Conformational Stability of Muscle Acylphosphatase: The Role of Temperature, Denaturant Concentration, and pH†

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ABSTRACT: The conformational stability (ΔG) of muscle acylphosphatase, a small α/β globular protein, has been determined as a function of temperature, urea concentration, and pH. A combination of thermally induced and urea-induced unfolding, monitored by far-UV circular dichroism, was used to define the conformational stability over a wide range of temperature. Through analysis of all these data, the heat capacity change upon unfolding (ΔC_p) could be estimated, allowing the determination of the temperature dependence of the main thermodynamic functions (ΔG, ΔH, ΔS). Thermal unfolding in the presence of urea made it possible to extend such thermodynamic analysis to examine these parameters as a function of urea concentration. The results indicate that acylphosphatase is a relatively unstable protein with a $\Delta G(H_2O)$ of 22 ± 1 kJ mol^{-1} at pH 7 and 25 °C. The midpoints of both thermal and chemical denaturation are also relatively low. Urea denaturation curves over the pH range 2−12 have allowed the pH dependence of ΔG to be determined and indicate that the maximum stability of the protein occurs near pH 5.5. While the dependence of ΔG on urea (the *m* value) does not vary with temperature, a significant increase has been found at low pH values, suggesting that the overall dimensions of the unfolded state are significantly affected by the number of charges within the polypeptide chain. The comparison of these data with those from other small proteins indicates that the pattern of conformational stability is defined by individual sequences and not by the overall structural fold.

Protein folding is one of the most intensively studied aspects of modern biochemistry. It has been realized, over the past 30 years, that many proteins can unfold cooperatively and reversibly under denaturing conditions, such as high temperature, chemical denaturants, or extremes of pH, with a virtually complete loss of native secondary and tertiary structure. The simplicity of the unfolding transition and its character of reversibility allow a detailed description of its thermodynamics; the two-state model, which assumes that at equilibrium the protein exists only in two alternative forms (fully folded or fully unfolded), frequently seems to be adequate to describe the unfolding transition caused by denaturing agents and has been used for the determination of thermodynamic parameters upon unfolding for a number of proteins (*1*, *2*).

In this work we have studied the conformational stability of acylphosphatase (AcP),[1] a small globular enzyme of 98 residues. It catalyzes the hydrolysis of acyl phosphates such as 1,3-bisphosphoglycerate, acetyl phosphate, carbamoyl phosphate, succinyl phosphate, and the β-aspartyl phosphate formed during the activity of membrane pumps (*3*−*7*). AcP has been purified and sequenced from several vertebrates as two isoenzymes sharing about 50% amino acid sequence identity (*8*, and references therein). The two isoenzymes are known as the muscle and erythrocyte AcP, indicating the tissues from which they were purified for the first time. The structure of muscle AcP has been determined by ^{1}H NMR data (*9*, *10*), whereas the erythrocyte isoenzyme structure has been determined more recently by X-ray crystallography (*11*). In both cases AcP is a single-domain protein with no disulfide bonds, consisting of two α-helices packed against an antiparallel five-stranded β-sheet. There is clear evidence that AcP unfolds in a highly cooperative and reversible manner when both heat and urea are used and that little persistent residual structure is maintained in the denatured protein (*12*). All of these characteristics prompted us to use

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[1] Abbreviations: AcP, acylphosphatase; ADA2h, activation domain of procarboxypeptidase A2; AdB, activation domain of procarboxypeptidase B; CD, circular dichroism; HPr, histidine-containing phosphocarrier protein; RNAase, ribonuclease.

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AcP for equilibrium unfolding studies and to obtain a full description of its overall stability.

An accurate determination of the conformational stability over a wide range of temperature, pH, and denaturant concentration is vital for the comprehension of the physical interactions that stabilize the functional structure of a protein. Surprisingly, there are very few proteins whose thermodynamic functions of unfolding have been studied in a thorough and complete manner. In this study we determine thermodynamic parameters of AcP unfolding as a function of temperature, urea concentration, and pH using far-ultraviolet circular dichroism as a spectroscopic probe to monitor unfolding. Our analysis focuses on the free-energy change upon unfolding (ΔG), the only parameter appropriate for fully describing the conformational stability of the folded form relative to the unfolded one. The analysis of our data and the comparison with those of other proteins allow us to gain insight into the effect of chemical denaturants on the thermodynamic parameters of protein stability under a variety of conditions.

MATERIALS AND METHODS

Protein Purification and Chemicals. AcP expression and purification was performed according to Modesti et al. (*13*). Protein purity was checked by determination of the enzyme specific activity (*14*) and SDS−PAGE, showing that the final content of AcP was higher than 95%. All chemicals used were of the highest analytical grade.

Thermally Induced Denaturation. Far-UV CD at 222 nm was used to monitor the thermal unfolding of AcP using a Jasco J-720 spectropolarimeter equipped with a temperature control system and cuvettes with 1-mm path lengths. The thermal unfolding curves were recorded using a heating rate of 0.5 °C/min from 20 to 80 °C or from 5 to 75 °C, depending on the approximately predicted temperature range of the transition. In all cases the temperature course was monitored by positioning a thermocouple inside the sample. The protein concentration of all samples was 0.3−0.4 mg/mL in buffer solutions with pH values ranging from 2.00 to 7.00 and urea concentration ranging from 0 to 4 M. The reversibility of the unfolding transitions was checked by the measurement of the CD signal at room temperature upon cooling immediately after the conclusion of the transition.

Urea-Induced Denaturation. Far-UV CD at 222 nm was also used to monitor the urea-induced unfolding of AcP. Urea denaturation curves were obtained at different temperatures (ranging from 5 to 41 °C) and at different pH values (from 2.50 to 11.50). Each urea denaturation curve was obtained by following the CD signal of 25−30 samples containing 0.4 mg/mL of protein and a concentration of urea varying from 0 to 9 M. For the analysis as a function of temperature, all samples were in 50 mM acetate buffer, pH 5.5. For the analysis carried out as a function of pH, several buffer solutions at different pH values were used (see below), but care was taken in maintaining a constant ionic strength. The pH was checked again for a mixture of 5−10 samples around the transition midpoint as suggested by Pace et al. (*15*). In some cases, the measured pH value varied by 0.10 pH units and the second value was considered the true value in the analysis. In all cases the CD signal was recorded after an incubation period of 3 h in order to let the protein reach equilibrium. Reversibility of unfolding was checked by diluting a sample at high urea concentration into the buffer solution and recording its ellipticity at 222 nm. At high pH values, the transition was also followed by fluorescence with excitation and emission wavelengths of 280 and 335 nm, respectively, on a Perkin-Elmer LS 50 B spectrofluorimeter.

Buffers and Urea Solutions. For the analysis performed as a function of pH, the following buffer solutions were used: 50 mM citric acid pH 2.40−2.80; 50 mM formic acid pH 3.00−3.80; 50 mM acetic acid pH 4.00−5.50; 50 mM cacodylic acid pH 6.50; 50 mM *N*-ethylmorpholine pH 7.50; 50 mM boric acid pH 8.50−9.50; 50 mM sodium carbonate pH 10.50−11.00; sodium hydroxide/40 mM sodium acetate pH 11.50. In the preparation of these buffer solutions, particular care was taken to avoid the addition of chloride, phosphate, or sulfate anions, which are known to bind to the active site of native AcP (*16*), potentially altering its stability. There is no evidence at present that any chemicals used in the present study bind preferentially to either the folded or unfolded form of AcP. Fresh urea stock solutions were prepared daily by dissolving urea in water and adding the appropriate chemical compound among those listed above in order to have a final 50 mM solution in all cases. The pH was then corrected to the desired value by adding sodium hydroxide, acetic acid, or trifluoroacetic acid. The urea concentration in the stock solutions was checked using a hand refractometer, ATAGO R5000, according to Pace (*17*).

Data Analysis. In both the thermally induced and urea-induced unfolding curves, the data before and after the transition were linearly fitted in order to determine the pre- and posttransition baselines. The apparent equilibrium constant of unfolding (K_{eq}) and free-energy change (ΔG) were estimated in the transition region by using

$$K_{eq} = (y_n - y)/(y - y_d) \quad (1)$$

$$\Delta G = -RT \ln(K_{eq}) \quad (2)$$

where y is the ellipticity observed at the given urea concentration or temperature and y_n and y_d are the ellipticities characteristic of the folded and unfolded protein, respectively, and extrapolated from the pre- and posttransition baselines to the urea concentration or temperature under consideration.

For all urea-induced denaturation curves, plots of ΔG against urea concentration could be fitted by linear least-squares analysis, according to the two-state model, by using

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

where m is a measure of the ΔG dependence on urea concentration and $\Delta G(H_2O)$ is the free-energy change in the absence of denaturant. This plot allows the determination, by the linear extrapolation method (*18*), of ΔG values also in the pre- and posttransition regions where the protein is fully folded or fully unfolded, respectively. The midpoint of denaturation, C_m, can also be determined from eq 3 and corresponds to the urea concentration where $\Delta G = 0$.

Santoro and Bolen (*19*) have pointed out that these methods of analyzing unfolding curves underestimate the experimental error in the parameters determined, because no error is assumed for the pre- and posttransition baselines.

To get a more realistic assessment of the error, they recommended the use of a nonlinear least-squares analysis to fit the data of a urea denaturation curve:

$$y = \{(y_f + m_f[\text{urea}]) + (y_u + m_u[\text{urea}]) \exp[-(\Delta G(H_2O)/RT - m[\text{urea}]/RT)]\}/\{1 + \exp[-(\Delta G(H_2O)/RT - m[\text{urea}]/RT)]\} \quad (4)$$

where y_f, m_f, y_u, and m_u are the slopes and intercepts of the pre- and posttransition regions and $\Delta G(H_2O)$ and m have the same meaning as in eq 3. Values of C_m, m, and $\Delta G(H_2O)$ were essentially identical using either eq 3 or 4.

In the case of thermally induced unfolding curves, the van't Hoff plot of $\ln(K_{eq})$ against $1/T$ was linearly fitted to

$$\ln(K_{eq}) = \Delta S_m/R - \Delta H_m/R(1/T) \quad (5)$$

ΔH_m and ΔS_m being the enthalpy and entropy changes upon unfolding at the half-denaturation temperature, T_m, respectively.

RESULTS

Equilibrium Unfolding. A complete characterization of the thermodynamics of AcP unfolding has been carried out at pH 5.50 by a combination of equilibrium heat and urea unfolding experiments performed under different conditions.

Figure 1 shows two representative unfolding curves followed by far-UV CD (222 nm). The transitions are characterized by the presence of a single sharp change in the ellipticity that is typical of a two-state model. All transitions showed a degree of reversibility higher than 90%. Figure 2A shows nine thermally induced unfolding curves, normalized to the fraction unfolded, f_u, obtained at urea concentrations ranging from 0 to 3.2 M at 0.4 M intervals. Thermodynamic parameters derived from fitting these curves to the van't Hoff analysis show that the increase of urea concentration causes a reduction of the thermoresistance of the protein (i.e., T_m value) concomitant with a slight but significant decrease of the enthalpy change upon unfolding.

In a complementary set of experiments, urea denaturation curves were acquired at temperatures ranging from 4 to 41 °C. Figure 2B and Table 1 show the results of this study. In this case an increase in temperature correlates with a diminishing of the protein resistance to chemical denaturation. Since the dependence of ΔG on urea concentration (m value) does not change significantly with temperature, the observed decrease of $\Delta G(H_2O)$ with temperature solely reflects the variation in C_m, the midpoint of urea denaturation.

Both of the approaches described above show that AcP does not exhibit, over the range of temperatures under study, the phenomenon of cold denaturation. In fact, the unfolding transition caused by urea shifts to higher urea concentration as the temperature decreases and the analysis was not possible at 4 and 10 °C because the transition occurs beyond the range of accessible urea concentrations. In addition, the absence of curvature in the pretransition regions of the heat denaturation curves at high urea concentration confirms this observation, since the existence of cold denaturation would have caused a value of f_u lower than 100% at low temperature. However, as a consequence of the positive value of ΔC_p of unfolding, the conformational stability of proteins is expected to decrease at sufficiently low temperatures, but evidently this occurs only below 0 °C for AcP.

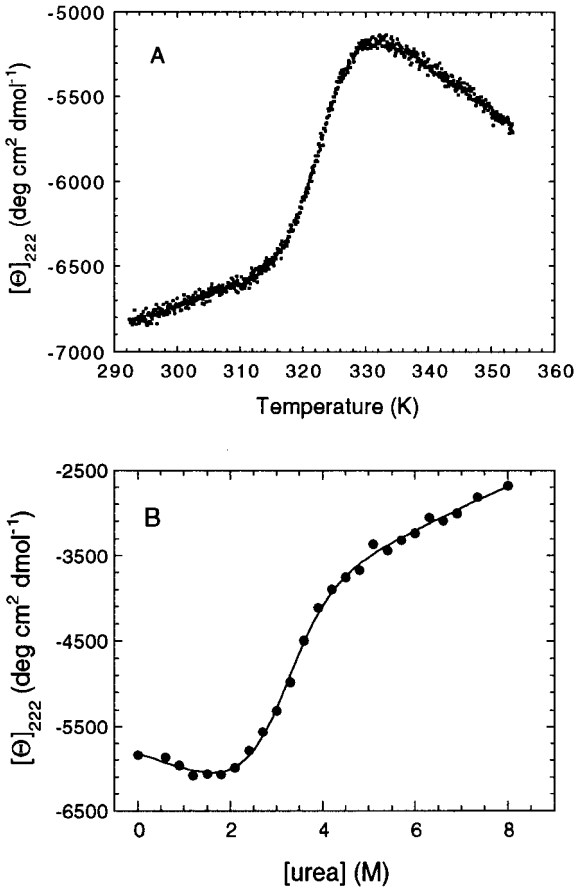

FIGURE 1: Representative thermal (A) and urea-induced (B) unfolding transitions for AcP monitored by CD at 222 nm. For the thermal transition, the sample contained 0.4 mg/mL AcP in 50 mM acetate buffer and 1.2 M urea, pH 5.50. Ellipticity was measured every 0.08 °C. The urea denaturation curve was obtained at 37 °C with samples containing 0.35 mg/mL AcP in the same buffer at different urea concentrations. The solid line through data in panel B represents the best fit to eq 4.

Stability Curves. To obtain a complete characterization of the thermodynamics of AcP as a function of temperature, the heat capacity change upon unfolding (ΔC_p) must be known in accordance with the standard thermodynamic relationships:

$$\Delta H(T) = \Delta H_m + \Delta C_p (T - T_m) \quad (6)$$

$$\Delta S(T) = \Delta S_m + \Delta C_p \ln(T/T_m) \quad (7)$$

$$\Delta G(T) = \Delta H_m(1 - T/T_m) + \Delta C_p[T - T_m - T \ln(T/T_m)] \quad (8)$$

where T_m is the temperature at which the protein is half-denatured ($\Delta G = 0$) and ΔH_m and ΔS_m are the enthalpy and entropy changes upon unfolding at T_m, respectively. In our study, a plot of ΔG against T over a wide range of temperature, called the stability curve by Becktel and Schellman (*20*), was achieved by the use of ΔG data from the transition region of a thermal denaturation and $\Delta G(H_2O)$ data from urea denaturation experiments carried out at different temperatures. The plots can be constructed at various urea concentrations with ΔG values at high temper-

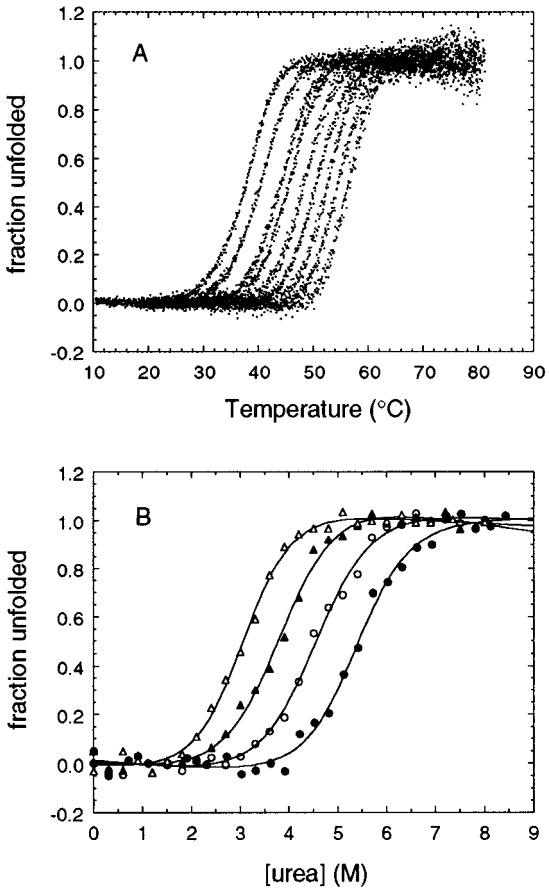

FIGURE 2: Thermally induced and urea-induced unfolding curves of AcP, followed by CD at 222 nm and converted to fraction unfolded using $f_u = (y - y_n)/(y_d - y_n)$, where y, y_n, and y_d have the same meaning as in eq 1. (A) Heat denaturation curves in 50 mM acetate buffer, pH 5.50. The curves shown from right to left were acquired at various urea concentrations ranging from 0 to 3.2 M with 0.4 M intervals. The spread of f_u at high temperatures is due to the vicinity of pre- and posttransition baselines in that region. (B) Urea denaturation curves of AcP (50 mM acetate buffer, pH 5.50) at temperatures of 15.5 °C (filled circles), 24.2 °C (empty circles), 32.6 °C (filled triangles), and 37.0 °C (empty triangles).

Table 1: Analysis of Urea Denaturation at Different Temperatures[a]

temp (°C)	C_m (M urea)	m (kJ mol^{-1} M^{-1})	$\Delta G(H_2O)$ (kJ mol^{-1})
4.0	not measurable	not measurable	not measurable
10.0	not measurable	not measurable	not measurable
15.5	5.36	5.22	28.0
20.2	4.91	5.14	25.3
24.2	4.54	5.31	24.1
28.0	4.32	5.22	22.5
32.6	3.70	5.26	19.5
37.0	3.04	6.09	18.5
41.3	2.72	4.71	12.8

[a] m and $\Delta G(H_2O)$ were estimated from plots of ΔG versus urea concentration fitted to eq 3. C_m is the concentration of urea corresponding to the midpoint of denaturation where $\Delta G = 0$. At low temperatures analysis was not possible due to the high stability of the protein. Experimental errors are 0.1 M for C_m, 0.5 kJ mol^{-1} M^{-1} for m, and ca. 10% for $\Delta G(H_2O)$.

atures from thermal unfolding curves obtained in the presence of urea and ΔG values at low temperatures calculated from urea denaturation curves by use of eq 3. Figure 3 shows such plots at different urea concentrations. Solid lines represent fits of data to eq 8, and the parameters derived are summarized in Table 2.

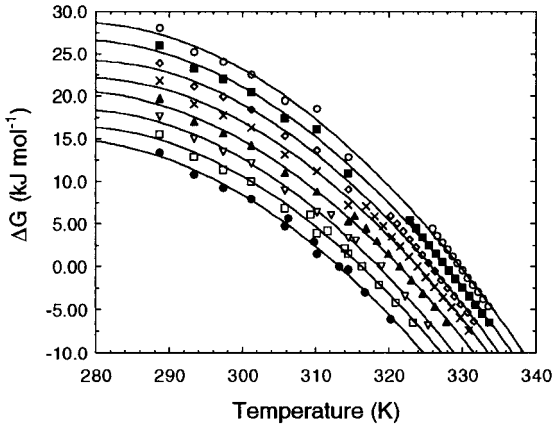

FIGURE 3: Temperature courses of conformational free energy of unfolding at pH 5.50 and at various urea concentrations (stability curves): 0 M (open circles), 0.4 M (filled squares), 0.8 M (open tilted squares), 1.2 M (crosses), 1.6 M (filled triangles), 2.0 M (open triangles), 2.4 M (open squares), and 2.8 M (filled circles). Each stability curve was obtained by the combination of ΔG data from the heat unfolding transition regions (high temperatures) and ΔG data calculated from isothermal urea denaturation curves with eq 3 (low temperatures). Solid lines represent fitting of every data set to eq 8 allowing the estimation of T_m, ΔH_m, and ΔC_p at several urea concentrations. The results of such a fitting procedure are reported in Table 2.

Table 2: Thermodynamic Parameters of AcP from the Analysis of Stability Curves at Different Urea Concentrations[a]

[urea] (M)	T_m (K)	ΔH_m (kJ mol^{-1})	ΔS_m (kJ mol^{-1} K^{-1})	ΔC_p (kJ mol^{-1} K^{-1})	T_s (K)	ΔG_s (kJ mol^{-1})
0.0	329.7	351	1.064	6.15	277.3	28.7
0.4	327.8	335	1.022	6.07	277.0	26.7
0.8	325.8	324	0.997	6.28	278.1	24.3
1.2	323.9	305	0.941	6.19	278.2	22.0
1.6	321.4	286	0.890	5.86	276.1	20.6
2.0	318.6	276	0.866	6.19	277.0	18.4
2.4	316.3	256	0.809	6.20	277.6	16.0
2.8	313.2	238	0.760	5.77	274.5	15.0

[a] Values of T_m, ΔH_m, and ΔC_p were derived from the stability curves (Figure 3) using eq 8. ΔS_m was subsequently calculated using $\Delta S_m = \Delta H_m/T_m$. T_s is the temperature of maximum free energy conformational stability and was obtained from eq 9. ΔG_s was thus determined by replacing T with T_s in eq 8. Experimental errors from the least-squares fits are as follows: 10% for ΔC_p (at high urea concentrations it increases to 20%), 5% for ΔH_m, 10% for ΔS_m, 0.5 and 2 K for T_m and T_s, respectively, and 5% for ΔG_s.

A number of interesting observations arise from this analysis. (I) The good fits to the data confirm the validity of this method based on the combination of ΔG estimates from heat and chemical denaturation experiments to express the equilibrium between the folded and unfolded states of protein. The good fits also allow us to get a reliable and averaged value of ΔC_p for the range of temperature under consideration. (II) Figure 3 allows the conformational stability of AcP to be defined over a wide range of temperature and urea concentrations. Furthermore, the determination of the parameters listed in Table 2 allows this two-dimensional characterization to be extended to enthalpy and entropy changes by the use of eqs 6 and 7. (III) The high values of ΔG at low temperatures, near the freezing point of water, are responsible for the absence of observable cold denaturation. (IV) Setting the first derivative of eq 8 equal to 0, we can determine the temperature, T_s, of maximum conformational stability of AcP and, consequently,

the maximum value of ΔG under these conditions (ΔG_s).

$$T_s = T_m/\{\exp[\Delta H_m/(\Delta C_p T_m)]\} \quad (9)$$

Values of T_s and ΔG_s, collected from each stability curve, are also listed in Table 2. T_s values fall in the narrow temperature range 1−6 °C and are largely independent of urea concentration.

ΔG, ΔH, and ΔS as a Function of Temperature and Urea Concentration. Equations 6−8 and the parameters listed in Table 2 determined from the analysis of the stability curves were utilized to determine the temperature dependencies of the three fundamental thermodynamic quantities. The large positive value of ΔC_p is responsible for the strong dependence of both ΔH and ΔS on temperature and for the higher value of ΔH with respect to $T\Delta S$ at lower temperatures. This is consistent with the fact that, at physiological temperatures, the native conformation of a protein is stabilized by a favorable enthalpic factor against an unfavorable entropy of folding (*2*).

The investigation of the relationship between the energetics of AcP unfolding and temperature can be extended to each of the urea concentration values under study. This allows the dependence of ΔH, ΔS, and ΔG on denaturant concentration to be determined at fixed values of temperature (data not shown). At high temperatures a slight dependence of ΔH on urea concentration has been found (e.g., −5.99 kJ mol^{-1} M^{-1} at 60 °C), whereas no significant variation of ΔH with urea was observed at lower temperatures. For ΔS the opposite behavior was found with small variations at low temperatures (e.g., 14.7 kJ mol^{-1} M^{-1} at 10 °C). The slopes of plots of ΔG against urea concentration provide estimates of *m* at various temperatures that are very similar to those listed in Table 1.

Estimate of ΔC_p. The values of the heat capacity change upon unfolding, listed in Table 2 and determined by the analysis of the stability curves, show that ΔC_p does not change significantly with increasing urea concentration. In the absence of urea, the calculated value of ΔC_p is 6.15 ± 0.60 kJ mol^{-1} K^{-1}. An alternative way to calculate ΔC_p is based on the Kirchoff equation:

$$d\Delta H_m/dT_m = \Delta C_p \quad (10)$$

The values of ΔH_m and T_m, determined from the van't Hoff analysis of the heat denaturation curves reported in Figure 2A, could be used to determine a plot of ΔH_m versus T_m, the slope of which yields a value of ΔC_p. This approach gives, however, only an apparent measurement of ΔC_p, as ΔH_m values obtained in the presence of urea underestimate the actual values due to a negative enthalpy interaction between urea and the protein surface area exposed to the solvent upon unfolding (*21*−*24*). Nevertheless, as explained above, our analysis allows us to determine the dependence of ΔH on urea concentration. This relationship was found to be linear at all temperatures, and thus, from our set of raw data corrected values of ΔH_m can be obtained in the absence of urea at the corresponding temperature. Figure 4 shows a Kirchoff plot with values of ΔH_m from heat denaturation experiments performed at different urea concentrations and pH values, the former being corrected as explained above. This approach provides a ΔC_p value of

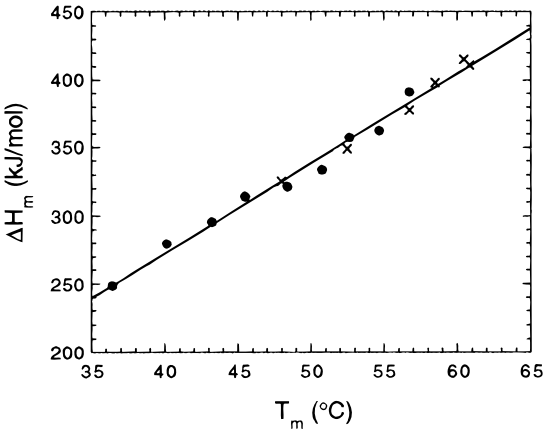

FIGURE 4: Plot of ΔH_m against T_m as determined from the analysis of heat denaturation curves at different pH values (crosses) and urea concentrations (filled circles). The latter values were calculated after taking into account the urea−protein enthalpy interaction (see the text for further details). The data were linearly fitted, and the slope provides a value of ΔC_p of 6.61 ± 0.70 kJ/mol.

6.61 ± 0.70 kJ mol^{-1} K^{-1}, in good agreement with that obtained from stability curves. However, deriving from a combination of heat and chemical denaturation, our best estimate of ΔC_p remains that obtained from the analysis of stability curves (6.15 ± 0.60 kJ mol^{-1} K^{-1}). This is the value we used for the analysis of the unfolding thermodynamics, described in the previous section, as a function of temperature and denaturant concentration.

Effect of pH on the Stability of AcP. The conformational stability of acylphosphatase has also been studied as a function of pH by means of urea denaturation curves performed at various pH values and monitored by far-UV CD and, in some cases, by fluorescence emission. Over the entire pH range analyzed, AcP unfolding was found to be highly reversible. At acidic pH the adequacy of a two-state model for the analysis of data has previously been demonstrated (*12*). At basic pH the steepness of the transition and the perfect coincidence of the urea denaturation curves as monitored by CD and fluorescence emission (data not shown) are consistent with a two-state transition also under these conditions. Figure 5 shows the analysis of C_m, *m*, and ΔG over a wide range of pH values. As can be seen, the pH dependencies of C_m and *m* are opposite. The lowest values of C_m are at both low and high pH, where the protein is expected to be unstable due to the protonation and deprotonation of acidic and basic residues, respectively. The highest stability of the protein against urea was found at pH 9.50 (C_m = 5.23 M), which is about 2 pH units lower than the isoionic point of acylphosphatase, 11.4 (*25*). Despite the behavior of C_m, the ΔG dependence on urea concentration (*m* value) was found to be high at extreme pH values and lower in the pH range 4.50−10.50, showing a minimum at pH 9.50 (m = 3.74 ± 0.15 kJ mol^{-1} M^{-1}). Thermally induced unfolding curves at various pH values give a pH dependence of T_m similar to that of C_m. T_m decreases rapidly as the pH is lowered from 4.50, whereas it does not vary consistently at neutral pH (data not shown). Heat denaturation is not completely reversible under basic conditions (*26*), and thus, the van't Hoff analysis must be limited only to neutral and acidic pH values where equilibrium unfolding conditions appear to persist.

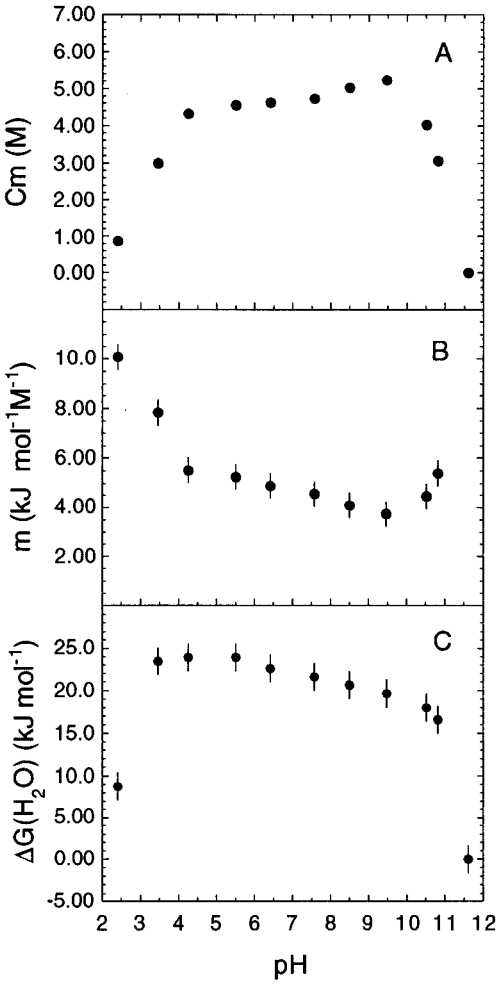

FIGURE 5: C_m (A), *m* value (B), and $\Delta G(H_2O)$ (C) as a function of pH for urea unfolding of AcP at 25 °C. Buffer solutions used in the analysis are reported in Materials and Methods. For C_m, standard deviation errors are approximately the size of symbols.

Figure 5C shows a plot of $\Delta G(H_2O)$ against pH. Several features are evident from the analysis of this figure. The first is that acylphosphatase is in its native state over a very wide range of pH values (2.50−11.00) with $\Delta G(H_2O)$ values greater than 10 kJ mol^{-1}. Outside this pH range, the stability of the enzyme collapses abruptly, most probably due to the excess of positive or negative charges present at low and high pH, respectively (*27*). Nevertheless, in the broad interval where the enzyme is fully folded, $\Delta G(H_2O)$ increases progressively with the decrease of pH with maximum values at pH 3.50−6.50, which is also approximately the pH range of maximum catalytic activity (*16*, *28*). The slope of a plot of $\Delta G(H_2O)$ against pH, such as that reported here, allows the number of protons taken up or released upon unfolding to be calculated (*29*, *30*). A reliable determination of this number at extremes of pH would require a much more detailed characterization of such a plot at these pH values. This is rather difficult to achieve because of the steepness of the plot in these two regions. However, our plot allows the qualitative conclusion that the release of protons at high pH outweighs the uptake at low pH due to the steeper slope in the former case. This is consistent with an isoionic point of 11.4 found for AcP (*25*). The slight slope observed at neutral to mildly basic pH could be caused by the difference in the pK_a value in the folded and unfolded states of one cysteine or tyrosine residue (see Discussion).

DISCUSSION

A comprehensive study of the unfolding of AcP is reported in this work. The most detailed analysis of the main thermodynamic quantities as a function of temperature and the interaction of AcP with urea has been carried out at pH 5.50 and under conditions of moderate ionic strength. This analysis has been supplemented by a measurement of the conformational stability of the protein over a wide range of pH values at 25 °C.

The basis of the overall study presented here is the assumption that the two-state model of folding is valid and correct for AcP when both urea and heat are used as denaturing agents. It has been previously reported that thermally and chemically induced unfolding curves of AcP are characterized by the presence of a single sharp transition (*12*). When normalized to the fraction of the native state, the transitions are perfectly superimposable using a variety of physical probes such as NMR, CD, and enzymatic activity measurements. Moreover, no changes are observed upon varying the protein concentration, indicating that aggregation is not significant. In the present study, intrinsic tryptophan fluorescence has also been used as a further probe to verify the coincidence of transitions at basic pH. Both thermal and chemical denaturation are reversible as demonstrated by the recovery of enzymatic activity (*31*) and CD signal characteristic of the folded state upon restoring the refolding conditions. Another key point for the applicability of our analysis is the assumption that the unfolded states generated by either urea or heat are effectively equivalent. In many cases, thermally denatured proteins have been shown to preserve a higher degree of residual structure than chemically denatured states (*32*, *33*). NMR and photo-CIDNP experiments suggest that this is not the case for AcP and indicate that very little persistent structure is present in the unfolded protein regardless of the method of denaturation (*12*). Furthermore, the same *m* values were obtained from urea-induced denaturation and from isothermal slices of heat denaturation curves (data not shown). Although we cannot rule out the existence of small structural differences between the two denatured proteins, these findings suggest that the two unfolded states must represent equivalent thermodynamic states, and thus, the difference in mean residue ellipticity in the two forms of denatured protein and found for AcP as well as for other proteins (*32*, *34*−*36*) is likely to be a baseline effect (*37*, *38*).

The description of the free-energy change of unfolding as a function of temperature represents the stability curve of the protein (*20*). Using a combination of thermal and urea unfolding experiments, the free-energy change upon unfolding was measured over a wide range of temperature, namely, 15−65 °C. Fitting data to the widely accepted eq 8 enables an estimate for the heat capacity change on unfolding to be made; this is the key parameter for a complete description of the energetics of the unfolding reaction (eqs 6−8). This procedure, using a simple spectroscopic probe to get a detailed insight into the thermodynamic parameters, was introduced by Pace and Laurentz (*39*) for the study of RNase A and adopted successfully, more recently, for other proteins

such as barstar (*36*) and HPr (*35*, *40*). The results reported here for AcP support the validity of this method as an alternative to the classical and widely used calorimetric techniques. Furthermore, while the ΔG of unfolding is determined in calorimetric studies by a long extrapolation performed with eq 8 from the high temperatures at which thermal denaturation occurs, our approach allows a full description of the stability curve, providing excellent experimental confirmation of the widely used expression itself. The applicability of eq 8 is subject to the assumption of a temperature-independent ΔC_p. Calorimetric studies have demonstrated that this parameter should not be treated as temperature-independent for a large body of proteins (*2*, *41*). Nevertheless, our study is limited to temperatures lower than 60 °C where a variation of ΔC_p greater than 20% has rarely been found. Besides, our data are consistent with an approximately invariant ΔC_p over the range of temperature considered, for two main reasons: the good fits of the ΔG data with eq 8 and the good agreement of our two best estimates of ΔC_p derived from different intervals of temperature (Figures 3 and 4).

One of the results of our study is the achievement of a comprehensive definition of AcP unfolding thermodynamics over a wide two-dimensional range of temperature and denaturant concentration. The parameters obtained from our analysis and their dependencies on temperature are compatible with the results obtained for other proteins (see ref *2* for a recent review). The enthalpy change of unfolding was found to increase linearly with temperature, a ΔC_p value of 6.15 ± 0.60 kJ mol^{-1} K^{-1} being the constant of proportionality (eq 6). Although ΔH was also found to be linearly dependent on denaturant concentration, this proved significantly less marked, and the highest changes were calculated at high temperatures (e.g., -5.99 kJ mol^{-1} M^{-1} at 60 °C). Similar findings are apparent for the entropy change upon unfolding, with a substantial variation with temperature (eq 7) and minor changes with urea. By contrast, the free-energy change upon unfolding exhibits a strong dependence not only on temperature (eq 9) but also on urea concentration with an abrupt decrease according to a constant of proportionality of 5.27 kJ mol^{-1} M^{-1} (*m* value, eq 3). This means that the destabilization of the native form of a protein accompanying the addition of denaturants is not caused by substantial changes of either ΔH or ΔS of unfolding, but that it arises simply from a favorable enthalpic factor being outweighed by an unfavorable entropic one.

The variations of the free-energy change with urea (*m* value) and the heat capacity change (ΔC_p) are those expected for a protein of the size of AcP (*1*, *42*, *43*). The results indicate that AcP is a relatively unstable protein among those showing full reversibility of the unfolding process both from the point of view of $\Delta G(H_2O)$ or of the midpoint of thermal and chemical denaturation. The low conformational stability of AcP cannot be ascribed to its small size, since ΔG of unfolding and the molecular weight of a protein are uncorrelated (*2*).

Study of the Acylphosphatase−Urea Interaction. At present, the mode by which chemical denaturants induce protein unfolding is not well understood. In our study, we have analyzed the variation of ΔG on urea concentration (*m* value) with temperature and pH. While no significant variation is found with temperature (Table 1), a consistent increase of more than 2-fold was observed from high to low pH values (Figure 5B). An alternative but similar model to the linear extrapolation model employed throughout the present work regards *m* as proportional to the number of additional sites available to interact with the denaturant after unfolding (*44*). Myers et al. (*1*) have pointed out that a linear positive relationship exists between *m* and the surface area exposed to solvent upon unfolding (ΔASA), calculated on the basis of known X-ray crystallographic structures of proteins and a random coil model for the unfolded conformation. Nevertheless, proteins with comparable ΔASA often exhibit consistent differences in *m*. This discrepancy suggests that other factors could play an important role in determining the magnitude of *m* (*1*, *15*). For example, the cleavage of disulfide bonds in RNase T1 leads to an increase of *m*, probably because the unfolded protein is on average more extended (*45*). The mounting evidence that chemically denatured proteins are not necessarily fully unfolded but retain at least local hydrophobic clusters (*33*, *46*, *47*) indicates that proteins may unfold to different extents. A study carried out with a range of denatured proteins using solvent perturbation difference spectroscopy (SPDS) shows that aromatic residues are more exposed to the solvent in those proteins which have a higher *m* value (*45*, *48*). All these observations appear to correlate *m* with the extent of surface area exposed in the unfolded protein. Our results suggest that the solvent exposure of chemically unfolded AcP does not change with temperature, *m* remaining constant in the range 15−60 °C. A similar result was obtained for barstar (*36*) and HPr (*35*, *40*). In contrast, *m* increases considerably at lower pH values for AcP. This was also found for the few other proteins studied in this way (*15*, *45*, *49*) and probably reflects the expansion of the unfolded state at low pH due to the electrostatic repulsion among the excess of positive charges. However, other possible explanations cannot be ruled out, and further investigation is necessary.

As far as the effect of denaturants on ΔC_p is concerned, earlier reports present a conflicting picture; while no trend was observed for AcP (present work), for β-lactoglobulin (*50*) and barstar (*36*), calorimetric studies have indicated that ΔC_p is higher in the presence of denaturants (*24*, *51*, *52*). Moreover, there are also reports of ΔC_p being lowered by the addition of denaturants (*35*, *53*). The extremely varied picture presented here and elsewhere indicates that the effect of chemical denaturants on ΔC_p may depend on the particular protein under study.

Effect of pH on the Stability of AcP. The study of the conformational stability of AcP over a wide range of pH was achieved by the analysis of urea-induced unfolding curves at various pH values. The analysis, performed according to Pace (*17*) and Santoro and Bolen (*19*), was possible throughout this interval of pH, since the transition appears to be highly reversible and to approach a two-state model. pH is known to influence the stability of a protein for two main reasons: (I) Changes of pH alter the ionization state of titratable groups leading to the formation/breakage of electrostatic repulsive/attractive interactions, and (II) pK_a values of chargeable groups are different in the folded and unfolded conformations; consequently, changes of pH leading to the titration of a group only in the unfolded form cause a destabilization of the overall native protein structure (*54*).

Figure 5 shows the results of our study. The mathematical analysis of a plot of ΔG versus pH (Figure 5C) is possible provided that the pK_a values of all the titratable residues are known in both the native and denatured forms of the protein. This information is not available for AcP to date, but the figure allows us to make some important remarks: the highly positive slope of the plot at very low pH indicates that pK_a values for acidic residues (i.e., aspartic and glutamic acid) are lower in the folded conformation than in the unfolded one (*30*, *54*). Similarly, the highly negative slope at high pH suggests that the pK_a values of basic residues (arginine and lysine) are higher in the folded form. According to Tanford (*30*), a simple calculation shows that a difference of only 0.4 pH units in the pK_a of a single histidine, cysteine, or tyrosine residue could account for the slight slope observed in the central part of the plot (pH 7−11). Muscle AcP lacks histidine residues (*25*) but contains one cysteine and several tyrosine residues that could be responsible for the observed decrease in stability.

Despite the observation that the maximum C_m value was found at basic pH, the highest conformational stability of AcP was estimated at mildly acidic pH (3.50−6.50). The strong dependence of *m* on pH is responsible for this apparent lack of correlation between C_m and $\Delta G(H_2O)$. According to Myers et al. (*1*) T_m and C_m do not represent a true index of the conformational stability of a protein, $\Delta G(H_2O)$ being the only parameter to account quantitatively for all of the interactions that stabilize the three-dimensional structure of a protein. Thus, T_m and C_m should be regarded as parameters simply indicating the resistance of a protein against heat and chemical denaturation, respectively. Since the isoionic point of AcP is 11.4 (*25*), the familiar assertion that the maximum conformational stability of a protein is around its isoionic point (*27*) does not apply to AcP when we regard $\Delta G(H_2O)$ as the main parameter reflecting the stability.

Comparison of AcP with Two Closely Related Proteins. A comprehensive study of protein unfolding thermodynamics as a function of temperature has been carried out also for HPr (*35*) and for the activation domains of procarboxypeptidase A2 and B (*55*, *56*). These proteins do not have any primary sequence homology but feature an apparently identical overall fold, consisting of two or three antiparallel α-helices packed against a four- or five-stranded antiparallel β-sheet. Thus, a comparative study of them represents a good opportunity to assess the importance of the folding pattern in determining the stability of proteins. Major differences in thermodynamic parameters can be found between the activation domains of procarboxypeptidases (ADA2h and AdB) and the other two proteins (Table 3). Surprisingly, the first two proteins show a relatively high thermal stability but a low stability to chemical denaturation (*55*, *56*). The low C_m and ΔH_m values are responsible for their conformational stability, which is significantly lower than that of AcP and HPr. By contrast, AcP and HPr have very similar characteristics, the only differences being a 7 °C higher T_m value for HPr and the absence of cold denaturation for AcP. Nevertheless, such a difference in T_m is not large, and the lack of cold denaturation for AcP can be ascribed to the $\Delta H_m/\Delta C_p$ ratio, which is higher for AcP than for HPr: a high $\Delta H_m/\Delta C_p$ ratio is responsible for cold denaturation occurring only below the freezing point of water and, hence, not being observable. What all these proteins have in common is the relatively low conformational stability when compared with values from a large body of proteins (*2*). This may in part be a consequence of the absence of disulfide bridges in all four proteins under consideration. Thus, the backbone hydrogen bonding and the structural constraints determined by the folding pattern of a protein do not represent the only determinants of the unfolding thermodynamics. The differences emerging from the comparison of the procarboxypeptidase activation domains with AcP and HPr clearly show that tertiary interactions, which are different within this group of proteins due to the absence of sequence identity, must play a significant role in determining the thermodynamic parameters for unfolding.

Table 3: Thermodynamic Parameters of Four Structurally Related Proteins[a]

protein	$\Delta G(H_2O)$ (kJ mol^{-1})	m (kJ mol^{-1} M^{-1})	C_m (M)	ΔH_m (kJ mol^{-1})	T_m (°C)	ΔC_p (kJ mol^{-1} K^{-1})
muscle AcP	22.1[b]	4.75[b]	4.68[b]	391[c]	56.7[c]	6.42[c]
HPr[d]	22.2	4.85	4.58	317	63.4	6.23
ADA2h[e]	17.0	4.0	4.2	199	77	3.6
AdB[f]	13.4	nd[g]	nd[g]	299	74.2	3.9

[a] Parameters regarding AcP were obtained from the present work, HPr from Nicholson & Sholtz (*35*), ADA2h from Villegas et al. (*55*), and AdB from Conejero-Lara et al. (*56*). [b] 50 mM *N*-ethylmorpholine, pH 7.0. [c] 50 mM acetate, pH 5.5. [d] 20 mM phosphate, pH 7.0. [e] 50 mM phosphate pH 7.0. [f] 20 mM phosphate, pH 7.5. Values are from differential scanning microcalorimetry experiments. [g] Not determined.

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