The equilibrium unfolding of *Azotobacter vinelandii* apoflavodoxin II occurs via a relatively stable folding intermediate

CARLO P.M. VAN MIERLO, WALTER M.A.M. VAN DONGEN, FRANK VERGELDT,
WILLEM J.H. VAN BERKEL, AND ELLES STEENSMA

Department of Biomolecular Sciences, Laboratory of Biochemistry, Wageningen Agricultural University,
Wageningen, The Netherlands

(RECEIVED April 27, 1998; ACCEPTED August 5, 1998)

Abstract

A flavodoxin from *Azotobacter vinelandii* is chosen as a model system to study the folding of α/β doubly wound proteins. The guanidinium hydrochloride induced unfolding of apoflavodoxin is demonstrated to be reversible. Apoflavodoxin thus can fold in the absence of the FMN cofactor. The unfolding curves obtained for wild-type, C69A and C69S apoflavodoxin as monitored by circular dichroism and fluorescence spectroscopy do not coincide. Apoflavodoxin unfolding occurs therefore not via a simple two-state mechanism. The experimental data can be described by a three-state mechanism of apoflavodoxin equilibrium unfolding in which a relatively stable intermediate is involved. The intermediate species lacks the characteristic tertiary structure of native apoflavodoxin as deduced from fluorescence spectroscopy, but has significant secondary structure as inferred from circular dichroism spectroscopy. Both spectroscopic techniques show that thermally-induced unfolding of apoflavodoxin also proceeds through formation of a similar molten globule-like species. Thermal unfolding of apoflavodoxin is accompanied by anomalous circular dichroism characteristics: the negative ellipticity at 222 nm increases in the transition zone of unfolding. This effect is most likely attributable to changes in tertiary interactions of aromatic side chains upon protein unfolding. From the presented results and hydrogen/deuterium exchange data, a model for the equilibrium unfolding of apoflavodoxin is presented.

Keywords: apoflavodoxin; circular dichroism; equilibrium unfolding; fluorescence; molten globule-like species; NMR; protein stability; three-state model

In contrast to most protein folds, the flavodoxin-like fold is shared by many (i.e., nine) superfamilies (Brenner, 1997). These nine superfamilies exhibit little or no sequence similarity and comprise

a broad range of unrelated proteins with different functions like catalases, chemotactic proteins, lipases, esterases, and flavodoxins. They are all characterized by a five-stranded parallel β -sheet surrounded by α -helices at either side of the sheet. Of these proteins, a flavodoxin is chosen by us as a model system to study protein folding and stability. General rules governing protein folding are only beginning to emerge and there is a strong need for additional well-characterized protein systems to obtain a better understanding of the fundamental rules describing protein folding. By studying the folding of flavodoxin, we extend the number of proteins under investigation and expect to learn the rules by which other, if not all, proteins with a flavodoxin-like topology fold.

Flavodoxins are a group of small flavoproteins that function as low-potential one-electron carriers and contain a noncovalently bound FMN cofactor (Mayhew & Tollin, 1992). The protein investigated by us is flavodoxin II from *Azotobacter vinelandii* (strain ATCC 478), henceforth designated flavodoxin. Its gene has been cloned in *Escherichia coli* and brought to high expression in our laboratory. The protein consists of 179 amino acid residues and belongs to the class of "long-chain" flavodoxins (Tanaka et al., 1977). Upon removal of the flavin, apoflavodoxin is generated. Apoflavodoxin molecules were shown to rapidly dimerize via the

Reprint requests to: Carlo P.M. van Mierlo, Department of Biomolecular Sciences, Laboratory of Biochemistry, Wageningen Agricultural University, Dreijenlaan 3, NL-6703 HA Wageningen, The Netherlands; e-mail: carlo.vanmierlo@nmr.bc.wau.nl.

Abbreviations: C69A, cysteine 69 replaced by alanine; C69S, cysteine 69 replaced by serine; CD, circular dichroism; C_m , concentration denaturant at the midpoint of unfolding; ΔC_p , denaturational heat capacity change; ΔG_{N-I} , Gibbs free energy of unfolding of the native state to the intermediate state; ΔG_{N-U}^0 , Gibbs free energy of unfolding of the native state to the unfolded state at zero denaturant concentration; ΔG_{N-U} , Gibbs free energy of unfolding of the native state to the unfolded state at zero denaturant concentration; $\Delta\Delta G_{N-U}$, change in free energy of unfolding between wild-type and mutant protein at C_m of wild-type protein; DEAE, diethylaminoethyl; FMN, riboflavin-5'-monophosphate; GndHCl, guanidinium hydrochloride; HSQC, heteronuclear single-quantum coherence; k_{ex} , measured amide proton exchange rate; m , $d(\Delta G)/d[\text{GndHCl}]$; m_{N-U} , $d(\Delta G_{N-U})/d[\text{GndHCl}]$; m_{N-I} , $d(\Delta G_{N-I})/d[\text{GndHCl}]$; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ppm, parts per million; T_m , temperature at the midpoint of unfolding; Tris-HCl, Tris-(hydroxymethyl)-aminomethane/HCl; wt, wild-type.

formation of an intermolecular disulfide bond involving the sole cysteine residue at position 69. Holoflavodoxin molecules also tend to dimerize but at a much lower rate. To avoid these complications, the single cysteine residue was replaced by either an alanine or a serine residue (Steensma et al., 1996).

Detailed knowledge about the conformational properties of a protein is a prerequisite to study its folding and stability. The excellent NMR characteristics of flavodoxins (solubility, relatively narrow line widths) enable the application of a variety of heteronuclear multidimensional NMR experiments (e.g., Wijmenga et al., 1996, 1997) and thus allow a detailed structural characterization of these proteins in solution (van Mierlo et al., 1990; Steensma et al., 1998). The use of NMR spectroscopy in the study of protein folding has proven to be particularly valuable. Native as well as nonnative states of proteins can be studied using this technique (van Mierlo et al., 1992a, 1992b; Shortle, 1996). In addition, it has been demonstrated that hydrogen exchange pulse labeling methods in combination with NMR spectroscopy give detailed information about the folding pathways of proteins (Roder et al., 1988) as do "real time" NMR experiments (Balbach et al., 1996). The excellent NMR properties of flavodoxin make it a good model protein to study protein folding and stability, and to enable the future application of the NMR methods just mentioned.

In our laboratory, the ^1H , ^{13}C , and ^{15}N backbone chemical shifts of holoflavodoxin were determined and the protein was shown to contain a five-stranded parallel β -sheet (order β_2 - β_1 - β_3 - β_4 - β_5), which is surrounded by five α -helices (Steensma et al., 1998). The tertiary structure of holoflavodoxin is the archetypal example of proteins with the α/β doubly wound topology (Orengo et al., 1994). The structural characteristics of apoflavodoxin, i.e., flavodoxin in the absence of the FMN cofactor, were determined using heteronuclear multidimensional NMR spectroscopy (van Mierlo et al., 1997; Steensma & van Mierlo, 1998). Despite the removal of the FMN cofactor, over 80% of the assigned $^1\text{H}^\alpha$, $^1\text{H}^\text{N}$, and ^{15}N resonance positions of *A. vinelandii* apoflavodoxin are identical to those of the holoprotein (Steensma & van Mierlo, 1998) as is shown in Figure 1. The observed NOE connectivities, the $^3J_{\text{HN-H}\alpha}$ coupling constants and the available chemical shift information show that large parts of the tertiary structure of holo- and apoflavodoxin are strictly conserved. Native apoflavodoxin has a stable, well-ordered core but its flavin binding region is shown to be flexible. This flexibility is a likely prerequisite to enable the flavin to enter the interior of the apoprotein during the final stages of apoflavodoxin folding. Apoflavodoxin contains five *trans* prolines, no disulfide bridges, and three tryptophan residues (Fig. 1). The stable nucleus of apoflavodoxin was probed using hydrogen/deuterium exchange experiments and local free energy differences for transient opening of the structure were determined (Steensma & van Mierlo, 1998).

In this paper, we expand the NMR results obtained until now, and report as a first step in the determination of the folding pathway of *A. vinelandii* flavodoxin, equilibrium unfolding studies on apoflavodoxin. The denaturant-induced unfolding of apoflavodoxin is demonstrated to be reversible. Apoflavodoxin thus can fold in the absence of the FMN cofactor under the experimental conditions used. The reversibility of apoflavodoxin unfolding is essential for the future study of flavodoxin folding using the NMR techniques mentioned above. Most importantly, the unfolding curves obtained for the denaturant-induced unfolding of wild-type, C69A, and C69S apoflavodoxin as monitored by circular dichroism spectroscopy are

Fig. 1. MOLSCRIPT cartoon drawing (Kraulis, 1991) of the X-ray structure of *Azotobacter chroococcum* flavodoxin (Thorneley et al., 1994). The structure is colored according to the $^1\text{H}^\alpha$ chemical shift differences ($\Delta\delta$) between the holo- and the apoform of the homologous *A. vinelandii* apoflavodoxin II (Steensma & van Mierlo, 1998). Blue: $\Delta\delta \leq 0.1$ ppm; red: $\Delta\delta > 0.1$ ppm; grey: residues of which the $^1\text{H}^\alpha$ proton could not be assigned. Dynamical exchange between different conformations occurs in the flavin binding region of the molecule, which is colored red and grey. The three tryptophan residues in the protein are shown. W74 is colored red in accordance with the $\Delta\delta$ of its indole N-H proton. The indole N-H protons of W167 (which resides in helix α_5) and W128 have $\Delta\delta \leq 0.1$ ppm but are colored yellow for reasons of clarity.

shown not to coincide with those obtained by fluorescence spectroscopy. A similar result is obtained for the temperature-induced unfolding of the three apoflavodoxin variants. This implies that the folding of apoflavodoxin occurs via at least one relatively stable folding intermediate. The conformational properties of the folding intermediate are discussed and related with the hydrogen/deuterium exchange results obtained on apoflavodoxin by NMR spectroscopy.

Results

Identification of the flavodoxin

The flavodoxin investigated in this study is encoded by the *nifF*-gene in the major *nif*-gene cluster from *A. vinelandii* ATCC 478. Proteins involved in the nitrogen fixation by this bacterium are encoded by the *nif*-gene. Previously, three different flavodoxins, named flavodoxin I, II, and III, have been isolated from *A. vinelandii* ATCC 478 (Klugkist et al., 1986). These flavodoxins can be distinguished from one another via differences in a variety of physical and spectroscopic criteria, and they are expressed under different growth conditions. The *nifF*-encoded flavodoxin is identified as flavodoxin II as it specifically interacts on a Western blot with antibodies raised against flavodoxin II, but not with those raised against flavodoxins I and III.

Spectroscopic characterization of native and unfolded apoflavodoxin

NMR

The ^1H - ^{15}N HSQC NMR spectrum of a protein serves as a finger print of its conformational state. In 100 mM potassium pyrophosphate, pH 6.0, the ^1H - ^{15}N HSQC spectrum of the 179-residue apoflavodoxin shows a nice dispersion in chemical shifts characteristic for a folded protein (Fig. 2). Besides small, buffer concentration dependent, chemical shift changes, the spectrum is

Fig. 2. ^1H - ^{15}N HSQC spectrum (A) of native C69A apoflavodoxin from *A. vinelandii* and (B) of apoflavodoxin in the presence of 4 M guanidinium hydrochloride. Cross peaks of the indole N-H groups of the three Trp residues are indicated. The spectrum acquired in the presence of 4 M guanidinium hydrochloride has limited chemical shift dispersion in the ^1H dimension and is typical for that of an unfolded protein. Cross peaks corresponding to specific amide groups in the unfolded protein, e.g., indole N-H groups of Trp residues, show minimal chemical shift differences. Upon removal of the denaturant, an identical distribution of cross peaks as shown in the upper spectrum is obtained for refolded apoflavodoxin. The NMR samples contained 100 mM potassium pyrophosphate, 90% H_2O /10% $^2\text{H}_2\text{O}$, at pH 6.0, and the spectra are recorded at 25°C. The spectra were plotted at different contour levels. For further details see Materials and methods.

identical to the assigned spectrum of the protein in 150 mM potassium pyrophosphate, pH 6.0 (van Mierlo et al., 1997; Steensma & van Mierlo, 1998). The three indole N-H cross peaks arising from the three tryptophan residues of apoflavodoxin are indicated in Figure 2. Upon increasing the GndHCl concentration to 4 M, the chemical shift dispersion in particularly the ^1H dimension is strongly reduced and the ^1H - ^{15}N HSQC spectrum becomes typical of a denatured protein. The resonances cluster in a narrow ppm window as is indicated for the cross peaks arising from the three indole N-H groups of apoflavodoxin in Figure 2.

Fluorescence

Apoflavodoxin contains three tryptophan (Fig. 1) and five tyrosine residues, which cause its fluorescent properties. Upon excitation of the protein at 280 nm, the maximum fluorescence emission is observed at 333 nm for wild-type, C69A, and C69S apoflavodoxin (Fig. 3A). This suggests that the tryptophans are shielded from the solvent in native apoflavodoxin. Treatment of all

Fig. 3. Fluorescence and CD characteristics of native and unfolded apoflavodoxin from *A. vinelandii*. The protein concentration was approximately 4 μM and the spectra were recorded at 25°C in 100 mM potassium pyrophosphate, pH 6.0. **A:** Fluorescence emission spectrum of native C69A apoflavodoxin (—) and of apoflavodoxin in 4 M guanidinium hydrochloride (---), respectively. The excitation wavelength was 280 nm. **B:** Far-UV CD spectrum of native C69A apoflavodoxin (—) and of apoflavodoxin in 4.2 M guanidinium hydrochloride (---), respectively.

three variants of apoflavodoxin with 4 M GndHCl leads to a strong decrease in fluorescence intensity and to a shift of the fluorescence emission maximum to approximately 354 nm (Fig. 3A) indicative of protein unfolding.

Circular dichroism

The far-UV CD spectrum of apoflavodoxin in 100 mM potassium pyrophosphate, pH 6.0, is shown in Figure 3B. It has a minimum in ellipticity at 222 nm for all three variants of the protein and the spectrum is virtually indistinguishable from the one obtained for holoflavodoxin (data not shown). The far-UV CD spectrum shows that native apoflavodoxin contains a substantial amount of secondary structure consistent with the NMR results discussed before. Addition of 4.2 M GndHCl leads to a CD spectrum, which is characteristic for a protein of which all secondary structure is lost (Fig. 3B).

Altogether, the spectroscopic properties of apoflavodoxin as monitored by NMR, fluorescence emission, and circular dichroism show that the protein is folded in 100 mM potassium pyrophosphate pH 6.0. In its native state, apoflavodoxin has an α/β doubly wound topology (Fig. 1). Addition of 4 M GndHCl leads to a loss of tertiary structure reflected in the solvent exposure of the apoflavodoxin tryptophan side chains and to a loss of secondary structure. The finger print NMR spectrum confirms that the protein is denatured in 4 M GndHCl.

GndHCl-induced equilibrium unfolding followed by fluorescence and CD spectroscopy

The unfolding and the stability of the three variants of apoflavodoxin were monitored by CD and fluorescence spectroscopy using GndHCl as denaturant. Fluorescence emission of apoflavodoxin was recorded at 333 nm as a function of the concentration denaturant. Alterations in the tertiary structure of apoflavodoxin caused by the denaturant-induced unfolding of the protein are thus monitored. Figure 4A shows the equilibrium unfolding data obtained for C69A apoflavodoxin. A two-state mechanism of unfolding (Equation 9) in which only native and denatured molecules are taken into account (Pace, 1986) fits excellently to the unfolding data. Similar unfolding curves were obtained for wild-type and C69S apoflavodoxin (data not shown). The linear extrapolation of the free energy of unfolding in the transition zone to zero concentration denaturant is used for the determination of ΔG_{N-U}^0 (fluor), the stability of apoflavodoxin as determined by fluorescence spectroscopy. Stability values thus obtained for the apoflavodoxin variants center around 5 kcal mol⁻¹ and are identical within the limits of the relatively large, but realistic (Santoro & Bolen, 1988), error (Table 1). The slopes m coincide within error (Table 1), consistent with the expectation that complete unfolding of all three variants of apoflavodoxin results in a similar exposure of hydrophobic groups. As is generally observed, the C_m values, the concentration denaturant at the midpoint of unfolding, are most accurately determined (Table 1). They show that protein stability decreases in the order wild-type, C69A, and C69S apoflavodoxin. $\Delta\Delta G_{N-U}$ values were calculated using Equation 12 to avoid the relatively large errors associated with protein stabilities caused by the extrapolation from the transition region to zero molar concentration denaturant. $\Delta\Delta G_{N-U}$ is the difference between the free energy of unfolding of a mutant and the wild-type protein at the concentration denaturant corresponding to the midpoint of unfolding of the wild-type protein. The $\Delta\Delta G_{N-U}$ values show that C69A and C69S apofla-

Fig. 4. GndHCl-induced equilibrium unfolding of apoflavodoxin from *A. vinelandii*. **A:** The GndHCl-induced unfolding of apoflavodoxin is reversible. Unfolding (●) and refolding (○) data for C69A apoflavodoxin as determined by the relative tryptophan fluorescence at 333 nm. The excitation wavelength was 280 nm. **B:** The GndHCl-induced unfolding of apoflavodoxin followed by CD in the far-UV (222 nm). The solid curves show the best fit of Equation 9 to the apoflavodoxin unfolding data assuming a two-state model of unfolding of the protein. The protein concentration was approximately 4 μ M in 100 mM potassium pyrophosphate, pH 6.0, and the spectra were recorded at 25 °C.

vodoxin are marginally destabilized by 0.10 and 0.13 kcal mol⁻¹, respectively, compared to the wild-type protein (Table 1).

The transition zone measured for the fluorescence equilibrium refolding data of apoflavodoxin coincides with the one obtained for apoflavodoxin equilibrium unfolding (Fig. 4A). The local geometry around the tryptophan residues of native apoflavodoxin, which is distorted by protein unfolding, can therefore be reversibly restored. NMR spectroscopy supports the latter. The ¹H-¹⁵N HSQC NMR spectrum acquired after removal of the denaturant from an unfolded apoflavodoxin sample shows a distribution of cross peaks characteristic for native apoflavodoxin (Fig. 2). Each individual amide group of refolded apoflavodoxin has regained its original native local microenvironment. Hence, apoflavodoxin unfolding is reversible at the atomic level.

The ellipticity at 222 nm is usually correlated with the α -helical content of a protein. This ellipticity was followed as a function of the concentration GndHCl to primarily track changes in secondary

Table 1. Thermodynamic parameters^a for the GndHCl-induced^b unfolding transitions of wild-type, C69A, and C69S *A. vinelandii* apoflavodoxin II, analyzed according to a two-state mechanism of equilibrium unfolding

	Wild-type ^c	C69A ^c	C69S ^c
Fluorescence emission			
ΔG_{N-U}^0 (fluor) (kcal mol ⁻¹) ^d	4.89 ± 0.40	5.23 ± 0.30	5.04 ± 0.24
m (kcal M ⁻¹ mol ⁻¹) ^e	-3.11 ± 0.24	-3.39 ± 0.19	-3.29 ± 0.14
C_m (M) ^f	1.57 ± 0.02	1.54 ± 0.01	1.53 ± 0.01
$\Delta\Delta G_{N-U}$ (kcal mol ⁻¹) ^g		0.10 ± 0.07	0.13 ± 0.07
Circular dichroism			
ΔG_{N-U}^0 (CD) (kcal mol ⁻¹) ^d	4.79 ± 0.87	4.13 ± 0.56	4.70 ± 1.46
m (kcal M ⁻¹ mol ⁻¹) ^e	-2.51 ± 0.43	-2.37 ± 0.30	-2.70 ± 0.81
C_m (M) ^f	1.91 ± 0.05	1.75 ± 0.04	1.74 ± 0.09
$\Delta\Delta G_{N-U}$ (kcal mol ⁻¹) ^g		0.38 ± 0.16	0.46 ± 0.33

^aDetermined at 25 °C in 100 mM potassium pyrophosphate, pH 6.0.^bFollowed by fluorescence emission intensity at 333 nm or by ellipticity at 222 nm. See Materials and methods for sample preparation and data analysis.^cError limits represent the standard error of each parameter in a nonlinear, least-squares fitting procedure.^dGibbs free energy of unfolding at zero denaturant concentration calculated using Equation 9.^eCalculated using Equation 9.^fCalculated using Equation 11.^g $\Delta\Delta G_{N-U}$ is the difference between the free energy of unfolding of the mutant and of the wild-type protein at the C_m of the wild-type protein and has been calculated using Equation 12.

structure during the equilibrium unfolding of apoflavodoxin. The far-UV CD equilibrium unfolding data obtained for C69A apoflavodoxin are shown in Figure 4B. All three variants of apoflavodoxin give rise to CD unfolding data to which a two-state mechanism of unfolding (Equation 9) can excellently be fitted. In analogy to the fluorescence results, the ΔG_{N-U}^0 (CD), m , C_m , and the $\Delta\Delta G_{N-U}$ values extracted from these two-state fits are reported in Table 1. The associated error values are higher than those from fluorescence due to the lower quality of the far-UV CD data. However, it is clear from the data of Figure 4 that CD measurements show shallower unfolding transitions (lower m values) and larger C_m values than those obtained by fluorescence measurements. This demonstrates that the unfolding of apoflavodoxin does not follow a simple two-state process.

Analysis using a three-state model for apoflavodoxin unfolding

The normalized fluorescence and CD data (Fig. 5) clearly show that some species, which lacks the characteristic tertiary structure of native apoflavodoxin but with significant secondary structure, exists at GndHCl concentrations around the midpoint of unfolding as measured from the fluorescence data. For example, at about 2 M GndHCl virtually no native wild-type apoflavodoxin molecules are present as judged from the fluorescence emission intensity but still a significant CD signal is observed (Fig. 5).

In its most simple form, apoflavodoxin folding can be described by the following three-state model:

in which a relatively stable folding intermediate is present at equilibrium.

The free energy of unfolding a protein is assumed to be linearly dependent on the denaturant concentration (Pace, 1986):

$$\Delta G_{N-U} = \Delta G_{N-U}^0 + m_{N-U}[D] \quad (2)$$

with ΔG_{N-U} the free energy of unfolding at a particular concentration of denaturant, ΔG_{N-U}^0 the free energy of unfolding at zero concentration denaturant, m_{N-U} the slope at the midpoint of the unfolding transition which is a measure for the exposure of hydrophobic groups upon protein unfolding, and $[D]$ the concentration of denaturant, respectively. A similar equation should account for the free energy of formation of partly unfolded folding intermediates from native molecules (Ikeguchi et al., 1992; Sanz & Fersht, 1993):

$$\Delta G_{N-I} = \Delta G_{N-I}^0 + m_{N-I}[D] \quad (3)$$

with definitions analogous to the ones described for Equation 2. Note that $m_{N-U} < m_{N-I}$ since the increase in exposure of buried groups on the complete unfolding of a protein must be larger than that on the formation of a partly unfolded intermediate (Sanz & Fersht, 1993).

In the three-state model for unfolding of apoflavodoxin (Equation 1), the equilibrium constants K_{N-U} and K_{N-I} are defined as $K_{N-U} = [U]/[N]$, and $K_{N-I} = [I]/[N]$. Consequently, the fraction of native protein molecules at equilibrium, $f_N = [N]/([N] + [I] + [U]) = 1/(1 + K_{N-I} + K_{N-U})$, which is rewritten as

$$f_N = \frac{1}{1 + \exp - [\Delta G_{N-I}/RT] + \exp - [\Delta G_{N-U}/RT]} \quad (4)$$

Similarly, the fractions of intermediate and unfolded states are given by

$$f_I = \frac{\exp - [\Delta G_{N-I}/RT]}{1 + \exp - [\Delta G_{N-I}/RT] + \exp - [\Delta G_{N-U}/RT]} \quad (5)$$

$$f_U = 1 - f_N - f_I \quad (6)$$

Fluorescence measurements inform about the loss of tertiary interactions upon unfolding and monitor the disappearance of the folded state of apoflavodoxin, as should be described by Equations 4 and 6.

tion 4. CD measurements monitor the appearance of the unfolded state of apoflavodoxin (described by Equation 6) if both the folded and intermediate forms have similar CD signals. Consequently,

using these equations, the fluorescence and CD experimental data are described by

$$Y_{\text{obs}}^{\text{CD}} = (\alpha_N + \beta_N[D]) + f_U((\alpha_U - \alpha_N) + (\beta_U - \beta_N)[D]) \quad (7)$$

$$Y_{\text{obs}}^{\text{FI}} = (\alpha'_U + \beta'_U[D]) + f_N((\alpha'_N - \alpha'_U) + (\beta'_N - \beta'_U)[D]) \quad (8)$$

with $Y_{\text{obs}}^{\text{FI}}$ the observed fluorescence emission intensity at 333 nM, $Y_{\text{obs}}^{\text{CD}}$ the observed CD ellipticity at 222 nM, and α and β the intercepts and slopes of the pre- and post-unfolding regimes.

In analogy to the approach of Sanz and Fersht (1993), and as a first approximation: (1) $m_{\text{N-U}}$ was calculated from the fluorescence unfolding data (about $-3 \text{ kcal M}^{-1} \text{ mol}^{-1}$ (Table 1)); (2) $m_{\text{N-I}}$ was firstly guessed as $m(\text{fluorescence}) - m(\text{CD})$ (about $-1 \text{ kcal M}^{-1} \text{ mol}^{-1}$ (Table 1)); (3) $\Delta G_{\text{N-U}}$ was taken from the fluorescence data. The intercepts and slopes of the pre- and post-unfolding regimes were taken from the fit of a two-state unfolding model to the data according to Santoro and Bolen (1988) using Equation 9. These latter parameters were kept fixed in the simultaneous fit of Equations 7 and 8 to the CD and the fluorescence data, respectively. This “global analysis” approach used a standard nonlinear least-squares method based on the Marquardt–Levenberg algorithm (Press et al., 1988) implemented in IDL (RSI, Boulder, Colorado). Due to the presence of local minima, the fit results were dependent on the initial m and ΔG values. Consequently, a refinement was implemented: starting values of m and ΔG were taken from a Gaussian distribution around their initial values and subsequently the “global analysis” approach was applied. This procedure was repeated several thousand times and the fit resulting in the lowest χ^2 was used to extract $\Delta G_{\text{N-U}}^0$, $m_{\text{N-U}}$, $\Delta G_{\text{N-I}}^0$, and $m_{\text{N-I}}$ values, which are reported in Table 2. The results of the fits for the changes in fluorescence and ellipticity correspond nicely with both the experimental fluorescence and the CD data obtained for wild-type, C69A, and C69S apoflavodoxin (Fig. 5).

The fractions of native, intermediate state, and unfolded molecules as a function of the concentration denaturant are shown in Figure 6 for C69A apoflavodoxin. The three-state model predicts that the maximum accumulation of intermediate state C69A apoflavodoxin is at 1.72 M GndHCl and equals 22% of the total amount of molecules present. A maximum fraction of 37% wild-type apoflavodoxin folding intermediate at 1.76 M GndHCl and of 24% C69S intermediate at 1.70 M GndHCl are calculated.

Thermally-induced unfolding

To investigate whether apoflavodoxin thermal unfolding involves an equilibrium folding intermediate species, temperature-induced unfolding curves were acquired. The apoflavodoxin variants were heated at a heating rate of $0.5^\circ\text{C min}^{-1}$. According to both CD and fluorescence spectroscopy, a further decrease in the heating rate did not result in a decrease of the thermal transition midpoint T_m of a $2 \mu\text{M}$ C69A apoflavodoxin sample. The reversibility of thermal unfolding of C69A apoflavodoxin as judged from the reappearance of its original fluorescence emission spectrum was typically about 60%. Since apoflavodoxin is in the latter method at temperatures that exceed T_m by up to 19°C (Fig. 7A) for an extended period of time, the reversibility is relatively low, but still substantial. Equation 14 which describes the temperature dependence of the experimental observables according to a two-state mechanism of thermal unfolding (see Materials and methods) was fitted to the CD and fluorescence unfolding data.

Fig. 5. Guanidinium hydrochloride unfolding profiles of (A) wild-type, (B) C69A, and (C) C69S apoflavodoxin from *A. vinelandii* as measured by fluorescence at 333 nM (●) and by ellipticity at 222 nM (○). Results of the fits for the changes in fluorescence and ellipticity calculated for a three-state transition (Equations 1–8) are shown in solid and dashed lines, respectively. The raw data are presented, including the slopes in the baselines, except that the values are normalized to 1 and 0 for the fluorescence and the ellipticity of the native and unfolded proteins, respectively. The CD measurements show shallower unfolding transitions (lower m values) and larger C_m values than those obtained by fluorescence measurements (Table 1). Apoflavodoxin is at 25°C in 100 mM potassium pyrophosphate, pH 6.0.

Table 2. Thermodynamic parameters^a for the GndHCl-induced^b unfolding transitions of wild-type, C69A, and C69S *A. vinelandii* apoflavodoxin II analyzed according to a three-state mechanism of equilibrium unfolding

	Wild-type ^c	C69A ^c	C69S ^c
ΔG_{N-U}^0 (kcal mol ⁻¹) ^d	7.36 ± 0.26	6.23 ± 0.20	6.44 ± 0.35
m_{N-U} (kcal M ⁻¹ mol ⁻¹) ^e	-4.27 ± 0.13	-3.84 ± 0.12	-3.97 ± 0.21
ΔG_{N-I}^0 (kcal mol ⁻¹) ^f	4.11 ± 0.31	4.42 ± 0.37	4.36 ± 0.52
m_{N-I} (kcal M ⁻¹ mol ⁻¹) ^e	-2.44 ± 0.21	-2.50 ± 0.25	-2.50 ± 0.35

^aDetermined at 25 °C in 100 mM potassium pyrophosphate, pH 6.0.^bFollowed by fluorescence emission intensity at 333 nM or by ellipticity at 222 nM. See Materials and methods for sample preparation and data analysis.^cError limits represent the standard deviation of each parameter in a nonlinear, least-squares fitting of the changes in fluorescence and ellipticity calculated for a three-state transition (Equations 1–8).^dGibbs free energy of unfolding at zero denaturant concentration calculated using Equations 1–8.^eCalculated using Equations 1–8.^fGibbs free energy of unfolding native apoflavodoxin to a partly unfolded folding intermediate at zero denaturant concentration calculated using Equations 1–8.

The temperature dependent fluorescence emission changes observed at 350 nM for C69A apoflavodoxin are illustrated in Figure 7A. Similar data are obtained for both wild-type and C69S apoflavodoxin. A single transition, which fits excellently to the two-state mechanism for thermally-induced unfolding (Equation 14), is observed. Thermal midpoints of unfolding (T_m) are: 47.7 ± 0.2 °C for wild-type, 48.6 ± 0.1 °C for C69A, and 49.1 ± 0.2 °C for C69S apoflavodoxin, respectively. The marginal differences measured in T_m suggest a virtually equal stability of C69A and C69S apoflavodoxin with the wild-type protein having the lowest stability.

The thermally-induced unfolding of all three variants of apoflavodoxin as followed by the ellipticity at 222 nM shows anomalous behavior (Fig. 7B). Beyond T_m , a cooperative increase in the negative amplitude of the ellipticity at 222 nM is observed. A similar observation has recently been made for the cellulose-binding domain of xylanase XynA from the hyperthermophilic bacterium *Thermotoga maritima* (Wassenberg et al., 1997). Evidently, the effect observed does not reflect an increase in helicity; rather, it is most

likely attributable to changes in the tertiary interactions of aromatic amino acid side chains (Wassenberg et al., 1997). The T_m values extracted from the fits of Equation 14 to the CD data are: 61.7 ± 0.3 °C for wild-type, 64.9 ± 0.4 °C for C69A, and 62.9 ± 0.3 °C for C69S apoflavodoxin, respectively. Protein stability now seems to decrease in the order C69A, C69S, and wild-type apoflavodoxin.

Figure 7C shows the fractions native C69A apoflavodoxin molecules as a function of the temperature. From the noncoincidence of the fluorescence and CD thermal unfolding data, it is clear that the thermal unfolding of apoflavodoxin does not obey a two-state mechanism of unfolding. In analogy to the GndHCl-induced unfolding of apoflavodoxin, a species that lacks the characteristic tertiary structure of native apoflavodoxin, but with significant secondary structure, exists at temperatures around the thermal midpoint of unfolding as measured from the fluorescence data. For example, at 60 °C, more than 10 °C above the thermal midpoint of unfolding of C69A apoflavodoxin as determined by fluorescence spectroscopy, virtually no native protein molecules are present, but still a significant CD signal is observed (Fig. 7).

Several differential scanning calorimetry (DSC) experiments were done using apoflavodoxin. Despite the variety of buffer conditions used and upon minimizing the protein concentration to the 15 μM DSC detection limit of our DSC apparatus, the apoflavodoxin thermal unfolding reaction remained irreversible. Consequently, a quantitative thermodynamic analysis of the DSC data is not feasible and ΔC_p values could not be extracted. Hence, we did not analyze the CD and fluorescence thermal unfolding data of apoflavodoxin (Fig. 7) according to a three-state mechanism of unfolding. The apoflavodoxin aggregation observed in the DSC experiments is most likely caused by the exposure of hydrophobic groups in the folding intermediate discussed, in combination with the relatively high protein concentrations necessary to perform these experiments.

Discussion

Presence of a relatively stable apoflavodoxin folding intermediate

In the present study, as a first step in the investigation of how *A. vinelandii* flavodoxin folds, denaturant- and thermally-induced

Fig. 6. Simulated fractions of native (---), intermediate state (—), and unfolded (•••) C69A apoflavodoxin molecules as a function of the concentration guanidinium hydrochloride. The fractions are calculated for a three-state apoflavodoxin unfolding transition using Equations 4–6 and the parameters of Table 2.

Fig. 7. Thermal unfolding of C69A apoflavodoxin from *A. vinelandii*. **A:** Thermal unfolding of apoflavodoxin as monitored by the change in fluorescence emission at 350 nm. The fluorescence excitation wavelength was 280 nm. **B:** Thermal unfolding of apoflavodoxin as monitored by the change in ellipticity at 222 nm. The solid curves shown are the best fit of Equation 14 to the apoflavodoxin thermal unfolding data assuming a two-state model of unfolding of the protein. **C:** Fraction folded C69A apoflavodoxin molecules versus temperature as monitored by the change in fluorescence emission at 350 nm (●) and by the change in CD at 222 nm (○). Both fractions are calculated using the fit parameters of the individual unfolding curves presented in (A) and (B), respectively. The apoflavodoxin concentration in all experiments is approximately 2 μ M. Apoflavodoxin is in 100 mM potassium pyrophosphate, pH 6.0, and the heating rate is 0.5 $^{\circ}$ C/min.

equilibrium unfolding studies on apoflavodoxin are reported. Both fluorescence and NMR spectroscopy reveal that the GndHCl-induced unfolding of apoflavodoxin is fully reversible (Figs. 2, 4A). Apoflavodoxin can thus fold in the absence of the FMN cofactor under the experimental conditions used. The reversibility of apoflavodoxin unfolding is a prerequisite to enable the future application of hydrogen exchange pulse labeling methods in combination with NMR spectroscopy (Roder et al., 1988), and of “real time” NMR experiments (Balbach et al., 1996) to study apoflavodoxin folding in atomic detail. The availability of sequence-specific resonance assignments of native apoflavodoxin is the second prerequisite to be able to characterize the folding of this α/β doubly wound protein in atomic detail. These NMR assignments and the individual amide hydrogen/deuterium exchange rates were recently obtained and are reported elsewhere (Steensma & van Mierlo, 1998).

The unfolding profiles obtained for the GndHCl-induced unfolding of apoflavodoxin as monitored by circular dichroism and fluorescence spectroscopy do not coincide (Fig. 5). Apoflavodoxin unfolding can therefore not be described by a simple two-state mechanism. The deviation from classical two-state behavior could arise from guanidinium hydrochloride effects on a dimer-monomer transition of apoflavodoxin molecules. This explanation, however, seems unlikely as the amount of dimer present in the samples is low (see Materials and methods), and as we observe that denaturant-induced fluorescence and CD unfolding data of C69A holoflavodoxin, of which no dimer or high molecular weight form has been detected, do not coincide either (data not shown). In addition, the denaturant-induced unfolding data may also be consistent with a two-state mechanism of unfolding in which the denatured state changes continuously from a compact form to an unfolded form with increasing denaturant concentration. The latter can theoretically lead to noncoincident protein unfolding curves (Dill & Shortle, 1991). Although we cannot completely rule out the latter possibility, the unfolding profiles can be wholly described under the assumption that apoflavodoxin unfolding occurs via some species that lacks the characteristic tertiary structure of native apoflavodoxin as deduced from its fluorescence emission intensity, but with a significant amount of secondary structure as inferred from CD spectroscopy. Folding intermediates with such characteristics, often referred to as molten globule-like species, have been observed for only a few proteins (Ptitsyn, 1992).

We modeled the GndHCl-induced apoflavodoxin unfolding curves by assuming a three-state mechanism of unfolding (Equation 1). Such a relatively simple model suffices to explain the experimental data. Excellent fits to both the fluorescence and the circular dichroism data of wild-type, C69A, and C69S apoflavodoxin are achieved (Fig. 5). The resulting ΔG_{N-U}^0 values are larger than those calculated via a two-state fit to the fluorescence and CD unfolding data (Tables 1, 2). The C69A and C69S variants of apoflavodoxin are equally stable but destabilized relative to wild-type apoflavodoxin by approximately 1 kcal mol $^{-1}$ (Table 2). Despite the resulting destabilization, the replacement of the single cysteine residue of apoflavodoxin is essential to avoid complications due to intermolecular disulfide bond formation during the long heteronuclear multidimensional NMR experiments. We use the C69A variant in our NMR investigations of both holo- and apoflavodoxin (van Mierlo et al., 1997; Steensma et al., 1998; Steensma & van Mierlo, 1998).

In this study it is shown that the maximum accumulation of the intermediate folding species of C69A apoflavodoxin is at 1.72 M

GndHCl and equals 22% of the total amount of molecules present (Fig. 6). Its corresponding m_{N-1} value is less negative than m_{N-U} , as is also found for the other two apoflavodoxin variants. This is not unexpected since there must be a greater increase in exposure of buried groups on the complete unfolding of a protein than there is on the formation of a partly unfolded intermediate from the native state (Sanz & Fersht, 1993). The m_{N-1} value obtained for the C69A apoflavodoxin folding intermediate is within experimental error identical to the corresponding values of the wild-type and the C69S apoflavodoxin intermediate species (Table 2). This suggests that similar conformational changes occur upon formation of the intermediate species from the native state of the three apoflavodoxin variants, as m -values reflect the exposure of hydrophobic groups upon protein unfolding. Wild-type apoflavodoxin, however, has the most stable intermediate species (Table 2).

Thermally-induced unfolding of apoflavodoxin as monitored by circular dichroism and fluorescence spectroscopy results in unfolding curves that do not coincide (Fig. 7). Considerably differing thermal midpoints of unfolding are obtained using both techniques. This, again, clearly contradicts two-state unfolding behavior. During thermal unfolding a species with molten globule-like characteristics similar to the folding intermediate observed in the denaturant-induced equilibrium unfolding of apoflavodoxin is present. Fluorescence emission changes suggest a virtually equal stability of C69A and C69S apoflavodoxin with the wild-type protein having the lowest stability. Changes in ellipticity at 222 nM show a decrease in protein stability in the order C69A, C69S, and wild-type apoflavodoxin, respectively. Both results contradict with the order of stability as determined via the three-state fit of the GndHCl-induced unfolding data.

Significant differences exist between the denaturant-induced and the thermally-induced unfolding data as measured by the changes in ellipticity at 222 nM (Figs. 4B, 7B). The denaturant-induced unfolding curve shows a decrease in the negative ellipticity due to unfolding of the protein upon increasing the concentration GndHCl, as is generally observed. Anomalous behavior is seen during the thermal unfolding of apoflavodoxin: the negative ellipticity at 222 nM increases in the transition region of the unfolding curve (Fig. 7B). The effect is most likely attributable to changes in the tertiary interactions of aromatic side chains (Wassenberg et al., 1997). The 222 nM ellipticity values at high temperatures suggest the presence of some residual structure in the thermally unfolded state of apoflavodoxin, which will be discussed in relation to hydrogen/deuterium exchange rates below. The fluorescent, circular dichroism and NMR properties of GndHCl denatured apoflavodoxin suggest an unfolded state with little, if any, residual ordered structure.

Structural scheme for the equilibrium unfolding of apoflavodoxin

Hydrogen exchange measurements, which are usually performed under native conditions inform about local, subglobal, and global unfolding of proteins (Woodward, 1993; Englander et al., 1996). Individual amide proton exchange rates of both holo- and apoflavodoxin were determined by using NMR spectroscopy in combination with the hydrogen/deuterium exchange method (Steensma et al., 1998; Steensma & van Mierlo, 1998). Hydrogen exchange rates can be analyzed in terms of a structural unfolding model, in which exchange only takes place from an "open" form of the protein, but not from the "closed" form (Linderstrøm-Lang, 1955;

Hvidt & Nielsen, 1966). The measured amide proton exchange rates, k_{ex} , are dependent on the local free energies for transient opening of the flavodoxin structure, which in turn can be determined. The transition between the "open" and "closed" forms of a protein is a temperature-dependent process. Consequently, the hydrogen exchange results of apoflavodoxin are intimately related to the thermally induced unfolding data discussed. Local unfolding of a protein structure is characterized by hydrogen exchange rates, which are larger than the ones associated with global unfolding. Figure 8 shows a cartoon drawing of apoflavodoxin in which residues are colored according to the exchange rates of backbone amide protons of the C69A variant of the protein. The most slowly exchanging amide protons reside in the ordered core of the molecule and form the stable nucleus, whereas amide protons in the flexible flavin binding region exchange rapidly (Steensma & van Mierlo, 1998).

Exchange of the most protected amide protons has been shown to often occur via global unfolding of a protein (Woodward, 1993; Englander et al., 1996). We therefore infer that the structured part and stable nucleus of the apoflavodoxin folding intermediate comprises the regions colored blue in Figure 8. These regions involve roughly 28 residues in strands $\beta 1$, $\beta 3$, $\beta 4$, and $\beta 5a$ and in helices $\alpha 4$ and $\alpha 5$, respectively. The calculated local free energies for transient opening of the C69A apoflavodoxin structure (ΔG_{op}) range

Fig. 8. MOLSCRIPT cartoon drawing (Kraulis, 1991) of the X-ray structure of *A. chroococcum* flavodoxin (Thorneley et al., 1994). The structure is colored according to the exchange behavior of the backbone amide protons of the homologous *A. vinelandii* apoflavodoxin II (Steensma & van Mierlo, 1998). The most slowly exchanging amide protons reside in the ordered core of the molecule, which is colored blue, and form the stable nucleus. Red: $\log(k_{ex}) \geq -2$; green: $-5 < \log(k_{ex}) < -2$; blue: $\log(k_{ex}) \leq -5$; yellow: overlap of ^1H - ^{15}N HSQC cross peaks; grey: proline residue or residue of which the amide proton could not be assigned by NMR spectroscopy.

from 3 kcal mol⁻¹ to >9 kcal mol⁻¹ (Steensma & van Mierlo, 1998). The average ΔG_{op} of the stable nucleus of apoflavodoxin is calculated to be 7.0 kcal mol⁻¹ (van Mierlo & Steensma, 1998). This is significantly higher than the measured global stability ($\Delta G_{\text{N-U}}^0$) of 6.2 kcal mol⁻¹ (Table 2). Possible explanations for ΔG_{op} exceeding $\Delta G_{\text{N-U}}^0$ have been described by Steensma and van Mierlo (1998) and include among others proline isomerization in the unfolded state and an increased protein stability in ²H₂O than in H₂O. A definite explanation for the enhanced protection found in apoflavodoxin, however, has to await further investigation. The extremely slow exchange of the backbone amide protons of Tyr114, Phe117, and Lys118 in the C-terminal half of helix α_4 (exchange rates $< 2 \cdot 10^{-7}$ s⁻¹) indicates that the aromatic region Y₁₁₄-S-F-F-K₁₁₈ most likely contains residual structure in the thermally denatured state of the protein (Steensma & van Mierlo, 1998).

A model for the equilibrium unfolding of *A. vinelandii* apoflavodoxin is shown in Figure 9. The scheme is based on a three-state model of apoflavodoxin unfolding (Equation 1), which incorporates the spectroscopic data presented here and the hydrogen/exchange results presented elsewhere (Steensma & van Mierlo, 1998). We infer that the equilibrium folding intermediate present under the hydrogen exchange conditions is structurally more or less equivalent to the species detected in the GndHCl- and thermally-induced equilibrium unfolding experiments. Whether the equilibrium folding intermediate is similar to a kinetic intermediate in apoflavodoxin folding needs to be verified. As structure formation during protein folding coincides with protection from amide proton exchange, hydrogen exchange pulse labeling methods in combination with NMR spectroscopy should reveal the latter in the near future and enable the study of the sequential steps in flavodoxin folding.

Conclusion

We have demonstrated that both GndHCl- and thermally-induced unfolding of *A. vinelandii* apoflavodoxin II occurs via a relatively stable folding intermediate. This is in contrast with the observations that unfolding of apoflavodoxin from *Anabaena* PCC 7119 by urea and temperature follows a simple two-state mechanism (Genzor et al., 1996). The factors that cause the significant stabilization of the *A. vinelandii* apoflavodoxin folding intermediate need additional attention. Since the *A. vinelandii* apoflavodoxin intermediate species accumulates in substantial amounts under equilibrium circumstances in GndHCl, it should be possible to further characterize its structure and dynamics by techniques like NMR spectroscopy. The investigations of the folding of flavodoxin, which is an excellent model system to study protein folding, should lead to the rules by which other, if not all, proteins with a flavodoxin-like topology fold.

Materials and methods

Materials

Enzymes for cloning, isopropylthio- β -D-galactoside (IPTG), and tryptone were purchased from Life Technologies (Gaithersburg, Maryland), oligonucleotides and DEAE-Sepharose CL 6B were from Pharmacia Biotech (Roosendaal, The Netherlands), tryptone from Difco (Detroit, Michigan), GndHCl (ultrapure), potassium pyrophosphate, and protamine sulfate were from Sigma (Bornem, Belgium), Biogel P6-DG from Biorad (Veenendaal, The Netherlands), and ampicillin, dithiothreitol, DNase I, and Tris-HCl were from Boehringer Mannheim (Almere, The Netherlands). All other chemicals were from Merck (Amsterdam, The Netherlands) of the purest analytical grade available.

Fig. 9. Schematic drawing of the equilibrium unfolding of apoflavodoxin from *A. vinelandii*. The proposed scheme is based on the equilibrium unfolding experiments described in this paper and on hydrogen/deuterium exchange NMR experiments with native apoflavodoxin (Steensma & van Mierlo, 1998). Rigid parts of the protein structures are depicted in black, whereas flexible regions are shown in white. Native apoflavodoxin has a stable well-ordered core but its flavin binding region is flexible. The relatively stable intermediate found in the three-state unfolding transition of apoflavodoxin is characterized by the loss of tertiary interactions without the complete loss of secondary structure (i.e., it has the characteristics of a molten globule-like species). The unfolded state of apoflavodoxin presumably contains some residual structure of an aromatic cluster in the region Y114-K118, as a remnant of helix α_4 (Steensma & van Mierlo, 1998).

Isolation of the flavodoxin II gene from A. vinelandii ATCC 478, construction of an expression plasmid, and site-directed mutagenesis

Plasmid pDB6, which contains the *nifHDK* genes from *A. vinelandii* OP (Brigle et al., 1985), was used as a hybridization probe. This probe was used to isolate the major *nif*-gene cluster from *A. vinelandii* ATCC 478 on to a set of overlapping restriction fragments, which were cloned into *E. coli*. The *nifF* gene, which encodes a flavodoxin in *A. vinelandii* ATCC 478, was isolated from one of these recombinant plasmids on a 0.72 kb *SphI/NdeI* restriction fragment. This fragment, of which the *NdeI* site was made blunt-ended with Klenow polymerase, was inserted between the *SphI* and *SmaI* sites of pUC19 (Yanisch-Perron et al., 1985). The *nifF* gene was placed in the proper orientation toward and at a suitable distance from the *lacZ* promoter of the vector. All experiments described in this paper were done with flavodoxin produced from this recombinant plasmid in *E. coli* TG2 (Gibson, 1984).

The amino acid sequence of the recombinant flavodoxin, derived from the nucleotide sequence of the corresponding gene, is identical with that of the *nifF*-encoded flavodoxin from *A. vinelandii* OP (Bennett et al., 1988). N-terminal sequencing of the recombinant flavodoxin confirmed the presence of residues Ala(1)-Phe(7). The N-terminal methionine, however, is not found in the mature protein.

To prevent complications due to intermolecular disulfide bridge formation, the sole cysteine of *A. vinelandii* flavodoxin II was replaced by both an alanine and a serine residue. To achieve this replacement, the TGC-codon for Cys69 was substituted by GCC (alanine) or TCC (serine). Site-directed mutagenesis of the double-stranded pAV34 DNA was done according to a polymerase chain reaction protocol using *Taq* polymerase as modifying enzyme (essentially as described by Landt et al., 1990). The mutated genes were sequenced entirely to confirm the presence of the desired mutations and the absence of adventitious mutations.

Protein expression and purification

Recombinant wild-type, C69A, and C69S *A. vinelandii* flavodoxin II (strain ATCC 478) were expressed in *E. coli* TG2 (pAV34) and purified according to a modification of the procedures of Tollin and Edmondson (1980) and Hinkson and Bulen (1967). A 6-l culture was grown in a standard TY medium (Miller, 1972), which contains 50 mg/L ampicillin and 20 mg/L IPTG, while being shaken, for approximately 20 h at 37°C. The *E. coli* cells were subsequently harvested via centrifugation for 30 min at 7,000 g and 4°C. The greenish wet cell paste (about 30 g) was suspended at a ratio of 1 g/mL in 100 mM Tris-HCl, pH 8.0, containing 0.75 mM EDTA, 0.5 mM 1,4-dithiothreitol, 1.5 mM MgCl₂ and 1 mg DNase I from bovine pancreas. To disrupt the cells, the suspension was passed three times through a precooled French press, which had an estimated internal cell pressure of 14,000 to 17,000 psi. Unbroken cells and large cell debris were removed by centrifugation for 30 min at 33,000 g and 4°C. To remove nucleic acids and large protein complexes, the supernatant was treated with 0.5% (w/v) protamine sulfate and clarified by centrifugation for 30 min at 33,000 g and 4°C. The standard buffer, 100 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM 1,4-dithiothreitol, pH 8.0, was used in all subsequent purification steps. While being stirred, the supernatant was brought to 50% ammonium sulfate by dropwise

addition of a 100% ammonium sulfate solution. The resulting precipitate was removed by centrifugation for 30 min at 16,000 g and 4°C, and the supernatant was subsequently brought to 75% ammonium sulfate by slow addition of pulverized ammonium sulfate. Again, precipitate was removed by centrifugation.

The yellow supernatant was brought on to a DEAE-Sepharose Cl 6B column, which had been pre-equilibrated in 75% ammonium sulfate. After washing with this solution, the strongly bound flavodoxin was eluted with 1 M KCl. The yellow eluate was concentrated and brought into 50 mM KCl in standard buffer by ultrafiltration using an Amicon YM10 membrane (Beverly, Massachusetts). It was then again applied onto the DEAE-Sepharose column, which had been pre-equilibrated with the latter buffer. After washing, a linear gradient of 0.05–0.7 M KCl was applied. Flavodoxin elutes between 0.4 and 0.5 M KCl. The fractions containing flavodoxin were pooled and brought into 100 mM KCl by ultrafiltration. Finally, the protein solution was applied onto a High Load Q Sepharose column (Pharmacia, Uppsala, Sweden), equilibrated in 100 mM KCl in standard buffer. After washing and application of a linear gradient of 0.1–0.7 M KCl, flavodoxin eluted in pure form as indicated by an absorbency ratio A₂₇₄/A₄₅₂ of 4.6–4.8. Flavodoxin concentrations were determined using a molar absorption coefficient of 11.3 mM⁻¹ cm⁻¹ at 452 nm (Klugkist et al., 1986). Approximately 5 mg of flavodoxin was obtained per gram of wet cell paste. Purified flavodoxin was concentrated by ultrafiltration, lyophilized, and stored at –20°C.

Apoflavodoxin preparation

All three apoflavodoxin variants were prepared according to a slightly modified version of the original procedure described by Edmondson and Tollin (1971). The FMN was removed from the holoprotein by addition of a 30% (w/v) aqueous solution of trichloroacetic acid to a final concentration of 3%. Holoflavodoxin was in 50 mM potassium phosphate pH 7.0, 1 mM dithiothreitol, and 0.3 mM EDTA. The precipitated protein was collected by centrifugation and resuspended in a 3% (w/v) TCA solution in 50 mM potassium phosphate pH 7.0, 1 mM dithiothreitol and 0.3 mM EDTA. After stirring and subsequent centrifugation, a colorless apoprotein was obtained. All solutions were kept on ice, and the procedure was executed in the dark. Finally, the apoprotein was dissolved in 300 mM Tris-HCl pH 8.5, brought to 100 mM potassium pyrophosphate pH 6.0 via gel filtration, and stored under liquid N₂.

The concentration of apoflavodoxin was determined spectrophotometrically using the molar absorption coefficient of 29 mM⁻¹ cm⁻¹ at 280 nm (Barman & Tollin, 1972). At least 85% yield of apoflavodoxin was generally obtained. Gel filtration chromatography experiments give reproducible results for all three variants of the protein: apoflavodoxin is largely monomeric. About 13% of the total amount of apoflavodoxin is in a high molecular weight complex. In the presence of 1 mM dithiothreitol, approximately 6% of C69A and C69S apoflavodoxin, and approximately 10% of wild-type apoflavodoxin elute at volumes corresponding with a dimer state of the protein.

NMR spectroscopy

¹H-¹⁵N HSQC spectra of native and unfolded C69A apoflavodoxin were recorded on a Bruker AMX-500 spectrometer at

298 K as described previously (see Steensma et al., 1998; Steensma & van Mierlo, 1998). The native apoflavodoxin sample was concentrated in 100 mM potassium pyrophosphate, pH 6.0, by ultrafiltration using an Amicon centriprep concentrator. The sample contained 2.1 mM ^{15}N -labeled protein in 90% H_2O /10% $^2\text{H}_2\text{O}$. Internal [2,3- $^2\text{H}_4$] 3-trimethylsilylpropionate was present in minute amounts. Apoflavodoxin was unfolded in the NMR tube by controlled addition of 5.4 M GndHCl in 100 mM potassium pyrophosphate pH 6.0 in 90% H_2O /10% $^2\text{H}_2\text{O}$. The final protein concentration was 0.7 mM in 4.0 M GndHCl. In the ^1H dimension of the ^1H - ^{15}N HSQC experiments, 1,024 real data points were acquired whereas in the indirect ^{15}N dimension 256 complex data points were collected. The NMR data were referenced, processed, and analyzed as described previously (Steensma et al., 1998; Steensma & van Mierlo, 1998).

GndHCl: Induced unfolding

All unfolding studies were done in 100 mM potassium pyrophosphate containing 1 mM freshly prepared dithiothreitol at pH 6.0. The GndHCl stock solution (6 M) was prepared gravimetrically in a volumetric flask analogous to the procedure proposed by Pace (1986) and Pace et al. (1989). For each individual data point in the unfolding/refolding experiments, 100 μL of 40 μM apoflavodoxin in 100 mM potassium pyrophosphate at pH 6.0 was diluted into 900 μL of the appropriate denaturant concentration using a Hamilton syringe. The denaturant series was made volumetrically using Gilson pipettes. The stock solutions of GndHCl, buffer, and protein were made particle free via filtration through 0.22 μm Millipore filters.

The apoflavodoxin samples at the various denaturant concentrations were kept in the dark in a waterbath at $25 \pm 1^\circ\text{C}$. Denaturant-induced apoflavodoxin unfolding was well at equilibrium within 5 h as judged from CD and fluorescence spectra. All individual apoflavodoxin samples were therefore equilibrated for exactly 5 h. The reversibility of unfolding was demonstrated using 40 μM apoflavodoxin, which had been denatured for at least 4 h in 4 M GndHCl. Refolding was then started by diluting the unfolded apoflavodoxin stock to the appropriate GndHCl concentration until equilibrium was reached. All spectroscopic measurements were carried out in a thermostatted cuvette holder at $25 \pm 0.2^\circ\text{C}$.

The GndHCl-induced unfolding data as monitored by fluorescence and CD could both be individually analyzed according to a two-state model of unfolding in which only the native and the denatured states are populated (Pace, 1986; Pace et al., 1989; Schmid, 1989, 1992). The free energy of unfolding of a protein is assumed to be linearly dependent on the denaturant concentration. Taking the linear dependence of the pre- and post-unfolding data on denaturant concentration into account as proposed by Santoro and Bolen (1988), the linear extrapolation method can be fitted to the entire data according to:

$$Y_{\text{obs}} = \frac{(\alpha_N + \beta_N[D]) + (\alpha_U + \beta_U[D])\exp - [(m[D] + \Delta G_{N-U}^0)/RT]}{1 + \exp - [(m[D] + \Delta G_{N-U}^0)/RT]} \quad (9)$$

with Y_{obs} the fluorescence emission intensity at 333 nM, or the CD ellipticity at 222 nM, $[D]$ the concentration of GndHCl, m the slope at the midpoint of the unfolding transition, α and β the intercepts and slopes of the pre- and post-unfolding regimes and

ΔG_{N-U}^0 the free energy of unfolding at zero concentration denaturant, respectively, and RT is 0.59 kcal mol $^{-1}$. Using Equation 9, relatively large, but realistic errors are obtained for both m and ΔG_{N-U}^0 (Santoro & Bolen, 1988). In contrast, it is a general observation that the determination of the C_m value, the concentration denaturant corresponding to the midpoint of unfolding, is quite accurate. Therefore, it provides a sensitive measure of the difference in stability between wild-type and mutant proteins. In analogy to Jackson et al. (1993), it follows from the linear dependence of the free energy of unfolding on the denaturant concentration (Equation 2) that at C_m : $\Delta G_{N-U}^0 = -m \cdot C_m$; thus,

$$\Delta G_{N-U} = m([D] - C_m) \quad (10)$$

with ΔG_{N-U} the free energy of unfolding at a particular denaturant concentration. Consequently, it follows from Equations 9 and 10 that

$$Y_{\text{obs}} = \frac{(\alpha_N + \beta_N[D]) + (\alpha_U + \beta_U[D])\exp - [m([D] - C_m)/RT]}{1 + \exp - [m([D] - C_m)/RT]} \quad (11)$$

Equation 11 was fitted to the data by nonlinear, least-squares analysis using the general curve fit option of the Kaleidagraph program (version 2.1, Synergy Software, PCS Inc., Reading, Pennsylvania) to obtain values for C_m and their respective standard errors. The difference between the free energy of unfolding of the mutant and of the wild-type protein at the concentration denaturant $[D]$ corresponding to the midpoint of unfolding of the wild-type protein has been calculated from Equation 12 (Jackson et al., 1993):

$$\Delta\Delta G_{N-U} = m([D] - C_m) - m'([D] - C'_m) \quad (12)$$

where m' and C'_m are the m and C_m values of the mutant, respectively. The $\Delta\Delta G_{N-U}$ values obtained by this method are more accurate than the $\Delta\Delta G_{N-U}^0$ values as there is no long extrapolation from the transition region to 0 M GndHCl, which introduces a large error in the estimate of ΔG_{N-U}^0 .

Thermally-induced unfolding

All experiments were done using 2 μM apoflavodoxin in 100 mM potassium pyrophosphate at pH 6.0 and all samples contained 1 mM freshly prepared dithiothreitol. The samples were made particle free via filtration through 0.22 μm Millipore filters and degassed under vacuum just prior to the measurements. Unfolding of apoflavodoxin was monitored by fluorescence and CD as a function of temperature. The heating rate was varied until thermal unfolding of the protein was at equilibrium according to both techniques, i.e., a further decrease in heating rate did no longer result in a decrease of the transition midpoint (T_m).

Analogous to the derivation of an expression for the experimental observable in denaturant-induced unfolding (Santoro & Bolen, 1988), the general observation that pre- and post-unfolding baselines in melting curves depend approximately linearly on temperature is incorporated into an expression for the experimental observable in thermally-induced unfolding. In such an

expression, the change in free energy for thermally-induced protein unfolding, $\Delta G(T)$, is described by the modified Gibbs–Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln(T/T_m)] \quad (13)$$

where ΔH_m is the enthalpy change for unfolding measured at T_m , T is the absolute temperature, T_m the temperature at the midpoint of the transition, and ΔC_p is the difference in heat capacity between the unfolded and folded states (Becktel & Schellman, 1987; Pace et al., 1989). Under the assumption that ΔC_p is temperature independent (Privalov & Khechinashvili, 1974), a two-state mechanism of unfolding can be fitted to the individual thermal unfolding curves:

$$Y_{\text{obs}} = (\alpha_U + \beta_U T) + \frac{((\alpha_N + \beta_N T) - (\alpha_U + \beta_U T))}{(1 + \exp(((\Delta H_m/R)(1/T - 1/T_m)) + ((\Delta C_p/R)((T_m/T) - 1) + \ln(T/T_m))))} \quad (14)$$

where Y_{obs} is the measured CD or fluorescence signal, R is the gas constant, α and β the intercepts and slopes of the pre- and post-unfolding baselines, respectively.

The data were fitted to Equation 14 by nonlinear, least-squares analysis using the general curve fit option of the Kaleidagraph program (version 2.1, Synergy Software, PCS Inc.) to obtain values for T_m with their respective standard errors. ΔC_p , a parameter that has been difficult to determine experimentally, has been included in Equation 14. Fortunately, as described by Shih et al. (1995), the important fitted parameters of ΔH_m and T_m are relatively insensitive to the value of ΔC_p . The determination of reasonable and reproducible values of ΔC_p via the thermal unfolding curves was not possible. We used ΔC_p as an adjustable parameter while fitting the thermal unfolding data obtained by both CD and fluorescence to Equation 14. The standard errors in T_m values were typically ≤ 0.5 K for the CD data, and ≤ 0.2 K for the fluorescence data, respectively.

Fluorescence measurements

An Aminco SPF-500 fluorimeter was used in the GndHCl-induced unfolding studies whereas an Aminco 8000C fluorimeter was used in the thermal unfolding studies. Fluorescence emission spectra were recorded in dual wave mode and photon counting mode, respectively. Both spectrometers were thermostatted via an external water bath and the apoflavodoxin samples were excited at 280 nm.

In the denaturant-induced unfolding studies, fluorescence emission was recorded from 290 to 500 nm. Excitation and emission band passes were 4 and 2 nm, respectively. The maximal decrease in fluorescence upon unfolding was observed at 333 nm for all three apoflavodoxin variants. All data are corrected for the fluorescence emission observed for a series of blank GndHCl solutions.

In the thermally-induced unfolding studies, the change in fluorescence emission at 350 nm was followed as a function of the manually controlled sample temperature. The samples were heated using a scan rate of $0.5^\circ\text{C min}^{-1}$. The sample temperature was measured with a digital thermometer (Fluke, Tilburg) using a thermocouple which was inserted directly into the protein solution. The precision of temperature reading was within $\pm 0.1^\circ\text{C}$. The excitation light beam was shut off during the interval between the individual data collections. Excitation and emission band passes were both set at 4 nm.

CD measurements

CD measurements were performed with a Jasco spectropolarimeter (Tokyo, Japan), model J-715, equipped with a model PTC-348WI

Peltier temperature control system. The instrument was calibrated with 0.06% (w/v) ammonium d-10-camphor sulfonate. For each far-UV CD measurement, Starna (Hainault, England) quartz cells with 1 mm cell length were used. During all experiments the sample cell chamber was purged by dry N_2 at a gas flow rate of 4–5 L/min. All apoflavodoxin samples used were degassed under vacuum before loading into the sample chamber. The results are expressed as the mean residue ellipticity $[\theta]$, where $[\theta]$ is defined as $[\theta] = 100 \theta_{\text{obs}}(cl)^{-1}$; θ_{obs} is the observed intensity, c is the concentration in residue moles centimeters, and l is the length of the light path in centimeters.

For GndHCl unfolding curves, the ellipticity at 222 nm was recorded in the kinetic mode, taking an average of 61 points recorded in a 1-min trace. The ellipticity at 255 nm was recorded for each sample and used as a baseline value (Khorasanizadeh et al., 1993). For thermal unfolding curves, the ellipticity at 222 nm was recorded in the temperature scan mode of the Jasco CD spectrometer. The thermal step resolution was 0.1°C and the samples were heated from 0 to 95°C using scanning rates of 0.25, 0.5, and $1.0^\circ\text{C min}^{-1}$, respectively.

DSC measurements

Differential scanning calorimetry was performed on a MCS differential scanning calorimeter from Microcal with buffer in the reference cell and using scan speeds of 60 and 120°C h^{-1} . Buffers used were: 100 mM potassium pyrophosphate at pH 6.0, 50 mM potassium pyrophosphate and 50 mM sodium phosphate pH 6.0, and 100 mM sodium phosphate pH 6.0. Apoflavodoxin concentrations were 15, 20, and 51 μM in the buffers mentioned. All solutions were carefully degassed by stirring under vacuum for 15 min and the DSC experiments were performed as basically described by Meijberg et al. (1996).

Acknowledgments

We thank Dr. D. Dean (Virginia State University, Blacksburg, Virginia) for his kind donation of pDB6, and H. Borgeld and W.A.M. van den Berg for the construction of the pAV34 expression plasmid. We gratefully acknowledge the expert assistance of Dr. W. Meijberg during the differential scanning calorimetry experiments. The research of Carlo P.M. van Mierlo has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

References

- Balbach J, Forge V, Lau WS, van Nuland NAJ, Brew K, Dobson CM. 1996. Protein folding monitored at individual residues during a two-dimensional NMR experiment. *Science* 274:1161–1163.

- Barman BG, Tollin G. 1972. Flavine-protein interactions in flavoenzymes. Temperature-jump and stopped-flow studies of flavine analog binding to the apoprotein of *Azotobacter* flavodoxin. *Biochemistry* 11:4746–4754.
- Becktel WJ, Schellman JA. 1987. Protein stability curves. *Biopolymers* 26:1859–1877.
- Bennett LT, Jacobson MR, Dean DR. 1988. Isolation, sequencing and mutagenesis of the *nifF* gene encoding flavodoxin from *Azotobacter vinelandii*. *J Biol Chem* 263:1364–1369.
- Brenner SE. 1997. Population statistics of protein structures: Lessons from structural classifications. *Curr Opin Struct Biol* 7:369–376.
- Brigle KE, Newton WE, Dean DR. 1985. Complete nucleotide sequence of the *Azotobacter vinelandii* nitrogenase structural gene cluster. *Gene* 37:37–44.
- Dill KA, Shortle D. 1991. Denatured states of proteins. *Annu Rev Biochem* 60:795–825.
- Edmondson DE, Tollin G. 1971. Chemical and physical characterization of the Shethna flavoprotein and apoprotein and kinetics and thermodynamics of flavin analog binding to the apoprotein. *Biochemistry* 10:124–132.
- Englander SW, Sosnick TR, Englander JJ, Mayne L. 1996. Mechanisms and uses of hydrogen exchange. *Curr Opin Struct Biol* 6:18–23.
- Genzor CG, Beldarraín A, Gómez-Moreno C, López-Lacomba JL, Cortijo M, Sancho J. 1996. Conformational stability of apoflavodoxin. *Protein Sci* 5:1376–1388.
- Gibson TJ. 1984. *Studies on the Epstein-Barr virus genome*. Thesis. Cambridge, UK: University of Cambridge.
- Hinkson JW, Bulen WA. 1967. A free radical flavoprotein from *Azotobacter*. Isolation, crystallization, and properties. *J Biol Chem* 242:3345–3351.
- Hvidt A, Nielsen SO. 1966. Hydrogen exchange in proteins. *Adv Protein Chem* 21:287–386.
- Ikeguchi M, Sugai S, Fujino M, Sugawara T, Kuwajima K. 1992. Contribution of the 6–120 disulfide bond of alpha-lactalbumin to the stabilities of its native and molten globule states. *Biochemistry* 31:12695–12700.
- Jackson SE, Moracci M, el Masry N, Johnson CM, Fersht AR. 1993. Effect of cavity-creating mutations in the hydrophobic core of chymotrypsin inhibitor 2. *Biochemistry* 32:11259–11269.
- Khorasanizadeh S, Peters ID, Butt TR, Roder H. 1993. Folding and stability of a tryptophan-containing mutant of ubiquitin. *Biochemistry* 32:7054–7063.
- Klugkist J, Voorberg J, Haaker H, Veeger C. 1986. Characterization of three different flavodoxins from *Azotobacter vinelandii*. *Eur J Biochem* 155:33–40.
- Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Cryst* 24:946–950.
- Landt O, Grunert H-P, Hahn U. 1990. A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene* 96:125–128.
- Linderstrøm-Lang K. 1955. Deuterium exchange between peptides and water. *Chem Soc (London) Spec Publ* 2:1–20.
- Mayhew SG, Tollin G. 1992. General properties of flavodoxins. In: Müller F, eds. *Chemistry and biochemistry of flavoenzymes*, Vol. 3. Boca Raton, FL: CRC Press. pp 389–426.
- Meijberg W, Schuurman-Wolters GK, Robillard GT. 1996. Interdomain interactions between the hydrophilic domains of the mannitol transporter of *Escherichia coli* in the unphosphorylated and phosphorylated states. *Biochemistry* 35:2759–2766.
- Miller JH. 1972. *Experiments in molecular genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. 433 pp.
- Orengo CA, Jones DT, Thornton JM. 1994. Protein superfamilies and domain superfolds. *Nature* 372:631–634.
- Pace CN. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Meth Enzymol* 131:266–280.
- Pace CN, Shirley BA, Thomson JA. 1989. Measuring the conformational stability of a protein. In: Creighton TE, eds. *Protein structure: A practical approach*. Oxford, UK: IRL Press. pp 311–330.
- Press WH, Flannery BP, Teukolsky SA, Vetterling WT. 1988. *Numerical recipes in C. The art of scientific computing*. New York: Cambridge University Press.
- Privalov PL, Khechinashvili NN. 1974. A thermodynamic approach to the problem of stabilization of globular protein structure: A calorimetric study. *J Mol Biol* 86:665–684.
- Pittsyan OB. 1992. The molten globule state. In: Creighton TE, eds. *Protein folding*. New York: W.H. Freeman and Company. pp 243–300.
- Roder H, Elöve GA, Englander SW. 1988. Structural characterization of folding intermediates in cytochrome *c* by H-exchange labelling and proton NMR. *Nature* 335:700–704.
- Santoro MM, Bolen DW. 1988. Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl α -chymotrypsin using different denaturants. *Biochemistry* 27:8063–8068.
- Sanz JM, Fersht AR. 1993. Rationally designing the accumulation of a folding intermediate of barnase by protein engineering. *Biochemistry* 32:13584–13592.
- Schmid FX. 1989. Spectral methods of characterizing protein conformation and conformational changes. In: Creighton TE, eds. *Protein structure: A practical approach*. Oxford, UK: IRL Press. pp 251–285.
- Schmid FX. 1992. Kinetics of unfolding and refolding of single-domain proteins. In: Creighton TE, eds. *Protein folding*. New York: W.H. Freeman and Company. pp 197–241.
- Shih P, Holland DR, Kirsch JF. 1995. Thermal stability determinants of chicken egg-white lysozyme core mutants: Hydrophobicity, packing volume, and conserved buried water molecules. *Protein Sci* 4:2050–2062.
- Shortle DR. 1996. Structural analysis of non-native states of proteins by NMR methods. *Curr Opin Struct Biol* 6:24–30.
- Steensma E, Heering HA, Hagen WR, van Mierlo CPM. 1996. Redox properties of wild-type, Cys69Ala, and Cys69Ser *Azotobacter vinelandii* flavodoxin II as measured by cyclic voltammetry and EPR spectroscopy. *Eur J Biochem* 235:167–172.
- Steensma E, Nijman MJM, Bollen YJM, de Jager PA, van den Berg WAM, van Dongen WMAM, van Mierlo CPM. 1998. Apparent local stability of the secondary structure of *Azotobacter vinelandii* holoflavodoxin II as probed by hydrogen exchange: Implications for redox potential regulation and flavodoxin folding. *Protein Sci* 7:306–317.
- Steensma E, van Mierlo CPM. 1998. Structural characterisation of apoflavodoxin shows that the location of the most stable nucleus differs among proteins with a flavodoxin-like topology. *J Mol Biol* 282(3):653–666.
- Tanaka M, Haniu M, Yasunobu KT, Yoch DC. 1977. Complete amino acid sequence of azotoflavin, a flavodoxin from *Azotobacter vinelandii*. *Biochemistry* 16:3525–3537.
- Thorneley RNF, Ashby GA, Drummond MH, Eady RR, Hughes DL, Ford G, Harrison PM, Shaw A, Robson RL, Kazlauskaitė J, Hill HAO. 1994. Flavodoxins and nitrogen fixation: Structure, electrochemistry and post-translational modification by coenzyme A. In: Yagi K, eds. *Flavins and flavoproteins 1993*. Berlin: Walter de Gruyter & Co. pp 343–354.
- Tollin G, Edmondson DE. 1980. Purification and properties of flavodoxins. *Methods Enzymol* 69:392–406.
- van Mierlo CPM, Darby NJ, Creighton TE. 1992a. The partially folded conformation of the Cys30-Cys51 intermediate in the disulphide folding pathway of bovine pancreatic trypsin inhibitor. *Proc Natl Acad Sci USA* 89:6775–6779.
- van Mierlo CPM, Darby NJ, Keeler J, Neuhaus D, Creighton TE. 1992b. Partially folded conformation of the (30-51) intermediate in the disulphide folding pathway of bovine pancreatic trypsin inhibitor: ^1H and ^{15}N resonance assignments and determination of backbone dynamics from ^{15}N relaxation measurements. *J Mol Biol* 229:1125–1146.
- van Mierlo CPM, Lijnzaad P, Vervoort J, Müller F, Berendsen HJC, de Vlieg J. 1990. Tertiary structure of two-electron reduced *Megasphaera elsdenii* flavodoxin and some implications, as determined by two-dimensional ^1H -NMR and restrained molecular dynamics. *Eur J Biochem* 194:185–198.
- van Mierlo CPM, Steensma E. 1998. Stabilisation centres differ between structurally homologous proteins as shown by NMR spectroscopy. *J Mol Cat B: Enzymatic*. Forthcoming.
- van Mierlo CPM, Steensma E, van Dongen WMAM, van Berkel WJH. 1997. NMR studies on apoflavodoxin II from *Azotobacter vinelandii*. In: Stevenson KJ, Massey V, Williams CH Jr, eds. *Flavins and flavoproteins 1996*. Calgary, Canada: University of Calgary Press. pp 449–452.
- Wassenberg D, Schurig H, Liebl W, Jaenicke R. 1997. Xylanase Xyna from the hyperthermophilic bacterium *Thermotoga maritima*: Structure and stability of the recombinant enzyme and its isolated cellulose binding domain. *Protein Sci* 6:1718–1726.
- Wijmenga SS, Steensma E, van Mierlo CPM. 1997. Doubly sensitivity-enhanced 3D HCCH-TOCSY of ^{13}C -labeled proteins in H_2O using heteronuclear cross polarization and pulsed field gradients. *J Magn Reson* 124:459–467.
- Wijmenga SS, van Mierlo CPM, Steensma E. 1996. Doubly sensitivity-enhanced 3D TOCSY-HSQC. *J Biomol NMR* 8:319–330.
- Woodward C. 1993. Is the slow-exchange core the protein folding core? *Trends Biochem Sci* 18:359–360.
- Yanisch-Perron C, Vieira J, Messing J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103–119.