Core Extrusion of the Two [4Fe–4S] Centers of *C. pasteurianum* Ferredoxin in Aqueous Triton

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A new method was recently developed in this laboratory for core extrusion and site identification of iron–sulfur centers in proteins [1, 2]. The method is based on a reaction in which cysteinate ligands to the iron–sulfur core are replaced by aromatic thiolate added in excess [3]. Reactants and products containing aromatic thiolate are solubilized by the non-ionic detergent, Triton X-100. The method was shown to give quantitative core extrusion of the [2Fe–2S] center in spinach ferredoxin and to provide useful mechanistic information about the core extrusion process for this ferredoxin. Reported here are analogous results on the 2[4Fe–4S] containing ferredoxin from *C. pasteurianum* using the same method as well as a new modification thereof.

All experiments were conducted as previously described [1]. Figure 1 shows spectra resulting from anaerobic addition of a Triton/DMF/PhSH* mixture to an aqueous solution of *C. p.* Fd. Conditions after addition are listed in the legend to Fig. 1. The final spectrum, obtained ~3 hours after addition has λ_{max} 454 nm and A_{454}/A_{550} = 2.10 clearly identifying it as $[Fe_4S_4(SPh)_4]^{-2}$ whose spectral properties in this medium have been determined previously [1]. Using published extinction coefficients and correcting for dilution the value of n_t = mol $[Fe_4S_4(SPh)_4]^{-2}$/mol Fd is calculated to be 1.9, 5% less than the expected value of 2.0. Starting about 45 minutes after addition of the Triton/DMF/PhSH mixture, the spectra in Fig. 1 exhibit tight isosbestic points at ~366 and ~418 nm. Essentially the same results are obtained in the above medium when acetonitrile is substituted for DMF except that the reaction is complete after ~4 hours. A first order rate plot of the absorbance increase at 454 nm is consistent with a biphasic reaction. The appearance of isosbestic behaviour occurs near the end of the first phase.

For many applications one would prefer times much shorter than 3–4 hours for quantitative core extrusion. Additional experiments have shown that in contrast to the results with spinach Fd, lower ionic strength seems to decrease the rate of core extrusion for *C. p.* Fd in the aqueous Triton/DMF medium and increasing the DMF concentration from 15 to 20

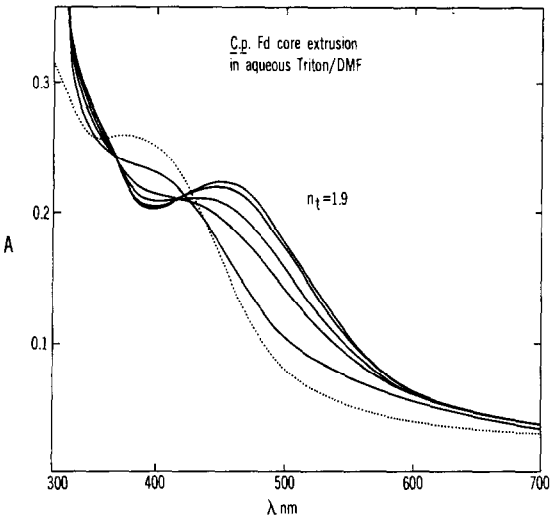

Fig. 1. (· · · ·), spectrum of *C. p.* Fd in 50 m*M* Tris-Cl pH 8.5. (——), spectra after addition of Triton/DMF/PhSH to the aqueous protein solution. Conditions after addition: 80:15:5 vol% aqueous buffer:DMF:Triton; [PhSH] = 50 m*M*; [Fd] = 135 μM; dilution factor = 1.25. Cell pathlength = 0.5 mm. In order of increasing A_{454} spectra were obtained 6, 48, 83, 145 and 197 minutes after addition. The final spectrum is that with the most intense absorbance at 454 nm and least intense at 400 nm.

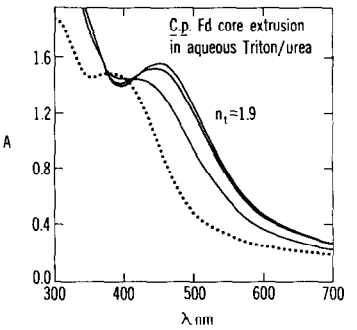

Fig. 2. (· · · ·), spectrum of stock *C. p.* Fd in 50 m*M* Tris-Cl pH 8.3 after dilution with buffer, [Fd] = 49.0 μM. (——), spectra of stock *C. p.* Fd in 50 m*M* Tris-Cl pH 8.3 after dilution with an aqueous Triton/urea/PhSH mixture resulting in 3.5 vol% Triton, 5.5 *M* urea, 50 m*M* PhSH and 49.0 μM Fd. Cell pathlength = 1.0 cm. Spectra were obtained 4, 14, and 34 minutes after dilution. Final spectrum is that with the most intense absorbance at 454 nm and least intense at 400 nm.

*Abbreviations: Triton, Triton X-100; DMF, N,N-dimethylformamide; PhSH, benzenethiol; Fd, ferredoxin; Et, $-C_2H_5$; Tris, tris(hydroxymethyl)aminomethane; DMSO, dimethylsulfoxide.

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vol% decreases core extrusion time by only ~20%. One of the main reasons for using Triton is to reduce the percentage of organic solvent compared to that used in other core extrusion methods [3]. Therefore, rather than increase the DMF concentration, a more traditional protein denaturant, urea was substituted for DMF.

Figure 2 shows spectra resulting from anaerobic addition of an aqueous Triton/urea/PhSH mixture to an aqueous solution of *C. p.* Fd such that the final urea concentration is 5.5 *M*. Other conditions are listed in the Figure legend. In this case spectral changes ceased within 30 minutes and isosbestic behavior is not clearly evident. The final spectrum gave λ_{max} 454 nm and A_{454}/A_{550} = 1.99. Spectral parameters determined in the same aqueous Triton/urea medium for $(Et_4N)_2[Fe_4S_4(SPh)_4]$ are: ϵ_{454} = 16,900 M^{-1} cm^{-1} and A_{454}/A_{550} = 2.02 ± 0.03. From the absorbances in Fig. 2 a value of n_t = 1.9 is calculated.

Additional experiments have shown that the rate of core extrusion is quite sensitive to concentrations of urea in the range of 4–6 *M*. Thus, with 4 *M* urea core extrusion is quantitative but only after ~5 hours (40 m*M* PhSH), and the spectra exhibit isosbestic behavior in this case similar to that in Fig. 1.

Within the range of 3.5–5 vol% of Triton used in this work, urea does not significantly affect the concentration of micelles since the critical micelle concentration of Triton X-100 measured in 5.5 *M* urea translates to 0.08 vol.% [4].

The results are consistent with the reaction scheme proposed previously for core extrusion in aqueous Triton [2]:

$$\text{native Fd} \xrightarrow{\text{Triton/urea(or DMF)}} \text{urea(or DMF)–modified Fd} \qquad (1)$$

$$\text{urea(or DMF)–modified Fd} + \text{PhSH}_{\text{micellar}} \longrightarrow [Fe_4S_4(SPh)_4]^{-2} + \text{apoFd} \qquad (2)$$

The data in Fig. 1 or in 4 *M* urea can be explained by assuming that the rate of reaction (2) is somewhat slower than the rate of reaction (1). A rapid reaction (1) followed by a slower two step process in reaction (2) containing an intermediate mixed ligand species $[Fe_4S_4(S\text{–}Cys)_{4-n}(SPh)_n]$ (n = 1, 2, or 3) could also account for the biphasic kinetics at low denaturant concentrations [3]. The lack of isosbestic behavior in Fig. 2 can be explained by assuming that at 5.5 *M* urea reactions (1) and (2) occur at about the same rate. It is quite possible that the modified *C. p.* Fd obtained in 15 vol% DMF (or 4 *M* urea) is different from that obatined in 5.5 *M* urea. Other workers have noted large and abrupt conformational changes in *C. p.* Fd only at concentrations of DMSO $\gtrsim$ 40 vol% [5, 6]. It is interesting to note that 40 vol% corresponds to ~5.5 *M* for both DMSO and DMF. In contrast significant changes in the environment of the cluster in spinach Fd exhibit a smooth dependence on DMF concentration in the range of 10–40 vol% [2]. Whatever the mechanism, the most important results for application of the method are that at 50 m*M* PhSH and 3.5–5 vol% Triton, quantitative core extrusion of the two [4Fe–4S] centers of *C. p.* Fd can be achieved within 3–4 hours when 15 vol% DMF is used as denaturant and within 30 minutes when 5.5 *M* urea is used as denaturant.

Substitution of urea for DMF also results in quantitative core extrusion of the [2Fe–2S] center from spinach Fd [7]. The results reported here together with those previously reported for spinach Fd [2] suggest that selective core extrusion and site identification could be achieved in complex iron–sulfur proteins by adjustment of denaturant concentrations over a fairly narrow range. Unlike DMF, the effects of urea on many proteins have already been well studied. Furthermore, the use of urea rather than high concentrations of DMF raises the distinct possibility that, after selective core extrusion, an enzyme could be reisolated by conventional chromatographic techniques.

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