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Kinetics and Equilibria of Active Site Core Extrusion from Spinach Ferredoxin in Aqueous *N,N*-Dimethylformamide/Triton X-100 Solutions[†]

Franco Bonomi and Donald M. Kurtz, Jr.*

ABSTRACT: The nature and reactivity of each species participating in core extrusion of the [2Fe–2S] cluster of spinach ferredoxin by benzenethiol have been investigated in a novel aqueous medium containing 10–40% (v/v) *N,N*-dimethylformamide (DMF) plus 5–6% (v/v) of the nonionic detergent, Triton X-100. By use of visible absorption and circular dichroism spectroscopies, it is found that prior to addition of benzenethiol, modifications of the ferredoxin induced by DMF are reversible. Both the extent and rate of modification are dependent on DMF concentration and on ionic strength. At ferredoxin concentrations near 0.1 mM, complete modification by DMF is shown to be unnecessary for quantitative core extrusion provided that the benzenethiol concentration is $\gtrsim 50$ mM and the DMF concentration is $\gtrsim 10\%$ (v/v). When benzenethiol is added after DMF, core extrusion occurs in two phases. Our interpretation is that the rapid phase corresponds to core extrusion of that portion of the ferredoxin already modified by DMF and that the slower phase corresponds to modification of the remaining portion of the ferredoxin by DMF. When DMF solutions containing benzenethiol and Triton X-100 in various ratios are mixed with aqueous solutions of spinach ferredoxin, the rate of core extrusion appears to be determined both by the rate at which the ferredoxin is modified and by the Triton/benzenethiol ratio. Under all conditions examined we observe significantly faster rates of core extrusion from spinach ferredoxin in aqueous Triton/DMF than in 80/20 (v/v) hexamethylphosphoramide/water, a previously used core extrusion medium. Our results suggest a catalytic role for the micellar phase.

Core extrusion of the active centers of iron–sulfur proteins has proven to be a useful analytical tool for the determination of the type and number of [2Fe–2S] and [4Fe–4S] centers in both simple and complex iron–sulfur proteins (Gillum et al., 1977; Averill et al., 1978; Coles et al., 1979; Kurtz et al., 1979). The method is based on a reaction in which cysteine thiolate ligands to the iron–sulfur center in the holoprotein are replaced by PhS^-:

$$\text{holoprotein} + \text{PhSH} \rightarrow [Fe_nS_n(SPh)_4]^{2-} + \text{apoprotein}$$

where $n = 2, 4$. Distinctive features of the method include the use of high concentrations of organic solvent (80 vol %) in order to ensure adequate solubility of the added thiol and of $[Fe_nS_n(SPh)_4]^{2-}$ as well as unfolding of the proteins to a sufficient extent for the extrusion reactions to take place. High concentrations of added thiol are mandatory in order to prevent decomposition or dimerization of the extruded cluster.

Recently one of us (Kurtz, 1982) has devised a new method of core extrusion using much lower concentrations of organic solvent in the presence of the nonionic detergent Triton X-100. We have now investigated in further detail the nature and reactivity of each species participating in the active site core extrusion from spinach ferredoxin under these conditions.

Materials and Methods

Spinach ferredoxin was prepared essentially as previously described (Petering & Palmer, 1970) and stored at −24 °C in 0.15 M Tris-HCl,[1] pH 7.3, containing 1 M NaCl. Aliquots were desalted just before each experiment on a Sephadex G-25 fine column (0.8 × 12 cm) equilibrated with 50 mM Tris-HCl, pH 8.1. Apoprotein was prepared by treatment of the holoprotein with 10% trichloroacetic acid. The protein pellet was washed with 1% trichloroacetic acid and resuspended in the same buffer used for the native protein.

DL-Dihydrolipoic acid was prepared by $NaBH_4$ reduction of a methanolic solution of the oxidized form. The solution was then brought to pH 4.5 with HCl and dried in a rotary evaporator, and the reduced compound was dissolved in DMF. Molarity of this solution was assayed with 5,5′-dithiobis(2-

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[1] Abbreviations: DMF, *N,N*-dimethylformamide; Et, $-C_2H_5$; PhSH, benzenethiol; CD, circular dichroism; Fd, ferredoxin; Triton, Triton X-100; Tris, tris(hydroxymethyl)aminomethane.

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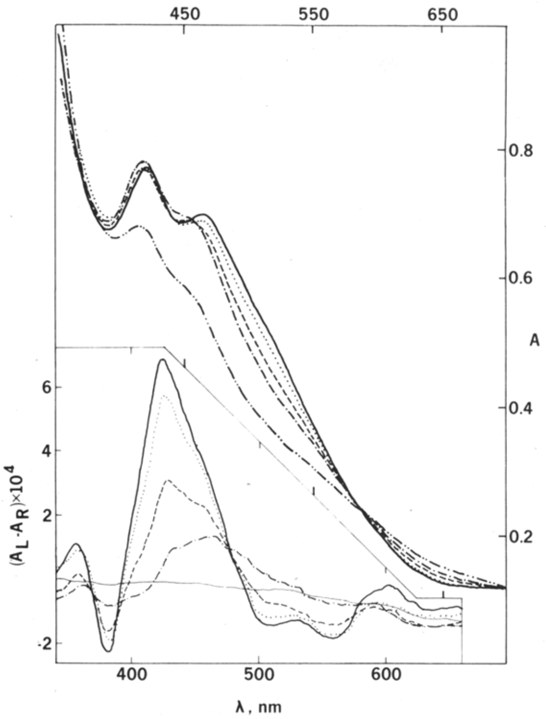

FIGURE 1: Absorption and CD spectra of spinach Fd in the presence of DMF. Absorption spectra of spinach Fd (0.074 mM) in 24 mM Tris-HCl, pH 8.0, were recorded in the absence (full line) or in the presence (broken lines) of 6% (v/v) Triton and of different concentrations of DMF. DMF concentrations are 12, 23, 35, and 46% (v/v) from top to bottom at 480 nm. (Inset) CD spectra of spinach Fd (0.075 mM). Concentrations of the reagents and symbols are the same ones used in the main figure. Thin line indicates base line. Both absorption and CD spectra were recorded 20 min after addition of DMF.

nitrobenzoic acid) (Ellman, 1959).

DMF was usually distilled from BaO at reduced pressure before use. Triton X-100 from Sigma was used as received.

All reactions were carried out at 25 °C. Where necessary reagents were mixed anaerobically as described by Kurtz (1982). Further details are given in the tables and figure legends. Procedures for synthesis and preparations of solutions and spectral parameters of $(Et_4N)_2[Fe_2S_2(SPh)_4]$ in aqueous Triton/DMF were the same ones used previously (Kurtz, 1982). Absorption spectra were recorded on either a Perkin-Elmer 554 or a Cary 118C spectrophotometer. Circular dichroism measurements were taken on either a Jobin-Yvon Model III dicrograph or a Jasco Model ORD/UV-5 ORD recorder equipped with a Sproul Scientific SS-20-2 CD modification.

Apparent pseudo-first-order rate constants were calculated from standard semilog plots which were linear for at least three half-lives. The ionic strengths cited in the text, tables, and figure legends include that contributed by 24 mM Tris-HCl which at pH 8.0 is ~16 mM.

Molarity of Triton X-100 was calculated from volume percentage by using an average molecular weight of 646 and density of 1.06 g/mL.

Results

Triton usually takes several minutes to dissolve in aqueous solutions at room temperature, and vigorous mixing can result in foaming. An advantage of conducting core extrusion reactions by addition of PhSH/DMF/Triton mixtures to aqueous protein solutions as described by Kurtz (1982) is that mixtures of Triton and organic solvent, when added to buffered aqueous solutions, dissolve in several seconds with little or no foaming.

Table I: Apparent Pseudo-First-Order Rate Constants for the Slow Phase of the Decrease in A_{482} upon Addition of DMF to Spinach Fd in 24 mM Tris-HCl, pH 8.0, Containing 6% (v/v) Triton[a]

% (v/v) DMF	μ (mM)[b]	k'_{obsd} (min^{-1})
12	16	0.12
23	35	0.66
23	54	0.62
23	73	0.55
35	16	0.65

[a] Final spinach Fd concentration = 0.075 mM. [b] Adjusted with 1 M KCl prior to addition of DMF.

Table II: Apparent Pseudo-First-Order Rate Constants for the Ellipticity Decrease at 431 nm upon Addition of DMF to Spinach Fd in 24 mM Tris-HCl, pH 8.0, Containing 6% (v/v) Triton[a]

% (v/v) DMF	μ (mM)[b]	k'_{obsd} (min^{-1})
12	16	0.75
23	16	1.6
23	54	0.55
35	16	4.3

[a] Final spinach Fd concentration = 0.075 mM. [b] Adjusted with 1 M KCl prior to addition of DMF.

The visible absorption and CD spectra of spinach Fd are not altered appreciably by addition of up to 6% (v/v) Triton X-100 to the protein solution in low molarity Tris buffer. Addition of DMF to the protein/detergent solution leads to characteristic modifications in both spectra (Figure 1). The absorbance decreases in the 450–600-nm region and increases slightly in the 600–700-nm region with an isosbestic point at 592 nm. The positive ellipticity at 431 nm decreases upon addition of DMF, and two isosbestic points are observed at 370 and 475 nm.

The time course of the decrease in absorbance and ellipticity following the addition of DMF to the protein/detergent solution was monitored at the wavelengths where the maximal differences arise, 482 and 431 nm, respectively (Figure 1 and Tables I and II). First-order kinetics were obtained at the reaction times we studied. Addition of DMF causes an initial rapid decrease in absorbance, the rate of which was too fast to measure on our spectrophotometer. This phase accounts for ~70% of the total decrease in absorbance at 12% (v/v) DMF and ~90% at 35% (v/v) DMF. The velocity of the slower phase of the absorbance decrease is not greatly influenced by the DMF concentration in the range 20–35% (v/v) (Table I). In contrast to the absorbance, loss of ellipticity upon addition of DMF is uniphasic, and its velocity is a complex function of the concentration of DMF (Table II). The CD spectra in Figure 2 demonstrate that addition of salt to solutions of spinach Fd after dilution with DMF partially reverses the loss in ellipticity. At 34% (v/v) DMF the loss in ellipticity observed at 60 mM ionic strength reduces to 57% of that observed at 16 mM. Very similar effects are observed in 23% (v/v) DMF.

These effects on the ellipticity appear to involve a competition between the rates of modification of spinach Fd by DMF and reversal of the modification by salt. Addition of DMF to 23% (v/v) causes a decrease in ellipticity at 431 nm which obeys first-order kinetics at ionic strengths of 16 and 54 mM with a lower rate constant at the higher ionic strength (Table II). Addition of DMF to 35% (v/v) causes a rapid decrease in ellipticity which obeys first-order kinetics at 16 mM ionic strength (Table II). However, at 60 mM ionic strength addition of DMF to 35% (v/v) causes, after the initial rapid decrease, a slower increase in ellipticity at 431 nm. The extent

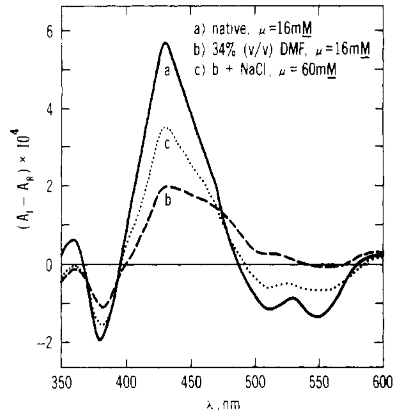

FIGURE 2: CD spectra of 0.078 mM spinach Fd in 24 mM Tris-HCl, pH 8.0, and 6% (v/v) Triton: (a) without DMF; (b) solution contains 34% (v/v) DMF, ionic strength = 16 mM; (c) addition of 1 M NaCl to the solution in (b) to a final ionic strength of 60 mM. Spectra were recorded 5 min after mixing and are corrected for base-line deviations.

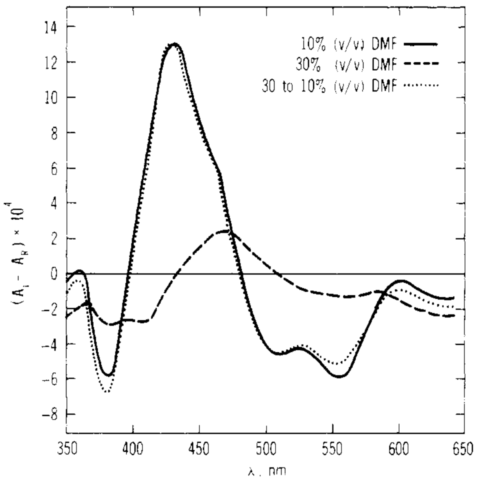

FIGURE 3: CD spectra of 0.144 mM spinach Fd in 24 mM Tris-HCl, pH 8.0, showing reversibility of the DMF modification by dilution. "30 to 10%" means that the 30% (v/v) DMF solution was rediluted to 10% (v/v). Solutions were incubated at room temperature for 20–30 min after each addition of DMF. These solutions contain no Triton. Spectra are corrected for differences in protein concentrations and base-line deviations.

of this reversal at equilibrium is similar to that illustrated in Figure 2.

The effect of ionic strength on the slow phase of the absorbance decrease is minimal (Table I). However, the total absorbance change of the "fast phase" decreases in magnitude with increasing ionic strength.

No loss or irreversible chemical alteration of the iron–sulfur cluster appears to occur up to a DMF concentration of ~35% (v/v). These conclusions can be drawn from the CD spectra in Figure 3 which show that the alterations which result from increasing the DMF concentration from 10 to 30% (v/v) can be reversed almost completely by rediluting the solution to 10% (v/v) DMF. The spectra in Figure 3 were obtained on solutions which contained no Triton. Addition of Triton to 6% (v/v) to the 10% (v/v) DMF solution resulted only in a further 6% decrease in ellipticity at 431 nm. When a solution of spinach Fd containing 34% (v/v) DMF and 6% (v/v) Triton (the spectrum of which is shown in Figure 2c) was applied to a Whatman DE-52 anion-exchange column and eluted with buffer containing 1 M NaCl, the optical parameters of the native protein were quantitatively restored. Ninety-two percent of the protein applied to the column was recovered based on absorbance at 420 nm. Thus, reversibility of the DMF modification is achieved in either the presence or absence of Triton.

Table III: Apparent Pseudo-First-Order Rate Constants for the Slow Phases of the Increase in A_{474} and the Decrease in Ellipticity at 431 nm upon Addition of PhSH (to 100 mM) to 0.075 mM Spinach Fd in 24 mM Tris-HCl, pH 8.0, Containing 6% (v/v) Triton and the Indicated Percentages of DMF[a]

% (v/v) DMF	μ (mM)[b]	k'_{obsd} (min^{-1}) A_{474}	k'_{obsd} (min^{-1}) CD$_{431}$
12	16	0.35	0.42
23	16	0.93	1.3
23	35	0.43	0.46
23	54	ND[c]	0.24
23	73	0.21	0.13

[a] About 20 min was allowed to pass between addition of DMF and addition of PhSH. [b] Adjusted with 1 M KCl prior to addition to DMF. [c] Not determined.

Anaerobic addition of PhSH to spinach Fd incubated in the presence of detergent and DMF results in the formation of $[Fe_2S_2(SPh)_4]^{2-}$ as shown previously (Kurtz, 1982). Core extrusion is quantitative at 100 mM PhSH used in the experiments described in Table III as long as the DMF concentration is ≳10% (v/v). The PhSH/protein mixture does not show any CD signal in the visible region after core extrusion is complete, whereas the absorption spectrum corresponds to that of an equimolar mixture of the apoprotein and $[Fe_2S_2(SPh)_4]^{2-}$ in the same medium. Rate of formation of $[Fe_2S_2(SPh)_4]^{2-}$ following addition of PhSH was monitored by measuring the absorbance increase at its λ_{max}, 474 nm. The loss of protein cluster interaction was followed by measuring the disappearance of ellipticity at 431 nm. An absorbance increase, too fast for us to measure, was followed by a slower increase which obeyed first-order kinetics (Table III). At 23% (v/v) DMF, the fast phase accounts for ~54% of the total absorbance increase at 16 mM ionic strength and ~39% at 73 mM ionic strength. The slower absorbance increase is accompanied by a decrease in ellipticity whose apparent pseudo-first-order rate constants closely match those for the absorbance increase (Table III). A fast phase of the ellipticity decrease accounts for ~10% of the total.

Finally, we focused our attention on the extrusion reaction when native Fd is added to a mixture of DMF/Triton/PhSH. Equilibrium studies allowed us to dêtermine the yield of the extrusion reaction from the equations

$$\text{absorbance} = C_e(\epsilon_{ec} + \epsilon_e) + C_m\epsilon_m$$

$$C_t = C_e + C_m$$

where C_t is the total Fd concentration, C_e is the concentration of the core-extruded (apo) ferredoxin, and C_m is the concentration of the DMF-modified Fd whose molar absorbance at the wavelength used (ϵ_m) was determined from data similar to those presented in Figure 1. The term $\epsilon_{ec} + \epsilon_e$ is the sum of the molar absorbances of $[Fe_2S_2(SPh)_4]^{2-}$ and of the apoprotein, respectively, in the solvent system used. The absorbance equation was checked at several wavelengths and found to fit the experimental data with an accuracy better than 2%. Extrusion yields are presented in Figure 4 as a function of the amount of PhSH added at two different DMF concentrations with the mole ratio of Triton to PhSH held fixed at 2/1. The yield increases only slightly at the higher DMF concentration.

When the extrusion reaction is started by mixing a DMF/PhSH/Triton solution with the protein, the absorbance increase at 474 nm follows first-order kinetics with no faster initial increase being detected. Apparent pseudo-first-order

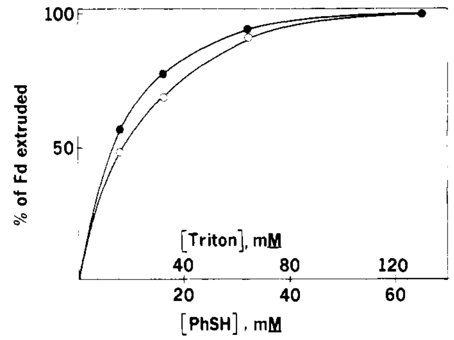

FIGURE 4: Equilibrium parameters of the core extrusion reaction. To 0.27 mL of a solution of water/DMF/Triton/PhSH (PhSH/Triton = 0.5 mol/mol) was anaerobically added 0.25 mL of 0.10 mM spinach Fd in 50 mM Tris-HCl, pH 8.1. Absorption spectra were taken until no further change was observed. For calculation of percentage yields, see text. Indicated concentrations are the final reagent concentrations. DMF concentrations in % (v/v) are 10 (open symbols) or 30 (closed symbols).

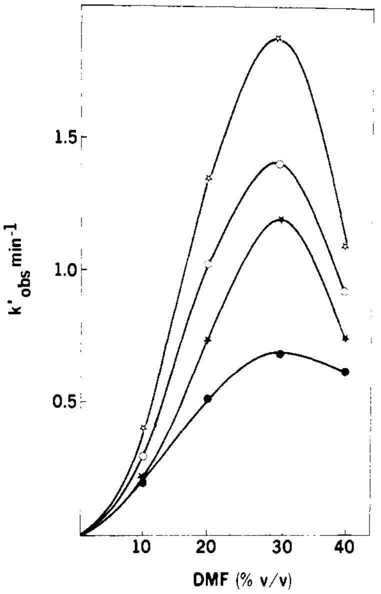

FIGURE 5: Rate constants for the core extrusion reaction as a function of concentration of DMF. Experiments were performed as described in the legend to Figure 4. The time course of the reaction was monitored by the absorbance increase at 474 nm. The mole ratio of PhSH to Triton is 1/1.5. PhSH concentrations are: 8 (closed circles), 16 (closed stars), 32 (open circles), and 64 mM (open stars).

rate constants increase with both the concentration of the thiol/detergent mixture and the concentration of DMF, falling suddenly when the DMF concentration is raised above 30% (v/v) (Figure 5). A result which is perhaps relevant to the rate decrease is that PhSH, added to give 100 mM, is incompletely soluble at room temperature in buffered aqueous solutions containing 10–30% (v/v) DMF unless Triton is added. However, in 40% (v/v) DMF, PhSH is soluble to at least 100 mM without Triton. Under these conditions $(Et_4N)_2[Fe_2S_2(SPh)_4]$ is quite stable. In aqueous solutions containing 0.18 mM $(Et_4N)_2[Fe_2S_2(SPh)_4]$, 64 mM PhSH, 6% (v/v) Triton, and 10–40% (v/v) DMF, A_{474} decreases by <1.3%/h. We observe a decrease in rate constant for core extrusion when the thiol concentration is kept constant and the detergent concentration is increased (Figure 6A). We observe an increase in the rate constant which appears to approach a plateau value when the thiol concentration is increased and the detergent concentration is kept constant (Figure 6B). These observations confirm and extend those made in the earlier study (Kurtz, 1982).

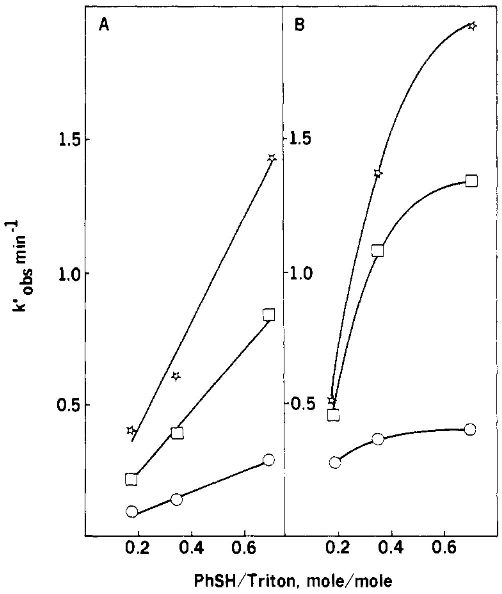

FIGURE 6: Rate constants for the extrusion reaction as a function of the PhSH/Triton ratio. Rate constants were determined as described in the legend to Figure 5. DMF concentrations (vol %) are 10 (circles), 20 (squares), or 30 (stars). (A) Effect of the variation of the Triton concentration at a fixed PhSH concentration of 16 mM. (B) Effect of variation of the PhSH concentration at a fixed Triton concentration of 95 mM [6% (v/v)].

In an attempt to differentiate reactivity of the Fd cluster toward thiols which may be present in a micellar phase, we substituted the dithiol, DL-dihydrolipoic acid, for PhSH. At a total thiol concentration where PhSH gives quantitative extrusion, this dithiol neither extrudes the cluster nor removes the iron from the protein in aqueous DMF/Triton. Instead it progressively bleaches the visible absorbance of the protein, with a concomitant increase in absorbance at 330 nm, where the oxidized form of lipoic acid absorbs. Final concentrations were 38.4 mM Tris-HCl, pH 8.1, 17% (v/v) DMF, 6% (v/v) Triton, 60 mM dihydrolipoic acid, and 0.077 mM spinach Fd. Full bleaching took about 40 min. This reaction does not occur in the absence of detergent and DMF, and spinach Fd can be reisolated in the native, oxidized form after reaction with lipoic acid by gel filtration and ion-exchange chromatography.

Discussion

The overall pattern of the data seems consistent with a reaction scheme of the type shown in Scheme I.

Scheme I

$$\text{native Fd} \xrightleftharpoons{\text{Triton/DMF}} \text{DMF-modified Fd} \qquad (1)$$

$$\text{DMF-modified Fd} + \text{PhSH}_{(\text{micellar})} \rightarrow [Fe_2S_2(SPh)_4]^{2-}{}_{(\text{micellar})} + \text{apoFd} \qquad (2)$$

Our failure to detect appreciable interaction between spinach Fd and Triton X-100 agrees with observations made on other water-soluble proteins (Helenius & Simons, 1975).

When the spectral changes associated with the addition of DMF to spinach Fd are considered, it is worth recalling that absorbance is due primarily to the nature of the prosthetic group while in this case ellipticity arises primarily from the interactions of the prosthetic group with its immediate environment. Our equilibrium CD measurements show that roughly half of the native cluster–protein interaction is lost at a DMF concentration of 3 M [23% (v/v)]. This concentration of DMF is well below that needed to reach the midpoint for denaturation of three globular heme proteins as measured by changes in optical parameters of the prosthetic group

(Herskovits et al., 1977). We are unaware of previous studies specifically examining the effects of DMF on spinach Fd. It is possible that the observed changes in optical parameters of spinach Fd correspond to less drastic conformational changes than those in the heme proteins. Cammack (1975) distinguishes two different degrees of modification in the environment of the [2Fe–2S] centers in two-iron ferredoxins. "Subtle effects" (decreased line widths) are produced in the EPR spectra of reduced two-iron ferredoxins by low concentrations of organic solvents such as methanol and Me_2SO whereas "drastic effects" (elimination of rhombicity) appear at $\gtrsim 50\%$ (v/v) of organic solvent. Using absorption and CD, Padmanabhan & Kimura (1970) differentiate four states during the process of aerobic denaturation of spinach Fd by urea or guanidine hydrochloride. The native "state I" changes after about 3 min to "state II" whose absorption and CD spectra closely resemble ours in 35% (v/v) DMF. Whatever the degree or exact nature of the modification, our observations of isosbestic points in the absorption and CD spectra and of reversibility speak in favor of an equilibrium between two protein conformations, the position of which depends on the concentration of DMF and on ionic strength. The DMF-modified Fd retains its cluster and thus its absorption but has lost most of the features giving rise to the native CD signal. An important conclusion from our work is that quantitative core extrusion occurs even though the equilibrium in Scheme I is not shifted completely to the right prior to addition of PhSH.

Absorbance changes following the addition of DMF are clearly biphasic. A rapid absorbance decrease at 482 nm is observed, its extent being greater the higher the concentration of DMF or the lower the ionic strength. A minor slow phase is then observed in which velocity is nearly independent of DMF concentration and of ionic strength. An explanation for these observations is that DMF alters the solvent polarity in the vicinity of the cluster, and thus the absorbance, even before any distortion in protein tertiary structure takes place. It is known for *S. platensis* Fd (Fukuyama et al., 1980) that the [2Fe–2S] cluster is located near the molecular surface and is surrounded mainly by hydrophobic residues which are essentially invariant among the chloroplast ferredoxins (Yasunobu & Tanaka, 1973). These residues could be the cause of spectral modifications arising from alteration of solvent polarity. Once the initial alteration in polarity has occurred, new relationships are established between amino acid residues and the cluster. Such establishments would account for the decrease in ellipticity upon addition of DMF.

High ionic strength preserves the native structure of spinach Fd as demonstrated by the absorbance and CD measurements of Petering & Palmer (1970). Their spectra of spinach Fd in 5 M urea and sodium chloride concentrations ranging from 20 mM to 1 M closely match those we obtain at fixed low ionic strength and varying concentrations of DMF. In our experiments comparable protection occurs in a lower range of ionic strength. At 34% (v/v) DMF an increase in ionic strength from 16 to 60 mM produces a protection comparable to the one observed in 5 M urea when the ionic strength goes from 0.3 to 1 M. It appears from both our kinetic and our equilibrium data that increased ionic strength increases the stability of the native protein–cluster interaction and decreases the sensitivity of the cluster environment toward changes in dielectric constant of the solvent.

Extrapolation of ln ΔA_{474} vs. time plots to $t = 0$ when PhSH is added to a solution of spinach Fd equilibrated with DMF/Triton suggests that the mole fraction of Fd already modified reacts immediately to give $[Fe_2S_2(SPh)_4]^{2-}$. This rapid phase produces only small changes in the CD at 431 nm which reflects the lower optical activity of the protein bound cluster in the DMF-modified Fd. Consistent with these conclusions is the lack of a rapid absorbance increase when a solution of native Fd is mixed with a solution of DMF/Triton/PhSH. The rate of the slower phase of formation of $[Fe_2S_2(SPh)_4]^{2-}$ remains approximately equal to that of the loss of native protein–cluster interaction (Table III). These observations are completely in accord with Scheme I if it is assumed that upon addition of PhSH, incorporation into the micelle is rapid and that the rate-limiting step in core extrusion at 60–100 mM PhSH is modification of the native protein by DMF. The rate of core extrusion initially increases with the concentration of DMF but decreases at DMF concentrations >30% (v/v) (Figure 5). The modification process may no longer be rate determining at >30% (v/v) DMF (Table II). It is also likely that micellization decreases at these higher DMF concentrations. Dimethyl sulfoxide is known to inhibit micellization of cetyltrimethylammonium bromide and to completely prevent micelle formation above 48% (v/v) (Ionescu et al., 1979). Very similar observations have been made on nonionic detergents with ethanol or dioxane as the inhibitor of micellization (Becher, 1967). Furthermore, in the absence of Triton PhSH becomes much more soluble in the bulk aqueous/organic phase above 30% (v/v) DMF. All of these observations are consistent with a rate decrease assuming that micelles containing PhSH are the reacting species. The decrease in measured rate is not due to decomposition of $[Fe_2S_2(SPh)_4]^{2-}$ once formed since the synthetic compound, $(Et_4N)_2[Fe_2S_2(SPh)_4]$, is quite stable under these conditions.

The preceding considerations coupled with the consistently faster rates of core extrusion from spinach Fd in aqueous DMF/Triton compared to 80/20 (v/v) hexamethylphosphoramide/water (Gillum et al., 1977) suggest a catalytic role for the micellar phase. Previous work has suggested that in the ligand exchange reactions which occur during core extrusion, thiol rather than thiolate is the reacting species (Dukes & Holm, 1974). In 80% (v/v) hexamethylphosphoramide it is likely that intermediate-mixed ligand species of the type $[Fe_2S_2(S\text{-}Cys)_{4-n}(SPh)_n]$ are present during the core extrusion process and that the sequential displacements of Cys-S^- by PhSH are the rate-determining steps (Gillum et al., 1977). In aqueous solutions with DMF concentrations up to 30% (v/v), PhSH is incompletely soluble unless Triton is added. The Triton presumably incorporates PhSH into a micellar phase. This phase may provide locally high concentrations of PhSH such that the sequential ligand displacements occur much more rapidly. If PhSH-containing micelles are the reacting species, their concentration, as well as the number of PhSH molecules per micelle, should influence the extrusion process. Figure 6A shows that the rate of core extrusion extrapolates to zero as the number of PhSH molecules per micelle is lowered by dilution with detergent at a fixed PhSH concentration. When the number of PhSH molecules per micelle is increased at a constant Triton concentration, the rate increases and appears to approach a plateau (Figure 6B), probably corresponding to saturation of the micelles with thiol. Increasing the DMF concentration increases the maximal rate because the rate of modification is increased. These observations are completely in accord with the proposed micellar role.

A reactivity different from that with PhSH is observed with DL-dihydrolipoic acid in aqueous Triton/DMF. If the spectral modifications we observe are due to reduction of the [2Fe–2S]

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center in the DMF-modified ferredoxin by the dithiol, an alteration in the redox properties of the reacting species must have occurred since reduction does not occur in aqueous solution. Whether this change is due to incorporation of the dithiol molecules into micelles, to partial denaturation of the protein, or to a combination of the two effects still has to be clarified. These observations suggest that some dithiols will not be useful for core extrusion in aqueous Triton/DMF.

At a starting pH range of 8.0–8.5 and a Fd concentration near 0.1 mM, optimal ranges for quantitative core extrusion from the active site of spinach Fd by PhSH can be summarized as 10–30% (v/v) DMF, 5–6% (v/v) Triton, and 50–100 mM PhSH, ≲50 mM ionic strength. Very recently, we have obtained quantitative core extrusion of the two [4Fe–4S] centers in *C. pasteurianium* Fd under these conditions (D. M. Kurtz, Jr., unpublished results). To our knowledge the studies reported here represent the first detailed descriptions of the kinetics and equilibria of core extrusion reactions as well as the nature of each species participating in these reactions. We think that such descriptions for simple ferredoxins can serve as guides to choosing optimal conditions for selective core extrusion and site identification in as yet incompletely characterized complex iron–sulfur proteins.

Acknowledgments

We express our gratitude to Dr. Silvia Pagani and Ron Utecht for experimental assistance.

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Tryptophan Residues of the γ Subunit of 7S Nerve Growth Factor: Intrinsic Fluorescence, Solute Quenching, and *N*-Bromosuccinimide Oxidation[†]

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ABSTRACT: The environment of tryptophan residues of the γ subunit derived from the 7S nerve growth factor has been studied by intrinsic fluorescence, solute quenching, and oxidation with *N*-bromosuccinimide (NBS). The native protein has a fluorescence emission maximum of 345 nm with a tryptophan quantum yield of 0.10. A red shift in the emission maximum occurs between 3 and 6 M urea; a 20% increase in quantum yield occurred at 7–8 M urea. NBS (20–24 mol of NBS/mol of protein) completely oxidized one tryptophan of the γ subunit, causing a 70% quenching of the intrinsic fluorescence, a complete loss of esterolytic activity toward synthetic substrates, an inability to combine with α and β subunits to re-form the 7S complex, and a shift of about 12% of the structure from β strands to random coil as determined by circular dichroism. About 80% of the fluorescing tryptophans are accessible to quenching by acrylamide but not to potassium iodide. These results suggest the presence of an exposed tryptophan contributing ≥70% of the native fluorescence, located near a negative charge, critical to the esterase activity and possibly to interaction with the β subunit to re-form 7S NGF.

Nerve growth factor (NGF)[1] as isolated from the mouse submaxillary gland is a 7S oligomer (M_r 130 000–140 000). The complex consists of three different subunits, α, β, and γ, in the stoichiometric ratio of 2:1:2 ($\alpha_2\beta\gamma_2$). The oligomer can be dissociated to the free subunits under a variety of conditions (Varon et al., 1968; Au & Dunn, 1977; Palmer & Neet, 1980b).

The subunits apparently have different functions. The β subunit, itself a homo dimer, is responsible for eliciting neurite

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[1] Abbreviations: BAPNA, N^α-benzoyl-DL-arginine-*p*-nitroanilide; EDTA, disodium salt of ethylenediaminetetraacetic acid; NGF, nerve growth factor; NBS, *N*-bromosuccinimide; TAME, tosylarginine methyl ester; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-6843$01.25/0