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Assembly of $[Fe_nS_n(SR)_4]^{2-}$ (n = 2, 4) in Aqueous Media from Iron Salts, Thiols, and Sulfur, Sulfide, or Thiosulfate plus Rhodanese

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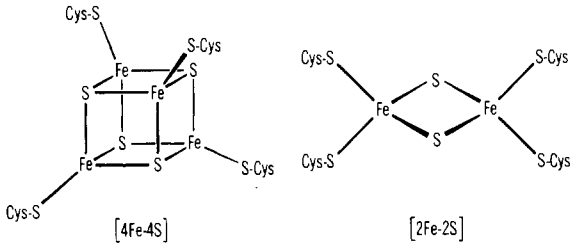
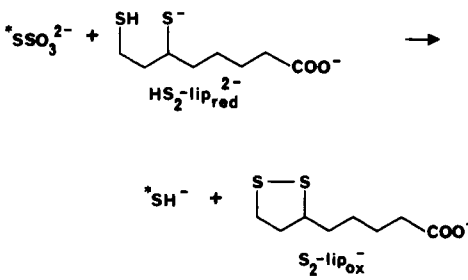
The high-yield syntheses in aqueous media of $[Fe_4S_4(SR)_4]^{2-}$ (R = Ph, CH_2CH_2OH) using sulfur, sulfide, or thiosulfate as the source of core sulfide are reported. From the reagent mole ratio 6/1/1 $HOCH_2CH_2SH/FeCl_2/S$ in pH ~8 aqueous buffer, a 65–70% yield of water-soluble $(n\text{-}Pr_4N)_2[Fe_4S_4(SCH_2CH_2OH)_4]$ can be obtained. This result demonstrates for the first time that synthetic analogues of biological [4Fe–4S] centers will spontaneously self-assemble in completely aqueous solvent in the absence of any protein. The spontaneous self-assembly and stability of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ in water raise the possibility that clusters containing the Fe_4S_4 core occur in vivo with natural thiols *outside of proteins*. With benzenethiol solubilized in water by Triton X-100, sulfide can be used in place of sulfur. A 70% spectrophotometric yield of $[Fe_4S_4(SPh)_4]^{2-}$ is achieved in aqueous Triton mixtures of 5/1/1 $PhSH/Fe^{3+}/S^{2-}$. The addition of the biologically relevant dithiol D,L-dihydrolipoic acid ($HSCH_2\overline{CH_2(H\text{-}S)}CHCH_2(CH_2)_3COOH$) at a dithiol/Fe mole ratio ≳2 greatly diminishes the rates of precipitation of iron sulfides and hydroxides and makes possible the assembly of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ using sulfide. When the outer sulfur of thiosulfate furnishes core sulfide, D,L-dihydrolipoate can serve as both reducing and sulfur-transfer agent. In this case the conversion to sulfide is catalyzed by the sulfurtransferase rhodanese. Yields of $[Fe_4S_4(SR)_4]^{2-}$ (R = Ph, CH_2CH_2OH) with the enzyme-mediated system routinely exceed 78% in solution and 60% as solid $(Et_4N)_2[Fe_4S_4(SPh)_4]$. $(Et_4N)_2[Fe_2S_2(SPh)_4]$ contaminated by $(Et_4N)_2[Fe_4S_4(SPh)_4]$ can be obtained from the thiosulfate/rhodanese system if Et_4NBr is added at the outset of the reaction. Under comparable conditions, the rates of reconstitution of *Clostridium pasteurianum* ferredoxin are 1–2 orders of magnitude faster than the rates of assembly of synthetic $[Fe_4S_4(SR)_4]^{2-}$ determined in this work. Thus, the *C. pasteurianum* ferredoxin polypeptide appears to accelerate the assembly of its own [4Fe–4S] centers.

The anions $[Fe_nS_n(SR)_4]^{2-}$ (n = 2, 4) are synthetic analogues of [2Fe–2S] and [4Fe–4S] active centers found in several iron–sulfur proteins.[1] Structures of these centers are shown schematically. In the case of the synthetic analogues, Cys is replaced



by R = alkyl or aryl. Syntheses and properties of the title clusters have been studied in detail in organic solvents and have produced a comprehensive picture of their assembly, including characterizations of intermediates and analyses of factors affecting pathways of assembly.[1-3] In the living cell, however, the assembly of Fe–S clusters and their insertion into the appropriate apoproteins probably takes place in a predominantly aqueous environment. Aqueous syntheses of both the binuclear and tetranuclear clusters with R = Ph has recently been achieved for the system 4/1/1 $PhSH/FeCl_3/S$.[4,5] Reactants and products in this system can be solubilized with use of the nonionic detergent Triton X-100. By proper adjustment of conditions, nearly quantitative yields of either the binuclear or tetranuclear cluster can be obtained from the same reagent mole ratio.

Although this system can be regarded as an improved model of the assembly of clusters in vivo, some of the reagents and conditions are obviously not the physiological ones. In the present work we have developed systems for the synthesis of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ in water without detergents or organic solvents. In addition we have defined conditions under which various donors of core sulfide can be used, namely sulfur, sulfide or the outer sulfur of thiosulfate. The sulfurtransferase rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) can catalytically generate sulfide from thiosulfate utilizing dithiols such as dithiothreitol or, as shown in reaction 1, D,L-dihydrolipoate ($HSCH_2\overline{CH_2(HS)}CHCH_2(CH_2)_3COO^- = (HS)_2\text{-}lip_{red}$).[6,7]



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A number of studies have shown that ferredoxins from several sources can be reconstituted by treating the apoproteins with iron salts and the catalytic rhodanese system.[8-12] The most recent studies have shown that apo- but not holoferredoxin protects rhodanese against inactivation. This pattern has been proposed to represent a feedback mechanism that limits the production of toxic sulfide.[11-13]

In the present work we have combined the techniques and methods used for the aqueous syntheses of the title clusters with the activity of rhodanese to obtain $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$,

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$[Fe_4S_4(SPh)_4]^{2-}$, and $[Fe_2S_2(SPh)_4]^{2-}$. Rhodanese-mediated assembly of synthetic analogues of the [2Fe–2S] and [4Fe–4S] centers presents the possibility of separating effects on the reconstitution reaction due to interaction with the apoferredoxins from those due to formation of the iron–sulfur clusters themselves.

Experimental Section

Rhodanese was isolated from bovine liver according to a published procedure[14] and stored at 0–4 °C as a crystalline suspension in 2 mM thiosulfate, 2.0 M ammonium sulfate, pH 7.6. This suspension was added as such in all the experiments, unless otherwise specified. Addition of this suspension (which dissolves immediately) introduces ~60 mM $(NH_4)_2SO_4$ into the reaction mixture. For some experiments rhodanese was added as a solution after isolation of the crystals by centrifugation and dissolution in buffer without $(NH_4)_2SO_4$. Rhodanese concentration was measured with use of $\epsilon_{280} = 57\,750\ M^{-1}\ cm^{-1}$. Thiosulfate reductase activity of rhodanese was measured as the initial rate of absorbance increase at 333 nm due to formation of lipoic acid ($\epsilon_{333} = 150\ M^{-1}\ cm^{-1}$).[15] At 25 °C, with 20–40 mM thiosulfate and 20–40 mM D,L-dihydrolipoate the turnover number of the enzyme ranged from 110 to 150. D,L-Dihydrolipoic acid was prepared by reducing the oxidized compound, usually at ~1 M, dissolved in H_2O and adjusted to pH 10 with dilute NaOH, with a 20-fold molar excess of sodium borohydride. After 20 min at room temperature, excess reductant was destroyed by careful acidification with 6 M sulfuric acid under a stream of Ar. Finally the pH of the solution was brought to 9.1 with solid Tris base. The yield of this reaction routinely exceeded 85%. The concentrations of D,L-dihydrolipoate and of the solutions of sodium sulfide (from reagent grade $Na_2S\cdot 9H_2O$ or NaHS[16]) were determined with dithiobis(nitrobenzoate).[17] D,L-Dihydrolipoate was added from concentrated stock solutions (~0.6 M) prepared as above. All other reagents were dissolved in 0.2 M Tris–sulfate (pH 9.1), which was the buffer used for all reactions. $Na_2S_2O_3$ and sodium sulfide were added as 1 M stock solutions.

All reactions were performed at room temperature, under a purified Ar atmosphere in either Schlenk-type glassware or septum-capped vials, with transferral of reagents via steel tubing or gastight syringe. $(Et_4N)_2[Fe_nS_n(SPh)_4]$ complexes (n = 2, 4) from these reactions were isolated as previously described.[4,5]

$(n\text{-}Pr_4N)_2[Fe_4S_4(SCH_2CH_2OH)_4]$. Syntheses of the Me_4N^+ salt in methanol by ligand substitution of $[Fe_4S_4(S\text{-}t\text{-}Bu)_4]^{2-}$ have been reported previously.[18,19] We have found two equally successful methods for preparation of the n-Pr_4N^+ salt in water from iron salts, 2-mercaptoethanol, and sulfur.

Method a. To 0.61 g (3.8 mmol) of $FeCl_3$ in 10 mL of CH_3CN is added 1.6 mL (22.7 mmol) of 2-mercaptoethanol. After 30 min of stirring the transparent green solution is transferred to a stirred mixture of 150 mL of aqueous 0.2 M Tris–sulfate (pH 9.1) and 0.12 g (3.9 mmol) of sulfur. After 6–8 h of stirring, the dark green-brown solution is filtered through a Celite pad and 1.0 g (3.8 mmol) of n-Pr_4NBr is added. Overnight storage at 4 °C results in black needlelike crystals in a pale red-brown solution. The pH at this point is ~7.9. The crystals are collected by filtration, washed with isopropyl alcohol and ether, and dried in vacuo. The yield is 0.65–0.70 g (65–70%). Anal. Calcd for $C_{32}H_{76}N_2O_4Fe_4S_8$: C, 37.21; H, 7.36; N, 2.72. Found: C, 37.26; H, 7.30; N, 2.77. The ^1H NMR spectrum in Me_2SO-d_6 (except for the cation resonances) and UV–vis spectrum in Me_2SO are identical with those published for the Me_4N^+ salt[18,19] and fully consistent with the above formulation.

Method b. To 160 mL of 0.2 M Tris–sulfate (pH 9.1) are added in order 0.13 g (4.0 mmol) of sulfur, 1.8 mL (25 mmol) of 2-mercaptoethanol, and 0.49 g (3.9 mmol) of $FeCl_2$. After 4–6 h of stirring the reaction mixture is filtered through a Celite pad and 1.1 g (4.0 mmol) of n-Pr_4NBr is added. Subsequent workup is the same as used in method a. Yields of 65–70% of pure material are obtained. Anal. Found: C, 37.19; H, 7.26; N, 2.59. Comparable yields are obtained when 0.1 M LiOH is substituted for buffer. We have found that the ^1H NMR spectrum in Me_2SO-d_6 provides a good criterion of analytical purity. The ratios of integrated areas of the resonances at δ 12.4 ($FeSCH_2CH_2OH$), 1.6 ($NCH_2CH_2CH_3$), and 0.9 ($NCH_2CH_2CH_3$) are 1:2.0 (±0.1):3.0 (±0.1) for analytically pure material.

Physical Measurements. Absorption spectra were recorded anaerobically at room temperature in 0.1 mm path length cuvettes on a Perkin-Elmer Model 554 spectrophotometer. Reaction rates were calculated from standard semilog plots of the decrease in absorbance at 620 nm, which were linear for at least 3 half-lives. The spectral parameters of $[Fe_4S_4(SPh)_4]^{2-}$ used for quantitation, $\epsilon_{454} = 17\,400\ M^{-1}\ cm^{-1}$ and purity ratio $A_{454}/A_{550} = 2.0 \pm 0.2$, were those used previously.[20] ^1H NMR spectra of isolated solids dissolved in Me_2SO-d_6 or CD_3CN were recorded on a Nicolet NT-300 spectrometer at ~22 °C. Shifts downfield of Me_4Si are reported as negative. The criterion of purity used for isolated solids of $(Et_4N)_2[Fe_nS_n(SPh)_4]$ (n = 2, 4) was the area ratio obtained from ^1H NMR in CD_3CN: m-H/cation $CH_2 = 2.0 \pm 0.2$. Proportions of $[Fe_2S_2(SPh)_4]^{2-}$ (d^{2-}) and $[Fe_4S_4(SPh)_4]^{2-}$ (t^{2-}) in isolated solids were determined from area ratios of the m-H resonances at −8.2 (t^{2-}) and −9.3 (d^{2-}) ppm.[2,5]

Results

Catalytic Behavior of Rhodanese in the Medium Used for the Synthesis of Fe–S Clusters. The thiosulfate reductase activity of rhodanese, as reaction 1, was tested in media similar to those used for the syntheses of iron–sulfur clusters discussed below, but with iron salts omitted. No evolution of sulfide was observed in the absence of rhodanese. Little if any inactivation of the enzyme was found to occur in the control runs over the first 20 min, a result different from that reported in previous work[12,13] likely because of the higher concentrations of thiosulfate and D,L-dihydrolipoate used in the present work. In the presence of 5 vol % Triton, the thiosulfate reductase activity of rhodanese is 65% of that of the control, but full activity is immediately recovered when the concentration of D,L-dihydrolipoate is doubled from 40 to 80 mM. Forty-five millimolar 2-mercaptoethanol slowly inactivates the enzyme, ~50% of control activity remaining after 15 min. This slow inactivation is consistent with a previous study.[21] Sixty millimolar PhSH in 5 vol % Triton causes a similar reduction in activity when compared to that with Triton alone. No thiosulfate reductase activity was detectable in the presence of tetraalkylammonium ions larger than n-Pr_4N^+ at 0.1 M. Even with n-Pr_4NBr the measured enzymatic activity was too low to allow effective utilization of the enzyme in the synthetic procedure. These results are illustrated in Figure 1 (included in the supplementary material). From the turnover numbers (see Experimental Section) and the concentrations of enzyme used, our results predict that sulfide will be generated at a maximum rate of 150–350 μM/min in the experiments described below.

Syntheses of $[Fe_4S_4(SR)_4]^{2-}$ (R = Ph, CH_2CH_2OH) in Aqueous Solutions Using Sulfur or Sodium Sulfide. Quantitative assembly of $[Fe_4S_4(SPh)_4]^{2-}$ from a buffered mixture of 4/1/1 PhSH/$FeCl_3$/S in aqueous solutions containing 5 vol % Triton X-100 requires ~6 h at pH 8.[4] A similar time scale occurs for the assembly of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ from a mixture of 6/1/1 $HOCH_2CH_2SH/FeCl_2/S$ in aqueous buffer (final pH ~8) with no organic solvent or detergent. Figure 2 (included in the supplementary material) shows the time course of absorbance changes for such a reaction. Use of $\epsilon_{374} = 15\,400\ M^{-1}\ cm^{-1}$ [18] gives a 92% spectrophotometric yield after 300 min, at which point $A_{296}/A_{374} = 1.34$. The published value for $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ is 1.35.[18] Workup of the reaction mixture as described in the Experimental Section (method b) led to a 70% yield of crystalline $(n\text{-}Pr_4N)_2[Fe_4S_4(SCH_2CH_2OH)_4]$. The stoichiometry is most likely that of reaction 2. Thus, the ~6/1 RSH/Fe mole ratio

$$4FeCl_2 + 4S + 10RS^- \rightarrow [Fe_4S_4(SR)_4]^{2-} + 8Cl^- + 3RSSR \quad (2)$$

used in the reaction mixture represents a 14-fold molar excess of RSH over $[Fe_4S_4(SR)_4]^{2-}$, even for a 100% yield. The excess 2-mercaptoethanol suppresses hydrolysis of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$.[18]

With sodium sulfide in place of sulfur, the reaction times with R = Ph decrease to a few minutes. Thus, mixing in buffered aqueous Triton (5 vol %) of 8 mM ferric ammonium citrate, 40

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Table I. Comparisons of Yields and Rates for Synthesis of $[Fe_4S_4(SR)_4]^{2-}$ Using $S_2O_3^{2-}$/Rhodanese or Sulfide[a]

	% yield (A_{454}/A_{550})	
	$S_2O_3^{2-}$/rhodanese[c]	sulfide[b]
2-mercaptoethanol	87.8 (1.81)	74.7 (1.72)
PhSH (+Triton)	96.3 (2.02)	78.4 (2.17)
	half-time, min	
	$S_2O_3^{2-}$/rhodanese	sulfide
2-mercaptoethanol	16.8	81.0
PhSH (+Triton)	31.8	135.0

[a] In all experiments iron (as ferric ammonium citrate) was 8–10 mM and sodium thiosulfate and sodium D,L-dihydrolipoate were each 20 mM. Thiols were 40 mM, and Triton, when present, was ~100 mM. Yields and rates were calculated spectrophotometrically as given in the footnote to Table II. Absorbance ratios A_{454}/A_{550} are of the final spectrum after PhSH/Triton treatment of those mixtures containing 2-mercaptoethanol. [b] Sodium sulfide was 11 mM. Spectra are shown in Figure 3 (supplementary material). [c] Rhodanese was added to a final concentration of 2.4 μM. Spectra are shown in Figure 4.

mM PhSH, and 13 mM NaHS resulted in the absorption spectrum (not shown) of $[Fe_4S_4(SPh)_4]^{2-}$ ~5 min after mixing. Further very small changes occurred over about a 1-h period, giving a final A_{454}/A_{550} = 2.0 and a 79% spectrophotometric yield of $[Fe_4S_4(SPh)_4]^{2-}$. Addition of Et_4NBr to 0.1 M resulted in a brown precipitate that, when collected by filtration, washed with water and ether, and dissolved in CD_3CN, showed the ^1H NMR spectrum of $(Et_4N)_2[Fe_4S_4(SPh)_4]$ contaminated with <5% of $(Et_4N)_2[Fe_2S_2(SPh)_4]$. With R = CH_2CH_2OH, only intractable black precipitates formed upon even gradual addition of NaHS to buffered aqueous solutions 20–25 mM in ferric ammonium citrate and 150–270 mM in 2-mercaptoethanol.

When D,L-dihydrolipoate is included in the reaction mixture prior to addition of sodium sulfide, the rate of assembly of $[Fe_4S_4(SR)_4]^{2-}$ decreases. However, rapid precipitation of iron sulfides is prevented in the presence of D,L-dihydrolipoate and assembly of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ becomes possible with sodium sulfide. Addition of sodium sulfide to solutions of ferric ammonium citrate, D,L-dihydrolipoate, and either 2-mercaptoethanol or PhSH results in progressive disappearance of the spectrum of the green Fe(III)/D,L-dihydrolipoate complex (λ_{max} 380 and 620 nm)[22] with the concomitant appearance of the spectra of, respectively, $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ and $[Fe_4S_4(SPh)_4]^{2-}$. The former dianion can be quantitatively converted to the latter by treatment with PhSH in Triton, as previously described.[20] These results are illustrated in Figure 3 (included in the supplementary material). Table I (right-hand side) reports yields, purity ratios, and time scales for these reactions. Thiosulfate presumably is a spectator ion in the solutions containing sodium sulfide; i.e., control experiments in which sulfide was omitted gave no detectable reaction on the time scales reported in Table I. $[Fe_4S_4(SPh)_4]^{2-}$ does form with a half-time of ~14 h from aqueous Triton mixtures of ferric salts, thiosulfate, D,L-dihydrolipoate, and benzenethiol. Yields in this case are similar to those reported in Table I. We have been unable to isolate any tractable material from reactions in aqueous solutions of ferric salts, D,L-dihydrolipoate, and sulfide.

Synthesis of $[Fe_4S_4(SR)_4]^{2-}$ via a Rhodanese-Mediated Reaction. Figure 4 illustrates the spectral time courses of reactions conducted identically with those described above, but with 2.4 μM rhodanese substituted for 11 mM Na_2S. Sulfide is generated via reaction 1 in these cases. The progression of spectra is quite similar to those described above with sulfide except that the reaction rates are 4–5 times faster. Yields, purity ratios, and half-times are listed in Table I. Addition of Et_4NBr to these reaction mixtures to a final concentration of 0.1 M gave rapid precipitation of fine, black crystals. These crystals were found to have the electronic and ^1H NMR spectra[2] of $(Et_4N)_2[Fe_4S_4(SPh)_4]$ after dissolution in CH_3CN and CD_3CN, respectively. No additional resonances were

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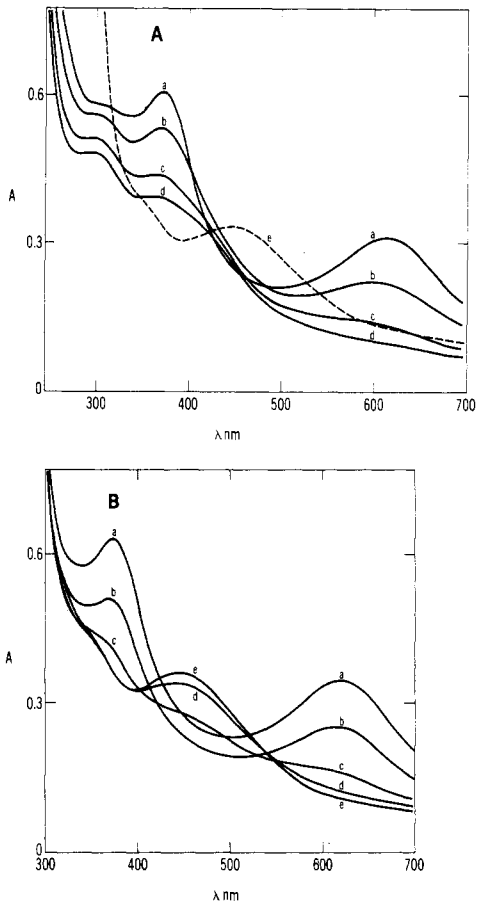

Figure 4. Spectral time course of the rhodanese-mediated synthesis of $[Fe_4S_4(SR)_4]^{2-}$. (A), R = CH_2CH_2OH) spectra of a buffered mixture of 20 mM thiosulfate, 9.9 mM ferric ammonium citrate, 40 mM 2-mercaptoethanol, and 20 mM D,L-dihydrolipoate before (a) and after the addition of 20 nmol (in 0 .25 mL) of rhodanese. The final volume was 7.5 mL. Spectra b–d were taken 10, 30, and 60 min, respectively after the addition of rhodanese. Then 1 mL of a PhSH/Triton mixture (1/15, v/v) was added, giving the spectrum shown as the dashed line (e). (B, R = Ph) Spectra of a buffered mixture of 20 mM thiosulfate, 8 mM ferric ammonium citrate, 40 mM PhSH, 100 mM (6 vol %) Triton, and 20 mM D,L-dihydrolipoate before (a) and after the addition of 20 nmol of rhodanese. The final volume was 7.6 mL. Spectra b–e were taken respectively 10, 60, 90, and 180 min after the addition of rhodanese.

Table II. Summary of Reaction Conditions, Yields, and Rates for Rhodanese-Mediated Synthesis of $[Fe_4S_4(SR)_4]^{2-}$ [a]

concn, mM[d]				
ferric ammonium citrate	10	8	4	4
D,L-dihydrolipate	20	20	12	12
sodium thiosulfate	20	20	20	20
2-mercaptoethanol	40		40	
benzenethiol/Triton		40/100		40/100
rhodanese	0.024	0.024	0.024	0.024
yield, %				
by spectrophotometry	87.8	96.3	79.5	77.8
by wt	60.4	84.5[c]	nd[b]	nd[b]
half-time, min	16.8	31.8	7.3	11.0

[a] Yields are of $[Fe_4S_4(SPh_4)]^{2-}$ (after addition of a 1.5–2.5-fold molar excess of PhSH/Triton to the samples containing 2-mercaptoethanol) calculated from absorbance at 454 nm or by weight of $(Et_4N)_2[Fe_4S_4(SPh)_4]$ after addition of Et_4NBr (to 0.1 M). Half-times were calculated from semilog plots of the fractional decrease in absorbance at 620 nm. [b] Not determined. [c] Average of three preparations. [d] Listed from top to bottom in order of addition.

observed in the NMR spectra from +40 to −40 ppm. Table II reports the results obtained when various conditions were used for the rhodanese-mediated syntheses of $[Fe_4S_4(SR)_4]^{2-}$. Control experiments show that assembly of $[Fe_4S_4(SR)_4]^{2-}$ does not take place on the time scales reported in Tables I and II, when mo-

nothiol is omitted. Under these conditions, the initial spectrum of Figure 4, with λ_{max} 620 nm, persists for several hours.

The rate of decrease in the concentration of Fe(III)/D,L-dihydrolipoate complex, measured as the decrease in A_{620} with time, showed good first-order behavior under all conditions reported in Tables I and II. Therefore, ΔA_{620} was used as a rough estimate of the rate of cluster assembly in order to provide a basis for comparing enzymatic with nonenzymatic rates. At D,L-dihydrolipoate/Fe(III) mole ratios higher than those reported in Tables I and II, A_{620} is greatly decreased prior to addition of rhodanese, presumably due to reduction of Fe(III) by the dithiol.[22] For this reason, at 20 mM D,L-dihydrolipoate and 2 mM ferric ammonium citrate, ΔA_{620} can no longer be used to measure the rate of formation of $[Fe_4S_4(SPh)_4]^{2-}$. However, the time scale and yield for the rhodanese-mediated reaction under these conditions, measured at 454 nm, are within the ranges reported in Table II.

Dithiothreitol can also be used to generate sulfide in a rhodanese-catalyzed reaction analogous to reaction 1. We have found that, at the dithiol/Fe mole ratios and concentrations used in our experiments, an Fe(III)/dithiothreitol complex of low solubility forms prior to addition of rhodanese. The resulting precipitate hinders spectral examination of the reaction solutions but does not prevent formation of $[Fe_4S_4(SPh)_4]^{2-}$.

Syntheses of $[Fe_2S_2(SPh)_4]^{2-}$ Using Sodium Sulfide or Rhodanese/Thiosulfate. For the system 4/1/1 PhSH/$FeCl_3$/S in aqueous Triton, we have shown previously that addition of tetraalkylammonium ions at the outset of the reaction results in increased proportions of $[Fe_2S_2(SPh)_4]^{2-}$ (d^{2-}) compared to $[Fe_4S_4(SPh)_4]^{2-}$ (t^{2-}) in the isolated solids.[4,5] Since, as shown above, rhodanese is inactivated by tetraalkylammonium ions larger than Et_4N^+, we limited our present studies to this cation. For the system ≥5/1/1 PhSH/Fe/S^{2-} in aqueous buffer with 5 vol % Triton and 0.1 M Et_4NBr, we obtained d^{2-}/t^{2-} mole ratios in the isolated solids of 0.06 (11/1 PhSH/$FeCl_3$, premixed in a small volume of CH_3CN), 0.27 (11/1 PhSH/Fe as ferrous ammonium sulfate), and 0.37 (5/1 PhSH/Fe as ferric ammonium citrate).

Results with rhodanese/$S_2O_3^{2-}$/D,L-dihydrolipoate in place of sodium sulfide are summarized in Figure 5. Significant proportions of $[Fe_2S_2(SPh)_4]^{2-}$ in the isolated solids were obtained only when Fe(II) was supplied at the outset, either as ferrous ammonium sulfate (Figure 5c) or as premixed $FeCl_3$ and PhSH, which results in Fe(II) and PhSSPh[4,5] (Figure 5d). The d^{2-}/t^{2-} mole ratios of ~0.7 obtained in these last two cases are significantly greater than those reported above using sodium sulfide. No significant change in the d^{2-}/t^{2-} mole ratio was observed when rhodanese was added as a buffered solution rather than as a crystalline suspension in $(NH_4)_2SO_4$.

Discussion

Synthetic Fe–S Cluster Assembly in Aqueous Solutions. With the successful assembly of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ from $HOCH_2CH_2SH/FeCl_2/S$ we have extended the range of conditions for high-yield syntheses of $[Fe_4S_4(SR)_4]^{2-}$ to completely aqueous solvent. The differences between our successful synthesis and an earlier study[23] in which the components of this cluster were mixed in water appear to be our uses of S instead of S^{2-} and anaerobiosis. The spontaneous self-assembly of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ in water and its stability near physiological pHs raise the interesting possibility that clusters containing the Fe_4S_4 core occur in vivo with natural thiols *outside of proteins*.[24]

Due to formation of water-insoluble iron sulfides, we have been unable to prepare $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ from aqueous mixtures containing 6–10/1 RSH/Fe and sulfide. Rapid precipitation of iron sulfides does not occur with benzenethiol in aqueous Triton. The average time required for completion of assembly of $[Fe_4S_4(SPh)_4]^{2-}$ decreases from several hours to a few minutes when sodium sulfide replaces elemental sulfur in aqueous Triton. It is quite likely that with solid sulfur the rate-limiting step of the assembly reaction is the dissolution of sulfur and its conversion into reactive species. Coucouvanis et al.[26] have shown that trisulfides (RSSSR), which contain a soluble form of (formally) S^0, rapidly react with $[Fe(SPh)_4]^{2-}$ in organic solvents to form $[Fe_2S_2(SPh)_4]^{2-}$.

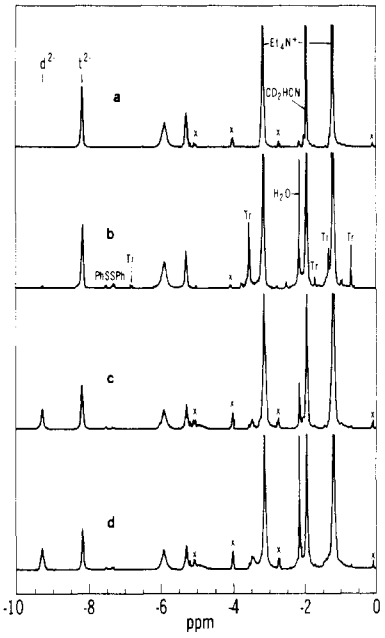

Figure 5. ^1H NMR spectra of CD_3CN solutions of solids obtained upon addition of rhodanese to reaction mixtures of various compositions. Reagent concentrations (included as Table III in the supplementary material) and workup are similar to those given in the text and Table I. Relative concentrations of $[Fe_2S_2(SPh)_4]^{2-}$ (d^{2-}) and $[Fe_4S_4(SPh)_4]^{2-}$ (t^{2-}) were calculated from area ratios of the *m*-H resonances of d^{2-} (−9.3 ppm) and t^{2-} (−8.2 ppm). For each spectrum the form in which the iron was supplied, the ratio PhSH/Fe, the concentration of Et_4NBr (supplied at the outset), the overall yield by weight (calculated as the percentage of the iron supplied), and the mole ratio d^{2-}/t^{2-} are as follows (a) ferric ammonium citrate, 4/1 PhSH/Fe, no Et_4NBr, overall yield 60.2%, d^2/t^{2-} = 0; (b) ferric ammonium citrate, 9/1 PhSH/Fe, 0.1 M Et_4NBr, overall yield 74.7%, d^{2-}/t^{2-} = 0.07; (c) ferrous ammonium sulfate, 12/1 RSH/Fe, 0.1 M Et_4NBr, overall yield 87.3%, d^{2-}/t^{2-} = 0.71; (d) ferric chloride premixed in CH_3CN with PhSH, 12/1 RSH/Fe, 0.1 M Et_4NBr, overall yield 86.7%, d^{2-}/t^{2-} = 0.66. No other resonances were obtained in any of the spectra from +40 to −40 ppm. All spectra exhibit an area ratio *m*-H/cation CH_2 = 2.0 ± 0.2. Peaks marked with × denote solvent impurity, and Tr denotes residual Triton.

When Et_4N^+ is included at the outset in the aqueous Triton reaction mixture, the proportion of $(Et_4N)_2[Fe_2S_2(SPh)_4]$ in the isolated solid increases. The considerably lower d^{2-}/t^{2-} mole ratios that we achieved in aqueous Triton with S^{2-} (0.06–0.37) in the present work, compared to S (1.5) in our previous study,[5] can be attributed to the more reducing nature of the reaction system when S^{2-} is employed. Under these conditions the tendency for formation of $[Fe_2S_2(SPh)_4]^{3-}$, which rapidly converts to $[Fe_4S_4(SPh)_4]^{2-}$, is likely to be greater.[27] $[Fe_2S_2(SPh)_4]^{2-}$ cannot be directly prepared in organic solvents with use of S^{2-}, presumably for the same reason.[28]

With S^{2-}, the presence of D,L-dihydrolipoate lowers the rates of both assembly of $[Fe_4S_4(SPh)_4]^{2-}$ and precipitation of iron sulfides. Presumably, it is the latter inhibition that permits the formation of $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ using sulfide. These effects of D,L-dihydrolipoate are due to complexation of Fe(II) and Fe(III) by the dithiol.[22]

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Synthetic Fe-S Cluster Assembly with Rhodanese. D,L-Dihydrolipoate was used to generate sulfide via reaction 1 for the bulk of these studies because of its presumed biological relevance and because of solubility problems encountered with Fe(III)/dithiothreitol mixtures. As shown in Table I, at comparable reagent concentrations we obtain a four- to fivefold higher rate and 15-20% higher yield for formation of $[Fe_4S_4(SR)_4]^{2-}$ using $S_2O_3^{2-}$/rhodanese in place of sulfide. Since the rates were calculated from the decrease in absorbance at 620 nm due to the Fe(III)/D,L-dihydrolipoate complex, the four- to fivefold increase suggests an interaction between this complex and rhodanese, perhaps at a site close to that of generation of sulfide. The demonstrated hydrophobicity and structural flexibility near the active site of rhodanese might permit such an interaction.[29,30]

We have been unable to obtain $[Fe_2S_2(SCH_2CH_2OH)_4]^{2-}$ (which is unknown) under any of several conditions. Therefore, to examine the rhodanese-mediated formation of $[Fe_2S_2(SR)_4]^{2-}$, we are limited at this point to the PhSH/Triton mixture used previously to obtain $[Fe_2S_2(SPh)_4]^{2-}$ in the presence of tetraalkylammonium ions.[4,5] Due to inhibition of rhodanese activity we are also limited to Et_4N^+. Figure 5 shows that, for the thiosulfate/rhodanese/D,L-dihydrolipoate system, a significantly higher d^{2-}/t^{2-} ratio is obtained in the isolated solids when iron is supplied as Fe(II) (~0.7) instead of Fe(III) (~0.06). This higher proportion may mean that, with Fe(II), D,L-dihydrolipoate tends to favor formation of an Fe_2S_2 core as does *o*-xylene-α,α'-dithiolate.[27,28]

Comparisons to Cluster Assembly in Ferredoxins. Our limited success in selective assembly of $[Fe_2S_2(SR)_4]^{2-}$ with S^{2-} or $S_2O_3^{2-}$/rhodanese prevents us from making meaningful comparisons with assembly of [2Fe-2S] centers in ferredoxins.[11] However, our results are consistent with the idea[5] that [2Fe-2S] sites form as part of a general $[Fe(S\text{-}Cys)_4]^{2-} \rightarrow [Fe_2S_2(S\text{-}Cys)_4]^{2-} \rightarrow [Fe_4S_4(S\text{-}Cys)_4]^{2-}$ assembly pathway in ferredoxins.

Clostridium pasteurianum ferredoxin contains two [4Fe-4S] centers and can be reconstituted from the apoprotein with use of either S^{2-} or $S_2O_3^{2-}$/rhodanese. With use of S^{2-} in the presence of D,L-dihydrolipoate under comparable conditions, the rates of reconstitution of *C. pasteurianum* ferredoxin ($t_{1/2}$ = 0.3-7 min) determined previously in this laboratory[12] are 1-2 orders of magnitude faster than the rates of assembly of synthetic $[Fe_4S_4(SR)_4]^{2-}$ ($t_{1/2}$ = 80-130 min) determined in the present work. The rates for reconstitution measure the recovery of the native structure of the ferredoxin, which can be no faster than the assembly of its [4Fe-4S] centers. Therefore, from this comparison of rates we conclude that the *C. pasteurianum* ferredoxin polypeptide accelerates the assembly of its own clusters. This perhaps predictable result has not been previously subjected to a direct experimental test.

Whether or not rhodanese actually catalyzes cluster assembly remains an open question. One function of rhodanese in vivo may be to generate toxic sulfide from nontoxic substrates in proximity to iron and apoferredoxin, thereby minimizing harmful or unproductive diffusion of sulfide through the cell. The possibility mentioned in the introduction of feedback regulation of rhodanese activity by the ferredoxin, the abundance and ubiquity of rhodanese, and the efficiency of the enzyme in terms of both rates and yields all point to its involvement in the delivery of core sulfide in vivo. The synthetic results presented above provide further support for this involvement.

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Registry No. $(n\text{-}Pr_4N)_2[Fe_4S_4(SCH_2CH_2OH)_4]$, 99148-42-2; $(Et_4N)_2[Fe_4S_4(SPh)_4]$, 55663-41-7; $(Et_4N)_2[Fe_2S_2(SPh)_4]$, 55939-70-3; $FeCl_3$, 7705-08-0; $FeCl_2$, 7758-94-3; S^{2-}, 18496-25-8; S, 7704-34-9; rhodanese, 9026-04-4; thiosulfate, 14383-50-7; D,L-dihydrolipoate, 7516-48-5; dithiothreitol, 3483-12-3; ferrous ammonium sulfate, 10045-89-3; ferric ammonium citrate, 1185-57-5.

Supplementary Material Available: Figures 1-3, depicting rhodanese activity under the synthetic conditions used in this work and spectral time courses for formation of $[Fe_4S_4(SPh)_4]^{2-}$ and $[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ in water using sulfide, and Table III, containing reagent concentrations for Figure 5 (5 pages). Ordering information is given on any current masthead page.

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Distal Histidine Coordination to Iron in Phthalocyanine-Reconstituted Myoglobin

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Reconstitution of apomyoglobin with tetracarboxy- (TcPc) and tetrasulfonated- (TsPc) (phthalocyanato)iron(II) complexes gives proteins containing both proximal and distal histidine bound to iron. MbTcPc binds isocyanides (RNC; R = benzyl, butyl, and tosylmethyl) at a rate independent of the concentration or nature of the isocyanide, $k = 1.7 \times 10^{-4}$ s^{-1} at 25 °C in phosphate buffer, pH 7. The rate-determining step in ligation to iron is proposed to involve a conformational change in which the E-helix is swung back approximately to its position in native Mb. Data for isocyanide and CO binding to $FeTsPcL_2$, L = methylimidazole, 2-methylimidazole, and pyridine, in water are similar to previously reported data for $FePcL_2$ in toluene. The rate constant for acid-catalyzed cleavage of the μ-oxo dimer $(FeTsPc)_2O$, $k = 5.6$ M^{-1} s^{-1} at $\mu = 0.11$, is over 100-fold slower than that for a similar water-soluble porphyrin dimer.

Introduction

Investigations of artificially reconstituted heme proteins in which the heme group is replaced with other metalloporphyrins[1,2] or metal complexes of phthalocyanines[2-4] or other tetradentate ligands[5] have led to an increased understanding of the role of the protein in modifying the reactivity of a metal complex. From a coordination chemist's perspective an apoprotein may be thought of as a rather large ligand that may introduce a number of effects by dominating the chemistry associated with the primary and secondary coordination spheres of the metal. A readily available protein such

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