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A NEW METHOD FOR EXTRUSION OF IRON-SULFUR CORES FROM ACTIVE CENTERS OF PROTEINS

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SUMMARY: A new method for identification and quantitation of [2Fe-2S] and [4Fe-4S] types of iron-sulfur centers in proteins is presented. The method relies on the solubilization of C_6H_5SH, $[Fe_2S_2(SC_6H_5)_4]^{-2}$ and $[Fe_4S_4(SC_6H_5)_4]^{-2}$ in aqueous solutions containing 5-17 vol % N,N-dimethylformamide by 5 vol % Triton X-100. Quantitative removal of the Fe_2S_2 core of Spinach ferredoxin is achieved in media containing 80 vol % water. Advantages over previous core extrusion methods include avoidance of toxic hexamethylphosphoramide, a smaller percentage of organic solvent, increased sensitivity and (for Spinach ferredoxin) decreased extrusion time.

INTRODUCTION

The core extrusion method has proven to be of considerable utility in the identification and quantitation of [2Fe-2S] and [4Fe-4S] types of Fe-S centers in proteins and enzymes (1-5). The method is based on the ligand exchange reaction 1, in which cysteine thiolate ligands to the Fe-S center in the holoprotein are replaced by PhSH. The reaction is conducted anaerobically, usu-

$$\text{holoprotein} \quad + \quad \text{PhSH} \longrightarrow [Fe_nS_n(SPh)_4]^{-2} \quad + \quad \text{apoprotein} \qquad 1$$

$$\text{I } (n=2), \text{ II } (n=4)$$

ally in 80:20 vol % HMPA:water, a medium simultaneously capable of unfolding protein structure and of solublizing a large excess of aromatic thiol. Fe_2S_2 and Fe_4S_4 cores of Fe-S centers are extruded from holoproteins by reaction 1 in the form of stable synthetic analogues (I and II) which can be identified and quantitated spectrophotometrically (1-3) as well as by ^{19}F NMR (using $p\text{-}CF_3C_6H_5SH$) (3-5) or by EPR (4).

Abbreviations. Fd, ferredoxin; DMF, N,N-dimethylformamide; Triton, Triton X-100; Me, $-CH_3$; Et, $-C_2H_5$; HMPA, hexamethylphosphoramide; PhSH, benzenethiol; the term "aqueous Triton" refers to predominantly ($\geq$ 80 vol %) aqueous solutions in which the remainder is some proportion of Triton and DMF.

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A new method of core extrusion via reaction 1 is presented herein which relies on the solubilization of I, II and PhSH in predominantly aqueous solutions by the nonionic detergent, Triton X-100 and which presents certain advantages over previous methods.

METHODS

Reagents. $[Fe_2S_2(SPh)_4](Et_4N)_2$ (I), $[Fe_4S_4(SPh)_4](Et_4N)_2$ (II) and $[Fe_4S_4(SCH_2CH_2OH)_4](Me_4N)_2$ (III) were synthesized by published procedures (6,7). Spinach Fd was purchased from Sigma Chemical Co. and desalted by passage over a small Sephadex G-25 column equilibrated with 50 m$\underline{M}$ Tris-Cl pH 8.3, immediately before use. Triton X-100 was purchased from Sigma and used without further purification. DMF (over BaO) and PhSH were distilled under reduced pressure.

All manipulations described below were carried out under argon on a vacuum manifold in either schlenk-type glassware or septum capped vials. Solutions were transferred via gas-tight syringes. Buffer is 50 m$\underline{M}$ Tris-Cl pH 8.3. All spectra were obtained using a Perkin-Elmer Model 554 recording spectrophotometer in double septum sealed quartz cuvettes of either 0.05 or 1 cm path lengths similar to a design described in reference 8.

Determination of spectral parameters of I and II in aqueous Triton. 10-20 m$\underline{M}$ stock solutions of I or II were prepared in DMF and contained a 50-100-fold molar excess of PhSH. Exact concentrations of I and II in the stock solutions were determined spectrophotometrically in DMF using published extinction coefficients (9). Solutions of I or II in aqueous Triton were prepared typically by injecting 0.040 mL of stock into 0.960 mL of a solution prepared by adding 0.04 mL DMF to 0.2 mL Triton and diluting with 3.6 mL buffer. The resulting solution was transferred to the double septum cuvette and spectra obtained between 700 and 350 nm. The final solvent composition was 90:5:5 vol % buffer:DMF:Triton. Molarities of I and II in these solutions were assumed to be those resulting from dilution of stock and ranged from 0.1-0.4 m$\underline{M}$. The calculated molar extinction coefficients in aqueous Triton are thus based on those in DMF. Unless otherwise noted, the final values for absorbance ratios and extinction coefficients are averages of three determinations.

Ligand exchange reactions of III in aqueous Triton. A solution of water soluble III was prepared by dissolving the solid in buffer containing 0.021 mL/mL (30 m$\underline{M}$) $HSCH_2CH_2OH$. A spectrum of this solution (Figure 2a) gave A_{296}/A_{374} = 1.32, close to the literature value of 1.35 (10), and a calculated concentration using $\varepsilon_{374} = 15{,}400\ M^{-1}cm^{-1}$ of 0.84 m$\underline{M}$. To 1.5 mL of this solution was injected 0.380 mL of a solution prepared by mixing 0.25 mL Triton, 0.725 mL DMF and 0.025 mL PhSH. The final solvent composition was approximately 80:15:5 vol % buffer:DMF:Triton and contained 50 m$\underline{M}$ PhSH. The same reaction was also carried out in a solution in which acetonitrile was substituted for DMF. The aqueous solution (1 mL) initially contained 0.84 m$\underline{M}$ III and 50 m$\underline{M}$ $HSCH_2CH_2OH$, and after dilution (to 1.25 mL) the final solvent composition was approximately 81:9.5:9.5 vol % buffer:CH_3CN:Triton (100 m$\underline{M}$ PhSH).

Active site core extrusion of Spinach Fd. After thorough degassing 0.5 mL Spinach Fd in buffer was transferred to the 1 cm cuvette and a spectrum obtained (Figure 3a). 0.15 mL of a solution prepared by mixing 0.25 mL Triton, 0.725 mL DMF and 0.025 mL PhSH was then injected and after thorough mixing, spectra were obtained at intervals of 10-20 minutes until no further spectral changes were evident (Figure 3b). The final solvent composition was approximately 77:17:6 vol % buffer:DMF:Triton and contained 50 m$\underline{M}$ PhSH. Three other extrusion reactions were carried out similarly with the final solvent compositions listed in Table I. The completeness of extrusion was judged from the value of n_d calculated as follows:

$$n_d = \frac{\text{mol I}}{\text{mol Fd}} = \frac{A_{474}/10{,}000\ M^{-1}cm^{-1}}{A_{420}/8870\ M^{-1}cm^{-1}} \times V_f/V_i$$

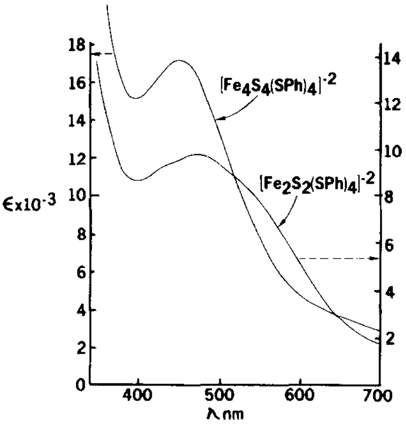


Figure 1. Visible absorption spectra of $[Fe_2S_2(SPh)_4]^{-2}$ and $[Fe_4S_4(SPh)_4]^{-2}$ in 90:5:5 vol % buffer:DMF:Triton containing excess benzenethiol.

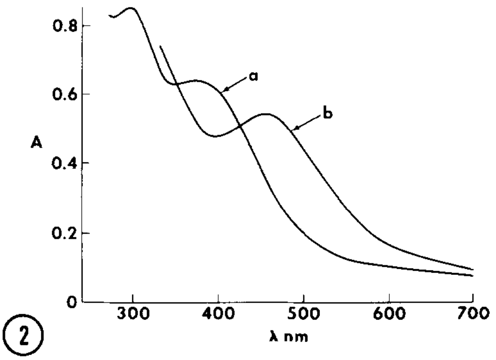
where V_f and V_i represent final and intial volumes, respectively. In all cases, the dilution factor V_f/V_i was either 1.25 or 1.30. The value of ε_{420} = 8870 $M^{-1}cm^{-1}$ is taken from reference 1.

RESULTS

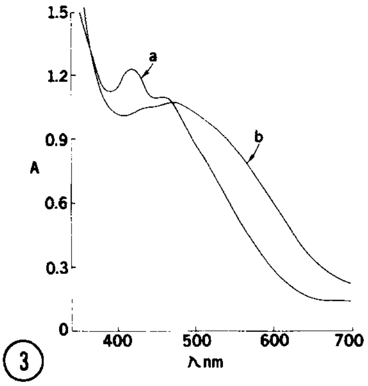
Determination of spectral parameters of I and II in aqueous Triton. I and II, which are insoluble in water, are soluble and stable for at least several hours in aqueous Triton in the presence of excess PhSH as judged by visible spectra. In the absence of excess PhSH, quantitative conversion of I to II was observed to occur in 90:5:5 vol % buffer:DMF:Triton, presumably via reaction 2, the one postulated to occur in 80:20 vol % HMPA:water (11). As in the latter

$$2[Fe_2S_2(SPh)_4]^{-2} \longrightarrow [Fe_4S_4(SPh)_4]^{-2} + 2PHS^- + PhSSPh \qquad 2$$

solvent, reaction 2 is rendered negligibly slow compared to extrusion time in aqueous Triton by maintenance of a mole ratio of PhSH/I $\geq$ 100, thus allowing correct site identification in reaction 1 for proteins containing only [2Fe-2S] or [4Fe-4S] active centers. Figure 1 shows spectra of I and II in 90:5:5 vol % buffer:DMF:Triton. The relevant spectral parameters determined in this medium for I are: λ_{max}, 474 nm (shoulder, ∿425 nm); ε_{474} = 10,000 $M^{-1}cm^{-1}$; A_{474}/A_{550} = 1.24 ± 0.02; A_{454}/A_{550} = 1.20 ± 0.01; A_{474}/A_{600} = 1.84 ± 0.04, the latter three values each being the average of eight determination. For II, the relevant parameters are λ_{max}, 454 nm; ε_{454} = 17,400 $M^{-1}cm^{-1}$; A_{454}/A_{550} = 2.05 ± 0.03.



② Figure 2. a) 0.84 mM $[Fe_4S_4(SCH_2CH_2OH)_4]^{-2}$ in buffer containing 30 mM $HSCH_2CH_2OH$ (0.05 cm path cell); b) solution in a) 15 minutes after a 1.25-fold dilution to a final solvent composition of 80:15:5 vol % buffer:DMF:Triton containing 50 mM PhSH. $A_{454}/A_{550} = 2.0$.



③ Figure 3. a) 0.140 mM Spinach Fd in buffer (1 cm path cell); b) solution in a) 10 minutes after a 1.3-fold dilution to a final solvent composition of 77:17:6 vol % buffer:DMF:Triton. These spectra represent expt 1 in Table I.

Ligand exchange reaction of III in aqueous Triton. To demonstrate the feasibility of reaction 1 in aqueous Triton, the ligand exchange reaction was carried out on III, which is water soluble, stable to hydrolysis in the presence of excess $HSCH_2CH_2OH$ and serves as a model for simple ferredoxins containing [4Fe-4S] active centers (10). Figure 2 shows spectra of such a reaction. A constant spectrum (Figure 2b) is reached in about 15 minutes which is unambiguously that of the expected product II. Upon application of $\varepsilon_{474} = 17{,}400\ M^{-1}cm^{-1}$ to figure 2b, the conversion of III to II is calculated to be 95% complete as is the same reaction carried out in a solution in which acetonitrile was substituted for DMF (spectra not shown). These nearly quantitative conversions were obtained from solutions containing only about a twofold molar excess of PhSH over $HSCH_2CH_2OH$.

Active site core extrusion of Spinach Fd. As a test of the method, Spinach Fd, which a variety of techniques have shown to contain one [2Fe-2S] center per protein molecule (1,12), was subjected to core extrusion in aqueous Triton. Figure 3 shows results of one such experiment and Table I lists relevant results from four such experiments using various percentages of Triton, DMF and buffer.

Table I

Expt.	[Spinach Fd]^a(m$\underline{M}$)	Solvent composition buffer:DMF:Triton (vol %)	[PhSH](m$\underline{M}$)	Extrusion time^b	n_d^{c}
1	0.140	77:17:6	50	10 min	1.00
2	0.041	80:15:5	50	15 min	1.02
3	0.149	80:10:10	100	40 min	0.97
4	0.149	90:5:5	80	>1.5 hrs	---

[a] Before dilution with Triton:DMF:PhSH.

[b] Approximate time required to reach a constant spectrum.

[c] See METHODS for equation used.

The spectral parameters in Figure 3b, namely λ_{max} = 474 nm, A_{474}/A_{550} = 1.23, A_{454}/A_{550} = 1.21, and A_{474}/A_{600} = 1.78, clearly identify it as that of I, whose Fe-S core has been extruded quantitatively from Spinach Fd as judged by n_d (Table I, expt 1). The average value of n_d for experiments 1-3 in Table I is 1.00 ± 0.03. Experiment 4 gave a spectrum which appeared to be intermediate between Spinach Fd and I even after 1.5 hours and extrusion was assumed to be incomplete at this point.

DISCUSSION

The results presented above demonstrate the feasibility of active site core extrusion via reaction 1 in aqueous Triton, at least for simple ferredoxins containing only [2Fe-2S] or [4Fe-4S] types of centers. Advantages of core extrusions of Spinach Fd in aqueous Triton over that in 80:20 vol % HMPA:water include avoidance of toxic HMPA, a smaller dilution factor (∿1.3 compared to 5) resulting in higher sensitivity, an extrusion time of 10-15 minutes (Table I, expts 1 and 2) compared to ∿90 minutes (1), and a lower percentage of organic solvent (∿20 vol % compared to 80 vol % or ∿4 mole % compared to 40 mole %) which may permit easier separation of extruded cores and reconstitution of the ferredoxin. Experiments 1, 2 and 4 in Table I show qualitatively that extrusion time is quite sensitive to the volume percentage of DMF, while experiments 2 and 3 show that increasing the volume percentage of Triton at the expense of DMF increases extrusion time. The highest volume percentage of DMF used for core extrusion of Spinach Fd (expt 1, Table I) corresponds to 2.2 M which is well below the concentration needed to reach the midpoint for denaturation of globular heme proteins (13) and perhaps indicates that complete denaturation is unnecessary for quantitative extrusion in these aqueous Triton solutions. The relatively high percentage of Triton compared to that normally used to solubilize membrane bound proteins appears to be necessary primarily to solubilize PhSH in the range of 50 mM.

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REFERENCES

1. Gillum, W. O., Mortenson, L. E., Chen, J. -S., and Holm, R. H. (1977) J. Am. Chem. Soc. 99, 584-595.
2. Averill, B. A., Bale, J. R. and Orme-Johnson, W. H. (1978) J. Am. Chem. Soc. 100, 3034-3043.
3. Wong, G. B., Kurtz, D. M., Jr., Holm, R. H., Mortenson, L. E., and Upchurch, R. G. (1979) J. Am. Chem. Soc. 101, 3078-3090.
4. Coles, C. J., Holm, R. H., Kurtz, D. M. Jr., Orme-Johnson, W. H., Rawlings, J., Singer, T. P., and Wong, G. B. (1979) Proc. Nat. Acad. Sci. USA 76, 3805-3808.
5. Kurtz, D. M., Jr., McMillan, R. S., Burgess, B. K., Mortenson, L. E., and Holm, R. H. (1979) Proc. Nat. Acad. Sci. USA 76, 4986-4989.
6. Christou, G., and Garner, C. D. (1979) J.C.S., Dalton, 1093-1094.
7. Reynolds, J. G., and Holm, R. H. (1980) Inorg. Chem. 19, 3257-3260.
8. Orme-Johnson, W. H., and Holm, R. H. (1978) Meth. Enzymol. 53, 268-274.
9. Mayerle, J. J., Denmark, S. E., De Pamphilis, B. V., Ibers, J. A., and Holm, R. H. (1975) J. Am. Chem. Soc. 97, 1032-1045.
10. Hill, C. L., Renaud, J., Holm, R. H., and Mortenson, L. E. (1977) J. Am. Chem. Soc. 99, 2549-2557.
11. Cambray, J., Lane, R. W., Wedd, A. G., Johnson, R. W., and Holm, R. H. (1977) Inorg. Chem. 16, 2565-2571.
12. Palmer, G. in "The Iron-Sulfur Proteins," Vol. II, W. Lovenberg, Ed., (1973) pp. 285-325, Academic Press, New York.
13. Herskovits, T. T., Behrens, C. F., Sinta, P. B., and Pandolfelli, E. R. (1977) Biochem. Biophys. Acta 490. 192-199.