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Articles

Equilibrium Dissociation and Unfolding of the Arc Repressor Dimer†

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ABSTRACT: The equilibrium unfolding reaction of Arc repressor, a dimeric DNA binding protein encoded by bacteriophage P22, can be monitored by fluorescence or circular dichroism changes. The stability of Arc is concentration dependent, and the unfolding reaction is well described as a two-state transition from folded dimer to unfolded monomer. The stability of the protein is decreased at low pH and increased by high salt concentration. The salt dependence suggests that two ions bind preferentially to the folded protein. In 10 mM potassium phosphate (pH 7.3) and 100 mM KCl, the unfolding free energy reaches a maximum near room temperature. The results suggest that at the low protein concentrations where operator DNA binding is normally measured, Arc is predominantly monomeric and unfolded.

The bacteriophage P22 Arc repressor is a small protein (53 residues) that binds to DNA in a sequence-specific manner (Knight et al., 1989). Because the *arc* gene is small and selections for and against activity have been designed (Bowie & Sauer, 1989; Vershon et al., 1986), the Arc system is well suited to genetic analysis. For example, the phenotypic effects of over 200 different missense mutations have been determined, and, as a set, these mutations affect every residue of Arc (Bowie & Sauer, 1989; Vershon et al., 1986). The Arc system is also amenable to structural studies. Crystallographic work is in progress (Jordan et al., 1985), and the secondary structure of the protein has been determined by NMR studies (Zagorski et al., unpublished results). Residues 6–14 are part of a β-sheet region while residues 16–28 and 35–47 form amphipathic α-helices. Finally, Arc is of interest from the standpoint of DNA binding. Genetic and biochemical experiments make it clear that residues in the β-sheet region are responsible for the specificity of operator DNA binding (Bowie & Sauer, 1989; Knight et al., 1989; Knight & Sauer, 1989; Vershon et al., 1986), indicating that Arc uses a novel structural motif for DNA recognition.

Arc is tetrameric when bound to operator DNA (B. Brown, unpublished results), but dimeric or monomeric in solution, depending upon conditions (Vershon et al., 1985). In this paper, we examine the dissociation and unfolding reactions of the Arc dimer. We show that these reactions are closely coupled, so that denaturation can be described in terms of the equilibrium populations of folded dimers and unfolded monomers. This two-state behavior suggests that folded monomers are thermodynamically unstable. Our results also suggest that the operator binding reaction in vitro proceeds, at least in part, from unfolded monomers to DNA-bound tetramers. Thus, mutations which affect protein stability will also affect DNA binding because the folding and dimerization reactions are an integral part of the overall DNA binding reaction.

Materials and Methods

Protein and Reagents. Arc was purified as described previously (Vershon et al., 1985, 1986) and stored frozen in a buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 0.1 mM EDTA. Protein concentrations were determined by using an extinction coefficient of 7800 M^{-1} cm^{-1} at 280 nm (Vershon et al., 1985). An 8 M stock solution of guanidine hydrochloride (GuHCl) was obtained from Pierce Chemical Co. Stock 8 M urea solutions were prepared freshly each day using high purity grade urea obtained from United States Biochemical Corp. and distilled, deionized water.

Urea and GuHCl Unfolding Monitored by Fluorescence. For denaturation experiments, protein samples were incubated for at least 1 h at each GuHCl or urea concentration prior to taking fluorescence measurements. For urea denaturation experiments at different temperatures, solutions were incubated for at least 30 min in a water bath at the appropriate temperature (maintained to ±1 °C) prior to fluorescence intensity measurements. This 30-min time period was more than sufficient to achieve equilibrium since no spectral changes were

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observed in the samples after a 5-min incubation at each temperature. In fact, unfolding and refolding of Arc were generally found to be complete within seconds under a variety of conditions. Urea or GuHCl denaturation curves obtained starting with fully folded protein were identical with renaturation curves starting with fully unfolded protein, indicating that these reactions are fully reversible.

Fluorescence measurements were made with a Perkin-Elmer MPF-3 spectrofluorometer in the early phases of this work and a Greg PC spectrofluorometer for the majority of the studies. For collection of emission spectra, the samples were excited at 280 nm, and the emission intensity was adjusted to about 80 000 photons detected per second at λ_{max}. Spectra were recorded in 1-nm wavelength increments, and the signal was acquired for 5 s at each wavelength. The spectrum of each sample was corrected by subtraction of the buffer alone. For urea and guanidine denaturation experiments, the samples were excited at 280 nm, and the emission intensity was recorded at 327 nm. The emission intensity of the protein solution in the absence of denaturant was adjusted to about 80 000 photons per second, and the emission intensity of each sample was acquired for 15 s. The absorbance at 280 nm for the highest Arc concentrations used in these experiments (16 μM) is only 0.125, and a smaller than 5% change in absorbance accompanies unfolding. Consequently, observations of the unfolding reaction by fluorescence should not be significantly altered by inner filter effects.

Thermal Denaturation Monitored by Circular Dichroism. Circular dichroism measurements were performed by using an AVIV Model 60DS spectropolarimeter. Thermal denaturation was monitored by changes in circular dichroism at 222 nm in 2.5 °C steps. The samples were equilibrated for 2.5 min at each temperature, and the signal was recorded for 1 min. This rate of heating was found adequate to achieve equilibrium at each temperature. The melts were found to be more reversible at low pH than at higher pH. At pH 4, the ellipticity at 222 nm returned to better than 95% of its starting value while at pH 7.3, the ellipticity returned to 80–90% of its starting value. When melts were held at the T_m for extended periods of time at either pH, the ellipticity at 222 nm remained constant. Hence, the partial irreversibility of the unfolding transitions appears to result from protein damage that occurs at high temperature. For spectra, multiple scans (10 or more) were obtained for each sample and averaged. Each spectrum was recorded in 1-nm wavelength increments and the signal acquired for 1 s at each wavelength.

Results

Probes of the Folding Equilibrium. Two spectroscopic methods can be used to monitor the unfolding reaction of Arc (Bowie & Sauer, 1989a,b; Vershon et al., 1985, 1986). Figure 1a shows the circular dichroism spectra of native Arc and heat-denatured protein. The peak of negative ellipticity at 222 nm in the native spectrum is characteristic of a high degree of α-helical character, and changes in this signal provide one method of following the unfolding reaction. Figure 1b shows the fluorescence emission spectra of native Arc and the protein denatured in urea. Arc contains a single tryptophan residue at position 14. The emission spectrum of the unfolded protein has a λ_{max} at 347 nm which is similar to that of tryptophan as a free amino acid. In contrast, λ_{max} of the folded protein is significantly blue-shifted to 327 nm, and this shift is accompanied by a large increase in intensity. This suggests that the tryptophan is buried in a hydrophobic environment in the folded protein (Teale, 1960). The fluorescence intensity change at 327 nm provides a second probe of Arc unfolding.

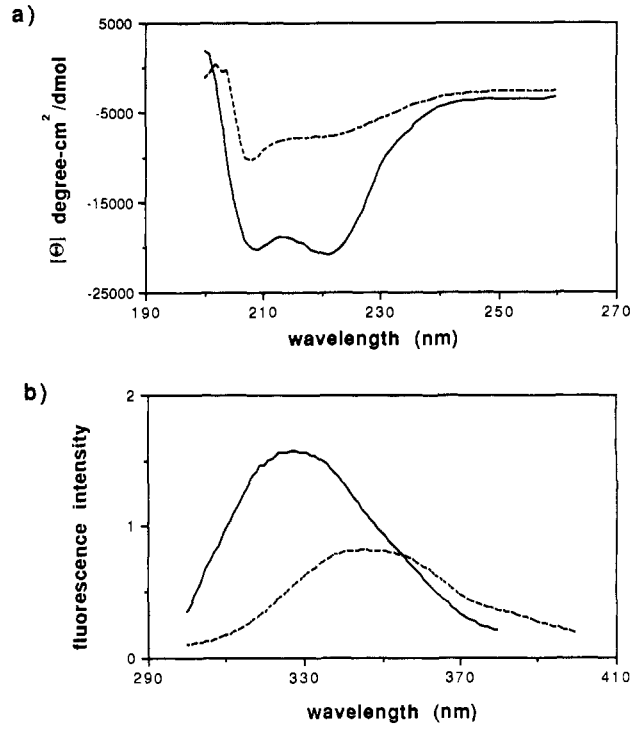

FIGURE 1: Circular dichroism and fluorescence spectra of native and denatured Arc. (a) CD spectra at 25 °C (solid line) and 80 °C (dotted line) of 16 μM Arc in 10 mM potassium phosphate (pH 7.3) and 100 mM KCl. (b) Fluorescence spectra of 1.6 μM Arc in 10 mM potassium phosphate (pH 7.3) and 100 mM KCl at 25 °C with (dotted line) or without (solid line) 4.8 M urea.

Denaturation Is Two-State. At the concentrations used in this work, Arc is dimeric under physiological conditions (Vershon et al., 1985). Thus, the overall unfolding reaction must start with the folder dimer (A_2) and end with two unfolded monomers (2U). There are, however, several possible descriptions of the unfolding reaction, depending on the relative stabilities of dimers and folded monomers (A). If both the folded monomer and folded dimer are significantly populated states in the denaturation transition zones, the overall unfolding reaction will be described by

$$A_2 \overset{K_1}{\rightleftharpoons} 2A \overset{K_2}{\rightleftharpoons} 2U \quad (1)$$

where

$$K_1 = [A]^2/[A_2] \text{ and } K_2 = [U]/[A]$$

In this case, one might see biphasic denaturation curves or nonsuperimposible transitions if the spectral probes used are differentially sensitive to the various species. As shown in Figure 2, however, this is not the case. The unfolding reaction follows a single and coincident transition, when followed by changes in either fluorescence or circular dichroism. This result suggests either that both CD and fluorescence respond in equivalent manners to the various equilibrium species or that only two states are significantly populated in the denaturation transition zone. The latter possibility is discussed below.

If the Arc dimer dissociates prior to the transition zone of the denaturation curves, only the equilibrium between folded and unfolded monomer would be observed. In this case, however, the observed reaction would be unimolecular, and the equilibrium populations of folded and unfolded protein would be independent of protein concentration. This possibility is ruled out by the experiments of Figure 3a,b, which show that the stability of Arc increases with higher concentrations.

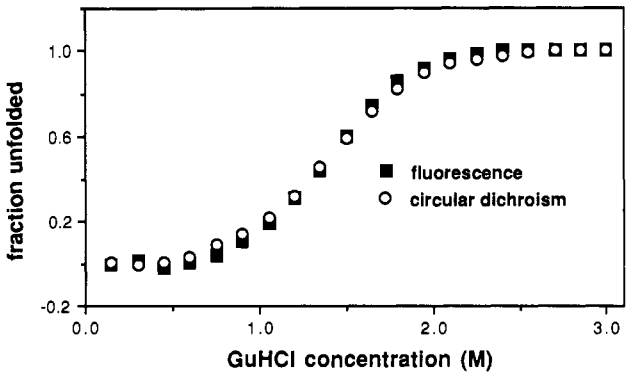

FIGURE 2: GuHCl denaturation curves monitored by fluorescence or circular dichroism. The data were obtained with 16 μM Arc in 50 mM Tris-HCl (pH 7.5) and 100 mM KCl, at 20 °C.

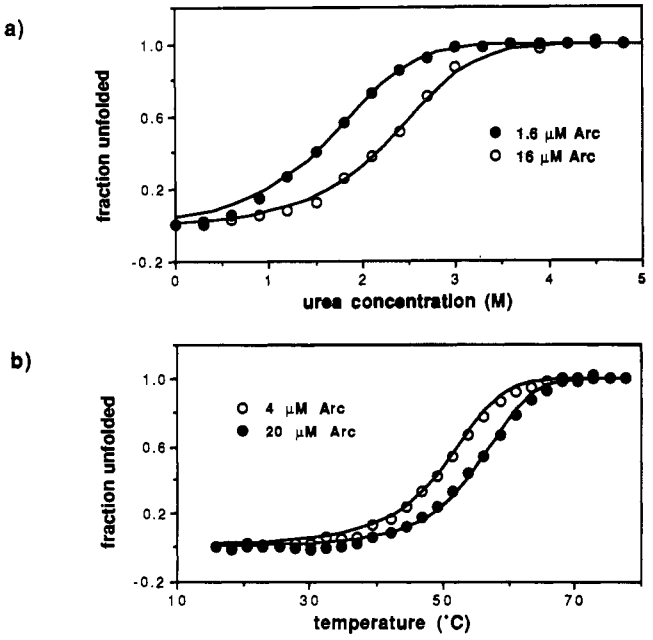

FIGURE 3: Concentration dependence of Arc denaturation. (a) Urea denaturation of 1.6 and 16 μM Arc in 10 mM potassium phosphate (pH 7.3) and 100 mM KCl at 25 °C. The unfolding reaction was monitored by changes in the intensity of fluorescence at 327 nm. Theoretical curves were calculated with eq 4 using $\Delta G_u^{H_2O}$ = 11.1 kcal/mol and m = −1.91 for 1.6 μM Arc and $\Delta G_u^{H_2O}$ = 11.0 kcal/mol and m = −1.91 for the 16 μM Arc concentration. These parameters were obtained from a least-squared fit to eq 4, using ΔG_u values determined in the transition zones of the two denaturation curves. (b) Thermal denaturation curves of 4 and 20 μM Arc in 10 mM potassium phosphate (pH 7.3) and 100 mM KCl. The unfolding reaction was monitored by circular dichroism changes at 222 nm. Theoretical curves were calculated from eq 5 using T_1 = 54 °C, ΔG_1 = 7.3 kcal/mol, ΔH_1 = 71 kcal/mol, and ΔC_p = 1.6 kcal/(mol·deg) (see text).

The dimer state must, therefore, be significantly populated in the transition zone.

If the folded monomer state is essentially unpopulated, unfolding could then be described as a concerted reaction:

$$A_2 \overset{K_u}{\rightleftharpoons} 2U \qquad (2)$$

where

$$K_u = [U]^2/[A_2] = 2P_t[f_u^2/(1 - f_u)] \qquad (3)$$

with P_t being the total protein concentration and f_u the fraction of unfolded protein. If this model provides a reasonable thermodynamic description of the denaturation reaction, then one should calculate the same value of K_u or ΔG_u (calculated as $-RT \ln K_u$) from experiments performed at different protein concentrations.

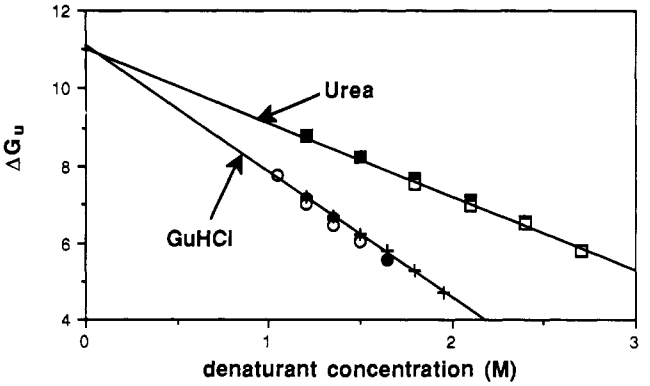

FIGURE 4: Unfolding free energy (ΔG_u) of Arc repressor as a function of urea concentration or GuHCl concentration. ΔG_u values were calculated by using the two-state assumption (eq 3). The urea denaturation free energies at Arc concentrations of 1.6 μM (filled squares) and 16 μM (open squares) were calculated by using the data shown in Figure 3. The least-squares line has a slope of −1.91 kcal/(mol·M) and an intercept at $\Delta G_u^{H_2O}$ = 11 kcal/mol. The data for GuHCl denaturation free energies were calculated from denaturation curves obtained with Arc concentrations of 4 μM (open circles), 16 μM (filled circles), and 32 μM (crosses) in 50 mM Tris-HCl (pH 7.5) and 100 mM KCl at 20 °C. The least-squares line has a slope of −3.27 kcal/(mol·M) and an intercept at $\Delta G_u^{H_2O}$ = 11.1 kcal/mol.

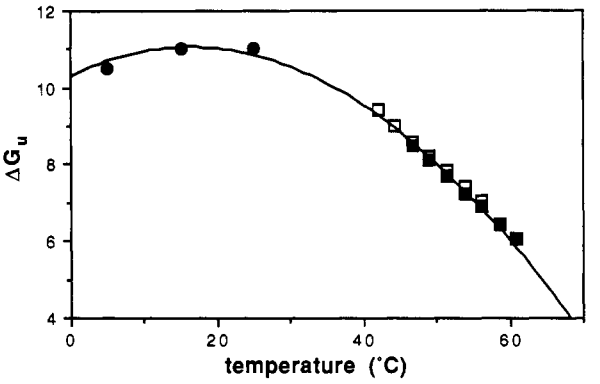

FIGURE 5: Unfolding free energy as a function of temperature. Squares represent unfolding free energies measured directly from the transition zones of the thermal denaturation curves shown in Figure 3 at Arc concentrations of 4 μM (open squares) and 20 μM (filled squares). Circles represent $\Delta G_u^{H_2O}$ values determined from an analysis of urea denaturation curves determined at various temperatures. The urea denaturation curves were obtained at 1.6 μM Arc concentrations in the same buffer used for the thermal denaturation curves. The curve shows the least-squares fit of the points to eq 5 as described in the text.

Figure 4 shows unfolding free energies, ΔG_u, calculated for different protein concentrations as a function of urea or GuHCl concentration. The free energies calculated at different protein concentrations assuming the two-state model are the same to within experimental error. Thus, the data are consistent with the two-state assumption (eq 3) in the transition zone of the urea and GuHCl denaturation curves. The data points for both denaturants were least-squares fit to the equation:

$$\Delta G_u = \Delta G_u^{H_2O} + m[\text{denaturant}] \qquad (4)$$

where $\Delta G_u^{H_2O}$ is the extrapolated unfolding free energy in the absence of denaturant. Both GuHCl and urea denaturation gave $\Delta G_u^{H_2O}$ values of about 11 kcal/mol ($K_u = 10^{-8}$ M). Thermal denaturation is also well approximated by the two-state model. The square symbols in Figure 5 show ΔG_u values calculated for the two thermal denaturation experiments shown in Figure 3b using the two-state assumption.

Temperature Dependence of the Unfolding Free Energy. The values of the thermodynamic parameters ΔG_u, ΔH_u, and

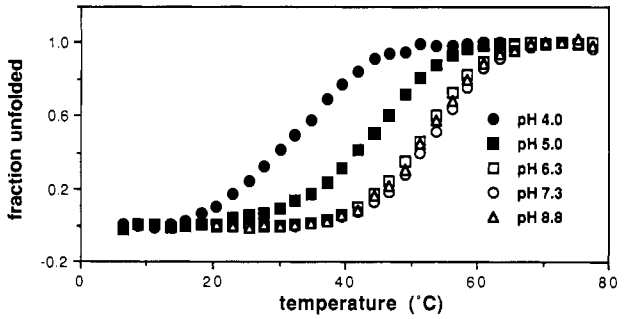

FIGURE 6: pH dependence of Arc stability. Thermal denaturation curves were obtained by using protein concentrations of 8 μM in 100 mM KCl and (i) 10 mM potassium acetate (pH 4.0), (ii) 10 mM potassium acetate (pH 5.0), (iii) 10 mM potassium phosphate (pH 6.3), (iv) 10 mM potassium phosphate (pH 7.3), and (v) 10 mM glycine (pH 8.8). The unfolding reaction was monitored by circular dichroism changes at 222 nm.

ΔS_u for denaturation vary as a function of temperature depending upon the value of ΔC_p, the constant-pressure heat capacity change for the reaction (Privalov, 1979). To estimate the value of ΔC_p for Arc denaturation, we have followed the recent suggestion of Pace and Laurents (1989) and used urea denaturation curves to determine unfolding free energies at temperatures well below the thermal transition zone. Urea denaturation curves were determined at 5, 15, and 25 °C and analyzed as discussed above to determine $\Delta G_u^{H_2O}$ values at each temperature. These values are plotted in Figure 5. Assuming that ΔC_p is approximately constant over the temperature range of the experimental data, the unfolding free energy, ΔG_T, at temperature T will be given by

$$\Delta G_T = \Delta H_1 - (T/T_1)(\Delta H_1 - \Delta G_1) + \Delta C_p[T - T_1 - T \ln (T/T_1)] \quad (5)$$

where ΔH_1 and ΔG_1 are the unfolding enthalpy and the unfolding free energy at some temperature T_1 in the thermal transition zone where ΔG_1 can be measured (Privalov, 1979). The data shown in Figure 5 were least-squares-fit to eq 5 using T_1 = 54 °C and ΔG_1 = 7.3 kcal/mol. The best fit was obtained with ΔC_p = 1.6 kcal/(mol·deg) and ΔH_1 = 71 kcal/mol. A theoretical curve using these values is also shown in Figure 5.

The data shown in Figure 5 indicate a slight (0.4 kcal/mol) decrease in Arc stability at 5 °C relative to 25 °C. To confirm the stability decrease at low temperature, we determined the fluorescence spectra at 2 and 20 °C of an Arc sample under conditions where the protein is about half-denatured [1.6 μM Arc, 1.5 M urea, 10 mM Tris (pH 7.3), and 100 mM KCl]. The spectrum at the low temperature was significantly red-shifted and had diminished intensity. Control experiments showed that this change could not be caused by temperature-dependent changes in the fluorescence intensity of the folded or unfolded protein (which increase with decreasing temperature) without a shift from folded to unfolded Arc. Thus, Arc indeed appears to be destabilized at the lower temperature.[1]

pH and Salt Dependence of Arc Stability. The stability of Arc is both pH and salt dependent. As shown in Figure 6, the thermal stability of Arc decreases significantly as the pH is lowered below 6. At a concentration of 32 μM, Arc was found to be completely unfolded at pH 2 and room temperature (data not shown).

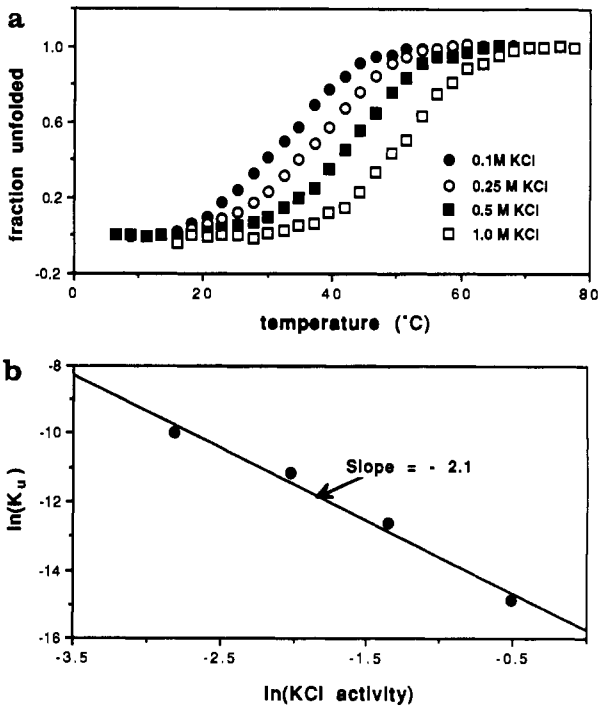

FIGURE 7: Salt dependence of Arc stability. (a) Thermal denaturation curves at protein concentrations of 8 μM in 10 mM potassium acetate (pH 4.0) and 0.1, 0.25, 0.5, and 1.0 M KCl. The unfolding reaction was monitored by circular dichroism changes at 222 nm. (b) Plot of ln K_u vs ln KCl activity at 40 °C. Equilibrium constants at 40 °C were estimated by linear extrapolation of van't Hoff plots of the data from the denaturation curves shown in (a).

Table I: Effect of Various Salts on the Thermal Stability of Arc Repressor[a]

salt	concn (M)	T_m (°C) at pH 4	T_m (°C) at pH 7.3
KCl	0.1	33	54
KCl	0.25	38	56
KCl	0.5	43	60
KCl	1.0	51	63
NaCl	0.5	44	
NH_4Cl	0.5	45	
$MgCl_2$	0.5	46	
KH_2PO_4	0.5	56	
Na_2SO_4	0.5	61	

[a] T_m is the temperature at which the protein is half-denatured. These experiments were performed at Arc concentrations of 8 μM in 10 mM potassium acetate (pH 4.0) or 10 mM potassium phosphate (pH 7.3).

Figure 7a shows melting curves for Arc at different concentrations of KCl. The protein is clearly stabilized by increasing salt concentration. Since potassium and chloride ions have relatively minor effects on hydrophobic forces (von Hippel & Wong, 1964), it would appear that salt stabilizes Arc because ions bind preferentially to the folded form of the protein. The difference in the number of ions bound to the folded state relative to the unfolded state can be obtained from the slope of a plot of ln a vs ln K_u, where a is the KCl activity and K_u is the apparent equilibrium constant for denaturation (Pace & Grimsley, 1988; Record et al., 1978). Such a plot is shown in Figure 7b for K_u values at 40 °C. The slope of the line is −2.1, indicating that two ions are bound to the folded dimer.

Table I lists the effect of various salts on the melting temperature of Arc. Because sulfate and phosphate ions can affect

[1] A worst case estimate of the error in ΔC_p can be obtained from the finding that ΔG_u reaches a maximum above 5 °C. Since $d\Delta G/dT = -\Delta S$, ΔS_u, the entropy of unfolding, must also reach zero above 5 °C. At 54 °C, ΔS_u = 0.2 kcal/(mol·deg), and the dependence of ΔS on temperature is given by $d\Delta S/dT = \Delta C_p/T$. If ΔS reaches zero at 5 °C, then ΔC_p (worst case) = 1.2 kcal/(mol·deg).

hydrophobic forces (von Hippel & Wong, 1964), it is not possible, in these cases, to assume that salt stabilization is caused solely by differential ion binding. Nevertheless, since Arc has a significant net positive charge and binds strongly to DNA and negatively charged ion-exchange columns, but not to positively charged ion-exchange columns, it would be surprising if the protein did not bind anions such as chloride, phosphate, and sulfate.

Discussion

A number of small DNA binding proteins appear to be folded only as dimers. These include the λ Cro protein and the Trp repressor. The crystal structure of Cro shows two distinct subunits, with a dimer interface formed by a region of antiparallel β-sheet (Anderson et al., 1981). While the structure of the Cro dimer appears to contain discrete domains, dissociation and denaturation of Cro dimers show two-state behavior (Pakula & Sauer, 1989), indicating that the folded Cro monomer must be unstable. For Trp repressor, the situation is different. In this case, the structural elements from each monomer are interwoven in the dimer and cannot be obviously separated into distinct folding units (Schevitz et al., 1985). As might be expected, Trp repressor also shows two-state dissociation and unfolding (C. R. Matthews, personal communication). In this case, however, unlike the λ Cro protein, it makes no sense to speak of folded monomers in a structural sense.

The results presented here show that the Arc dimer dissociates and unfolds in a concerted two-state reaction. Thus, the equilibrium constant measured in the transition zone of the denaturation curves reflects the overall stability of the Arc dimer relative to the unfolded monomer. It is possible that the Arc dimer consists of two unstable domains as seen in Cro repressor or as a single domain containing two polypeptide chains as seen in Trp repressor. In either case, the finding that folding and dimerization in Arc are tightly coupled suggests that Arc needs to form dimers to maintain a stably folded structure.

The dissociation–unfolding constant for Arc, K_u, has a value of about 10^{-8} M at 25 °C in 10 mM potassium phosphate (pH 7.3) and 100 mM KCl. Thus, the protein is expected to be largely dissociated below 10^{-8} M. Under similar buffer conditions, half-maximal binding of Arc to operator DNA is observed at a protein concentration of about 5×10^{-10} M (Vershon et al., 1989). Thus, Arc is almost certainly monomeric and probably unfolded at the concentrations where operator binding is measured. When bound to the operator under these conditions, Arc is tetrameric (B. Brown, unpublished results). Hence, operator binding is a complex reaction that involves folding and dimer formation, tetramer formation, and DNA binding. Clearly, effects on any of these coupled reactions will affect the level of binding observed, and interpreting the effects of solution conditions or amino acid substitutions on operator binding will be complicated. For example, Arc binding to operator DNA changes as a function of temperature, salt, and pH (Vershon et al., 1987), but as we have shown here, so does the stability of the dimer. In some cases, these effects are in opposite directions. For example, operator binding becomes weaker as the KCl concentration is raised (Vershon et al., 1987), whereas the dimer becomes more stable. Hence, the intrinsic salt dependence of dimer–operator binding must be greater than is indicated by the overall salt dependence of the binding reaction. The consequences of changes in pH, salt, and temperature on Arc stability reported here should be quite useful in helping to understand the overall operator binding reaction.

Acknowledgments

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References

Anderson, W. F., Ohlendorf, D. H., Takeda, Y., & Matthews, B. W. (1981) *Nature 290*, 754–758.

Bowie, J. U., & Sauer, R. T. (1989a) *J. Biol. Chem. 264*, 7596–7602.

Bowie, J. U., & Sauer, R. T. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2152–2156.

Jordan, S. R., Pabo, C. O., Vershon, A. K., & Sauer, R. T. (1985) *J. Mol. Biol. 185*, 445–446.

Knight, K. L., & Sauer, R. T. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 797–801.

Knight, K. L., Bowie, J. U., Vershon, A. K., Kelley, R. D., & Sauer, R. T. (1989) *J. Biol. Chem. 264*, 3639–3642.

Pace, C. N., & Grimsley, G. R. (1988) *Biochemistry 27*, 3242–3246.

Pace, C. N., & Laurents, D. V. (1989) *Biochemistry 28*, 2520–2525.

Pakula, A. A., & Sauer, R. T. (1989) *Proteins: Struct., Funct., Genet. 5*, 202–210.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.

Record, M. T., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.

Schevitz, R. W., Otwinowski, Z., Joachimiak, A., Lawson, C. L., & Sigler, P. B. (1985) *Nature 317*, 782–786.

Teale, F. W. J. (1960) *Biochem. J. 76*, 381.

Vershon, A. K., Youderian, P., Susskind, M. M., & Sauer, R. T. (1985) *J. Biol. Chem. 260*, 12124–12129.

Vershon, A. K., Bowie, J. U., Karplus, T. M., & Sauer, R. T. (1986) *Proteins: Struct., Funct., Genet. 1*, 302–311.

Vershon, A. K., Liao, S., McClure, W. R., & Sauer, R. T. (1987) *J. Mol. Biol. 195*, 323–331.

Vershon, A. K., Kelley, R. D., & Sauer, R. T. (1989) *J. Biol. Chem. 264*, 3267–3273.

von Hippel, P. H., & Wong, K. (1964) *Science 145*, 577–580.