of ΔC_p determined by other techniques.

In Figure 3, the experimental data from the urea and thermal unfolding curves for RNase T1 are shown along with theoretical curves calculated with eq 1 by using the T_m and ΔH_m values from Table I and the ΔC_p values from Table II.

Becktel and Schellman (1987) refer to these plots as protein stability curves and show that the temperature of maximum stability, T_S, where ΔS = 0, can be calculated by using $T_S = T_m \exp(-\Delta H_m/T_m \Delta C_p)$. The calculated values of T_S for the three curves in Figure 3 are −10, −3, and −5 °C. The T_S values of proteins generally fall between −10 °C for a relatively hydrophilic protein such as RNase A and 35 °C for a relatively hydrophobic protein such as β-lactoglobulin. Thus, a protein like β-lactoglobulin can be cold-denatured as shown 20 years ago by Pace and Tanford (1968), and more recently for phosphoglycerate kinase (Nijima et al., 1977), myoglobin (Privalov et al., 1986), and staph nuclease (Griko et al., 1988).

In summary, we propose the following method for determining ΔC_p: (1) use a thermal unfolding curve to measure T_m and ΔH_m as accurately as possible; (2) use urea unfolding curves to measure $\Delta G(H_2O)$ at several temperatures, at least 25 °C below T_m; and (3) calculate ΔC_p from T_m, ΔH_m, and $\Delta G(H_2O)$ by using the Gibbs–Helmholtz equation. This method will yield a reasonable estimate (±10–15%) of ΔC_p for protein unfolding and is especially useful because it does not require a calorimeter or large amounts of protein. On the basis of this method, we estimate that ΔC_p = 1650 ± 200 cal/(deg·mol) for the unfolding of RNase T1.

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Registry No. RNase, 9001-99-4; RNase T1, 9026-12-4.

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Characterization of Site-Directed Mutants in the *lac* Permease of *Escherichia coli*. 1. Replacement of Histidine Residues†

Irene B. Püttner, Hemanta K. Sarkar, Etana Padan,‡ Julius S. Lolkema, and H. Ronald Kaback*
Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110
Received September 28, 1988

ABSTRACT: Wild-type *lac* permease from *Escherichia coli* and two site-directed mutant permeases containing Arg in place of His35 and His39 or His322 were purified and reconstituted into proteoliposomes. H35-39R permease is indistinguishable from wild type with regard to all modes of translocation. In contrast, purified, reconstituted permease with Arg in place of His322 is defective in active transport, efflux, equilibrium exchange, and counterflow but catalyzes downhill influx of lactose without concomitant H^+ translocation. Although permease with Arg in place of His205 was thought to be devoid of activity [Padan, E., Sarkar, H. K., Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765], sequencing of *lac Y* in pH205R reveals the presence of two additional mutations in the 5′ end of the gene, and replacement of this portion of *lac Y* with a restriction fragment from the wild-type gene yields permease with normal activity. Permeases with Asn, Gln, or Lys in place of His322, like H322R permease, catalyze downhill influx of lactose without H^+ translocation but are unable to catalyze active transport, equilibrium exchange, or counterflow. Unlike H322R permease, however, the latter mutants catalyze efflux at rates comparable to that of wild-type permease, although the reaction does not occur in symport with H^+. Finally, as evidenced by flow dialysis and photoaffinity labeling experiments, replacement of His322 appears to cause a marked decrease in the affinity of the permease for substrate. The results confirm and extend the contention that His322 is the only His residue in the permease involved in lactose/H^+ symport and that an imidazole moiety at position 322 is obligatory. In addition, the observations are consistent with the idea that His322 functions as a component of a catalytic triad that is important for lactose/H^+ symport. In the following paper [Carrasco, N., Püttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry* (second paper of three in this issue)], the role of Glu325 is examined in detail, and in the third paper [Lee, J. A., Püttner, I. B., & Kaback, H. R. (1989) *Biochemistry* (third paper of three in this issue)], evidence is presented supporting the hypothesis that His322 and Glu325 are ion-paired.

lac permease of *Escherichia coli* is a hydrophobic, transmembrane protein encoded by the *lac Y* gene that catalyzes symport of a single β-galactoside molecule with a single H^+ [cf. Kaback (1983, 1986a,b) for reviews]. Thus, in the presence of an H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$, interior negative and/or alkaline),[1] *lac* permease utilizes free energy released from downhill translocation of H^+ with $\Delta\bar{\mu}_{H^+}$ to drive uphill accumulation of lactose against a concentration gradient. Conversely, in the absence of $\Delta\bar{\mu}_{H^+}$, movement of lactose down a concentration gradient drives uphill movement of H^+ with

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* To whom correspondence should be addressed.
‡Current address: Institute of Life Sciences, The Hebrew University, Jerusalem, Israel.

[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; $\Delta\Psi$, membrane potential; RSO, right side out; NPG, *p*-nitrophenyl α-D-galactopyranoside; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; *p*CMBS, *p*-(chloromercuri)benzenesulfonate; EMB, eosin–methylene blue; Mab, monoclonal antibody; kDa, kilodalton(s); $HgCl_2$, mercuric chloride; DTT, dithiothreitol.

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