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Conformational stability of apoflavodoxin

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Abstract

Flavodoxins are α/β proteins that mediate electron transfer reactions. The conformational stability of apoflavodoxin from *Anabæna* PCC 7119 has been studied by calorimetry and urea denaturation as a function of pH and ionic strength. At pH > 12, the protein is unfolded. Between pH 11 and pH 6, the apoprotein is folded properly as judged from near-ultraviolet (UV) circular dichroism (CD) and high-field ^{1}H NMR spectra. In this pH interval, apoflavodoxin is a monomer and its unfolding by urea or temperature follows a simple two-state mechanism. The specific heat capacity of unfolding for this native conformation is unusually low. Near its isoelectric point (3.9), the protein is highly insoluble. At lower pH values (pH 3.5–2.0), apoflavodoxin adopts a conformation with the properties of a molten globule. Although apoflavodoxin at pH 2 unfolds cooperatively with urea in a reversible fashion and the fluorescence and far-UV CD unfolding curves coincide, the transition midpoint depends on the concentration of protein, ruling out a simple two-state process at acidic pH. Apoflavodoxin constitutes a promising system for the analysis of the stability and folding of α/β proteins and for the study of the interaction between apoflavoproteins and their corresponding redox cofactors.

Keywords: calorimetry; flavodoxin; molten globule; protein folding; protein stability; redox proteins

Many proteins fold spontaneously in vitro but the mechanism of this process is not well understood (Creighton, 1992; Fersht, 1993; Dobson et al., 1994). One of the difficulties lies in our limited understanding of the noncovalent interactions that stabilize the folded and intermediate conformations of proteins. Data gathered in the course of recent years indicate that the folded conformation of a protein is marginally more stable than the unfolded conformations. This difference in stability is a delicate balance between numerous interactions that protein atoms may form either with solvent or with other atoms within the protein in any of its possible conformations. The folded state of proteins is usually the most amenable to structural analysis, and its energetics can be characterized by a combination of protein engineering (Serrano et al., 1992), calorimetry (Privalov, 1992), and other unfolding equilibrium studies (Pace et al., 1989). Equilibrium intermediates, which could be very similar to kinetic intermediates (Jennings & Wright, 1993), are more elusive. Their structure may be studied by NMR (Hughson et al., 1990; Redfield et al., 1994), but at a lower resolution; they do not seem to absorb much heat in order to unfold (Haynie & Freire, 1993) and, perhaps because they are often thought of as being only weakly cooperative, the analysis of their energetics by protein engineering is only at its beginnings (Hughson et al., 1991; Carra et al., 1994).

Flavodoxins are photosynthetic electron transfer proteins (Rogers, 1987) that carry a noncovalently bound flavin mononucleotide prosthetic group. The redox properties of this group are modulated by the apoprotein. Flavodoxins may appear in three different redox forms of presumably different stability. From a structural point of view, flavodoxins are archetypal examples of α/β proteins with an open β-sheet, a class of proteins for which only a few detailed stability studies are available (Filimonov et al., 1993; Jennings et al., 1993). We have chosen the flavodoxin from the cyanobacteria *Anabæna* PCC 7119 (Fig. 1) as a model protein for stability and folding studies. This protein appears well suited for the purpose: its crystal structure is known (Rao et al., 1992, for the holo form; Genzor et al., 1996, for the apo form), its gene has been cloned (Fillat et al., 1991), and the protein can be expressed in *Escherichia coli* with good yields. Flavodoxin contains four tryptophane residues, one *trans* proline, and no disulphide bridges. The main contributor to protein fluorescence is tryptophane 120 (C.G. Genzor & J.

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Abbreviations: ANS, 8-anilino-naphthalenesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; Tris, tris(hydroximethyl) amino methane; DSC, differential scanning calorimetry.

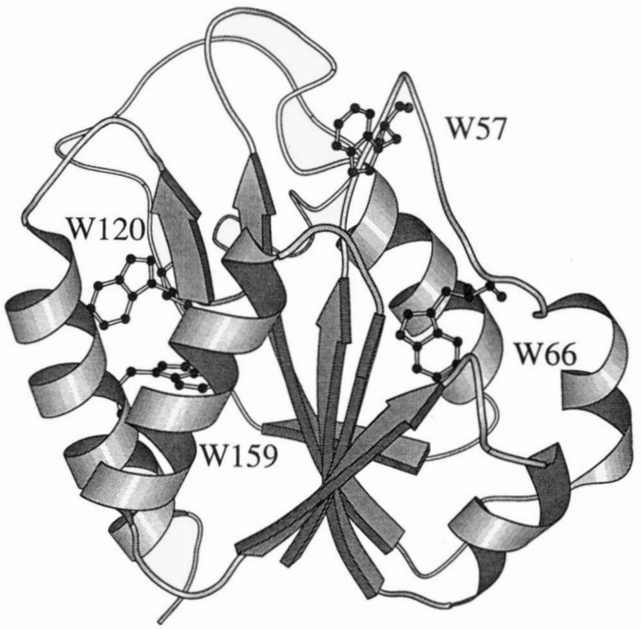

Fig. 1. Drawing of the X-ray structure of *Anabœna* PCC 7120 flavodoxin (Rao et al., 1992) drawn with MOLSCRIPT (Kraulis, 1991). The four tryptophane residues in the protein are shown. This flavodoxin is identical to that from *Anabœna* PCC 7119, whose recombinant form has been used in this study.

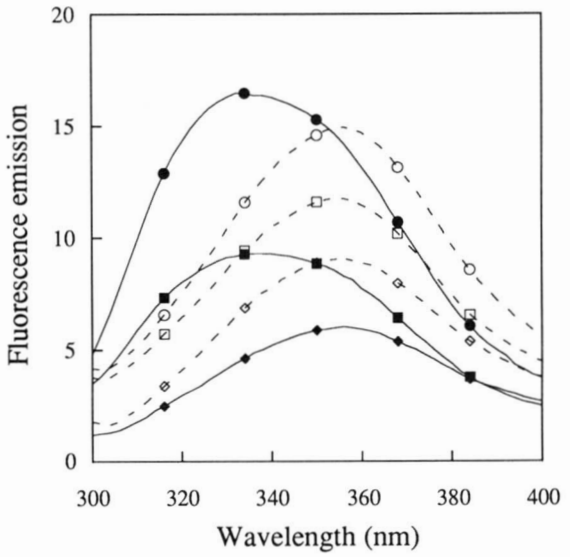

Fig. 2. Emission fluorescence spectra of apoflavodoxin at several pH values and urea concentrations. Solid lines and solid symbols, 0 M urea; dashed lines and open symbols, 8 M urea. ●, ○, pH 7; ■, □, pH 2.0; ◆, ◇, pH 12. All spectra were recorded at 25 °C. Protein concentration was 2.4 μM. Fluorescence is given in arbitrary units.

Sancho, unpubl.; see Fig. 1). In this paper, we undertake the study of the stability of the apoflavodoxin as a first step toward characterizing its interaction with the redox group. At neutral pH, apoflavodoxin from *Anabœna* is folded and undergoes reversible heat and urea denaturation complying to a two-state model. The protein thus can fold, at physiologic pH, in the absence of its redox cofactor. At acid pH, apoflavodoxin adopts a conformation with the properties of a molten globule. This conformation is also cooperatively unfolded by urea, but the transition midpoint depends on the concentration of protein, indicating that at low pH apoflavodoxin is not monomeric.

Results

Spectroscopic characterization of apoflavodoxin at several pHs

Fluorescence emission spectra of apoflavodoxin at pH 2.0, 7.0, and 12.0 are shown in Figure 2. At neutral pH, the emission maximum is at 335 nm. Addition of 8 M urea shifts the maximum to 355 nm, which is typical of unfolded proteins with fully exposed tryptophan residues. Fluorescence quantum yield is lower at pH 2.0 than at pH 7.0, but the maximum of emission remains close to 335 nm, indicating that the tryptophan residues are not fully exposed to the solvent. As in the previous case, addition of 8 M urea shifts the maximum to 355 nm. At pH 12.0, maximal emission occurs at 355 nm regardless of the presence of urea, suggesting that the protein is denatured at this pH.

Circular dichroism (CD) spectra in the far-ultraviolet (UV) indicate that, at both pH 7 and 2, apoflavodoxin contains a substantial amount of secondary structure (Fig. 3A). The differences between the two spectra might be due to a different contribution of aromatic residues at pH 7.0 and pH 2.0 (Vuilleumier et al., 1993). At either pH in the presence of 8 M urea, and at pH 12 with or without urea, the spectra suggest denatured conformations. CD spectra in the near-UV are negligible at pH 2 and 12 (Fig. 3B). Only at pH 7 do some of the aromatic residues appear to be in a rigid environment. The spectrum at pH 7 of the urea denatured protein is also flat.

^{1}H NMR spectra of apoflavodoxin in water at three pH values are shown in Figure 4. At pH 7 (Fig. 4C,D), dispersion of the amide protons and the presence of several high-field methyl resonances indicate a well-defined tertiary structure. In contrast, high-field resonances are no longer present in the spectra at pH 13.0 (Fig. 4A,B). The amide resonances are also absent, but this is probably due to fast exchange with the solvent. The spectra at pH 13.0 correlate well with what is expected for an unfolded protein at basic pH. At pH 2 (Fig. 4E,F), the high-field resonances have almost disappeared and dispersion of the aromatic and amide protons is reduced.

ANS binding as a function of pH

The hydrophobic fluorescence probe ANS increases its fluorescence intensity when bound to proteins. The affinity of ANS for proteins is greater when these have hydrophobic clusters that are exposed to the solvent, as is the case of molten globule intermediates (Semisotnov et al., 1991). We have followed the binding of ANS to apoflavodoxin as a function of pH. In the presence of apoflavodoxin, ANS fluorescence changes little from pH 10 to 5.5 (Fig. 5). In contrast, there is a sharp increase from pH 5 to 3.5, and the fluorescence remains at a maximum from pH 3.5 to 2.0. The high fluorescence of ANS may suggest the presence of a molten globule conformation of apoflavodoxin at acid pH.

While performing the ANS-binding experiment, we observed that solutions with pH values corresponding to the transition

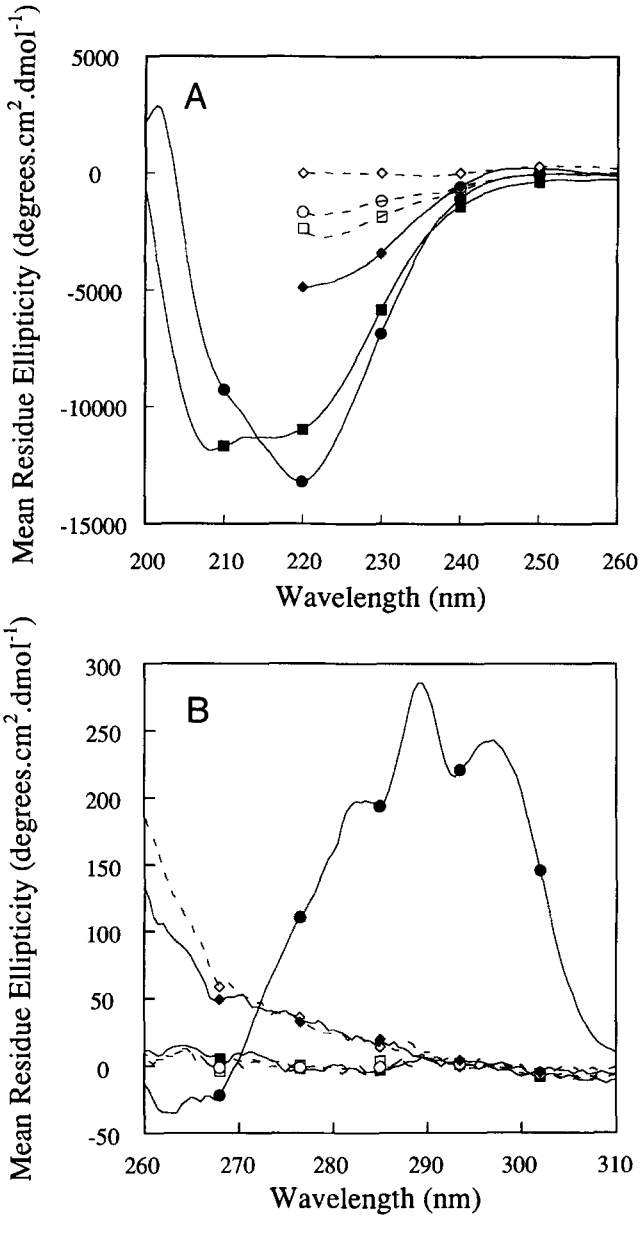

Fig. 3. CD spectra of apoflavodoxin at several pH values and urea concentrations. Solid lines and solid symbols, 0 M urea; dashed lines and open symbols, 8 M urea. ●, ○, pH 7; ■, □, pH 2.0; ◆, ◇, pH 12. **A:** Far-UV spectra. Protein concentration was 1.3 μM. **B:** Near-UV spectra. Protein concentration was 13.0 μM. All spectra were recorded at 25 °C in 25 mM sodium phosphate.

from low fluorescence to high fluorescence were turbid, which prompted us to determine the solubility of apoflavodoxin as a function of pH. Aliquots of the protein were incubated for 30 min in buffer solutions of different pH values and then centrifuged to remove protein aggregates. The remaining soluble protein was quantified from the optical density of the supernatants at 278 nm. The solubility of apoflavodoxin is very low near its isoelectric point (3.9, not shown). Outside the 3.5–5.0 pH interval, the solubility sharply increases from 2 μM to much higher values (not shown). A blank was performed to determine if light scattering due to protein aggregates contributed to the observed ANS fluorescence in the transition region in Figure 5. Fluorescence emission of apoflavodoxin at 495 nm in the absence of ANS is negligible in the experimental pH interval (see Fig. 5), which rules out that possibility.

Equilibrium urea denaturation of apoflavodoxin at neutral pH

Apoflavodoxin can be unfolded by moderate concentrations of urea. The conformational change of the protein can be conveniently followed from the decrease in emission fluorescence or from the change in ellipticity in the far-UV (Fig. 6). Both spectroscopic probes give similar unfolding curves that can be fitted to a two-state transition using Equation 8. At 25 °C, pH 7.0, and ionic strength 16.6 mM, half of the molecules are unfolded at 1.96 ± 0.01 M urea (fluorescence data; mean of four measurements ± SD) or 1.96 M urea (CD data; one measurement). Extrapolation of the fluorescence unfolding data to 0 M urea gives an average value of $\Delta G_w = -17.1 \pm 0.5$ kJ mol^{-1} with a slope $m = 8.71 \pm 0.25$ kJ mol^{-1} M^{-1}. The stability of apoflavodoxin at neutral pH does not depend on protein concentration from 0.5 to 25 μM (Fig. 10). The unfolding reaction is fully reversible (see Fig. 6).

Although the unfolding data fit well to a two-state equation, one cannot, however, exclude the accumulation of small amounts of equilibrium intermediates. The denaturant-induced unfolding reactions of proteins in which an intermediate is known to accumulate sometimes can produce unfolding curves that can be fitted to a two-state transition (Pace et al., 1989; Barrick & Baldwin, 1993). In order to study whether this is the case here, we have measured the unfolding of apoflavodoxin by urea in the presence of ANS. The fluorescence of the probe is slightly higher when it is mixed with folded apoflavodoxin than with urea-unfolded apoflavodoxin and, hence, the unfolding reaction can be followed using solely ANS fluorescence. The enhancement of ANS fluorescence by apoflavodoxin at neutral pH is, however, marginal compared with that afforded by apoflavodoxin at pH 2.0 (Fig. 5): under our experimental conditions, the increase in ANS fluorescence emission on binding to apoflavodoxin at pH 2.0 is 160 times higher than the increase produced on binding to apoflavodoxin at pH 7.0. Consequently, if an intermediate with the properties of the conformation at pH 2.0 accumulated in the unfolding transition at neutral pH, ANS binding would reveal its presence even at a low concentration of intermediate. Unfolding of apoflavodoxin at pH 7.0, as detected by ANS fluorescence, closely follows a two-state transition and yields ΔG_w and m values similar to those found in the absence of ANS (not shown). In Figure 7, the experimental data are compared with a simulated unfolding curve in which an intermediate accumulates in the transition midpoint. The simulated curve was calculated under the following assumptions: (1) at the point of maximal accumulation, the concentration of intermediate is 1% of total protein; (2) the fluorescence of ANS bound to the intermediate, S_I, is the same as that of ANS bound to apoflavodoxin at pH 2; also, the binding affinity of ANS to the intermediate S_I is the same as the affinity of ANS to the low pH form; (3) each of the m slopes for the transitions from folded to intermediate and from intermediate to unfolded (m_{FI} and m_{IU} in Equations 2 and 3) are half of the measured slope of the global process (m in Equation 8); (4) the fluorescence of the folded and unfolded states vary linearly with urea

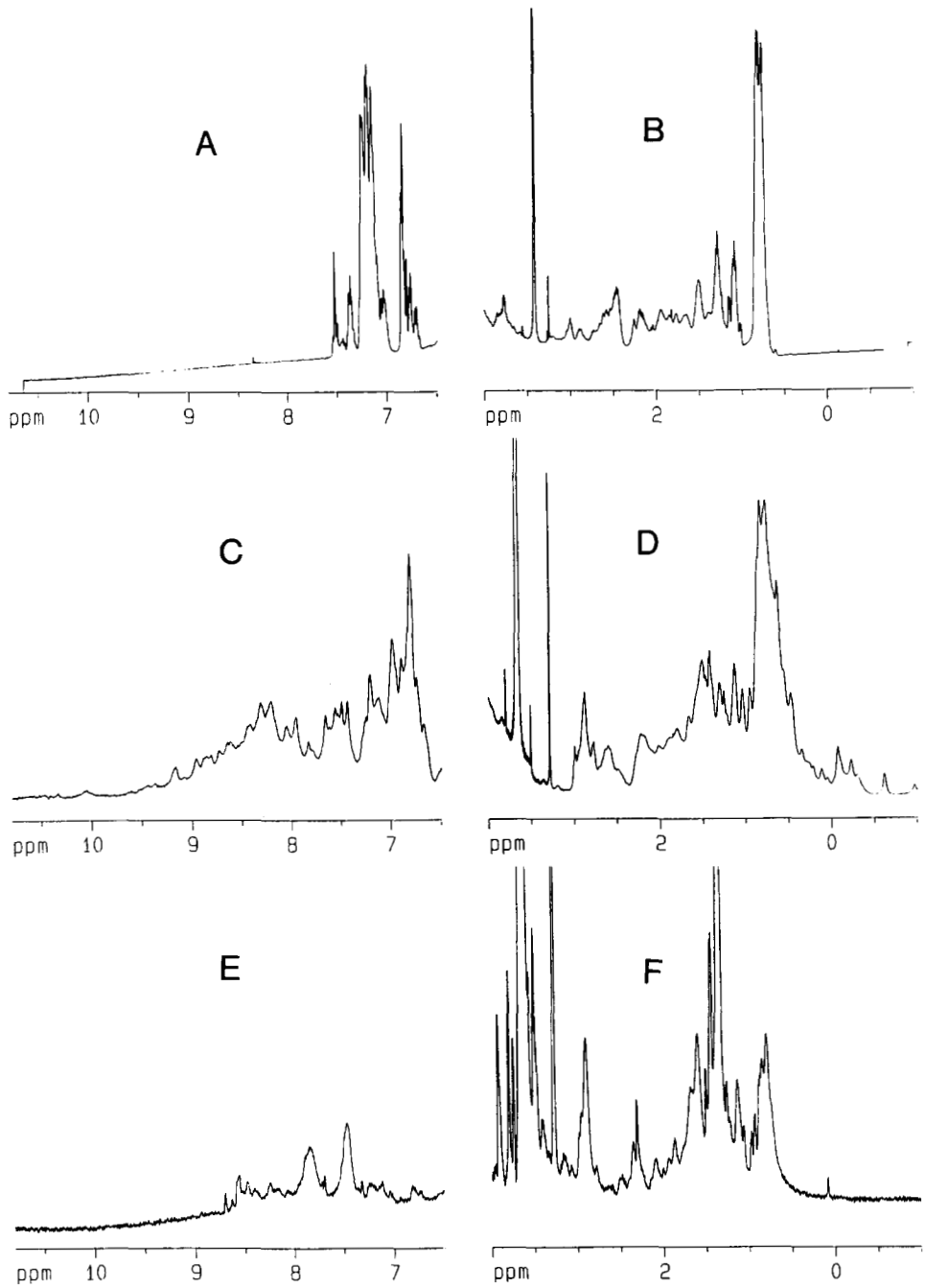

Fig. 4. ^{1}H-NMR spectra of apoflavodoxin at several pH values. **A,B:** pH 13. **C,D:** pH 7.0, 50 mM sodium phosphate. **E,F:** pH 2.0, 28 mM sodium phosphate. All spectra were measured at 20 °C.

concentration with slopes m_F and m_U, as calculated in a previous fitting of the data to Equation 8. For simplicity, the fluorescence of ANS bound to the intermediate was considered to be urea independent in the transition region. The following equation was used that is an adaptation of the three-state equation of Barrick and Baldwin (1993).

$$S = \frac{S_F + m_F D + S_I Z_{FI} + (S_U + m_U D) Z_{FI} Z_{IU}}{1 + Z_{FI} + Z_{FI} Z_{IU}}, \quad (1)$$

where Z_1 and Z_2 are given by Equations 2 and 3:

$$Z_{FI} = e^{-(\Delta G_{FI} - m_{FI} D)/RT} \quad (2)$$

$$Z_{IU} = e^{-(\Delta G_{IU} - m_{IU} D)/RT}, \quad (3)$$

where ΔG_{FI} and ΔG_{IU} are the energy differences between the folded and intermediate, and between the intermediate and unfolded conformations, respectively. Other terms in Equa-

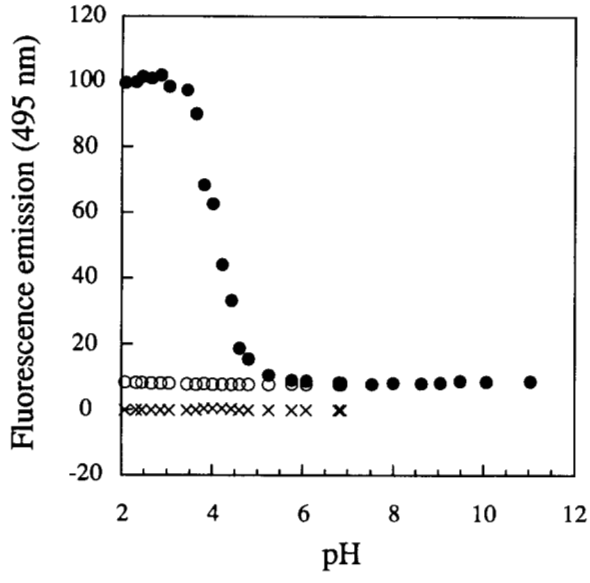

Fig. 5. ANS binding to apoflavodoxin as a function of pH. Emission fluorescence at 495 nm (excitation at 396) was measured at 25 °C. ●, 250 μM ANS plus 1 μM apoflavodoxin; ○, 250 μM ANS; ×, 1 μM apoflavodoxin. Buffers were 10 mM ionic strength sodium citrate, pH 2.0–6.0, and 50 mM sodium phosphate or 50 mM 2-[*N*-cyclohexylamino]-ethanesulfonic acid, pH 7.0–11.0. Fluorescence is given in arbitrary units.

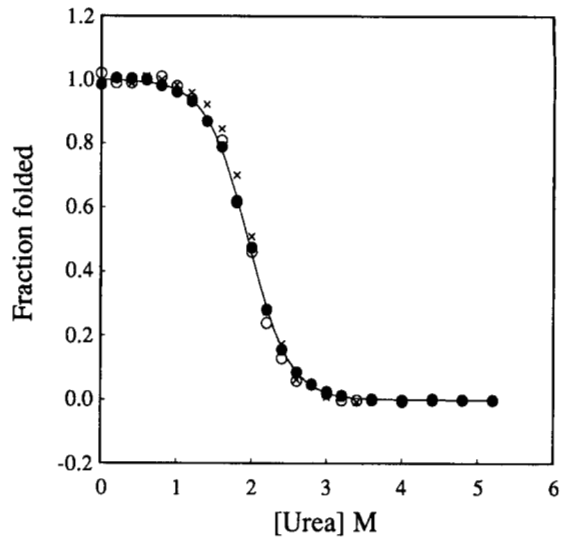

Fig. 6. Urea denaturation of apoflavodoxin at pH 7.0 followed by emission fluorescence (●) or far-UV CD (○) at 25 °C. The fraction of folded protein is represented versus urea concentration after fitting the data to Equation 8. Fluorescence emission was recorded at 320 nm (excitation at 280 nm) and ellipticity was measured at 226 nm. Protein concentration was 2.5 μM and the buffer 50 mM MOPS (fluorescence) or 5 mM MOPS with 15 mM NaCl (dichroism). A refolding experiment followed by emission fluorescence (×). A 25 μM apoflavodoxin solution was equilibrated in 500 mM MOPS containing 4 M urea for 30 min and then diluted 10-fold into urea solutions of different concentrations. The emission fluorescence was measured after 1 h equilibration.

tions 1, 2, and 3 have the same meaning as in Equation 8. The simulation indicates that accumulation of an intermediate (at 1%) would produce a significant perturbation of the unfolding curve that is not observed in actual data [see Semisotnov et al. (1991), for data on other proteins in which such perturbations have been found]. The same result is obtained when some of the assumptions are changed. For example, if the fluorescence of folded, intermediate, and unfolded states are all considered as urea-independent; or if the m_{FI} and m_{IU} slopes are varied from $0.1m$ and $0.9m$ to $0.9m$ and $0.1m$, respectively, the deviation of the simulated curve from a two-state transition is similarly noticeable (not shown).

Calorimetry of apoflavodoxin

The thermal unfolding of apoflavodoxin is nearly pH-independent from pH 6.0 to 9.0 (Table 1), with $T_m = 57.3 \pm 0.1$ °C, $\Delta H = 264 \pm 3$ kJ/mol, and $\Delta C_p = 5.6 \pm 0.1$ kJ/mol K. The reversibility of these transitions was about 50–60%, as measured from the relative area recovery seen in a second scan performed after cooling inside the calorimetric cell. If the heating was terminated at temperatures before T_m, the second scan showed reversibilities higher than 90%. A thermodynamic treatment of the unfolding data is only possible if the process is not under kinetic control. The reversibility of the thermal transition merely shows that the native structure is partially recovered after cooling. More convincing evidence for thermodynamic equilibrium during the unfolding is the independence of the calorimetric profiles on the scan rate [see, for example, Sánchez-Ruiz et al. (1988) and Sánchez-Ruiz (1992)]. Apoflavodoxin thermal transitions from pH 6.0 to 9.0 are, indeed, independent on the scan

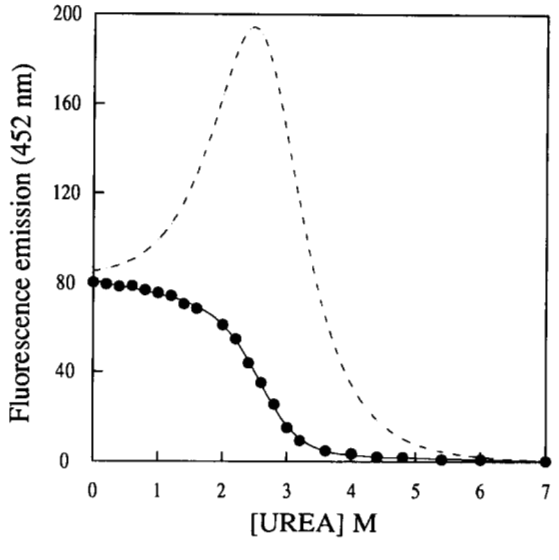

Fig. 7. Urea denaturation of apoflavodoxin at pH 7.0 followed by emission fluorescence of bound ANS (●, experimental data points; solid line, fitting of data to Equation 8) and a simulated curve assuming accumulation of a molten-globule like intermediate (dashed line). Samples contained 5 μM apoflavodoxin and 100 μM ANS in 50 mM MOPS buffer. They also contained 250 mM KCl, which improved the quality of the data, but similar results were obtained in the absence of KCl (not shown). The simulated curve was calculated assuming that maximal accumulation of the intermediate represents 1% of the total protein and that it takes place at the transition midpoint as described in Results.

Table 1. *Thermodynamic characteristics of apoflavodoxin denaturation as a function of pH in different buffer solutions*

Buffer (mM)	pH	T_m (°C)	ΔH^{cal} (kJ/mol)	R[a]	ΔC_p (kJ/mol K)
Phosphate					
20	6.0	57.2	260	0.98	5.56
50	6.0	57.3	262	1.01	5.57
20	7.0	57.4	264	0.97	5.68
50	7.0	57.3	265	1.02	5.69
20	8.0	57.3	264	0.97	5.48
50	8.0	57.3	266	0.99	5.49
50	9.0	57.3	268	0.97	5.73
Glycine					
50	10.0	48.9	224	1.07	5.82
50[b]	11.0	40.3	206	–	–

[a] $R = \Delta H^{cal}/\Delta H^{vH}$.
[b] The transition was irreversible.

rate (from 0.25 to 2 K min^{-1}), suggesting that the denaturation process is always under equilibrium conditions.

The van't Hoff enthalpies of denaturation of apoflavodoxin have been calculated assuming that the native structure is a monomer that undergoes a two-state transition. The average ratio, R, between the calorimetric and the calculated van't Hoff enthalpies is 0.99 ± 0.02 (see Table 1), showing that the thermal unfolding of apoflavodoxin is a two-state transition in this pH interval. The T_m of the thermal transitions is independent of the protein concentration (between 1.0 and 4.0 mg/mL), confirming the absence of aggregation phenomena.

It was not possible to study the thermal unfolding of the protein between pH 3.5 and 5.0, due to its low solubility in this pH interval. In the acid region, pH 2.0, no calorimetric transition was detected. At pH values higher than 9.0, the T_m and ΔH were lower than in the neutral region, the transition being irreversible at pH 11.0 (see Table 1).

The influence of ionic strength was investigated at pH 7.0 in a 50 mM sodium phosphate buffer by adding increasing amounts of KCl (Fig. 8). The transitions showed calorimetric to van't Hoff ratios around unity (1.00 ± 0.04), again indicating that the process was two-state (Table 2). The reversibilities are higher in the presence (70–80%) than in the absence of KCl and the calorimetric profiles are also independent on scan rate and protein concentration (not shown). The average ΔC_p value, obtained directly from the calorimetric curves, is 5.6 ± 0.1 kJ/mol K (Table 2). A plot of the enthalpy values in Table 2 versus T_m yields a straight line with a slope of ΔC_p = 5.8 kJ/mol K (not shown).

Experimental error in the values of ΔC_p precludes a direct extrapolation in the form of a $\Delta C_p(T)$ function. The temperature dependence of ΔC_p can, however, be evaluated approximately from the difference between the heat capacity values of the folded and unfolded states. The heat capacity of the unfolded protein, $C_p^{unf}(T)$, can be calculated from the amino acid composition, assuming that all residues are fully exposed to the solvent in this state (Makhatadze & Privalov, 1990). Polynomial fitting of the calculated values for apoflavodoxin at several temperatures gives Equation 4.

$$C_p^{unf}(T) = -48.20 + 0.4756T - 0.000614T^2. \quad (4)$$

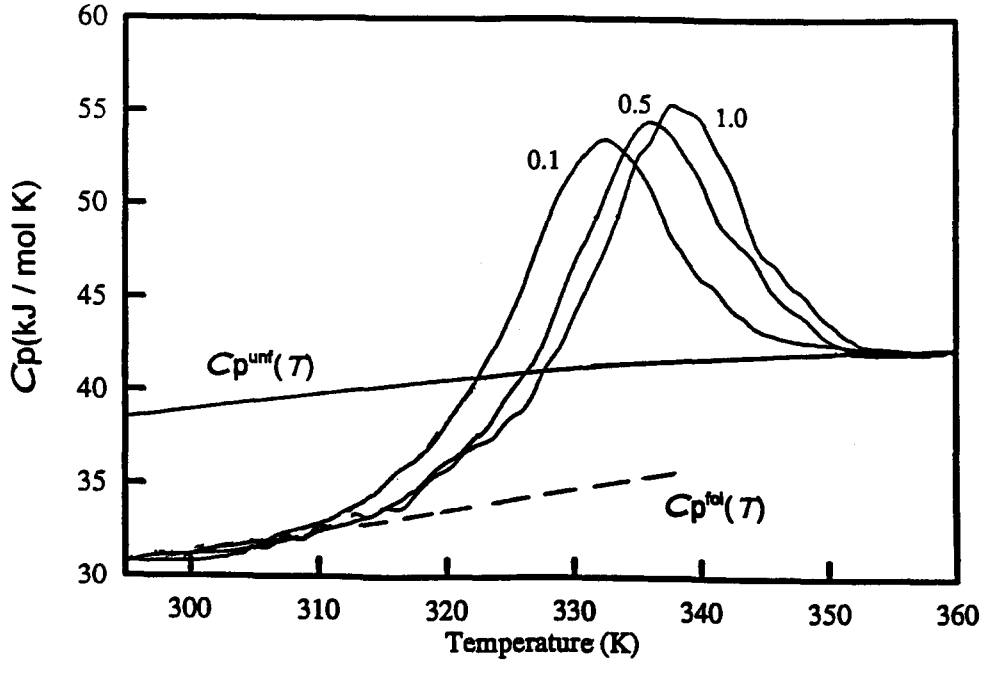

Fig. 8. Temperature dependence of the partial heat capacity of apoflavodoxin in a 50 mM sodium phosphate, pH 7.0, containing the KCl concentrations (M) indicated in the figure. $C_p^{unf}(T)$ and $C_p^{fol}(T)$ are the calculated curves for the unfolded and folded protein, respectively (see Results).

Table 2. *Influence of the ionic strength on the thermodynamic parameters for apoflavodoxin in a 50 mM sodium phosphate buffer at pH 7.0*

KCl (M)	T_m (°C)	ΔHcal (kJ/mol)	R[a]	ΔC_p (kJ/mol K)
0	57.4	265	1.02	5.69
0.1	59.4	276	0.97	5.50
0.2	60.7	283	0.93	5.63
0.3	61.6	291	1.03	5.64
0.5	62.8	295	0.99	5.77
0.75	64.9	308	1.06	5.74
1.0	66.3	317	1.03	5.79
2.0[b]	66.8	160	—	—

[a] $R = \Delta H^{cal}/\Delta H^{vH}$.
[b] The transition was irreversible.

This heat capacity function has been plotted in Figure 8 (solid line), showing that the calculated values agree well with the experimentally measured ones at high temperatures. Inspection of Figure 8, in the low temperature region, clearly shows that the heat capacity of the folded state, $C_p^{fol}(T)$, is also temperature-dependent. This dependence can be described by Equation 5 and has been plotted as a dashed line in Figure 8.

$$C_P^{fol}(T) = -13.87 + 0.1500T. \quad (5)$$

Taking together the temperature dependence of the folded and unfolded states (Equations 4 and 5), the following expression has been calculated for $\Delta C_p(T)$ in kJ/mol K:

$$\Delta Cp(T) = -34.33 + 0.3256T - 0.000614T^2. \quad (6)$$

The $\Delta S(T)$ and $\Delta G(T)$ functions have been calculated as described in Materials and methods, either assuming a constant ΔC_p or one described by Equation 6. The thermodynamic values so calculated for the unfolding process at 25 °C are given in Table 3. Although the ΔH and ΔS values derived from each assumption are very different, the ΔG values coincide within experimental error, and they also agree well with the value determined by urea unfolding studies (see above).

Equilibrium urea denaturation of apoflavodoxin at pH 2.0

The unfolding of apoflavodoxin by urea at pH 2.0 is cooperative and reversible (Fig. 9). Fluorescence emission and CD unfolding curves are very similar (Fig. 9) when recorded under identical conditions. For example, the transition midpoint at 25 °C and 16.6 mM ionic strength occurs at 3.80 ± 0.01 M urea (fluorescence data; mean of four measurements ± SD) or 3.81 (CD data; one measurement) when the concentration of protein is 5 μM. One could, in principle, think of fitting the data to a two-state equation and calculate the stability of this conformation in water. If the simple two-state model applies to this process, then the same stability value should be found regardless of the concentration of protein used. This is, indeed, observed for apoflavodoxin at pH 7.0: the transition midpoint does not change from 0.5 to 25 μM; the slope of the transition (Equation 7) is the same within this concentration range, and thus the calculated stability in water does not depend on protein concentration (Fig. 10). In contrast, at acidic pH, apoflavodoxin transition midpoint increases from 2.3 M urea (at 0.5 μM apoflavodoxin) to 4.7 M urea (at 25 μM apoflavodoxin). If simple two-state model equations are applied to the unfolding of apoflavodoxin at pH 2.0 in this protein concentration range, the slope is found to vary from 3 to 14 kJ mol^{-1} M^{-1}, and the calculated apparent stability in water from 8 to 54 kJ mol^{-1} (Fig. 10). These results clearly show that, at pH 2.0, apoflavodoxin is not a monomer and that its unfolding behavior is not described by a simple two-state model involving monomers.

Discussion

Spectroscopic properties of the different conformations of apoflavodoxin

The fluorescence of ANS is strongly enhanced by apoflavodoxin at acidic pH (Fig. 5). Although this suggests that apoflavodoxin may adopt a molten globule conformation at acidic pH, native proteins are also known that bind to ANS and enhance its flu-

Table 3. *Thermodynamic parameters for the thermal unfolding of apoflavodoxin at 298.15 K as a function of [KCl] in 50 mM sodium phosphate buffer at pH 7.0*

[KCl] (M)	ΔH[a] (kJ/mol)	ΔS[a] (kJ/mol K)	ΔG[a] (kJ/mol)	ΔH[b] (kJ/mol)	ΔS[b] (kJ/mol K)	ΔG[b] (kJ/mol)
0	75.1	0.197	16.3	27.0	0.041	14.4
0.1	74.1	0.189	17.7	25.7	0.033	15.6
0.2	73.4	0.184	18.6	24.8	0.027	16.7
0.3	73.1	0.181	19.2	27.5	0.033	17.6
0.5	74.1	0.180	20.1	24.4	0.021	18.2
0.75	73.7	0.174	21.9	25.4	0.018	20.0
1.0	73.5	0.168	23.4	26.7	0.018	21.3

[a] Values obtained assuming a constant ΔC_p = 5.8 kJ/mol K.
[b] Values obtained assuming that ΔC_p is described by Equation 6.

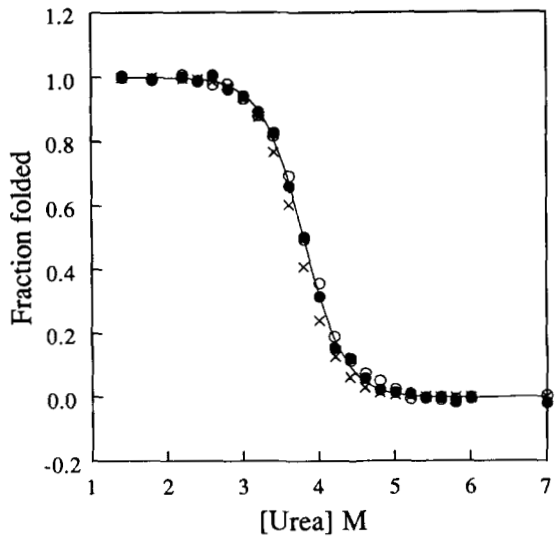

Fig. 9. Urea denaturation of apoflavodoxin at pH 2.0 followed by fluorescence emission (●) or far-UV CD (○) at 25 °C. Fraction of folded protein is represented versus urea concentration after fitting of data to Equation 8. Fluorescence emission was recorded at 320 nm (excitation at 280 nm) and ellipticity was measured at 226 nm. Protein concentration was 5 μM and the buffer 35 mM sodium phosphate. A refolding experiment followed by emission fluorescence (×). A 50-μM apoflavodoxin solution was equilibrated for 1 h in 350 mM sodium phosphate, pH 2.0, containing 6 M urea. The unfolded protein was then diluted 10-fold into urea solutions of different concentrations. After 48 h, the fluorescence emission was measured. Four experiments were averaged.

orescence (Semisotnov et al., 1991). The CD spectra of apoflavodoxin at different pH values are more revealing. At acidic pH, the far-UV CD spectrum is typical of an α/β protein (Fig. 3), but the corresponding near-UV CD spectrum is almost flat. These spectroscopic features indicate the presence, at acidic pH, of a substantial amount of secondary structure, together with the lack of the rigid environment in which aromatic residues are often found in native proteins. At neutral pH, the CD spectrum of apoflavodoxin shows the presence of both secondary structure and tertiary interactions, as characteristic of well-folded proteins.

The fluorescence spectrum at neutral pH (Fig. 2) indicates that some tryptophan residues are shielded from solvent. At acid pH, the fluorescence emission maximum is also blue shifted from the position of the emission maxima of denatured proteins (355 nm), suggesting that the conformation of apoflavodoxin, at acid pH, is still compact enough for the tryptophan residues to be inaccessible to solvent. The NMR data (Fig. 4) also show that, at neutral pH, the protein is properly folded with well-defined tertiary interaction between aliphatic and aromatic side chains (high field signals), whereas at acid pH these interactions are practically absent.

All the spectroscopic evidence available thus indicates that, at pH 7, the conformation of apoflavodoxin is similar to that of well-folded proteins, with a hydrophobic core and a rigid environment for some aromatic side chains. We shall refer to this conformation (predominant from pH 11 to 6, not shown) as native apoflavodoxin. At acidic pH, the situation is different; although the secondary structure is formed and there is a core in which some tryptophan residues are buried, tertiary interactions

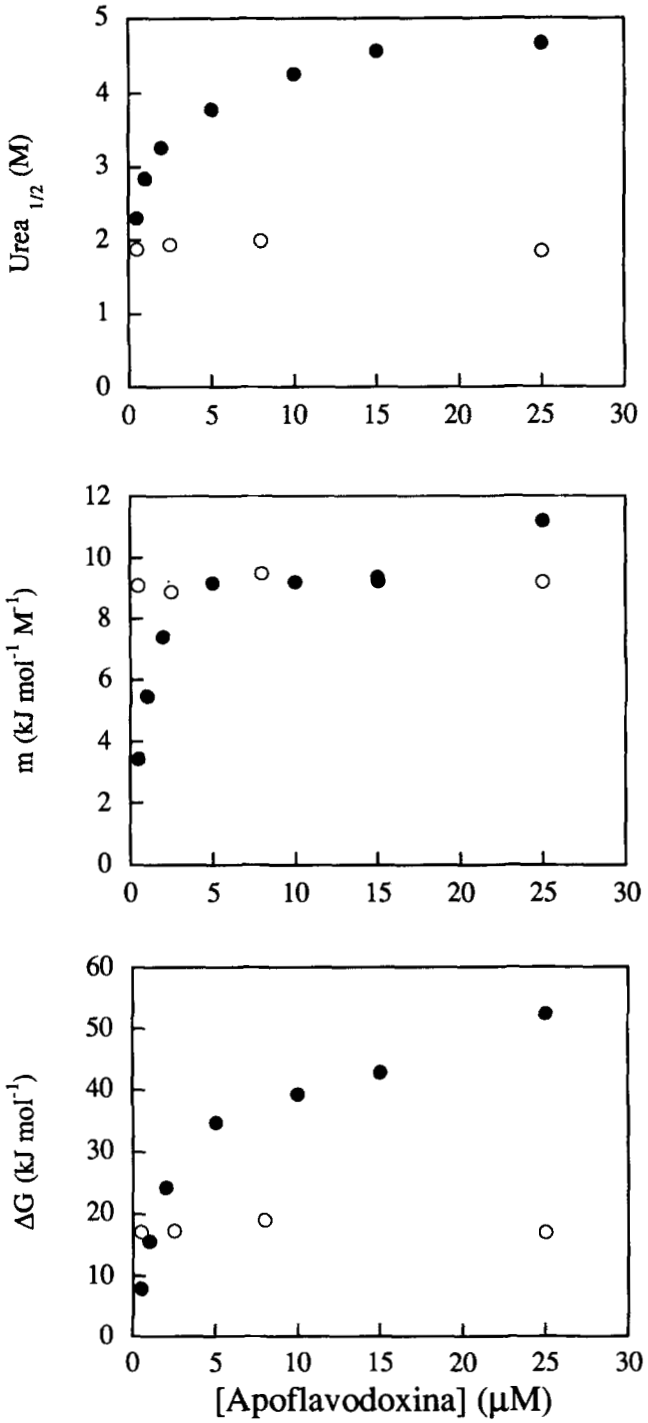

Fig. 10. Urea denaturation of apoflavodoxin at pH 7.0 (○) and pH 2.0 (●) as a function of protein concentration. **A:** Transition midpoints. **B:** Calculated slopes *m* according to Equation 8. **C:** Extrapolated protein stability in water according to Equation 8. All experiments were performed at 25 °C in 50 mM MOPS recording fluorescence emission at 320 nm (excitation at 280 nm).

involving aromatic residues are missing or weakened. The spectroscopic properties of apoflavodoxin at acidic pH and the binding of ANS in this pH region suggest that the conformation of apoflavodoxin at low pH is similar to that of molten globules

(Pane, 1994). We shall refer to this conformation (dominant from pH 3.5 to 2.0, not shown) as molten globule apoflavodoxin. At pH values higher than 12, the fluorescence, CD, and NMR spectra indicate that the protein is denatured and the same is true at neutral and acid pH values in the presence of urea (Figs. 2, 3, and 4).

Evidence for two-state transition of native apoflavodoxin: Stability of this conformation

A folded protein at equilibrium with its unfolded conformation in the absence of populated intermediates must satisfy certain criteria: any spectroscopic probe used to monitor the unfolding transition should yield the same midpoint and the same degree of cooperativity, and the calorimetric and van't Hoff enthalpies of denaturation should be equal. We have followed the urea-induced unfolding of native apoflavodoxin at pH 7.0 by the decrease in fluorescence at 320 nm and by the decrease in absolute ellipticity at 226 nm. With either probe, half denaturation occurs at the same urea concentration and, in both cases, the calculated value of the slope m (Equation 7) is the same within experimental error. The thermal denaturation experiments indicate that the calorimetric enthalpy of denaturation is identical, within experimental error, to the van't Hoff enthalpy from pH 6 to 10 and, at pH 7 (50 mM sodium phosphate), from 0 M to 1 M KCl. Taken together, these data provide strong evidence for a two-state transition between native and unfolded apoflavodoxin in the neutral region. The coincidence of the calorimetric and van't Hoff enthalpies and the lack of concentration effects in the thermal and in the urea unfolding experiments indicate that native apoflavodoxin is a monomer under our experimental conditions.

Determination of the heat capacity allows calculation of the free energy of denaturation as a function of temperature. Our data indicate that, at 25 °C, ΔG_w is 14.4 kJ mol^{-1} (variable ΔC_p) or 16.3 kJ mol^{-1} (constant ΔC_p), which agrees fairly well with the value extrapolated to 0 M urea from the urea induced denaturation measurements (17.1 ± 0.5 kJ mol^{-1}). This suggests that the urea-denatured and the temperature-denatured states are the same.

Although all our data are consistent with a two-state model, we are aware of the fact that small amounts of intermediates may escape detection. In an attempt to determine an upper limit for the concentration of a hypothetical intermediate that could be present in our system, we followed the urea-induced denaturation of native apoflavodoxin by the fluorescence of bound ANS (see Results). If an intermediate accumulates at pH 7, and it is able to bind and enhance the fluorescence of ANS, it will produce a perturbation of the sigmoidal fluorescence denaturation curve. We did not detect such a perturbation. Assuming that the hypothetical intermediate would enhance ANS fluorescence as much as molten globule flavodoxin (at pH 2.0), our simulated curve (Fig. 7) indicates that its concentration must be lower than 1% of total protein. This, again, supports a simple two-state model for the urea-induced unfolding of native apoflavodoxin and makes it unlikely that the molten globule conformation found at low pH participates as an equilibrium intermediate in the unfolding of the protein at neutral pH. The possibility still remains that ANS binding to apoflavodoxin at pH 2 only occurs at the interfaces between monomers. If this were the case, small amounts of monomeric molten globule-like intermediates at neutral pH could pass undetected.

Unfolding of molten globule apoflavodoxin: Protein concentration effect

The calorimetric study of molten globule apoflavodoxin (pH 2) has not been possible because no thermal transition was detected between 15 and 80 °C. A similar behavior has been found for other molten globules [see Pain (1994), for review], and has been explained by a low enthalpy or a low cooperativity of the thermal transition from molten globule to fully denatured protein. In our case, CD indicates that almost as much secondary structure is present at 80 °C as at 25 °C (not shown), suggesting that a thermal transition from molten globule to fully denatured protein does not occur below 80 °C. The same behavior has been reported recently for the inhibitory protein barstar (Khurana & Udgaonkar, 1994). Equilibrium urea denaturation studies, however, show (Fig. 9) that molten globule apoflavodoxin can be reversibly unfolded to a denatured state lacking both secondary structure and a hydrophobic core, as judged from the CD and fluorescence evidence (Figs. 2, 3). The transition is cooperative and reversible, and both fluorescence and CD data fit well the two-state equation of Santoro and Bolen (1988) and yield similar results. This appears to indicate that molten globule apoflavodoxin denatures in a simple two-state fashion. If this were true, the difference in free energy between the molten globule and the denatured state could be calculated by extrapolating the unfolding data to 0 M urea. However, when this extrapolation is done with data obtained at concentrations of apoflavodoxin higher than 5 μM (Fig. 10), two striking results are obtained. First, it turns out that the apparent conformational stability of molten globule apoflavodoxin at pH 2 is higher than that of the native protein at pH 7. This is a surprising result for a conformation in which many well-defined tertiary interactions seem to have disappeared although a similar case has been reported for molten globule and native barstar (Khurana & Udgaonkar, 1994). Second, the slope m of the unfolding (see Equation 7) is similar to that of the unfolding of the native conformation. Because m is a measure of the increase in the exposure of hydrophobic residues to solvent on unfolding, this m value seems to indicate (the acid and neutral unfolded states being equal) that the hydrophobic residues are equally buried in the molten globule as in the native protein, which conflicts with the ANS binding experiments (Fig. 5). Moreover, comparative analyses of the unfolding of several proteins for which a molten globule intermediate exists shows that the slope of the transition molten globule/unfolded is always smaller than that of the transition between native and unfolded protein (Ptitsyn & Uversky, 1994). The fact that the transition midpoint and the apparent stability of molten globule apoflavodoxin are concentration dependent (Fig. 10) indicates that the molten globule is not a monomeric species and provides an explanation for these anomalous results. The increase in apparent stability at increasing concentrations of protein implies that the unfolding transition is coupled to dissociation of associated monomers (Neet & Timm, 1994). This could also explain the relatively high value of m. Although a monomeric molten globule is expected to expose more hydrophobic residues than the corresponding native conformation and therefore should exhibit lower m values on unfolding, association of monomers in molten globule apo-

flavodoxin could have a compensating effect because it would decrease the exposed hydrophobic surface of the associated monomers. Association phenomena in the molten globule could also explain why it is not thermally denatured at temperatures where native apoflavodoxin is fully unfolded; because the interactions between monomers in the molten globule are likely to be of a hydrophobic nature, the probable effect of heating is to strengthen the association, thus opposing unfolding. Some of the properties of molten globule apoflavodoxin (denaturant-induced reversible denaturation but lack of thermal unfolding below 80 °C, higher apparent stability than the native conformation, and association) have also been reported for the molten globule of the inhibitory protein barstar (Khurana et al., 1995).

Linearity of plots of ellipticity or fluorescence emission as a function of protein concentration is sometimes taken as a strong indication that the protein being studied is a monomer in the concentration range analyzed. It is worth mentioning that apoflavodoxin gives linear plots ($R = 0.999$) of both spectroscopic probes from 0.25 to 5.0 μM at both pH 7.0 and pH 2.0 (data not shown). This spectroscopic linearity test thus may be misleading because it can fail to detect the association of the molten globule. Measurement of transition midpoints of unfolding versus protein concentration seems to be a better test.

Thermodynamic properties of native flavodoxin

The difference in heat capacity between unfolded and folded states of proteins is mainly explained by an increase in hydrophobic surface area on unfolding. Assuming that all hydrophobic groups are exposed to the solvent in the unfolded state, a linear relationship between the number of apolar contacts in the native state of several proteins (contacts at <4 Å, determined from their known tertiary structures) and their ΔC_p values has been reported (Privalov & Khechinashvili, 1974; Privalov, 1979). From this relationship and the ΔC_p value for the unfolding of apoflavodoxin, we calculate that there should be approximately 87 apolar contacts in the native state of apoflavodoxin. This is a very low value for a protein that contains 168 amino acid residues and, more importantly, it disagrees with the actual value calculated from the X-ray structure of apoflavodoxin (Genzor et al., 1996), which is about 200. The discrepancy between the two values could be explained if the unfolded state of apoflavodoxin were not fully exposed to solvent. However, the agreement between the stability calculated from urea and from temperature unfolding experiments and the agreement between the measured heat capacity of the unfolded protein and that calculated assuming full exposure of its residues argues against this possibility.

The enthalpy of unfolding as a function of temperature has been calculated, as described in Materials and methods, assuming either that ΔC_p does not change with temperature or that it is given by Equation 6. Results of the calculation are shown in Figure 11. The two curves fit well the experimental data, although there are clear discrepancies between the two assumptions at low and high temperatures. The extrapolated values of the specific heat of unfolding (Δh_m) of globular proteins have been reported to intersect close to 385 K, showing a common value of Δh_m of about 54 J/g, when ΔC_p is taken as temperature-independent (Privalov & Khechinashvili, 1974; Privalov et al., 1989); a similar common value has been found under the assumption that ΔC_p is a function of temperature (Privalov & Gill, 1988; Privalov et al., 1989; Doig & Williams, 1992). In the present case, considerably smaller values of about 20–30 J/g are obtained. Examples of a similar behavior have been reported for small proteins, such as histones (Tiktopulo et al., 1982), and the discrepancies have been attributed to a smaller number of hydrogen bonds and/or nonpolar contacts per residue in these folded proteins. However, these explanations cannot apply to apoflavodoxin (Genzor et al., 1996). Other discrepancies of higher Δh_m values than the average one have also been reported (Martínez et al., 1994; Ruiz-Arribas et al., 1994), but as yet they remain unexplained.

A strong correlation between ΔH and ΔC_p has also been reported for many proteins at 298 K (Murphy et al., 1990, 1992; Spolar et al., 1992). Again, the values found for apoflavodoxin ($\Delta H_{m,r} = 380$ cal/mol res., and $\Delta C_{p,r} = 11$ cal/mol res. K) are

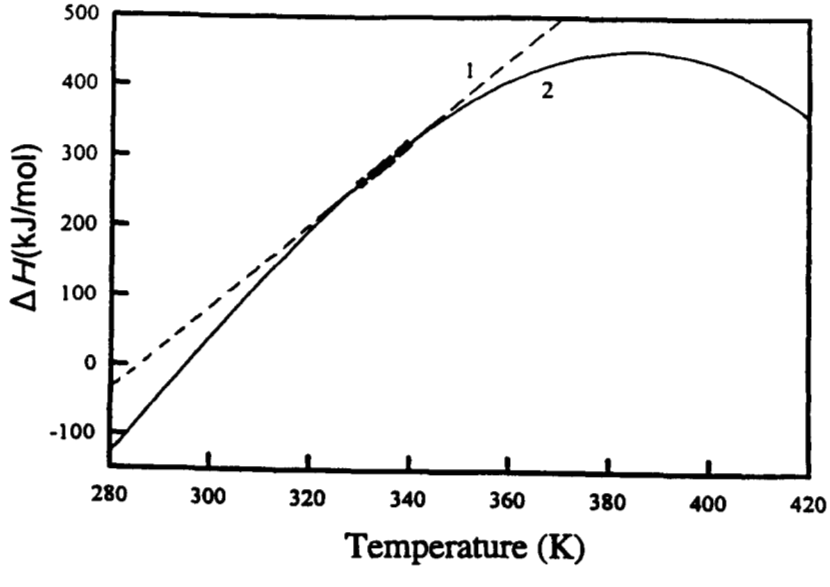

Fig. 11. Experimental dependence of the molar calorimetric enthalpy on T_m for apoflavodoxin in 50 mM sodium phosphate (◆) plotted along with the $\Delta H(T)$ functions calculated assuming: (1) a constant ΔC_p of 5.8 kJ K^{-1} mol^{-1} (dashed line); (2) a variable ΔC_p given by Equation 6 (solid line).

smaller than the expected ones from such a correlation. As for the other empirical correlations, we do not know the reason of this discrepancy, but we can rule out that it is due to factors such as an erroneous extinction coefficient, the occurrence of aggregation in the concentration range used in this experiment, deviation from the two-state model, or a kinetically controlled unfolding mechanism (see Results).

The highest stability of native apoflavodoxin (calculated from Equations 11, 13, and 14) occurs at around 25 °C. This temperature is high compared with those found for hydrophilic proteins (Pace & Laurents, 1989). Although the maxima values of ΔG are relatively small (from 14 to 21 KJ/mol), they clearly indicate that apoflavodoxin can fold to a stable conformation in the absence of the redox cofactor. This explains why substantial amounts of soluble recombinant apoflavodoxin can be obtained, along with holoflavodoxin, under certain culture conditions (Fillat et al., 1991). The low stability of apoflavodoxin permits its accurate determination from urea denaturation experiments, because only a short extrapolation to 0 M urea is required. Finally, we observe a strong stabilizing effect of increasing ionic strength (Table 3) that may be useful to allow the study of destabilized protein variants.

Conclusions

Removal of the redox cofactor from *Anabœna* flavodoxin yields an apoprotein that, at neutral pH, is well folded and monomeric. The protein thus can fold in the absence of its redox cofactor at physiologic pH values. In this pH region, the unfolding of apoflavodoxin by urea or heat is a two-state process and the unfolded state appears to be fully denatured. Apoflavodoxin is singular in that its specific heat capacity is very low in spite of being well folded. The conformational stability of apoflavodoxin at pH 7 and low ionic strength is fairly low. This permits the accurate calculation of the stability from urea denaturation experiments because only a short extrapolation to 0 M urea is required. Increasing the ionic strength stabilizes the folded conformation, which can be useful for the analyses of destabilizing mutations. At acidic pH, apoflavodoxin adopts a conformation with the properties of a molten globule that can be reversibly unfolded by urea. Although the unfolding of this conformation appears to be two-state by some criteria, the dependence of the calculated stability on the concentration of protein proves that the process is more complex and involves the dissociation of associated monomers. This molten globule conformation of apoflavodoxin is not an intermediate in the equilibrium urea unfolding of native apoflavodoxin at neutral pH. In summary, apoflavodoxin is a promising model for the analysis of the stability and folding of α/β proteins and for the study of the interactions between apoflavoproteins and their redox cofactors.

Materials and methods

Reagents

Urea Ultrapure was from Boehringer Mannheim. MOPS, ANS, and flavin mononucleotide were purchased from Sigma. Isopropyl-β-thiogalactopyranoside was obtained from Promega and DE-52 from Whatman. All other chemicals were reagent-grade.

Protein purification

Flavodoxin was purified by a scaled-up adaptation of the method described by Fillat et al. (1991). A 2.5-L culture of *E. coli* cells (PC2495 or TG1), harboring the plasmid pTrc99a containing the gene for the cyanobacterial flavodoxin from *Anabœna* PCC 7119, was grown overnight in Luria broth medium supplemented with ampicilline (50 mg/mL). A 50-L fermenter, filled with 45 L of the same medium, was inoculated with the culture, and cells were grown at 37 °C, pH 7.2, and 70% of maximum pO_2, until the optical density of the culture at 620 nm reached 1.2. At this point, isopropyl-β-thiogalactopyranoside was added (5 g) and the fermentation was continued for 8–12 h. After cooling, cells were harvested by centrifugation in a JCF-Z continuous rotor from Beckman. The cell-paste (200–600 g) was washed into 0.15 M NaCl and frozen. Thawed cells were resuspended in five volumes of 50 mM Tris/HCl, pH 8, containing 1 mM EDTA, 1 mM β-mercaptoethanol, and 1 μM phenylmethanesulfonyl fluoride (Tris buffer). This suspension was placed in an ice-water bath and sonicated 5 × 45 s, with 30-s intervals between each treatment. Flavin mononucleotide was added (50 mg) and the extract was centrifuged for 1 h at 13,000 rpm in a J-14 Beckman rotor. The supernatant was brought to 65% ammonium sulfate saturation, centrifuged as above to remove unwanted proteins, and loaded on a DE-52 column (5 × 25 cm) equilibrated in 65% saturated ammonium sulfate in Tris buffer. The column was washed with the same buffer until most free excess flavin mononucleotide was eluted. Flavodoxin was then eluted with 2 L of a linear gradient from 65% to 0% saturated ammonium sulfate in Tris buffer. Fractions with a ratio of $OD_{464}/OD_{280} \geq 0.15$ were pooled, dialyzed, against 3 × 5 L of 50 mM Tris/HCl, pH 8.0, and loaded onto a DE-52 column (2.6 × 50 cm) equilibrated in the same buffer. Flavodoxin was eluted with 2 L of a linear salt gradient (0–0.5 M NaCl). Fractions with $OD_{464}/OD_{280} \geq 0.16$ were pooled. This preparation was homogeneous as judged from SDS-PAGE.

Preparation of apoflavodoxin

The flavin mononucleotide group was removed from the holoprotein by treatment with trichloroacetic acid (Edmondson & Tollin, 1971). To a flavodoxin solution (4 mg/mL) containing 1 mM dithiothreitol, trichloroacetic acid was added (at 0 °C in the dark) up to 3% (w/v). Apoflavodoxin was pelleted by centrifugation and resuspended in 1 mM dithiothreitol/3% trichloroacetic acid to remove the remaining flavin nucleotide. The washing step was repeated until the apoprotein pellet appeared colorless and the resuspended protein showed no absorption in the visible region. Alternatively, apoflavodoxin was prepared by dropping the pH to 2, followed by dialysis or gel filtration at the same pH.

Protein concentration

The molar extinction coefficient of apoflavodoxin, at 280 nm in 20 mM sodium phosphate, pH 6.5, was determined from its amino acid composition and its absorbance in that buffer and in 6 M guanidine hydrochloride (Gill & von Hippel, 1989). A value of 34,100 M^{-1} cm^{-1} was obtained.

ANS binding

Binding of ANS to apoflavodoxin was measured as a function of pH by recording emission fluorescence at 495 nm, with excitation at 396 nm. Apoflavodoxin and ANS concentrations were 1 μM and 250 μM, respectively. Buffers were: from pH 2 to 6, sodium citrate (10 mM ionic strength), and, from pH 6 to 10, 50 mM sodium phosphate or 50 mM 2-[*N*-cyclohexylamino]-ethanesulfonic acid.

ANS binding to apoflavodoxin at pH 7 was also measured as a function of urea concentration in an attempt to detect potential molten globule-like intermediates appearing at low concentration in the unfolding region. In this experiment, apoflavodoxin was 5 μM, ANS 100 μM, and the buffer 50 mM MOPS.

Emission fluorescence, CD, and NMR

Spectra of apoflavodoxin at several pH values and urea concentrations were recorded on a Kontron SFM25 fluorimeter, a Jasco 710 spectropolarimeter, or a Bruker AMX-500 spectrometer. In the NMR measurements, water suppression was achieved by selective presaturation, placing the carrier on the H_2O or HOD resonance.

Equilibrium urea denaturation

Samples were prepared by mixing 900-μL urea solutions [previously aliquoted with a positive displacement dispenser (Brand) and kept frozen] with 100-μL aliquots of buffered apoflavodoxin [dispensed with a transferpettor (Brand)]. Unfolding was followed, after equilibration at 25 °C for 30 min, by emission fluorescence at 320 nm (excitation at 280) or by CD at 226 nm. Temperature was monitored with a thermocouple immersed in the cuvette before measurement. Data were analyzed, assuming a two-state equilibrium, according to the method of Pace (1986), as modified by Santoro and Bolen (1988). In this method, the free energy of unfolding, ΔG, is considered to be a linear function of the concentration of denaturant:

$$\Delta G = \Delta G_w - mD, \tag{7}$$

where ΔG_w is the free energy of unfolding in water, D is the molar concentration of denaturant, and m is a proportionality constant. The spectroscopic signals of the folded S_F and unfolded states S_U are assumed to vary linearly with urea concentration, m_F and m_U being the corresponding slopes. Under these assumptions the observed spectroscopic signal follows Equation 8:

$$S = \frac{S_F + m_F D + (S_U + m_U D)e^{-(\Delta G_w - mD)/RT}}{1 + e^{-(\Delta G_w - mD)/RT}}, \tag{8}$$

where R is the gas constant, T the absolute temperature, and D, ΔG_w, and m have the same meaning as in Equation 7.

Equilibrium unfolding data were fitted to Equation 8 and best fits for m and ΔG_w were obtained.

Differential scanning calorimetry (DSC) measurements

DSC was performed using a DASM-4 microcalorimeter with digital control (cell volume 0.47 mL), at heating rates from 0.25 to 2 K/min and protein concentration in the range of 1.0 to 4.0 mg/mL. An extra pressure of 1.5 atm was maintained during all DSC runs to prevent degassing of the solutions on heating. The molar partial heat capacity of apoflavodoxin, C_p, was evaluated according to standard procedures (Privalov & Potekhin, 1986), taking 0.679 mL/g as the partial specific volume [calculated from the amino acid composition by the method of Makhatadze et al. (1990)] and using a value of 19.0 kDa for the molecular mass of the apoflavodoxin. Each sample was extensively dialyzed against its corresponding buffer before measurement. The baseline of the instrument was routinely recorded before or after the experiments with both cells filled with buffer. Reversibility of the unfolding was checked by sample reheating after cooling inside the calorimetric cell, and by the independence of the thermograms on the scan rate.

Analysis of DSC data

Protein stability as a function of temperature, $\Delta G(T)$ (Becktel & Schellman, 1987), was calculated from the transition temperatures, T_m, the experimental transition enthalpies at T_m, $\Delta H(T_m)$, and the heat capacity changes, ΔC_p. Two cases were considered. In the first one, ΔC_p was assumed to be independent of solution conditions and temperature, and $\Delta G(T)$ was calculated from Equations 9, 10, and 11.

$$\Delta H(T) = \Delta H(T_m) + \Delta C_p(-T - T_m) \tag{9}$$

$$\Delta S(T) = \frac{\Delta H(T_m)}{T_m} + \Delta C_p \ln(T/T_m) \tag{10}$$

$$\Delta G(T) = \Delta H(T) - T\Delta S(T) \tag{11}$$

In the second, ΔC_p was considered to be temperature dependent, following Equation 12, where T is the absolute temperature, as described in Results:

$$\Delta C_p = a + bT + cT^2 \tag{12}$$

In this case, $\Delta G(T)$ was calculated from Equations 11, 13, and 14:

$$\Delta H(T) = \Delta H(T_m) + a(T - T_m) + \frac{1}{2} b(T^2 - T_m^2) + \frac{1}{3} c(T^3 - T_m^3) \tag{13}$$

$$\Delta S(T) = \frac{\Delta H(T_m)}{T_m} + a \ln(T/T_m) + b(T - T_m) + \frac{1}{2} c(T^2 - T_m^2). \tag{14}$$

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