Displacement of Iron–Sulfur Clusters from Ferredoxins and Other Iron–Sulfur Proteins

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Abstract: The displacement of iron–sulfur clusters from low molecular weight ferredoxins has been carried out in binary and ternary mixed aqueous-organic solvent systems incorporating dimethyl sulfoxide, hexamethylphosphoramide, *N*-methylformamide, tetramethylene sulfone, formamide, *N,N*-dimethylformamide, and *N*-methylpyrrolidinone. Thiols examined in the displacement reaction include thiophenol, α,α'-dimercapto-*o*-xylene, α,2-dimercaptotoluene, 3,4-dimercaptotoluene, 1,8-dimercaptonaphthalene, 2,2′-dimercaptobiphenyl, 5-dimethylaminonaphthalene-1-thiol, and *p*-dimethylaminothiophenol. Although dithiols with varying "bite" (S–S) distances do not selectively displace $Fe(SR)_4$, $Fe_2S_2(SR)_4$, and $Fe_4S_4(SR)_4$ cores, specific ligands given spectra with the three cluster types that differ sufficiently so that the quantitative estimations of the clusters may be made, if the solvent system is chosen to minimize the rate of interconversion of the iron–sulfur cluster types. A favorable example, and also a good solvent for many proteins, is 80% hexamethylphosphoramide–20% H_2O (v/v) with thiophenol as the displacing agent. In this system, and in the absence of oxygen, Fe_2S_2 cores are stable as the thiophenolate complexes, with absorbance maxima at 452 and 478 nm, respectively, and with millimolar absorbances of 4.6 and 4.9 per Fe atom, respectively. Ten representative iron–sulfur proteins were tested to demonstrate the generality of the method, and all save one gave results compatible with previous assignments of iron–sulfur cluster structures. Iron–sulfur protein III of *Azotobacter vinelandii*, which has been proposed to contain two Fe_4S_4 clusters, but which is anomalous in that the redox potentials of the two centers differ by 0.7 V, appeared to yield approximately one Fe_4S_4 cluster per molecule by this method; the balance of the iron did not give an easily identifiable species. This highlights the fact that the method depends on having only known types of clusters present in the sample. In the case of proteins containing one or more iron–sulfur centers of known type and in normal ligation in the protein, the method is rapid and quantitative once an unfolding solvent appropriate to the protein is found.

Introduction

Recent developments in the chemistry of iron–sulfur proteins have made it likely that chemical methods may be devised for the determination of the type and quantity of iron–sulfur center in a given protein. Thus, the finding that low molecular weight iron–sulfur proteins are reversibly denatured by Me_2SO[2] (if O_2 is excluded)[3] and the observation that low molecular weight iron–sulfur clusters undergo facile thiol ligand exchange[4] led Bale[5] and Que et al.[6] to explore the possibility of displacing iron–sulfur clusters from ferrodoxins with organic thiols in Me_2SO–water solutions. Since iron–sulfur proteins larger than ferredoxins (mol wt <24 000) are in our experience insoluble in the 4:1 Me_2SO–water mixtures useful in unfolding ferredoxins, we introduced the use of other solvents such as HMPA–water (4:1) and formamide–Me_2SO–water (3:1:1), in which many proteins are more soluble. Soluble systems are required for accurate spectrophotometric estimation of the displaced clusters. We have briefly reported on the use of these systems in studying hydrogenase[7] and the nitrogenase Fe protein[8] from *Clostridium pasteurianum*, and the result, that these proteins contain Fe_4S_4 clusters, has been confirmed by others[9] using these "exotic" solvent systems. Recently, Hill et al.[10] have studied the cluster content of an iron–sulfur flavoprotein, using difference spectroscopy to eliminate interference by the flavin chromophore. Because of the apparent usefulness of the displacement procedure, we have subjected it to detailed scrutiny, considering the effect of solvent composition, type of displacing thiol, pH, oxidation state of the protein, and reactant compositions. In this paper we report the results of these studies along with our conclusions about the critical factors affecting a successful and quantitative cluster displacement reaction. We give standard curves for quantitative estimations in several solvent systems. We also give results of cluster displacement experiments with ten low molecular weight iron–sulfur proteins, six of which had not been previously examined.

Experimental Section

Methods. Syntheses and solution preparations were carried out in glass apparatus equipped with stopcocks,[11] such that the apparatus could be alternately evacuated and flushed with prepurified nitrogen to remove oxygen. Anaerobic transfers of solution were effected by cannulae and rubber serum stoppers, or by specially designed gas-tight Hamilton syringes. Displacement reactions were run and spectra of iron–sulfur compounds obtained in double-septum cuvettes and tonometers (Figure 1) under an atmosphere of purified argon.[12] For a typical displacement reaction, 200 μL of a solution of iron–sulfur protein (600 μM Fe) and buffer (150 mM) was diluted with 800 μL of a 12.5 mM solution of thiol in the desired organic solvent at room temperature; the final concentrations were therefore Fe, 120 μM; buffer, 30 mM; thiol, 10 mM; organic solvent, 80% (v/v). In the case of Me_2SO and tetramethylene sulfone, the rapid oxidation of thiols by solvent[13] required that the thiol solution be prepared immediately before use in each experiment; this was accomplished by dilution of aliquots of stock 1–2 M thiol solutions in *N,N*-dimethylacetamide. Optical spectra were obtained in 1-cm cuvettes using a Cary 14 spectrophotometer which had been calibrated with the 550-nm band of reduced cytochrome *c*. Proton resonance spectra were recorded on a Varian T-60 spectrometer. Melting points were measured in Pyrex capillaries using a hot oil bath equipped with a calibrated thermometer, and are corrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn., and by Microtech Laboratories, Inc., Skokie, Ill.

Materials. Thiophenol, *tert*-butyl mercaptan (2-methylpropane-2-thiol), and α,α'-dibromo-*o*-xylene were obtained from Aldrich and used without further purification. The following compounds were prepared or purified according to published procedures: *p*-nitrothiophenol,[14] α,α'-dimercapto-*o*-xylene,[4b] $(Me_4N)_2[Fe_4S_4(S\text{-}t\text{-}Bu)_4]$,[15] $(Ph_4As)_2[Fe_4S_4(S\text{-}t\text{-}Bu)_4]$,[4a] $(Et_4N)_2[Fe_4S_4(SPh)_4]$,[16] $(Et_4N)_2[Fe_2S_2(SPh)_4]$,[4b] and $(Ph_4As)_2[FeS(SCH_2)_2C_6H_4]_2$.[4b] *p*-Dimethylaminothiophenol[17] was stored as its air-stable HPF_6 salt or as the disulfide, and the free thiol generated in the absence of air just before use. 1,8-Dimercaptonaphthalene was a gift from Mr. D. Roberts.

5-Dimethylamino-1-mercaptonaphthalene (Dansyl Mercaptan). This compound was prepared from dansyl chloride[18] by lithium aluminum hydride reduction to the disulfide, obtained as highly fluorescent

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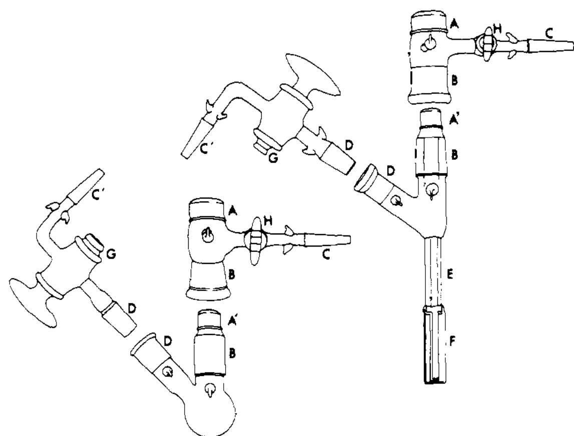

Figure 1. Double-septum-seal anaerobic apparatus for use in cluster displacement. A and A' are rubber serum stoppers which isolate the top chamber, formed by B (₮ 24/25 joint), from the bottom chamber. The top chamber is evacuated and flushed through C (₮ 7/15) via microstopcock H. The bottom chamber (left, a tonometer for preparing anaerobic reagents; right, a 10 mm path × 4 mm width silica spectrophotometer cell (F)) is evacuated through C' via a 2-mm vacuum stopcock (G) joined to the main body of the apparatus by D (₮ 12/18). Solutions are introduced into the apparatus through D, or after evacuation and flushing with O_2-free (<1 ppm) gas, through the tandem septum seals. A small amount of the solution may be ejected into the top compartment, to waste the O_2-contaminated portions of the solution within the syringe tip, following which the second septum is pierced and the solution transferred to the lower chamber.

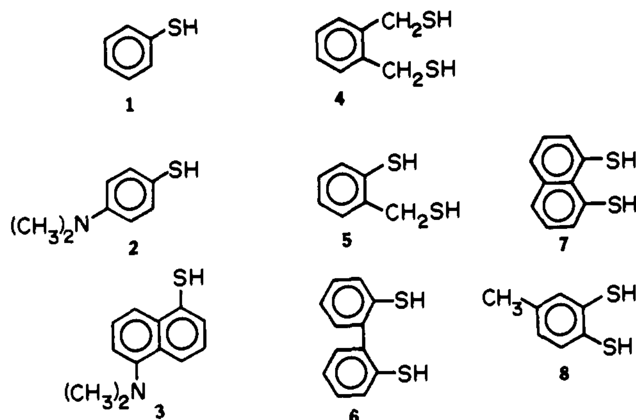

Figure 2. Thiols investigated in this work.

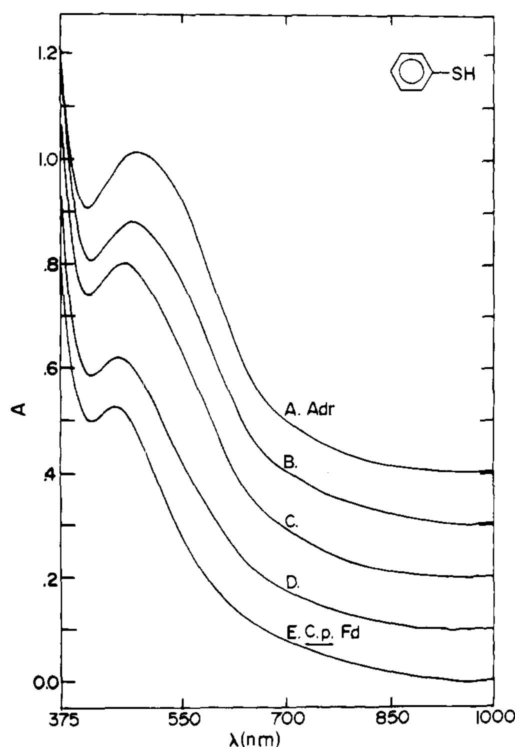

Figure 3. Spectra of *C. pasteurianum* ferredoxin and bovine adrenodoxin buffer (pH 9.0), 23 mM PhSH, 120 μM total Fe concentration. (a) 60 μM Adr; (b) 45 μM Adr + 3.75 μM *C.p.* Fd; (c) 30 μM Adr + 7.5 μM *C.p.* Fd; (d) 15 μM Adr + 11.25 μM *C.p.* Fd; (e) 15 μM *C.p.* Fd.

(yellow) pale yellow crystals from 2-propanol: mp 122.5-125.5 °C; NMR ($CDCl_3$) δ 8.2 (t, 2), 7.2 (m, 4), 2.85 (s, 6). Anal. Calcd for $C_{24}H_{24}N_2S_2$: C, 71.24; H, 5.98; N, 6.93; S, 15.85. Found: C, 70.97; H, 6.36; N, 6.92; S, 15.82. Further reduction with Zn/HCl followed by precipitation with NH_4PF_6 gave the HPF_6 salt of dansyl mercaptan as a pale yellow powder. The free thiol is a yellow solid melting below room temperature, and is rapidly oxidized by air.

α,2-Dimercaptotoluene. This compound was prepared from *o*-mercaptobenzyl alcohol[19] by oxidation to the disulfide, treatment with HBr in acetic acid to give the bis(benzyl bromide), reaction with thiourea in ethanol, and subsequent alkaline hydrolysis, followed by reduction of the disulfide and vacuum distillation to give α,2-dimercaptotoluene as a pale yellow to clear oil: bp 60-65 °C (0.1 mmHg); NMR ($CHCl_3$) δ 1.85 (t, 1), 3.6 (s, 1), 3.8 (d, 2), 7.2 (m, 4). Anal. Calcd for $C_7H_8S_2$: C, 53.80; H, 5.16. Found: C, 54.22; H, 5.39.

2,2'-Dimercaptobiphenyl. This compound was prepared via biphenyl-2,2'-disulfonate, in turn obtained from the reductive coupling of diazotized *o*-aminobenzenesulfonate with cuprous ion in aqueous ammonia.[20] Final purification was by sublimation (70 °C, 0.02 Torr) to give white crystals: mp 78-79.5 °C (lit.[21] 78-79 °C); NMR ($CDCl_3$) δ 3.3 (s, 2), 7.3 (m, 8).

Proteins. Eight-iron ferredoxins from *Chromatium vinosum*, *Clostridium acidi-urici*, and *Clostridium pasteurianum*,[22] the four-iron ferredoxin from *Bacillus polymyxa*,[23] Fe-S proteins II[24] and III[25] from *Azotobacter vinelandii*, bovine and porcine adrenodoxins,[26] spinach ferredoxin,[26] and the "paramagnetic protein" of *C. pasteurianum*[27] were all isolated by the methods referred to. The high-potential iron-sulfur protein of *C. vinosum* was generously provided by R. G. Bartsch, while a partially purified concentrate containing the ferredoxin from this organism was provided by H. Winter. The rubredoxin from *C. acidi-urici* was purified as described for the *C. pasteurianum* protein[28] and was identified by its absorbance and EPR spectra.

Results

The results obtained are presented in three parts: first, a survey of displacement reactions from small proteins containing iron-sulfur prosthetic groups of known identity, using a variety of thiols and reaction conditions; second, a discussion of the means by which the prosthetic groups of proteins containing mixtures of Fe-S clusters can be identified under specific conditions; and third, a demonstration that the method can be applied to a variety of Fe-S proteins, including several for which the identity of the Fe-S chromophore was previously not established.

Survey of Thiols and Conditions. Spectral data for displacement reactions with the thiols discussed below are given in Table I. All extinction coefficients mentioned in the text are per iron, ε/Fe, since in several cases the degree of oligomerization of a given species is unclear. The thiols investigated are shown in Figure 2.

A. Thiophenol. Figure 3a shows the spectrum of $[Fe_2S_2(SPh)_4]^{2-}$ obtained from adrenodoxin in 80% HMPA, pH 9.0, 23 mM PhSH, and Figure 3e shows the spectrum of $[Fe_4S_4(SPh)_4]^{2-}$ obtained from *C. pasteurianum* ferrodoxin at the same total Fe concentration. Both spectra are stable (if oxygen is rigorously excluded) for at least 24 h under these conditions. Lower concentrations of organic solvent (i.e., 55 or 70%), while sufficient to unfold the proteins and remove the Fe-S prosthetic groups, result in *rapid* conversion of dimer to

Table I

Fe source	Solvent[a]	Thiol	Concn of thiol	pH[b]	Time after mixing, min	λ_{max}, nm	ϵ/Fe × 10^{-3}	Comments
Adr	80% Me_2SO	**1**	23	9.0	20	485	5.3	Stable
	80% HMPA	**1**	23	9.0	72	478	4.9	Stable
	80% HMPA	**1**	120	9.0	2	480	4.9	Stable
	60% formamide/20% Me_2SO	**1**	24	9.0	2	473	5.0	Unstable
	79% HMPA/1% py	**1**	12		110	478	5.0	Stable
C.p. Fd	80% Me_2SO	**1**	23	9.0	8	452	4.4	Stable
	80% HMPA	**1**	23	9.0	8	452	4.6	Stable
	60% formamide/20% Me_2SO	**1**	24	9.0	2	445	4.2	Stable
Adr	80% HMPA	**2**[c]	12	9.0	50	535	3.9	Dimer → tetramer rapidly
C.p. Fd	80% HMPA	**2**[c]	12	9.0	60	535	3.1	Stable
Adr	80% HMPA	**3**[d]	12	9.0	24	548	5.4	Stable
C.p. Fd	80% HMPA	**3**[c]	12	9.0	2	535 (sh)	2.9	Almost featureless
C.a.-u. Rb	80% HMPA	**4**	12	9.0	9	485	7.9	Unstable
Adr	80% Me_2SO	**4**	12	9.0	10	587, 415	2.4, 5.6	Stable
Adr	80% NMP	**4**	12	9.0	2	587, 410	2.6, 5.7	Stable
C.p. Fd	80% Me_2SO	**4**	12	9.0	10	417	4.7	Stable
C.p. Fd	80% NMF	**4**	12	8.0	2	410	3.9	Unstable
C.p. Fd	80% HMPA	**4**	12	9.0	47	412	4.3	Stable
C.a.-u. Rb	80% HMPA	**5**	10	9.0	9	450 (sh)	19.3	Stable
Adr	80% HMPA	**5**	10	7.4	2	530	4.1	Less stable at high pH
C.p. Fd	80% HMPA	**5**	12	7.4	2	450 (sh)	4.8	Stable
C.a.-u. Rb	80% HMPA	**6**	12	9.0	10	600 (sh), 450 (sh)		Almost featureless
Adr	80% HMPA	**6**	12	7.7	8	520, 420	4.0, 9.3	Also 540 (sh). Very unstable at high pH.
C.p. Fd	80% HMPA	**6**	12	7.7	2	450 (sh)	3.9	Unstable
Adr	80% NMP	**7**	19	9.0	15	750	4.8	Stable
C.p. Fd	80% HMPA	**7**	12	9.0	120	722, 775 (sh)	4.5	Stable
C.a.-u. Rb	80% HMPA	**8**	120	9.0	8	562, 482	10.2, 10.7[e]	Stable
Adr	80% NMP	**8**	19	9.0	8	495	8.1[e]	Stable
C.p. Fd	80% HMPA	**8**	120	9.0	8	840, 562, 482	1.0, 7.5, 5.5[e]	Stable

[a] All solvent compositions v/v. [b] pH of aqueous component. [c] Free thiol generated from the disulfide plus dithiolthreitol. [d] Free thiol generated from the disulfide plus β-mercaptoethanol. [e] Apparent extinction coefficients based on total Fe concentration.

tetramer. Similar spectra are obtained in other solvents, although the exact positions of the maximum and the extinction coefficient vary slightly (Table I). In addition, the spectra seem to depend on the thiol concentration in some cases, although this was not investigated in detail. For example, in 80% HMPA or in 60% HMPA–20% NMF, the spectrum obtained from adrenodoxin is different at low (<15 mM) and high (>100 mM) concentrations of thiophenol. Although λ_{max} (478–480 nm) and the extinction coefficient (4.9×10^3 M^{-1} cm^{-1}/Fe) do not change, the intensity of the long-wavelength shoulder (~550 nm) is noticeably greater at high thiophenol concentrations, imparting a deeper purple tint to the solutions.

The effect of a coordinating solvent, pyridine, was also examined. As indicated in Table I, inclusion of 1% pyridine (124 mM) had no effect on the displacement of $[Fe_2S_2(SPh)_4]^{2-}$ from adrenodoxin, but when 70% pyridine was used as the solvent for the displacement reaction with very high thiophenol concentrations (10%, 0.97 M), the solution rapidly became colorless.

Treatment of *C. acidi-urici* rubredoxin in 80% HMPA with 24 mM thiophenol gave a bright purple solution which bleached to colorless within 2 min.

B. Other Monothiols. Spectra obtained with *p*-dimethylaminothophenol (**2**) and 5-dimethylaminonaphthalene-1-thiol (dansyl mercaptan) (**3**) under comparable conditions are shown in Figures 4 and 5, respectively. Only the species obtained with dansyl mercaptan are relatively stable to solvolysis or interconversion.

C. α,α'-Dimercapto-*o*-xylene (*o*-Xylyldithiol). This ligand gives a characteristic spectrum with the dimeric Fe–S cluster, with peaks at 590 and 415 nm in the visible region (Figure 6e), whether in 80% Me_2SO or NMF or in a 60% formamide–20% Me_2SO mixture. In certain solvents, however, the spectrum is radically altered. For example, the methanol solution from which the synthetic dimer is isolated gives only a weak shoulder at 585 nm. Similarly, in aqueous HMPA mixtures, but not in HMPA alone, the 590-nm peak is abolished and the features at 450 and 415 nm are seen as shoulders on a gradually rising absorption. This behavior does not depend on the pH of the aqueous component between pH 8 and 9, nor on the presence of excess *o*-xylyldithiol in solution.

The *o*-xylyl dimer is the most oxygen-sensitive species encountered in this work, and this sensitivity gave some insight into its chemical stability. In almost all experiments, the intensity of the initial spectrum decreased by up to 15% during the first hour after mixing, presumably owing to reaction with residual oxygen in the solvent or adsorbed onto the glass. Then the intensity of the 415- and 587-nm peaks invariably began to increase, approaching the original values on standing overnight. In an experiment where a known amount of oxygen was subsequently injected, the same process occurred. It thus appears that, after oxidative degradation, the *o*-xylyl dimer is reassembled in the presence of excess thiol.

Formation of the *o*-xylyl dimer was also observed in the spectra of the tetrameric species with this ligand. Thus, treatment of clostridial ferredoxin with 10 mM *o*-xylyldithiol in 80% Me_2SO gives the characteristic spectrum of a tetramer with a benzyl thiol ligand (Figure 6e). Upon standing for 1 h

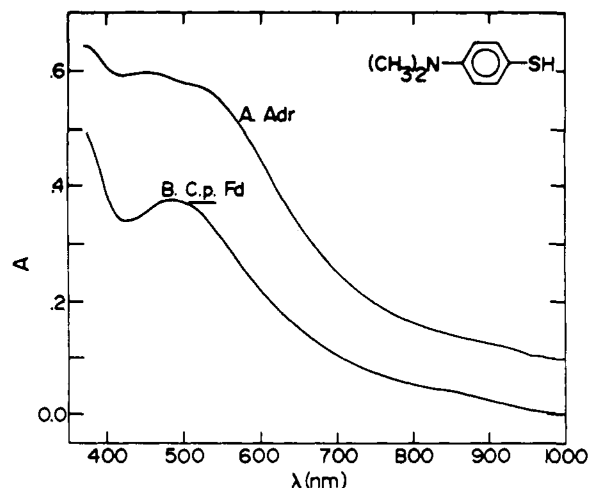

Figure 4. Spectra of *C. pasteurianum* ferrodoxin and bovine adrenodoxin treated with *p*-dimethylaminothiophenol in 80% (v/v) HMPA. Conditions: 30 mM Tris buffer (pH 9.0), 6 mM *p*-dimethylaminophenyl disulfide, 12 mM 2-mercaptoethanol, 120 μM Fe. (a) 60 μM Adr; (b) 15 μM *C.p.* Fd.

or more at room temperature, a shoulder at 587 nm slowly develops. The rate at which this occurs is sensitive to solvent polarity and to pH, being quite rapid (<30 min) in 60% formamide–20% Me_2SO and in 80% NMF, and slowed by a factor of 2–4 upon going from pH 9.0 to 8.0 aqueous component (30 mM Tris in each case).

Treatment of 60 μM *C. acidi-urici* rubredoxin with 12 mM *o*-xylyldithiol in 80% HMPA, pH 9.0, resulted in a deep red-purple solution. The spectrum (Figure 6f) showed bands at 340, 485, and ~675 nm, and decreased in intensity rapidly on standing at room temperature.

D. Other Dithiols. Both α,2-dimercaptotoluene (**5**) (Figure 7) and 2,2′-dimercaptobiphenyl (**6**) (Figure 8) give characteristic spectra with the dimeric Fe–S cluster, but essentially featureless spectra with the tetrameric cluster and with rubredoxin. Maximum stability of the new dimers is observed at pH ~7.4–7.7, although only that derived from **6** is sufficiently stable for use in analysis. In contrast, the major species produced in the reaction of either 1,8-dimercaptonaphthalene (**7**) (Figure 9) or 3,4-dimercaptotoluene (**8**) (Figure 10) with adrenodoxin or *C. pasteurianum* ferredoxin is the same regardless of the nature of the starting material. In the case of **8**, the major species (λ_{max} 482, 562 nm) is formed in the absence of sulfide, while a broad peak at 840 nm is seen only with the bacterial ferredoxin.

Displacements from Mixtures of *C. pasteurianum* Ferredoxin and Adrenodoxin. A. Thiophenol. Treatment of mixtures of *C. pasteurianum* ferredoxin and adrenodoxin with thiophenol in 80% Me_2SO results in the spectra shown in Figures 3a–e. In these experiments, the total iron concentration was constant at 120 μM; the fraction of iron present in dimer and tetramer was varied from 1.00 to 0 in 0.25 increments. Inspection of the spectra shows the eypected shift in wavelength with changing composition (Figure 11c). Thus, the composition of any mixture of the two proteins can be estimated simply from the position of the peak. The basis for a more sensitive method of determining the composition of such mixtures is shown in Figure 11a, where the ratio of the absorbance at 540 nm to that at 440 nm is plotted vs. composition. This ratio varies smoothly from 0.53 for 100% Fe_4S_4 to 1.01 for 100% Fe_2S_2. Owing to the spectral shifts discussed above, this plot holds only for spectra in 80% Me_2SO. An analogous plot for spectra in 80% HMPA is also linear, with A_{540}/A_{440} of 0.50 for 100% Fe_4S_4 and 0.90 for 100% Fe_2S_2.

B. *o*-Xylyldithiol. The spectra obtained for mixtures of *C.*

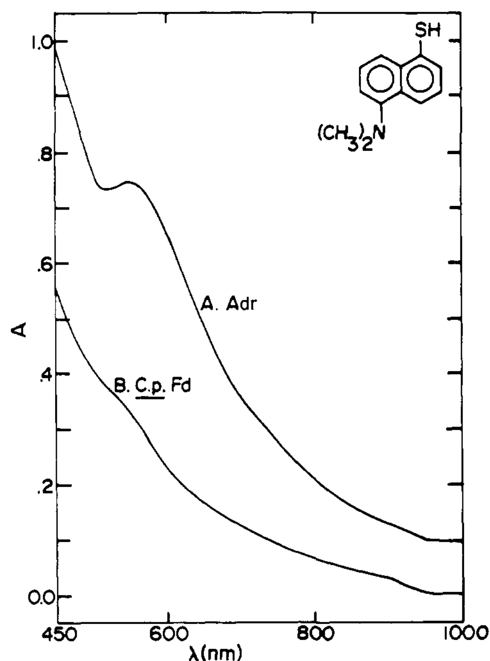

Figure 5. Spectra of *C. pasteurianum* ferredoxin and bovine adrenodoxin treated with dansyl mercaptan in 80% (v/v) HMPA. Conditions: 30 mM Tris buffer (pH 9.0), 6 mM dansyl disulfide, 120 μM Fe. (a) 60 μM Adr, 12 mM 2-mercaptoethanol; (b) 15 μM *C.p.* Fd, 12 mM dithiothreitol.

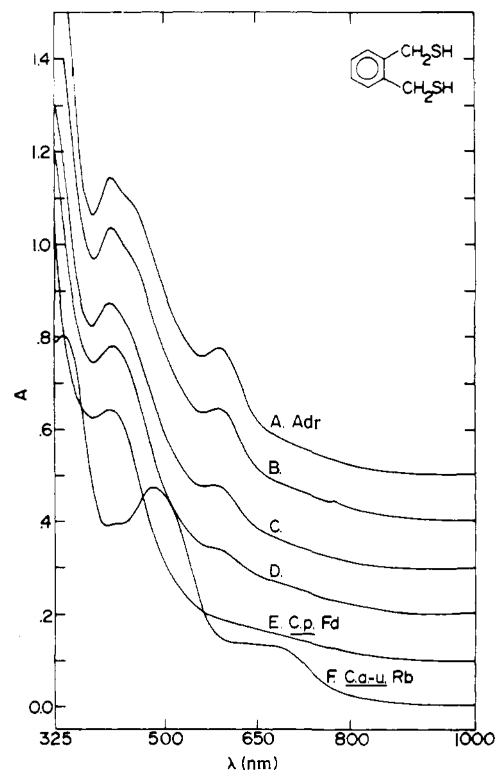

Figure 6. Spectra of *C. pasteurianum* ferredoxin, bovine adrenodoxin, and *C. Acidi-urici* rubredoxin treated with *o*-xylyldithiol in 80% (v/v) Me_2SO (a–e) and HMPA (f). Conditions: 30 mM Tris buffer (pH 9.0), 12 mM *o*-xylyldithiol, 120 μM Fe (a–e), 60 μM Fe (f). (a) 60 μM Adr; (b) 45 μM Adr + 3.75 μM *C.p.* Fd; (c) 30 μM Adr + 7.5 μM *C.p.* Fd; (d) 15 μM Adr + 11.25 μM *C.p.* Fd; (e) 15 μM *C.p.* Fd; (f) 60 μM *C.a.-u.* Rb.

pasteurianum ferredoxin and adrenodoxin with this thiol in 80% Me_2SO are shown in Figures 6a–e. Since the major peak is at 415 nm for both dimeric and tetrameric species, only a decrease in the relative intensity of the 590-nm peak with decreasing percentage of dimer is observed. This can be quanti-

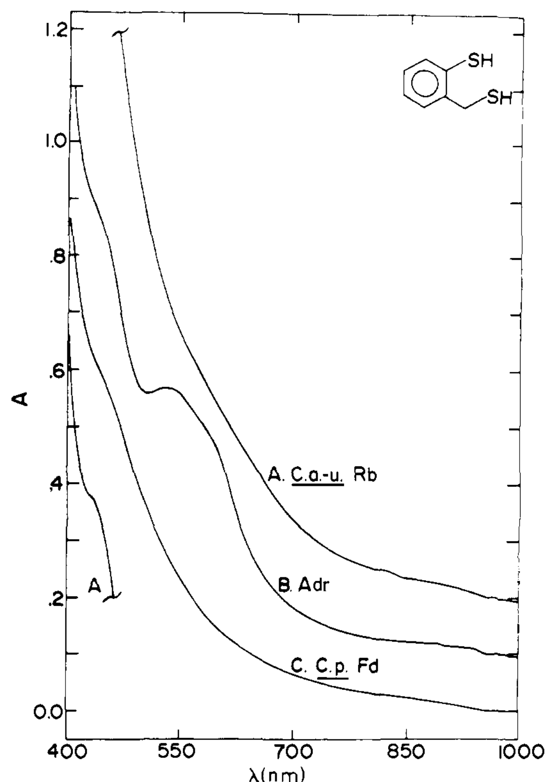

Figure 7. Spectra of *C. pasteurianum* ferredoxin, bovine adrenodoxin, and *C. acidi-urici* rubredoxin treated with α,2-dimercaptotoluene in 80% (v/v) HMPA. (a) 30 mM Tris buffer (pH 9.0), 10 mM dithiol, 60 mM *C.a.-u.* Rb (60 μM Fe); (b) 30 mM Tris buffer (pH 7.4), 24 mM dithiol, 60 μM Adr (120 μM Fe); (c) 30 mM Tris buffer (pH 7.4), 12 mM dithiol, 15 μM *C.p.* Fd (120 μM Fe).

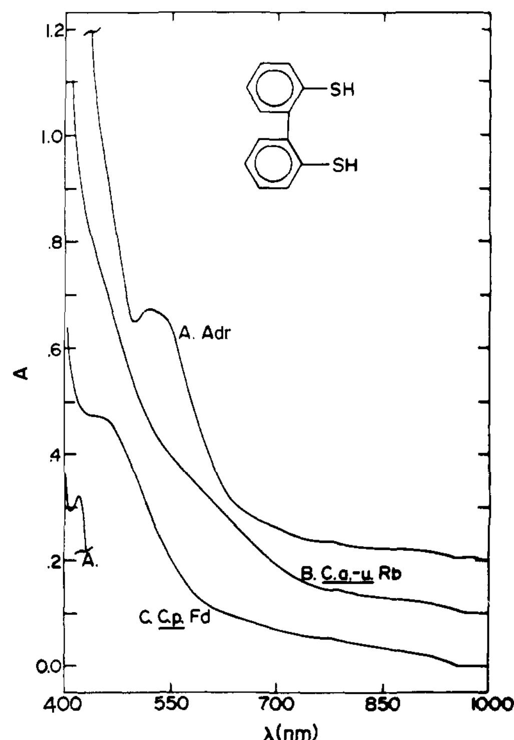

Figure 8. Spectra of *C. pasteurianum* ferredoxin, bovine adrenodoxin, and *C. acidi-urici* rubredoxin treated with 2,2'-dimercaptobiphenyl in 80% (v/v) HMPA. Conditions: 30 mM Tris buffer, 12 mM dithiol, 120 μM Fe (a,c), 60 μM Fe (b). (a) 60 μM Adr, pH 7.7; (b) 60 μM *C.a.-u.* Rb, pH 9.0; (c) 15 M *C.p.* Fd, pH 7.7.

Table II

Protein	Mol wt	Fe/mol	Fe_4S_4	Fe_2S_2
C. pasteurianum ferredoxin	6000	8	2.0	0[a,b]
Chromatium ferredoxin	10 000	8	1.93	0.38[a]
B. polymyxa ferredoxin I	9 000	4	0.92	0.16
Chromatium $HiPip_{red}$	10 000	4	0.97	0.21[a]
Chromatium $HiPip_{ox}$	10 000	4	1.10	*a*
Adrenodoxin	12 000	2	0	1.0[b,c]
Spinach ferredoxin	11 000	2	0.02	1.09[c]
C. pasteurianum paramagnetic protein (Cp^3)	24 000	2	0.03	1.07[c]
A. vinelandii iron-sulfur protein II	24 000	2	0.08	0.96[c,d]
A. vinelandii iron-sulfur protein III	16 000	8	1.43 1.13	1.01[e] 2.08[c]

[a] 24 mM PhSH in 80% Me_2SO. [b] Standard. [c] 12 mM *o*-xyl$(SH)_2$ in 80% Me_2SO. [d] 24 mM PhSH in 80% NMF. [e] 120 mM PhSH in 60% HMPA, 20% NMF.

tated by comparison with a plot of A_{590}/A_{400} vs. composition (Figure 11b); the ratio varies from 0.17 for 100% Fe_4S_4 to 0.46 for 100% Fe_2S_2. Again, the limiting absorbance ratios vary with the solvent system. For example, in 80% NMF and in 60% formamide-20% Me_2SO, the ratios vary from 0.46 for 100% Fe_2S_2 to 0.25 and 0.21, respectively, for 100% Fe_4S_4, owing to the more rapid formation of small amounts of dimer in NMF (vide supra).

Displacements from Small Iron-Sulfur Proteins. A series of small (mol wt ≤24 000) proteins known to contain 2, 4, or 8 Fe-S per molecule were examined using the displacement technique discussed above. The proteins investigated, the conditions for the displacement reactions, and the results are summarized in Table II. Thus, *Chromatium* ferredoxin, *B. polymyxa* ferredoxin I, and *Chromatium* high-potential iron protein are shown to contain 2, 1, and 1 Fe_4S_4 clusters per molecule, respectively. Treatment of oxidized and reduced HiPIP (corresponding to $[Fe_4S_4(SR)_4]^-$ and $[Fe_4S_4(SR)_4]^{2-}$) gives the same result within experimental error. Similarly, spinach ferredoxin, *C. pasteurianum* paramagnetic protein (Cp^3), and *A. vinelandii* iron-sulfur protein II are shown to contain the dimeric iron-sulfur cluster indicated by their iron content. The last of these, AvII, is insoluble in 80% Me_2SO; consequently, the displacement reaction was run in 80% NMF, and appropriate controls were used to construct a standard curve as in Figure 11.

Treatment of *A. vinelandii* iron-sulfur protein III[25] with 24 mM thiophenol in 80% Me_2SO gave the expected peak at 452 nm, but the spectrum showed distinct shoulders at 580 and 515 nm (Figure 12) as well. These additional features were stable for several hours, and are not characteristic of either dimeric or tetrameric clusters under these conditions. Although use of 120 mM thiophenol in 60% HMPA-20% NMF gave the same initial spectrum, the long-wavelength features decreased rapidly in intensity and were totally absent after 80 min. Treatment with *o*-xylyldithiol in 80% Me_2SO gave no unusual spectral features. The iron-sulfur cluster compositions shown for this protein in Table II result from a spectral analysis identical with that employed for the other proteins.

A brief description of this analysis is in order. The concentration of the proteins was determined by published extinction coefficients and, in some cases, by Fe analysis.[29] After the ratio of absorbance at the appropriate wavelengths was measured, referral to Figure 11a,b or similar plots for the specific solvent system used gave the mole fraction of dimeric and tetrameric species in the sample. Then, reference to a plot of A_{440} or A_{400} (for thiophenol and *o*-xylyldithiol, respectively) vs. composition at a known total iron concentration gave the expected ab-

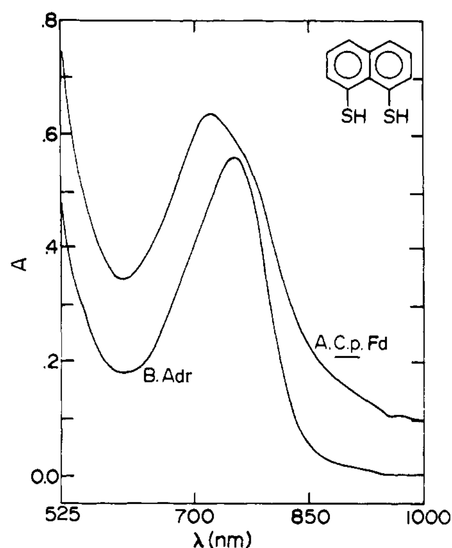

Figure 9. Spectra of *C. pasteurianum* ferredoxin and bovine adrenodoxin treated with 1,8-dimercaptonaphthalene, in 80% (v/v) organic solvents. Conditions: 30 mM Tris buffer (pH 9.0), 120 μM Fe. (a) 15 μM *C.p.* Fd, 19 mM dithiol, HMPA; (b) 60 μM Adr, 12 mM dithiol, NMP.

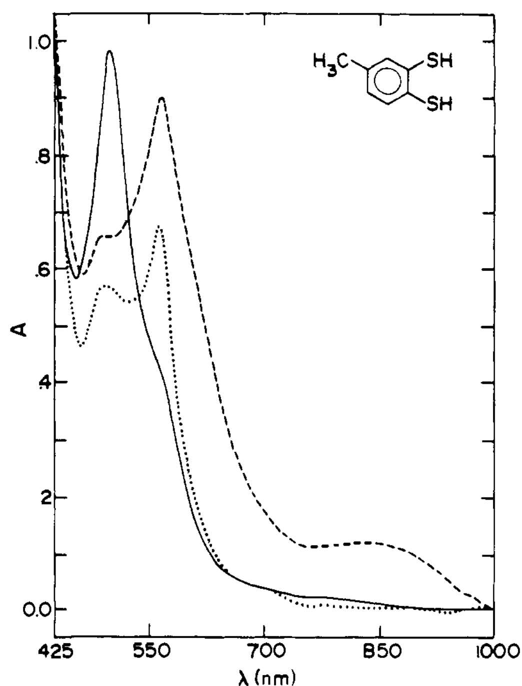

Figure 10. Spectra of *C. pasteurianum* ferredoxin, bovine adrenodoxin, and ferrous-NTA treated with 3,4-dimercaptotoluene in 80% (v/v) organic solvents. Conditions: 30 mM Tris buffer 9Ph 9.0). (—) 60 μM Adr (120 μM Fe), 19 mM dithiol, NMP; (- - - -) 15 μM *C.p.* Fd, (120 μM Fe), 120 mM dithiol, HMPA; (····) 60 μM Fe^{2+}-NTA, 139 mM dithiol, HMPA.

sorbance for the observed sample composition at that total iron concentration. (With thiophenol in 80% Me_2SO, the extinction coefficient per iron at 440 nm varies from 4.4 mM^{-1} cm^{-1} for pure tetramer to 4.7 mM^{-1} cm^{-1} for pure dimer; with *o*-xylyldithiol in 80% Me_2SO, the corresponding values are 4.5 and 5.1 mM^{-1} cm^{-1} at 400 nm, respectively.) The observed absorbance gives the actual total iron concentration, from which the actual concentrations of Fe_4S_4 and Fe_2S_2 units are readily calculated. The data in Table II are the ratio of these concentrations to the concentration of protein employed in the experiment, calculated by diluting a stock solution of known concentration. Owing to uncertainties in dilution of small

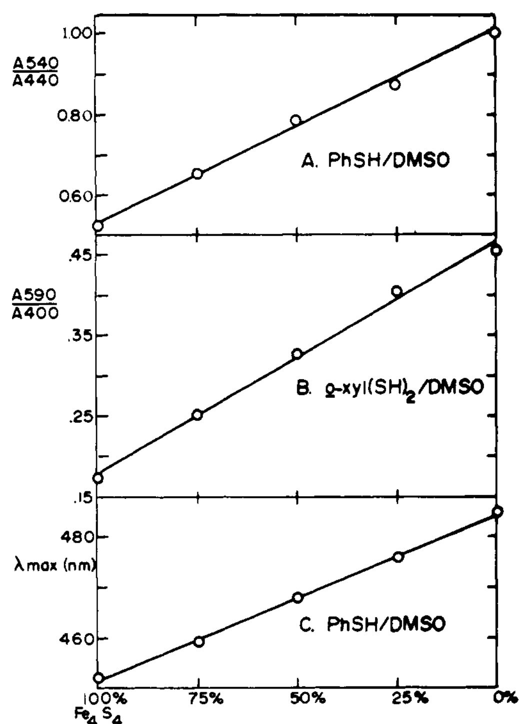

Figure 11. Plots of spectral data for mixtures of *C. pasteurianum* ferredoxin and bovine adrenodoxin treated with thiols as indicated in 80% (v/v) Me_2SO, pH 9.0 (spectra shown in Figures 2, 5). Abscissa is % Fe present as tetramer (100% = 15 μM *C.p.* Fd, 0% = 60 μM Adr) at constant [Fe] = 120 μM). (a) Ratio of absorbance at 540 and 440 nm for mixtures treated with 23 mM thiophenol; (b) ratio of absorbance at 590 and 400 nm for mixtures treated with 12 mM *o*-xylyldithiol; (c) dependence of λ_{max} on cluster composition in mixtures treated with 23 mM thiophenol.

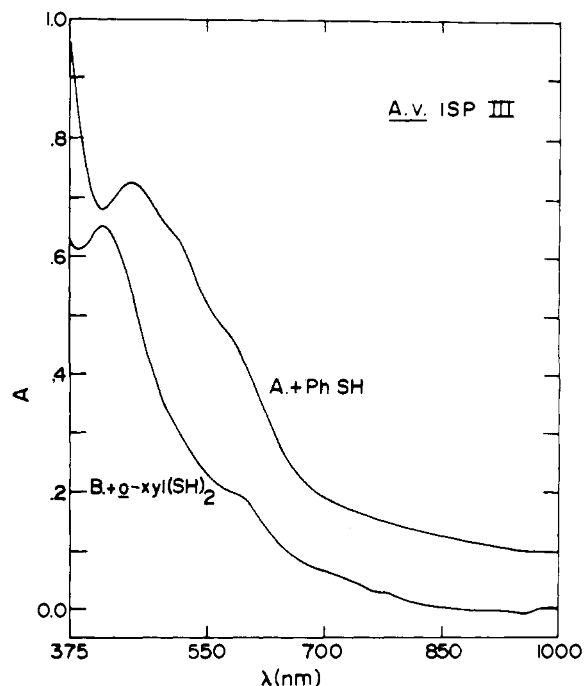

Figure 12. Spectra of *A. vinelandii* iron-sulfur protein III treated with 24 mM thiophenol (a) and 12 mM *o*-xylyldithiol (b) in 80% (v/v) Me_2SO, 30 mM Tris buffer (pH 9.0), at 120 μM Fe.

volumes of proteins and the reproducibility of the absorption spectra on subsequent scans, these results are estimated to be accurate to within 10%. This is substantiated by those cases in which the experiments were repeated several times; repeti-

tion was not possible for some proteins owing to limited amounts of sample.

Discussion

Survey of Displacement Reactions with *C. pasteurianum* Ferredoxin, Porcine Adrenodoxin, and *C. acidi-urici* Rubredoxin with Selected Thiols. The three proteins listed above, all available in relatively large amounts, were selected as prototype sources for displacement of 4Fe-4S, 2Fe-2S, and 1Fe-OS prosthetic groups, respectively. Displacement of these species was investigated under a variety of conditions with thiophenol and *o*-xylyldithiol. The other mono- and dithiols were then examined under the optimum conditions for these two; if preliminary results were encouraging, conditions were varied to obtain maximum stability of the displaced iron-sulfur species.

Thiophenol was initially selected for its avidity for iron-sulfur clusters relative to alkyl mercaptans[4a] and the apparent ease with which the 2Fe-2S and 4Fe-4S clusters with this ligand could be distinguished spectrally.[4b] A survey of the displacement reaction with *C. pasteurianum* ferredoxin and adrenodoxin in a variety of organic solvents and as a function of pH showed that both Fe-S cores could be removed from the proteins at relatively low thiophenol concentrations (ca. 10 mM) in 55% organic solvents (55% Me_2SO was the minimum concentration found by Bale to significantly perturb the optical and EPR spectra of adrenodoxin[5]). The dimeric Fe-S species is not stable under these conditions, however, being converted to the tetramer so rapidly as to preclude spectral identification; variation of solvent or pH in 55% Me_2SO had little effect. On raising the concentration of organic solvent to 80% (v/v), the dimeric species is stable against dimerization to the tetramer for several days at room temperature (conditions: pH ≥ 8.5, [thiol] ≥ 10 mM) in all solvents except formamide and *N*-methylformamide. In these last two solvents, the absorption maxima are significantly blue shifted, and both dimeric and tetrameric species hydrolyzed relatively rapidly, making it difficult to assess the extent of dimer-tetramer interconversion. The stability of the dimer with regard to dimerization appears to be critically dependent on the organic solvent concentration. For example, at 70% Me_2SO, the system behavior is very similar to that at 55% Me_2SO. There is thus an abrupt increase in stability of the dimeric species somewhere between 70 and 80% Me_2SO. It may be relevant to point out that 80% Me_2SO corresponds to a mole fraction of water of 0.5, or a 1:1 ratio of organic solvent to water. The ability of HMPA, with its higher molecular weight, to afford equal stability to the dimer at 80% (v/v) solvent levels (mole fraction water 0.71) may be related to its superior hydrogen-bond acceptor properties compared to other organic solvents.[30,31] These results are in good agreement with those obtained by Que et al.[6] under similar conditions (~0.8 mM PhSH, 80% Me_2SO, pH 8.5), except for a 5-6-nm shift in λ_{max} to shorter wavelength for both dimeric and tetrameric species. It is unlikely that this is due entirely to differences in calibration of the spectrophotometers employed, since the corresponding spectral data for displacements with *o*-xylyldithiol (vide infra) show only a 2-3-nm shift in the same direction.

In an attempt to maximize the spectral difference between dimers and tetramers with the same ligand, the effect of extending the conjugation of the aromatic thiol was examined. The first obvious candidate, *p*-nitrothiophenol, is unsuitable, since it is partially dissociated at the pH employed, and the anion absorbs strongly in the visible region. *p*-Dimethylaminothiophenol gives very different spectra with tetrameric and dimeric Fe-S clusters, but it has not yet been possible to find conditions under which the dimer with this ligand is stable for any appreciable time. In contrast, a similar ligand, 5-dimethylaminonaphthalene-1-thiol (dansyl mercaptan), affords distinctive spectra with both dimeric and tetrameric clusters, and these species are stable for several hours. This thiol was originally synthesized in order to allow the use of fluorescence spectroscopy in the assay of iron-sulfur cluster types. One would expect significant perturbations or possible quenching of the ligand fluorescence upon binding to iron-sulfur chromophores. In practice, this has not been observed because the requirement for a large excess of free thiol to stabilize the iron-sulfur species in mixed aqueous-organic medium results in a very high background fluorescence.

Historically, the first ligand to be discovered to stabilize the Fe_2S_2 chromophore was α,α'-dimercapto-*o*-xylene (*o*-xylyldithiol).[32] It was also successfully employed in displacement reactions with 2- and 8-Fe ferrodoxins by Bale[5] and Que et al.[6] In the present work it was found that the dimeric species with this ligand is surprisingly stable to hydrolysis in 55% Me_2SO or 80% NMF. In fact, it appears that the dimer is the most stable iron-sulfur species in mixed aqueous-organic solvent systems, pH ca. 9, in the presence of excess thiol, since the tetrameric cluster is slowly converted to dimer under these conditions. (This is in distinct contrast to the case with thiophenol, where the tetramer is the more stable species.) Conversion of tetramer to dimer is minimized at lower pH (7.5-8.0) in the *o*-xylyldithiol system, suggesting that the reaction proceeds via basic hydrolysis of the iron-sulfur cluster, perhaps assisted by the uncoordinated thiol of the monodentate dithiol. (The tetramer with monothiol ligands, e.g., benzyl mercaptan, is quite stable at pH 9.0-9.5.)

The spectral results for the dimer are in good agreement with those reported earlier[6] for the same solvent system, but those for the tetramer show a discrepancy. Que et al. report an extinction coefficient of 5.6×10^3/Fe at 417 nm for the tetrameric species with *o*-xylyldithiol;[6] in this work a value of 4.7×10^3/Fe, also at 417 nm, was obtained. That this latter value is correct is suggested by several lines of evidence. First, the same value ($\pm 0.2 \times 10^3$) is obtained from either *C. pasteurianum* ferredoxin in 80% Me_2SO or $[Fe_4S_4(S\text{-}t\text{-}Bu)_4]^{2-}$ in anhydrous Me_2SO. Second, the extinction coefficient reported for $[Fe_4S_4(SCH_2Ph)_4]^{2-}$ is 4.65×10^3/Fe at 420 nm;[16] if the second thiol of the *o*-xylyl ligand is indeed not coordinated, one would expect similar spectral properties. Third, as the tetramer is converted to the dimer, the absorbance at 417 nm increases as the peak at 587 nm develops. This would be expected if the dimer (ϵ_{417} 5.6×10^3/Fe) does indeed have a higher extinction coefficient than the corresponding tetramer. Fourth, in experiments with $[Fe_4S_4(S\text{-}t\text{-}Bu)_4]^{2-}$ in 100% Me_2SO, it was observed that addition of less than stoichiometric amounts of dithiol gave a presumably polymeric species with increased absorbance at 417 nm and below. Upon subsequent addition of excess dithiol, this was only slowly converted to the normal tetramer spectrum. Thus, the observed spectrum may be sensitive to the order and method of mixing, especially if this results in initial displacement of the tetramer at relatively low dithiol concentrations.

A particularly inconvenient property of the *o*-xylyldithiol dimer is the sensitivity of its absorption spectrum to certain solvents or solvent mixtures. As cited above, in methanol or in mixtures of water and HMPA the spectrum is relatively featureless, in contrast to the well-defined spectrum obtained in all other solvents or solvent mixtures investigated. Thus, although the spectrum in anhydrous HMPA is especially well resolved, addition of only 4-20% aqueous buffer results in complete loss of the spectral features. The origin of this effect is not clear, since methanol and aqueous HMPA mixtures are not obviously similar in properties.

The *o*-xylyldithiol ligand has also been reported to form a rubredoxin-like FeS_4 core[33] with Fe(III). Treatment of clostridial rubredoxin with the dithiol gave a spectrum very similar to that reported for the synthetic compound. Whether the

rather rapid loss of the spectrum is due to hydrolysis of the chromophore or reduction by excess thiol has not been examined; the conditions employed for this reaction are thus probably not optimal.

Several other dithiols were examined in the displacement reactions, with three goals in mind: (1) possible selective removal of one type of iron-sulfur species from a protein by a geometrically constrained dithiol; (2) development of alternate methods for iron-sulfur cluster identification, with possibly greater hydrolytic and/or oxidative stability, greater spectral differences between mono-, di- and tetranuclear species with the same ligand, and lack of spectral sensitivity to certain solvents; (3) determination of whether *o*-xylyldithiol is indeed unique in its ability to stabilize the Fe_2S_2 species, as has been reported.[4b] Since both a benzyl dithiol and aryl monothiols can separately stabilize the Fe_2S_2 unit, combinations of alkylaryl dithiol and aryl dithiols of varying bite (S-S distance) were synthesized and examined.

Both 2,2′-dimercaptobiphenyl (**6**) and α,2-dimercaptotoluene (**5**) form new, apparently dimeric, iron-sulfur species with characteristic spectra upon reaction with adrenodoxin in aqueous-organic solvents. The complexes are similar in their extreme sensitivity to base hydrolysis at pH >8, but only that with **6** is sufficiently stable at low pH (ca. 7.4) to allow its use as an analytical probe. These ligands are also similar in that they both give relatively featureless spectra with 4Fe-4S and 1Fe-OS proteins. The initial reaction products of **5** with adrenodoxin and clostridial ferredoxin slowly react further to give what appears to be the same spectral species, of unknown structure, with an intense peak at 440 nm. In contrast, the decomposition mode of both dimer and tetramer with **6** appears to be simply hydrolysis, resulting in bleached spectra and liberation of sulfide. In view of the extreme sensitivity of, for example, the thiophenolate dimer to solvent composition and pH, it is certainly conceivable that neither of these ligands has yet been investigated under optimum conditions for stability of the displaced iron-sulfur species.

The final two dithiols investigated, 1,8-dimercaptonaphthalene (**7**) and 3,4-dimercaptotoluene (**8**), give the same spectral results with either 4Fe-4S or 2Fe-2S starting materials. Although the identity of the species responsible for the 740-750-nm peak with **7** is not definitely established, the most likely candidate seems to be a tetrameric iron-sulfur species with a dithiol coordinated to each iron, as in Balch's complexes[34,35] $[Fe_4S_4(S_2C_2R_2)_4]^{2-}$. The variation of results obtained with **8** was apparently not due to the nature of the iron-sulfur source, but rather to the overall redox state of the solution. This, together with the observation that these peaks are obtained even in the absence of sulfide, indicates that the species involved may be simple iron dithiolene complexes in a redox equilibrium.[36] The complex $[Fe(S_2C_6H_3Me)_2]_2^{2-}$ has been reported to be red-purple, but no spectral data in the visible region were given.[37] Finally, the peak at 840-850 nm is seen only when the starting material contains the 4Fe-4S unit, suggesting that it may be due to a tetrameric species similar to Balch's.[34,35] Thus, reaction of **8** with iron-sulfur proteins is a complex process, involving at least partial degradation of the polynuclear prosthetic groups.

Analysis of Mixtures of *C. pasteurianum* Ferredoxin and Adrenodoxin. Displacement reactions of the two most generally useful thiols (thiophenol and *o*-xylyldithiol) with mixtures of 4Fe-4S and 2Fe-2S containing proteins were examined, in anticipation of the possible simultaneous occurrence of such species in certain proteins. As shown in Figure 3, with thiophenol only a single peak is observed in mixtures containing both dimeric and tetrameric clusters; the position of the maximum is a linear function of cluster composition (Figure 11c). An alternative method of analyzing the composition of mixtures relies on a simple line-shape analysis. Since the dimer with thiophenol has a pronounced long-wavelength shoulder, while the absorption of tetramer falls off smoothly beyond λ_{max}, the ratio of the absorbance at 540 nm to that at 440 nm is very sensitive to variations in the cluster composition. These wavelengths do not correspond to λ_{max} of either species or to the exact position of the shoulder, but were selected to give a maximum difference in the ratio for pure tetramer and pure dimer. Measurement of this ratio for an unknown solution gives the dimer/tetramer ratio in the solution directly. In order to obtain the absolute concentration of each species, it is necessary to refer to a second plot, that of the absorbance at a fixed wavelength as a function of composition for a fixed total iron concentration. In the case of thiophenol in 80% Me_2SO, the absorbance at 440 nm increases by less than 10% on going from 100% tetramer to 100% dimer, minimizing the error in this procedure. The absolute concentration of each species may then be calculated from the ratio of the observed absorbance and the absorbance of the standard curve. A similar analysis can be employed with *o*-xylyldithiol, using 590 and 400 nm as the key wavelengths. A point which cannot be overemphasized is the necessity of obtaining standard curves for each solvent or set of conditions; the line shapes of the iron-sulfur species are sufficiently sensitive to these effects that application of one standard curve to another solvent system is, in general, not possible.

Displacement Reactions with Small Iron-Sulfur Proteins Using Thiophenol and *o*-Xylyldithiol. In order to assess the generality of the method, the displacement reaction was applied to a series of small iron-sulfur proteins. Although the identity of the prosthetic groups of most of these proteins had been previously inferred from various physical techniques, it was desirable to demonstrate the applicability of the displacement method to all spectral and chemical classes of proteins.[38] This would decrease the likelihood of a special environment or reactivity of an iron-sulfur group giving erroneous results when applying the technique to an unknown protein. As shown in Table II, all three spectral and EPR classes of 2Fe-2S protein (adrenodoxin, *C. pasteurianum* paramagnetic protein, and spinach ferredoxin) give identical results, as does *A. vinelandii* ferredoxin II. Since these are all known by analysis to contain only two Fe and two S^{2-} per mol, the results come as no great surprise. Nonetheless, they do demonstrate that, *whatever the chemical differences which give rise to the observed spectral differences, these do not affect the general ligand substitution reactions of the 2Fe-2S chromophore.* Similarly, *Chromatium* high-potential iron protein[34] and, by analogy to *M. aerogenes* ferredoxin,[40] *C. pasteurianum* ferredoxin are known from x-ray crystallographic analysis to contain 4Fe-4S prosthetic groups; the same has been inferred for *Chromatium* and *B. polymyxa* ferredoxins. Again, regardless of the source, and, in the case of high-potential iron protein, regardless of oxidation state, all proteins containing tetrameric iron-sulfur species gave essentially the same spectra upon treatment with thiols in denaturing organic solvents. A point worth mentioning in connection with these proteins is the apparently high content of dimeric cluster resulting from the analysis discussed above (0.38, 0.16, 0.21 2Fe-2S units per mol of *Chromatium* ferredoxin, *B. polymyxa* ferredoxin I, and *Chromatium* high-potential iron protein). The data for *B. polymyxa* ferredoxin do not indicate that 92% of the protein molecules investigated contained one tetrameric cluster and 8% contained two dimeric units; within experimental error, the homogeneous protein contains only tetrameric units.

The single protein which did not give clear-cut results is *A. vinelandii* iron-sulfur protein III (AvIII). That this protein possesses anomalous features is indicated by the work of Sweeney et al.,[41] who interpreted their EPR and optical titration data as indicating that the protein contains two 4Fe-4S clusters with midpoint potentials for the 2-/1-redox process

of +340 and −420 mV. The former value is that expected for a high-potential iron protein;[38] the latter is thus shifted to negative potential by over 700 mV. While the chemical basis of this shift is not known, the results clearly indicate that one of the iron–sulfur prosthetic groups has abnormal properties. Howard, Lorsbach, and Que[42] have reported preliminary results on the displacement of the iron–sulfur chromophores from AvIII with thiophenol in 80% Me_2SO. Based on the position of the absorption maximum (458 nm) and the extinction coefficient of 4400 cm^{-1} M^{-1}/Fe for $[Fe_4S_4(SPh)_4]^{2-}$, these authors concluded that AvIII contained 1.89 4Fe–4S clusters per mol. In the present work, treatment of AvIII with thiophenol under standard conditions (24 mM thiol in 80% Me_2SO, pH 9.0) resulted in the anomalous spectrum shown in Figure 12. The long-wavelength features at 515 and 580 nm are not seen for any combination of dimer and tetramer in the standard spectra (Figure 3) and suggest the presence of a new iron–sulfur chromophore. Uncritical application of the spectral analysis to this spectrum gives 1.35 4Fe–4S and 1.87 2Fe–2S units per molecule. In an attempt to determine whether the spectrum resulted from an iron–sulfur chromophore attached to the protein by a relatively nonlabile ligand, with the other cluster ligands replaced by thiophenol, the experiment was repeated at 120 mM thiophenol in a 60% HMPA–20% NMF solvent system. In this case, the initial spectra were very similar to those of the previous experiment, but the anomalous features decreased in intensity with time, yielding a "normal" spectrum after ca. 90 min at room temperature. Analysis of this spectrum (using the appropriate standard curve) gives 1.43 4Fe–4S and 1.01 2Fe–2S units per molecule. This does not agree with the results obtained by simple inspection of the spectrum: the λ_{max} (452 nm) and the absorbance at 452 nm are in excellent agreement with a complement of two 4Fe–4S units per molecule. The use of an alternative ligand, *o*-xylyldithiol, was also examined in an attempt to resolve the issue. Treatment of AvIII with 12 mM *o*-xylyldithiol in 80% Me_2SO results in a spectrum with no new features; application of the spectral analysis gives 1.13 4Fe–4S and 2.08 2Fe–2S units per molecule.

The results obtained with AvIII illustrate one of the limitations of the thiol displacement method, namely, that *it is applicable only to proteins containing normal Fe–S units attached to the protein via sulfhydryl groups or other labile ligands*. The method may break down when novel iron–sulfur species with altered reactivity are encountered, such as appears to be the case with AvIII. Que (personal communication) confirms the presence of the same transient long-wavelength features in the spectra obtained in his laboratory, indicating that this is not an artifact of our preparation. The present results suggest that the protein contains one easily removable, typical 4Fe–4S unit (presumably that associated with the normal midpoint potential of +340 mV[41]). The identity of the prosthetic group(s) which contain the other four irons and labile sulfides is unknown. While EPR results[41] indicate a second tetramer with an abnormal redox potential, the displacement data suggest that the alternative possibility of two strongly interacting dimeric species of abnormal structure must not be disregarded. *A. vinelandii* iron–sulfur protein III is thus the first example of the possible existence in nature of an iron–sulfur prosthetic group materially different from the three basic types known to exist in small proteins and synthesized as nonprotein analogues by Holm and co-workers.[43]

General Considerations. At present, it seems unlikely that it is possible to design a thiol ligand which will selectively remove only one of the three types of iron–sulfur species from a protein. Instead, geometrically constrained dithiols, *acting as monodentate ligands when present in excess*, tend to remove all iron–sulfur species made accessible by unfolding a protein with organic solvents. In the absence of a method for selectively removing only a single type of cluster, separating it from apoprotein, and then identifying the displaced cluster spectrally, one must perforce employ thiols which simultaneously remove all iron–sulfur prosthetic groups (and possibly other chromophores) and then determine the composition of the resulting mixture. Of the three ligands shown to be generally useful above (thiophenol, *o*-xylyldithiol, 2,2′-dimercaptobiphenyl), each has its advantages and drawbacks. Thus, thiophenol is probably the single most useful reagent for determining 2Fe–2S and 4Fe–4S groups, but it does not reveal the presence of rubredoxin-like noncluster iron. Only *o*-xylyldithiol gives a characteristic spectrum with this latter species. In addition, *o*-xylyldithiol is also the reagent of choice for confirming the presence of dimeric centers, owing to the highly characteristic spectrum, but with tetrameric units the spectrum is relatively undistinguished. In solvents where the *o*-xylyldithiol ligand is not applicable (e.g., aqueous HMPA),2,2′-dimercaptobiphenyl is a useful alternative for the dimeric species. A point which must be emphasized is the desirability of confirming results obtained with one ligand by using a second, if possible. For example, had *A. vinelandii* iron–sulfur protein III been examined using only *o*-xylyldithiol, the anomalous nature of one of its prosthetic groups would not have been apparent.

In summary, the following guidelines are proposed for application of the technique described in this paper to more complex iron–sulfur systems.

(1) Since free iron–sulfur species are all extremely air sensitive, a gas train to ensure adequate anaerobicity is required. The presence of a few nanomoles of oxygen will destroy a large proportion of the displaced prosthetic groups when working on the scale described above. Serum stoppered bottles, suitable for working with air-sensitive proteins in the presence of dithionite, are in general *not* suitable.

(2) Stabilization of the displaced iron–sulfur species with regard to hydrolysis requires careful control of the pH of the aqueous component (optimum pH varies for each thiol), high organic solvent concentrations, and relatively large excess concentration of thiol. In general, highly polar solvents such as formamide and *N*-methylformamide seem to give more rapid hydrolysis than relatively less polar solvents such as Me_2SO, DMA, and HMPA. Since no evidence for any harmful effect (other than olfactory) of high thiol levels has been found, it is possible that even 0.1 M may not be optimal.

(3) Because of the use of conventional absorption spectroscopy, the technique works best when the apoprotein is soluble in the medium employed. Preliminary results indicate that many larger proteins are not soluble in aqueous Me_2SO.[7,8] This may necessitate a tedious search for the proper solvent. In such cases, the appropriate low molecular weight proteins must be used to construct a standard curve.

(4) The displacement reaction must be performed on a relatively *oxidized* form of the protein to be investigated. Two lines of evidence indicate that the reduced 2Fe–2S unit is not stable. First, in this and other laboratories, attempts to reduce the synthetic species with stoichiometric amounts of a one-electron donor with a potential between the first and second reduction potentials of the dimer[4b] do not result in any EPR signal attributable to the dimer. Rather, formation of partially reduced tetramer is the result.[44] Second, in experiments investigating the transfer of iron–sulfur clusters from one protein to another apoprotein, used of reduced adrenodoxin as the starting material resulted in quantitative formation of the corresponding tetrameric species.[45] Moreover, the reduced form of the mononuclear iron–*o*-xylyldithiol complex is essentially colorless.[33] Thus, the sodium dithionite customarily used to protect oxygen sensitive proteins must be removed and the protein oxidized, preferably to a resting potential above 0.0 V (relative to SHE).

(5) The case of complex proteins containing other cofactors which absorb in the visible region is probably best dealt with using difference spectroscopy.[46] It is possible to destroy the iron–sulfur prosthetic groups with mersalyl, for example, in organic solvents, and, after adding thiol, use this solution as a reference. When the additional cofactor is a heme, inclusion of 1% pyridine in the solvent gives the pyridine hemochromogen with identical spectra in both reference and sample, without affecting the stability of the iron–sulfur clusters.[44]

(6) Certain large proteins unfold quite slowly under the displacement conditions. It may be possible to markedly accelerate this reaction, using slightly elevated temperatures to take advantage of the well-known cooperative nature of protein denaturation, without adversely affecting the stability of the iron–sulfur species.

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References and Notes

(1) To whom correspondence should be addressed.
(2) Abbreviations: Me_2SO, dimethyl sulfoxide; HMPA, hexamethylphosphoramide; NMF, *N*-methylformamide; DMF, *N,N*-dimethylformamide; NMP, *N*-methylpyrrolidinone; DMA, *N,N*-dimethylacetamide; NTA, nitrilotriacetate; Adr, adrenodoxin; Rb, rubredoxin; Fd, ferredoxin.
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(46) In the case of trimethylamine dehydrogenase, Hill et al.,[10] using difference spectroscopy, were able to identify the Fe–S prosthetic group as an Fe_4S_4 cluster.