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Quantitative Extrusions of the Fe_4S_4* Cores of the Active Sites of Ferredoxins and the Hydrogenase of *Clostridium pasteurianum*

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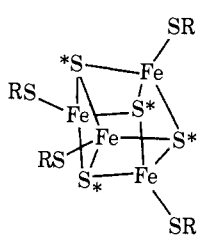
Abstract: Previous research has demonstrated that the complexes $[Fe_4S_4(SR)_4]^{2-}$ and $[Fe_2S_2(SR)_4]^{2-}$, synthetic analogues of the $[Fe_4S_4$*$(S\text{-}Cys)_4]$ and $[Fe_2S_2$*$(S\text{-}Cys)_4]$ active sites of oxidized ferredoxin proteins (Fd_{ox}), undergo facile ligand substitution reactions with added thiols at ambient temperature. These reactions have been applied to the extrusion of intact Fe_4S_4* and Fe_2S_2* cores of protein sites in the form of their spectrally characteristic R = Ph analogues. In 4:1 v/v HMPA/H_2O medium (aqueous component pH 8.5) essentially quantitative core extrusion with benzenethiol has been accomplished for *C. pasteurianum* Fd_{ox} and Fd_{ox} + Fd_{red}, *B. stearothermophilus* Fd_{ox}, reduced *Chromatium* high-potential protein, and the Fe protein of *C. pasteurianum* nitrogenase, all of which contain 4-Fe sites. With the methodology developed for the lower molecular weight proteins, the extrusion method has been applied to a preparation of hydrogenase from *C. pasteurianum*, prior analysis of which indicated ~11.2 g-atom of Fe and S*/60 500 g. Recovery of total Fe in the form of the $[Fe_4S_4(SPh)_4]^{2-}$ extrusion product is in excellent agreement with calculated initial concentrations. General experimental guidelines for the successful application of the core extrusion technique are considered, with particular attention to problems posed by spontaneous dimer → tetramer extruded analogue conversion, coextrusion of mixed types of Fe-S sites, and the presence of extraneous iron in protein preparations. The first two factors were examined by extrusion of mixtures of *C. pasteurianum* Fd_{ox} and spinach Fd_{ox} and of spinach Fd_{ox} + Fd_{red}. It is concluded that the sole detectable extrusion product of hydrogenase, $[Fe_4S_4(SPh)_4]^{2-}$, conveys the correct nature of the active sites in this enzyme preparation, which most probably contains three 4-Fe sites of essential composition $[Fe_4S_4$*$(S\text{-}Cys)_4]$.

Previously reported and ongoing research[2-4] has resulted in the synthesis and structural and electronic characterization of analogues of the three currently recognized types of active sites in soluble iron-sulfur redox proteins of relatively low molecular weight.[5,6] In terms of their minimal composition these sites may be specified as $[Fe(S\text{-}Cys)_4]$ (Rd), $[Fe_2S_2$*$(S\text{-}Cys)_4]$ (2-Fe Fd), $[Fe_4S_4$*$(S\text{-}Cys)_4]$[7] (4-, 8-Fe Fd, HP[8]). From x-ray diffraction results the active sites of *P. aerogenes* 8-Fe Fd_{ox}[6,9] and *Chromatium* HP_{red}[6,10] possess Fe_4S_4*S_4 clusters containing Fe_4S_4* *cores* of cubane-type stereochemistry which are essentially congruent with the cores of the isoelectronic 4-Fe analogues $[Fe_4S_4(SR)_4]^{2-}$[2,11] (**1**). The 2-Fe analogues (**2**) possess a planar Fe_2S_2* core[12] which,



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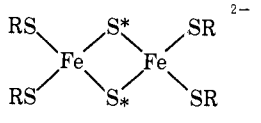

2

on the basis of comparative analogue-protein physical properties,[12,13] is doubtless present with similar dimensions in isoelectronic 2-Fe Fd_{ox} proteins.

At present analogue complexes **1** and **2** are known to undergo two types of reactions in which core structures remain intact: electron transfer[2] and thiolate substitution. The latter reaction type includes cases of replacement of thiolate with X upon reaction with electrophilic RCOX,[14] direct displacement of thiolate with inorganic anions and water in aqueous solution,[15] and substitution of thiolate by reaction with thiol.[11b,12,16-19] Thiolate substitution reactions are pertinent to the present investigation, and the reactions

$$[Fe_4S_4(SR)_4]^{2-} + 4R'SH \rightleftarrows [Fe_4S_4(SR')_4]^{2-} + 4RSH \quad (1)$$

$$[Fe_2S_2(SR)_4]^{2-} + 4R'SH \rightleftarrows [Fe_2S_2(SR')_4]^{2-} + 4RSH \quad (2)$$

proceed readily in nonaqueous or partially aqueous media at ambient temperature. Reaction 1 has been the more thoroughly studied and exhibits the following properties: (i) equilibrium is attained rapidly; (ii) equilibrium[11b] and kinetic[19] substitution tendencies of thiols R'SH roughly parallel their aqueous acidities, such that complete substitution of an R =

Table I. Properties of *C. pasteurianum* Hydrogenase Preparations

Property	Values
Molecular weight, daltons	60 500 (no subunits)[a]
Fe, g-atom/mol	~12;[a] 11.2;[c] 4.0-4.5[b]
S*, g-atom/mol	11.2;[c] 4.5-4.7[b]
Half-cystine	12[a]
Reduced form	
ϵ_M[d] (400 nm)	25 300;[a] 28 800;[c,i] ~27 000[e]
ϵ_{Fe}, g-atom Fe	2100 (12);[a] 2600 (11.2);[c] ~6300 (4.3)[e]
EPR: *g* values	2.079-1.892;[f,h] 2.098-1.898[b]
spins/mol	1.6-1.8;[f] 0.7-1.0[b]
Oxidized form	
ϵ_M[d] (400 nm)	33 500;[c,j] ~29 000[e]
ϵ_{Fe}, g-atom Fe	2800 (12);[a] 3000 (11.2);[c] ~6700 (4.3)[e]
EPR: *g* values	2.099, 2.046, 2.005;[f,g] 2.099, 2.041, 2.001[b]
spins/mol	0.7;[f,g] 0.07[b]
H_2ase activity, μmol H_2/min/mg protein	505;[c] 304-378[b]

[a] Reference 27. [b] Reference 22. [c] Preparation used in this work. [d] M^{-1} cm^{-1}. [e] *Estimated* from Figure 1 of ref 22. [f] Reference 34. [g] Observed at $E_h \geq -330$ mV. [h] Observed at $E_h \leq -400$ mV. [i] Under H_2. [j] Aerobic.

alkyl tetramer at ca. 10^{-3} M can be achieved by addition of $\lesssim$50% mol excess of aryl thiol/tetramer;[11b,16-18] (iii) initial (and, presumably, succeeding) thiolate substitution is bimolecular with rate constants ca. $1-10^3$ M^{-1} s^{-1}, and proceeds via proton transfer from R'SH to coordinated RS^- followed by loss of RSH and coordination of $R'S^-$.[19] Qualitative observations of reaction 2 indicate that properties i and ii apply to it as well. It has further been shown that $[Fe_2S_2{*}]^{2+}$ [12] and $[Fe_4S_4{*}]^{2+}$ [11] cores exhibit no significant dimensional changes when terminal ligands are alkyl- vs. arylthiolates, and that the simplest arylthiolate complexes, $[Fe_2S_2(SPh)_4]^{2-}$ and $[Fe_4S_4(SPh)_4]^{2-}$, can be distinguished from each other (and from their alkylthiolate relatives) by absorption spectra and polarographic half-wave potentials.[12,17]

Because of retention of core structure in thiolate substitution reactions and their simplicity of execution, these reactions lend themselves to significant chemical and biological applications. Reaction scheme 3, among others,[18] utilizes property ii and

$$[Fe_4S_4(S\text{-}t\text{-}Bu)_4]^{2-} + t\text{-Boc-(Gly-Cys-Gly)}_4\text{-NH}_2 \longrightarrow$$

$$[Fe_4S_4(SPh)_4]^{2-} \xleftarrow{PhSH} [Fe_4S_4(t\text{-Boc-(Gly-Cys-Gly)}_4\text{-NH}_2)]^{2-} \quad (3)$$

illustrates incorporation of peptide structure around, and its subsequent removal from, an Fe_4S_4* core which is liberated in the form of the thoroughly characterized benzenethiolate tetramer dianion.[11b,12,17] As emphasized on earlier occasions,[11b,12,16,18] these reactions offer the possibilities of protein reconstitution from apoprotein and preformed (synthetic) cores, and removal of intact cores from proteins in the form of synthetic analogues (reaction 4), which may be independently

$$\text{holoprotein} + \text{RSH} \rightarrow \left\{ \begin{matrix} [Fe_2S_2(SR)_4]^{2-} \\ \text{and/or} \\ [Fe_4S_4(SR)_4]^{2-} \end{matrix} \right\} + \text{apoprotein} \quad (4)$$

prepared.[11,12] The latter reaction has recently been accomplished in partially aqueous media with a number of 2-, 4-, and 8-Fe Fd proteins[20-22] and has been described as active site core extrusion[20] or displacement.[21,22] Provided experimental conditions are adjusted to prevent hydrolytic or oxidative decomposition[23] or dimer → tetramer core oligomerization,[20] identification of the extruded analogue in turn identifies the core structure of the active site.

Based on a large body of accumulated physical data[5] there is in general little difficulty in identifying 1-, 2-, and 4-Fe sites in soluble low molecular weight Fe-S proteins by use of conventional spectroscopic methods (absorption, MCD, EPR) on dilute fluid or frozen solutions. This is not necessarily the case with higher molecular weight enzymes such as, e.g., nitrogenase,[24] succinate dehydrogenase,[25] and hydrogenase.[26] Thus, the most intriguing and potentially valuable application of the extrusion technique is that of active site core structural determination using reactions in dilute solution at ambient temperature. The first published demonstration that core extrusion can be effected with a higher molecular weight iron-sulfur enzyme is that of Erbes et al.,[22] who have shown that the Fe-S* content of *Clostridium pasteurianum* W5 H_2ase may be liberated in the form of a 4-Fe analogue. Properties of this enzyme[22,27] reported to be purified to homogeneity are collected in Table I. As may be seen there is apparent difference between the Fe and S* contents of the enzyme subjected to extrusion[22] (~4 Fe) and those of another enzyme preparation[27] (~12 Fe) from the same organism. In view of these results the present investigation has been undertaken for the principal purposes of (i) development of procedures for quantitative core extrusion using as controls smaller proteins of known composition, and (ii) application of these procedures to the *C. pasteurianum* H_2ase preparation with the higher Fe and S* content in order to provide further definition of the number of Fe-S* sites and their structural type(s) in this enzyme, which plays a central role in microbial metabolism.[26]

Experimental Section

Materials. Hexamethylphosphoramide[28] (HMPA, Aldrich Chemical Co.) was stirred under dinitrogen with metallic sodium and the resulting blue solution fractionally distilled. The fraction boiling at 90 °C (6 mm) was collected and stored at −20 °C under dinitrogen. Benzenethiol was fractionally distilled and the fraction boiling at 168 °C (1 atm) was collected and stored under dinitrogen. Trischloride buffer was prepared from Trizma Base (Sigma Chemical Co.) and concentrated hydrochloric acid. All other chemicals were of reagent grade quality. $(Ph_4As)_2[Fe_4S_4(SPh)_4]$ [11b] and $(Et_4N)_2[Fe_4S_4(S\text{-}t\text{-}Bu)_4]$ were prepared by the method of direct tetramer synthesis;[11a] $(Et_4N)_2[Fe_2S_2(SPh)_4]$ was synthesized by the procedure described elsewhere.[12] *C. pasteurianum* ferredoxin[29] (A_{390}/A_{280} 0.80) and spinach ferredoxin[30] were isolated as previously described. *Chromatium vinosum* HP_{red}[31] (A_{280}/A_{388} 2.57) and *Bacillus stearothermophilus* Fd_{ox}[32] were gifts from Drs. R. G. Bartsch and D. O. Hall, respectively. Solutions of the latter two proteins were desalted on a Sephadex G-25-150 column (0.9 × 30 cm) prior to use. The Fe protein of *C. pasteurianum* nitrogenase was obtained by the procedure of Zumft and Mortenson.[33]

Hydrogenase was purified from nitrogen-fixing cells of *C.*

Table II. Active Site Core Extrusion of Iron-Sulfur Proteins[n]

Protein	Fe, g-atom/mol	Protein concn, μM	Mol ratio PhSH/Fe[a]	$[Fe_4S_4(SPh)_4]^{2-}$, μM	n	A_{458}/A_{550}[b]
C. pasteurianum Fd_{ox}	8	5.2	230	9.9	1.9	*c*
		11	110	20.4	1.9	*c*
		19	290, 570	40.1, 39.4	2.1, 2.0	1.78, 1.79
		21	12, 58[d]	38.8, 41.7	1.9, 2.0	1.79, 1.89
		39	290	79.4	2.0	1.97
		97	110	193	2.0	2.01
		192	57[e]	389	2.0	1.92
C. pasteurianum Fd_{ox} + Fd_{red}[f]	8	20	120[g]	38	1.9	1.86
B. stearothermophilus Fd_{ox}[h]	4	50	980	50.5	1.0	1.86
Chromatium HP_{red}[i]	4	33	1220	34.2	1.0	1.86
C. pasteurianum N_2ase Fe protein[j]	4	45	120[k]	35.7	0.93	1.80
$[Fe_4S_4(S\text{-}t\text{-}Bu)_4]^{2-}$	4	1370[l]	100	1360	0.99	2.13
$[Fe_4S_4(SPh)_4]^{2-}$	4	6–1100[l]				2.18 (0.16)[m]

[a] 1 μl PhSH = 9.7 μmol, other reagents added to 1 ml of extrusion solutions given in footnotes. [b] Absorbancies corrected for background absorbance of added components. [c] Reaction followed by monitoring A_{458} only. [d] Figure 1. [e] Figure 2. [f] Fd_{ox} + 0.10 μmol $Na_2S_2O_4$/ml, Figure 5. [g] Thiol addition followed by 0.29 μmol of $Fe(CN)_6^{3-}$/ml. [h] 3:2 v/v HMPA/H_2O. [i] Figure 4. [j] Preparation contained 3.4 g-atom Fe/55 000 g by prior analysis; protein concentration determined by Folin-Lowry method. [k] Thiol addition followed by 0.09 μmol of $Fe(CN)_6^{3-}$/ml. [l] Analogue concentration. [m] Standard deviation from ten measurements. [n] Conditions: 4:1 v/v HMPA/H_2O (50 mM TrisCl, aqueous pH 8.5), 25 °C, *l* = 0.05–1 cm.

pasteurianum as described by Chen and Mortenson[27] except that, as in more recent work,[34] a second hydroxyapatite column has been added to improve the resolution from a leading and trailing component, thus improving the yield of purified enzyme. Hydrogenase, purified in this manner (A_{400}/A_{276} 0.37), exhibited a single sharp band at the 60 000 dalton position and a minor band at about the 50 000 dalton position when subjected to disc gel electrophoresis in the presence of sodium dodecyl sulfate. From a scan of the stained protein bands of the disc gel, the area under the hydrogenase peak was found to contain better than 95% of the total protein. Activity of the hydrogenase used in this study (Table I), determined from its catalysis of H_2 evolution in the presence of 1 mM methyl viologen and excess $Na_2S_2O_4$,[27] was 540 μmol H_2/min/mg protein. This solution was concentrated anaerobically by ultrafiltration and its activity after concentration was 505. Protein was analyzed by the method of Lowry.[35] The stock hydrogenase solution used in this work (prior to HMPA dilution) was found to contain 4.65 mg of protein/ml or 76.9 μM. Iron analysis was performed by a modification[27] of the method of Fortune and Mellon[36] and yielded 11.2 g-atom/mol. Sulfide was determined by a modification of the method of Brumby et al.[37] and gave 11.2 g-atom/mol; details of the modified method will be reported separately.[38]

Active Site Core Extrusion. Preparations of protein solutions were carried out using a glass manifold system with provision for admittance of Ar subsequent to removal of traces of dioxygen by passage through a tower of BASF R3-11 catalyst maintained at 120 °C. The system was equipped with vacuum stopcocks to which were attached 2-in. lengths of thick-walled vacuum tubing. Connection of the manifold system to solutions was made by gas-tight insertion of a syringe barrel (protruding lip removed) equipped with a 25-gauge stainless steel needle into the vacuum tubing. In a typical experiment involving ferredoxin proteins a stock protein solution was diluted to the desired concentration with 50 mM TrisCl aqueous buffer solution, pH 8.5. A portion of this solution was transferred by syringe to an Ar-filled cuvette attached via a septum cap to the system, and was then subjected to eight to ten cycles of degassing by alternately reducing the pressure to 100 μ and admitting Ar at 1 atm. The protein solution was cooled in ice and similarly cooled and degassed HMPA was added by syringe to give a 4:1 v/v HMPA/H_2O solution, which was then subjected to three to four additional degassing cycles. The spectra of this solution and those formed by subsequent addition of degassed components via microliter syringes were recorded. Hydrogenase was stored as frozen solution pellets in liquid nitrogen. As the residual liquid nitrogen evaporated, a vial containing the pellets was attached to the manifold system and the thawing solution was subjected to ten degassing cycles. The procedure at this point was the same as just described. Further experimental details are given in Tables II and III and in figure legends. All spectra were measured at 25 °C in matched quartz cuvettes of path lengths 0.05–1 cm using a Cary Model 17 recording spectrophotometer.

Concentrations of TrisCl buffer solutions containing proteins known to have 4-Fe active sites were determined using the following molar extinction coefficients: *C. pasteurianum* Fd_{ox}, ϵ_{390} 30 000;[39] *Chromatium* HP_{red}, ϵ_{388} 16 100;[31] *B. stearothermophilus* Fd_{ox}, ϵ_{390} 15 000 M^{-1} cm^{-1} (assumed). All values are near the most precisely determined value of ϵ_{390} 30 600 M^{-1} cm^{-1} (15 300/4-Fe site) for *C. acidi-urici* Fd_{ox}.[40] Concentrations of spinach Fd_{ox} were determined using ϵ_{420} 8870 M^{-1} cm^{-1}, derived from a published value[41a] corrected to the protein molecular weight determined from its amino acid sequence.[41b] The ratio of the corrected and original values is 0.92. Solutions of $(Ph_4As)_2[Fe_4S_4(SPh)_4]$ in 4:1 v/v HMPA/H_2O (50 mM TrisCl, aqueous pH 8.5) with PhSH/Fe mole ratios of ~100:1 were shown to obey Beer's law at λ_{max} 458 nm. The following values were used to calculate concentrations of analogues or total iron upon completion of an extrusion reaction: $[Fe_4S_4(SPh)_4]^{2-}$, ϵ_{458} 17 200; $[Fe_2S_2(SPh)_4]^{2-}$, ϵ_{482} 11 900 M^{-1} cm^{-1}. In these calculations the absorbancy values A_{458} and A_{482} were corrected for possible background absorption by species added to the extrusion solutions, or produced by reaction of these species, by determining the spectra of these species singly or as mixtures (Tables II and III) at the concentrations present in initial and final solutions and at times when the extrusion reaction was complete. Apoprotein absorption was checked using a mersalyl-treated solution of *C. pasteurianum* Fd_{ox} and found to be negligible in the visible region. In all cases corrections were ≤4% of observed A_{458} values. In certain of the figures spectra of solutions recorded at times when extrusion was not yet complete are those of slowly reacting rather than steady-state systems. Indicated times refer to initiation of a spectral scan, some of which were recorded at a rate of 1 nm/s over the 300–700-nm interval. However, in all cases the 400–500-nm region was recorded at this scan rate.

Results and Discussion

In considering the active site core extrusion reactions to be described, the series (eq 5) of total oxidation level equivalencies of 4-Fe analogues and protein sites[2,17] is of significance. Isoelectronic species are arranged in columns. In addition, $[Fe_2S_2(SR)_4]^{2-}$ ≡ 2-Fe Fd_{ox} and $[Fe_2S_2(SR)_4]^{3-}$ ≡ 2-Fe Fd_{red}.[2,12] Thus, for example, removal of the Fe_4S_4* core of Fd_{ox} or HP_{red} in the form of the analogue dianion in reaction 4 is a process involving no change in core oxidation level.

$$\underset{Fd_{red}}{[Fe_4S_4(SR)_4]^{3-}} \rightleftharpoons \underset{Fd_{ox}}{[Fe_4S_4(SR)_4]^{2-}} \rightleftharpoons \underset{HP_{ox}}{[Fe_4S_4(SR)_4]^{-}} \qquad (5)$$

$HP_{s\text{-}red}$ HP_{red}

Table III. Active Site Core Extrusion of *C. pasteurianum* H_2ase[l]

			Initial				Final			
Soln	H_2ase, μM[a]	Fe, g-atom/mol	Fe, μM	4-Fe sites[d]	A (λ_{max})	ϵ_{Fe} (λ_{max})	A_{458}[g]	Fe, μM	n	A_{458}/A_{550}[g]
(A)[h]	15	11.2[b]	168	2.8	0.610(408)[e]	3 600[f]	0.750	174	2.9	1.96
		12[c]	180	3.0		3 400				
(B)[i]	15	11.2[b]	168	2.8	0.612(390)	3 600	0.723	168	2.8	1.84
		12[c]	180	3.0		3 400				
(C)	11	11.2[b]	123	2.8	*j*		0.545	127	2.9	1.97
		12[c]	132	3.0						
	15	8[c]	120	2		5 100[k]	0.516		2	
	15	4[c]	60	1		10 200[k]	0.258		1	

[a] Calculated from Folin–Lowry protein determination and 60 500 g/mol. [b] Experimental value/60 500 g. [c] Assumed value/60 500 g. [d] Assumed value based on stated H_2ase concentration. [e] In nm. [f] In l. g-atom^{-1} cm^{-1}. [g] Corrected for background absorption of other components. [h] Figure 7. [i] Figure 8. [j] PhSH added immediately to solution, initial H_2ase spectrum not recorded. [k] Based A = 0.61 observed for (A) and (B). [l] Conditions: 4:1 v/v HMPA/H_2O (50 mM TrisCl, aqueous pH 8.5), 1 ml of solution, l = 1 cm, 25 °C; (A) H_2ase solution exposed to air for 1 min, 2 μl (19 μmol) of PhSH, 1 μl of 29 mM $Na_3Fe(CN)_6$ added anaerobically; (B) anaerobic, 2 μl of PhSH + 2 μl of 29 mM $Na_3Fe(CN)_6$ added; (C) anaerobic, same as (B).

Experimental Conditions for Extrusion Reactions. A variety of physical studies, the results of which have been well summarized elsewhere,[42] has delineated the stability of secondary, tertiary, and active site structures of Fe–S proteins in aqueous solution (pH ~ 7–9) at ambient temperature. Several experiments conducted under these conditions (in the absence of denaturants) using 2-Fe and 8-Fe Fd_{ox} proteins and aryl thiols, extrusion reagents of first choice based on the substitution tendencies[11b,19] noted above, failed to reveal the occurrence of extrusion reaction 4 at any appreciable rate. However, these proteins, particularly those containing 4-Fe sites, exhibit significant changes in properties in mixed aqueous–nonaqueous solvents compared with the case in purely aqueous solution. Thus *C. pasteurianum* Fd_{ox} in Me_2SO/water mixtures in which the Me_2SO content exceeds about 50% v/v shows broadened low-field contact-shifted ^1H NMR resonances and changes in the high-field region suggestive of random coil arrangements,[43] removal of EPR fine structure[44] in Fd_{red} spectra indicating loss of spin–spin interaction[45] between two doublet-state sites, and red shifts of the 390-nm Fd_{ox} chromophore band[20,46] and cathodic displacement of Fd_{ox}/Fd_{red} potentials,[46] such that these two properties approach those of isoelectronic 4-Fe analogues in purely nonaqueous media.[17,18] Further, HP_{red} can be reduced (by dithionite) to the "super-reduced" form $HP_{s\text{-}red}$ in 80% Me_2SO, but not in water.[47] While none of these effects can as yet be interpreted in detail at the molecular level, all are consistent with some extent of unfolding of protein tertiary structure. This appears to result in disruption of protein structural and environmental effects at or near the active site(s), with the consequence that the latter become more exposed to the medium and assume certain properties similar to those of the tetranuclear analogues **1**, especially those derived from cysteinyl peptides.[18] Under these conditions the similarity of well documented reactions 1–3 to reaction 4 is quite apparent, with the unfolded protein structure probably allowing more ready access of added thiol to the active site at which protonation of coordinated S–Cys by thiol presumably initiates the extrusion process.[19] These considerations led to the successful extrusion of 2-Fe and 8-Fe Fd_{ox} active sites in 80% Me_2SO.[20]

Since the initial report of active site core extrusion,[20] it has been our experience that the 80% HMPA aqueous medium introduced by Erbes et al.[22] is more broadly applicable than Me_2SO/H_2O mixtures in dissolving a variety of Fe–S proteins and enzymes without detectable active site decomposition over periods of several hours, so long as strictly anaerobic conditions are maintained. Consequently, this medium has been employed in this and related studies currently in progress in these laboratories. In the experiments described below, the principal intent has been to define conditions leading to quantitative core extrusion of 4-Fe sites using *C. pasteurianum* Fd_{ox} as the primary control. These conditions were then applied to H_2ase from the same organism. Effects of variation of medium composition and pH of the aqueous solvent component, maintained constant at 80% HMPA and 8.5, respectively, were not explored nor were attempts made to define conditions leading to the most rapid *and* quantitative extrusions. The main variables were protein concentration and thiol/Fe mole ratio. Throughout, the extrusion reagent is benzenethiol. The final conditions, summarized in Tables II and III, afford quantitative core extrusion in convenient times ($\lesssim$1 h), and may be useful in selecting conditions for extrusion of other proteins. The liberated analogue, $[Fe_4S_4(SPh)_4]^{2-}$, in the extrusion medium possesses a prominent absorption maximum at 458 nm (ϵ 17 200), which is substantially red-shifted compared to protein bands at 400–410 nm, and thus is readily identified and quantitated. Results of quantitative core extrusions of the smaller proteins are collected in Table II. The quantity of principal interest is n, the moles of $[Fe_4S_4(SPh)_4]^{2-}$ liberated per mole of protein. Values of n were calculated from final, time-independent spectra at times up to 60 min after thiol addition, and are based on spectrophotometrically determined protein concentrations, assuming exactly stoichiometric quantities of iron in the proteins.

Active Site Core Extrusions. (a) Analogue Reaction. To provide initial demonstration of the efficacy of ligand substitution reactions under conditions employed here, $[Fe_4S_4(S\text{-}t\text{-}Bu)_4]^{2-}$ was treated with a 100-fold mole excess of PhSH/Fe and the spectrum of the solution recorded within several minutes and 30 min after mixing. A_{458} values were negligibly different and n = 0.99.

(b) *C. pasteurianum* Fd_{ox}. All physical properties of this prototype clostridial ferredoxin are fully consistent with the presence of two $[Fe_4S_4{*}(S\text{-}Cys)_4]$ active sites, as has been established for *Peptococcus aerogenes* Fd_{ox} by x-ray diffraction.[6,9] Extrusion reactions were investigated at 5–192 μM protein concentrations and at PhSH/Fe mole ratios varying from 12:1 to 570:1. Shown in Figures 1 and 2 are spectra of reaction mixtures with PhSH/Fe ratios of 57:1 and protein concentrations of 21 and 192 μM, respectively. The initial protein band near 400 nm is immediately displaced to longer wavelengths upon addition of PhSH, and the spectra continued

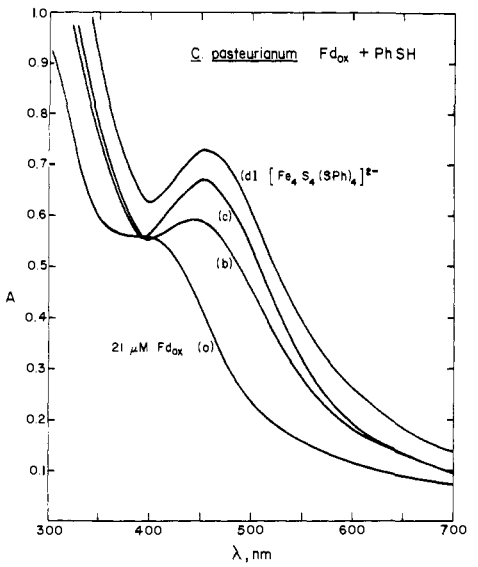

Figure 1. Active site core extrusion of *C. pasteurianum* Fd_{ox} in 4:1 v/v $HMPA/H_2O$ (50 mM TrisCl, pH 8.5), l = 1 cm, 25 °C: (a) 21 μM Fd_{ox}; (b) 1 ml of solution a + 0.2 μl of PhSH 6 min after mixing; (c) solution b after 54 min; (d) solution c + 0.8 μl of PhSH 4 min after mixing. Spectrum d was constant with time.

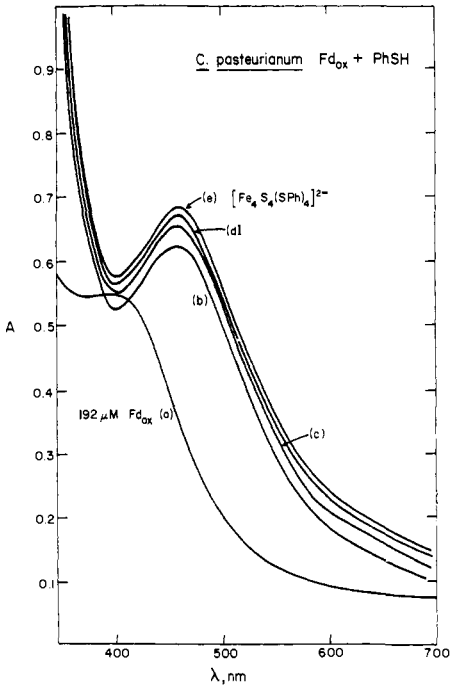

Figure 2. Active site core extrusion of *C. pasteurianum* Fd_{ox}; experimental conditions are the same as in Figure 1 except that l = 0.1 cm: (a) 192 μM Fd_{ox}; (b) 1 ml of solution a + 9.0 μl of PhSH 1 min after mixing; (c) solution b 6 min after mixing; (d) solution b 13 min after mixing; (e) solution b 25 min after mixing. Spectrum e was constant with time.

to change until the spectrum of $[Fe_4S_4(SPh)_4]^{2-}$ was fully developed. Time course curves for the extrusion reactions are given in Figure 3. As would be expected, protein ligand substitution reactions are slower for this protein than for analogues. The time dependencies represented in this figure are not to be construed as strict kinetic curves for the formation of $[Fe_4S_4(SPh)_4]^{2-}$, inasmuch as A_{458} values have not been corrected for absorbance contributions from intermediate mixed ligand species $[Fe_4S_4{*}(S\text{-}Cys)_{4-n}(SPh)_n]$ such as may be formed prior to liberation of the benzenethiolate tetramer dianion. These results serve to define time-concentration

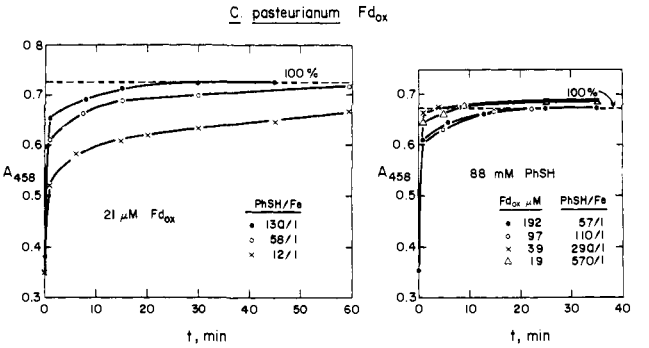

Figure 3. Time course of the active site core extrusion of *C. pasteurianum* Fd_{ox}. Left: time dependence of A_{458} at constant Fd_{ox} and varying PhSH/Fe mole ratios; complete extrusion of two 4-Fe sites corresponds to A_{458} = 0.719 (l = 1 cm). Right: time dependence of A_{458} at constant PhSH concentrations and varying PhSH/Fe mole ratios. All path lengths were chosen such that complete extrusion corresponds to A_{458} = 0.664.

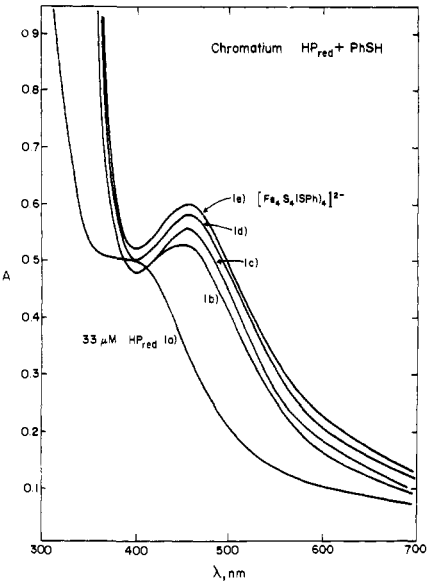

Figure 4. Active site core extrusion of *Chromatium* HP_{red}; experimental conditions are the same as in Figure 1: (a) 33 μM HP_{red}; (b) 1 ml of solution a + 1.5 μl of PhSH 1 min after mixing; (c) solution b after 4 min; (d) solution c + 3 μl of PhSH 1 min after mixing; (e) solution d + 12 μl of PhSH immediately after mixing. Spectrum e remained constant with time.

conditions for core extrusion, with a particularly useful guideline being that *complete extrusion is achieved with the mole ratio PhSH/Fe ~ 100:1 at times of ~20–30 min after introduction of thiol.* Under all conditions employed in this work n = 1.9–2.1.

(c) *Chromatium* HP_{red}. As shown by x-ray diffraction,[10] this protein contains one $[Fe_4S_4{*}(S\text{-}Cys)_4]$ site, which is situated in the interior of the structure and surrounded by nonpolar residues so as to prevent direct contact of the cluster with solvent. When dissolved in 80% HMPA at 33 μM, the well-defined band at 388 nm in aqueous solution is replaced by a plateau-like feature centered near 400 nm (ϵ_{400} 15 000), suggesting some change in the environment of the chromophore. The spectral behavior of this protein upon addition of benzenethiol is shown in Figure 4, and is similar to that for the preceding case. Complete extrusion (n = 1.0) was achieved within 15 min with a final PhSH/Fe mole ratio of 1200:1.

(d) *B. Stearothermophilus* Fd_{ox}. The physical properties of this 4-Fe protein, recently isolated by Mullinger et al.,[32] depart in some respects from those of members of the more extensive class of 8-Fe Fd proteins. Certain of these differences, e.g., in

the low temperature Mössbauer and EPR spectra of the reduced form, have been interpreted in terms of a single $[Fe_4S_4{*}(S\text{-}Cys)_4]$ site with the attendant absence of intramolecular magnetic interactions with a second such site. This view is supported by the extrusion technique. Although the aqueous solution band maximum at 390 nm did not shift appreciably in the 60% HMPA reaction medium used in this case, extrusion proceeded readily. At 50 μM protein concentration and the mole ratio PhSH/Fe $\sim$ 980:1, active site core extrusion was found to be complete (n = 1.0) after 30 min. Complete extrusion also occurs in 80% HMPA; the complete study of the extrusion reactions of this protein will be reported separately.[48] This and previous results[32] allow secure classification of this protein as one of a relatively small number of ferredoxins (e.g., those from *B. polymyxa*[49] and *Desulfovibrio desulfuricans*[50]) which contain a single cubane-type 4-Fe active site.

(e) ***C. pasteurianum*** $\mathbf{Fd_{ox} + Fd_{red}}$. In order to investigate the core extrusion properties of mixtures of oxidized and reduced 4-Fe sites, such as was anticipated to be encountered in H_2ase,[34] solutions of Fd_{ox} in 80% HMPA were 50–80% reduced with excess dithionite. Spectra of an ca. 50% reduced protein solution subjected to extrusion are shown in Figure 5; experimental details are given in Table II and are similar to those of other experiments on more fully reduced solutions. Intensity reduction in the visible region is characteristic of the Fd_{red} chromophore, which is isoelectronic with analogue trianions (series 5). Thiol addition to the partially reduced protein solution resulted in the development of the spectrum of $[Fe_4S_4(SPh)_4]^{2-}$ within several minutes. In every case examined (mole ratios PhSH/Fe $\cong$ 120:1, $S_2O_4^{2-}$/Fd $\cong$ 5:1) the A_{458} value at this stage corresponded to 75–77% of the absorbance for complete extrusion of a Fd_{ox} solution of the same concentration. Addition of ferricyanide (mole ratio $Fe(CN)_6^{3-}$/Fd $\cong$ 15:1) resulted in an enhancement of the intensity of the analogue dianion spectrum, with the final time-invariant A_{458} values corresponding to n = 1.9.

Appearance of the $[Fe_4S_4(SPh)_4]^{2-}$ spectrum with an intensity inconsistent with the amount of residual Fd_{ox} sites, prior to addition of an oxidant, was unexpected. In view of the possibility that Fd_{red} sites may have been extruded as $[Fe_4S_4(SPh)_4]^{3-}$, whose low redox potential ($E_{1/2}(2-,3-)$ = −1.04 V in DMF vs. SCE[17]) testifies to its extreme ease of oxidation, certain reactivity properties of this trianion were examined. These experiments are facilitated by the recent preparation of analytically pure tetramer trianion salts;[4] solutions of $(Et_4N)_3[Fe_4S_4(SPh)_4]$ were prepared by weight and manipulated anaerobically. In anhydrous aprotic solvents the visible spectra consist of shoulders at $\sim$340 and $\sim$400 nm, with no defined feature at or near 460 nm, and correspond to that obtained by electrochemical reduction of $[Fe_4S_4(SPh)_4]^{2-}$ in acetonitrile.[51] Based on visible spectral observations the trianion salt in the 80% HMPA extrusion medium was immediately oxidized upon dissolution, and an A_{458} value 92% of that calculated for the 0.80 mM solution was developed. Similar experiments using 1.03–1.42 mM solutions containing benzenethiol (mole ratio PhSH/Fe $\sim$ 100:1) led to A_{458} values 86–89% of the limiting values for complete oxidation to the dianion. Complete oxidation was readily achieved by addition of a small excess of ferricyanide ($\sim$1.5:1) based on the amount of trianion salt used. Judging from published rate studies in aqueous solution, the redox system in the complete extrusion medium (including thiol) is not necessarily simple. Ferricyanide, diphenyl disulfide (from ferricyanide oxidation of thiol[52]), and sulfite (from dithionite reduction of Fd_{ox}) are possible oxidants of the analogue trianion, and sulfite is a reductant for ferricyanide.[53] Spontaneous oxidation of $[Fe_4S_4(SPh)_4]^{3-}$ in the absence of thiol suggests that the solvent system itself acts as an oxidant. Whatever the exact nature of the redox process under extrusion conditions,[54] its operation represents a considerable practical advantage. As already demonstrated Fd_{ox} cores are readily extruded. The amount of ferricyanide required to obtain full development of the $[Fe_4S_4(SPh)_4]^{2-}$ spectrum in the Fd_{ox} + Fd_{red} mixtures within a convenient time period (ca. 15–30 min) necessitates only a minor correction to A_{458} values, from which n is calculated. Reactions of $[Fe_4S_4(SR)_4]^{3-}$ complexes in mixed protic/aprotic media are currently under active study and results will be presented elsewhere.

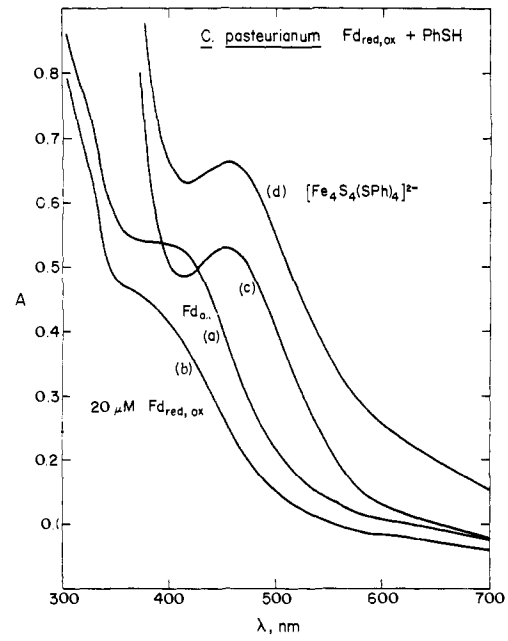

Figure 5. Active site core extrusion of *C. pasteurianum* Fd_{ox} + Fd_{red}; experimental conditions are the same as in Figure 1: (a) 20 μM Fd_{ox}; (b) 1 ml of solution a + 2 μl of 50 mM $Na_2S_2O_4$; (c) solution b + 2 μl of PhSH 2 min after mixing; (d) solution c after 15 min + 10 μl of 29 mM $Na_3Fe(CN)_6$ 8 min after mixing. Further addition of 5 μl of ferricyanide solution caused no spectral change. The A_{458} value of solution c is 77% of the value for complete extrusion.

Hydrogenase. Various properties of recent H_2ase preparations from *C. pasteurianum*, including that used in this study, are summarized in Table I. Visible spectra of a 15 μM aqueous solution showing the "reduced" and "oxidized" forms are given in Figure 6. These designations are referenced here to the conditions affording these spectra, and do not imply that Fe–S centers in each form are necessarily entirely reduced or oxidized. The featureless spectrum of the reduced form is typical of 2-Fe and 4-Fe Fd_{red} chromophores, while the appearance of a shoulder near 400 nm in the spectrum of the oxidized form is suggestive of the presence of perhaps one 4-Fe Fd_{ox}-type site. The reduced spectrum may be correlated with the larger number of spins/mole as determined from integration of EPR signals (Table I), which are indicative of sites isoelectronic with those in Fd_{red} proteins.[5] Neither spectrum is clearly diagnostic of chromophore structure.[55]

The number and structural type(s) of sites in H_2ase has been investigated by the core extrusion technique in 80% HMPA using procedures described above for the lower molecular weight proteins. Experimental conditions are similar to those employed by Erbes et al.,[22] who have reported that $[Fe_4S_4(SPh)_4]^{2-}$ is formed by extrusion of a preparation with apparently lower Fe and S* contents based on a molecular weight of 60 000 daltons. Extrusions were performed using reduced enzyme solutions briefly exposed to air (A) and maintained anaerobic throughout (B, C); these solutions contained only trace quantities of dithionite. Experimental details are given in Table III and Figures 7 and 8.

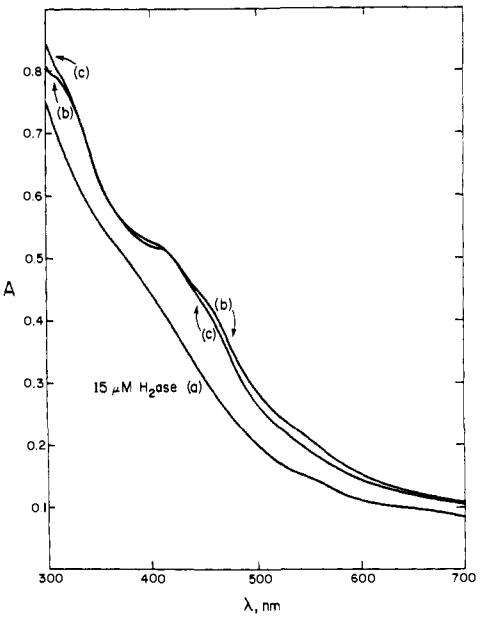

Figure 6. Spectra of *C. pasteurianum* H_2ase in 50 mM TrisCl, pH 8.5, l = 1 cm: (a) 15 μM H_2ase incubated under H_2; (b) 1 ml of solution a exposed to air for 30 s, then made anaerobic and the spectrum recorded immediately; (c) 1 ml of solution a + 12 μl of 29 mM $Na_3Fe(CN)_6$ ca. 5 min after mixing.

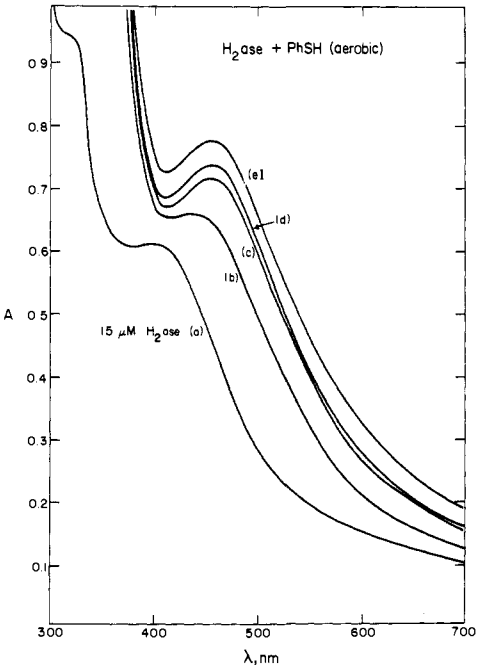

Figure 7. Active site core extrusion of *C. pasteurianum* H_2ase; experimental conditions are the same as in Figure 1: (a) 1 ml of 15 μM H_2ase solution after exposure to air for 1 min; (b) solution a made anaerobic + 2 μl of PhSH 2 min after mixing; (c) solution b 5 min after mixing; (d) solution b 15 min after mixing; (e) solution d + 1 μl of 29 mM $Na_3Fe(CN)_6$ 5 min after mixing. Further addition of 1 μl of ferricyanide solution caused no spectral change. The A_{458} value of solution d is 95% (12 Fe) or 101% (11.2 Fe) of that for complete extrusion of 4-Fe sites based on the indicated g-atom Fe/mol.

The course of the extrusion process for 15 μM H_2ase solution (A) is shown in Figure 7. After aerial oxidation and *before* thiol addition the enzyme spectrum, compared to that of the oxidized form in water, is structurally informative. It is significant that the maximum at 405 nm and ϵ_{Fe} values (3400 and 3600 for 12 and 11.2 g-atom Fe/mol) are both close to those of

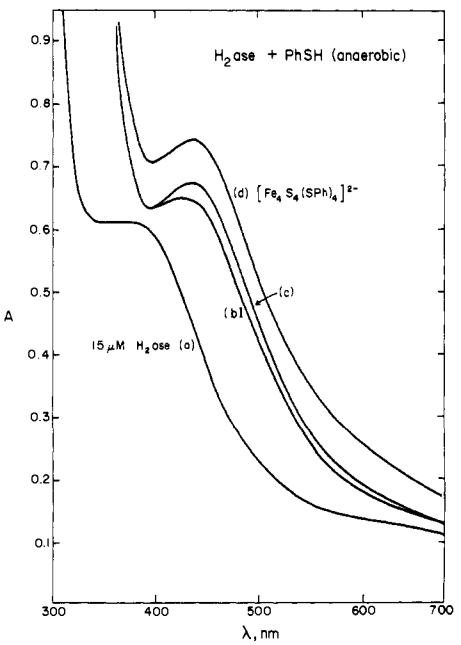

Figure 8. Active site core extrusion of *C. pasteurianum* H_2ase under anaerobic conditions; experimental conditions are the same as in Figure 1: (a) 15 μM H_2ase solution; (b) 1 ml of solution a + 2 μl of PhSH 5 min after mixing; (c) solution b after 10 min + 1 μl of 29 mM $Na_3Fe(CN)_6$ 2 min after mixing; (d) solution c + 1 μl of 29 mM $Na_3Fe(CN)_6$ 2 min after mixing. Further addition of 1 μl of ferricyanide solution caused no spectral change. The A_{458} value of solution b is 84% (12 Fe) or 90% (11.2 Fe) of that for complete extrusion calculated as in Figure 7.

proteins with 4-Fe sites measured in 80% HMPA (Figures 1, 2, and 4). For HP_{red} ϵ_{Fe} is 3850 (390 nm) and for *C. pasteurianum* Fd_{ox} ϵ_{Fe} 3560 ± 200 (395 nm) using nine solutions in the range 19–192 μM. The spectrum contains no resolved features such as are observed for oxidized 1-Fe and 2-Fe sites at wavelengths longer than 400 nm.[5] The spectrum of spinach Fd_{ox}, which contains a typical 2-Fe chromophore, is shown for comparison in Figure 9. In 80% HMPA the band at 420 nm ($\epsilon \sim 9200$) persists, but the 465-nm band is blue-shifted and appears as a shoulder at ~455 nm. These observations suggest that H_2ase in this form contains $[Fe_4S_4(S\text{-}Cys)_4]$ sites largely oxidized to a level isoelectronic with Fd_{ox} and HP_{red}, a matter supported by extrusion. Spectrum d of Figure 7, recorded prior to addition of an oxidant, is that of $[Fe_4S_4(SPh)_4]^{2-}$ and corresponds to ≳95% of A_{458} for complete extrusion of 4-Fe sites. The final time-invariant spectrum (e) was obtained after the addition of a small amount of ferricyanide (mole ratio $Fe(CN)_6^{3-}/H_2$ase ≅ 2:1).

Anaerobic extrusion of 15 μM H_2ase solution B is depicted in Figure 8, which reveals that the reduced form, as the analogue trianion, is appreciably oxidized when placed in 80% HMPA solution. Spectrum b of Figure 8 corresponds to ≳84% of A_{458} for complete extrusion of 4-Fe sites. Again, a final time-invariant spectrum (d) was developed after addition of ferricyanide (mole ratio $Fe(CN)_6^{3-}/H_2$ase ≅ 4:1). Anaerobic solution C was treated in much the same manner, except that thiol was added immediately after preparation in order to initiate extrusion before possible enzyme decomposition or Fe-S site reorganization. A constant spectrum was obtained after 10 min whose A_{458} value corresponds to ≳93% of the value for complete extrusion. Addition of ferricyanide yielded the final A_{458} value.[56]

Two additional features of the H_2ase extrusion reactions deserve comment. The PhSH/Fe mole ratios employed were 100–150:1, in keeping with the guideline developed from Fd_{ox} extrusions. More important, however, are the band maximum and band shape of those spectra designated as final in the ex-

trusion reactions of solutions A–C. These correspond closely to the spectral features of isolated pure salts of $[Fe_4S_4(SPh)_4]^{2-}$ when measured in the extrusion medium with a PhSH/Fe mole ratio of ~100:1. From these and other results to be described in following sections, it is concluded that this H_2ase preparation contains only 4-Fe (Fe_4S_4*) sites. The specification of the core unit is intended as a qualification of this statement, inasmuch as the extrusion method directly identifies only the core substructure of the active site and not the terminal ligands.[57] However, there being no evident case of deviation of a 4-Fe site from the essential composition $[Fe_4S_4(S\text{-}Cys)_4]$, it is entirely likely that this description applies to the sites of H_2ase.

Data pertinent to quantitation of the active site core extrusion of hydrogenase are collected in Table III. As in related work,[22] protein concentrations (mg/ml) were determined by the Folin–Lowry method,[35] and molarities were calculated here using a molecular weight of 60 500 daltons. Initial Fe concentrations for each solution were calculated from 11.2 g-atom/mol, the experimental value, or 12 g-atom/mol, the nearest integral multiple of four. The final Fe concentrations, independently determined from the $[Fe_4S_4(SPh)_4]^{2-}$ spectra, are within experimental error of either initial concentration. The results, as those on the lower molecular weight proteins, point to another utility of the extrusion method, viz., rapid determination of a minimal (extrudable) Fe content. Inasmuch as we have yet to encounter a case with proteins of known composition where extrudable Fe does not equal total Fe, this method may prove to be a valuable analytical technique. The values of n, based only on the stated protein concentrations, are 2.8 or 2.9. As required by the initial and final Fe concentrations and the presence of only 4-Fe sites, these values agree to ±0.1, the estimated experimental uncertainty in n, with the number of sites calculated for a H_2ase molecule from initial concentrations. Alternatively, the value of 11.2 g-atom Fe/mol might be viewed as an indication of protein contamination, in which case the true Fe content per mole is 12 g-atom and the effective H_2ase concentration is ca. 0.93 of the values in Table III. In this event n = 3.1 (A), 3.0 (B), and 3.1 (C), calculated from final Fe concentrations. As shown in the table, assumed Fe contents of 4 or 8 g-atom/mol in our 15 μM H_2ase solutions would yield ϵ_{Fe} values which are unacceptably high for 4-Fe Fd_{ox}-type chromophores, and predicted A_{458} values after extrusion which are totally inconsistent with experiment. While there are obvious experimental uncertainties associated with protein concentrations,[58] the present results indicate that this preparation of H_2ase contains more than one, and most probably three, $[Fe_4S_4(S\text{-}Cys)_4]$ sites.

Extrusion Reactions: General Experimental Considerations. The foregoing descriptions of extrusion reactions make clear a number of experimental conditions necessary to the successful application of these reactions, chief among which are (i) disruption of protein tertiary structure in a solvent medium in which the protein is soluble and stable; (ii) anaerobicity at all stages of the reaction due to the oxygen sensitivity of analogues and proteins in aqueous–nonaqueous solutions; (iii) employment of soluble thiols whose analogue chromophores are substantially red-shifted compared to protein absorption; (iv) addition of sufficient thiol and, where appropriate, oxidant to promote quantitative extrusion in a conveniently short time period. In addition to these rather obvious points, there are several other experimental matters of general importance which are next considered, with specific reference to H_2ase extrusion reactions.

(a) Mixed 2-Fe/4-Fe Sites in Proteins. With the higher molecular weight Fe–S proteins there is the possibility of mixtures of the three structural types of sites ($[Fe(S\text{-}Cys)_4]$, $[Fe_2S_2(S\text{-}Cys)_4]$, $[Fe_4S_4(S\text{-}Cys)_4]$) found separately in the lower molecular weight redox proteins,[5] as well as the presence

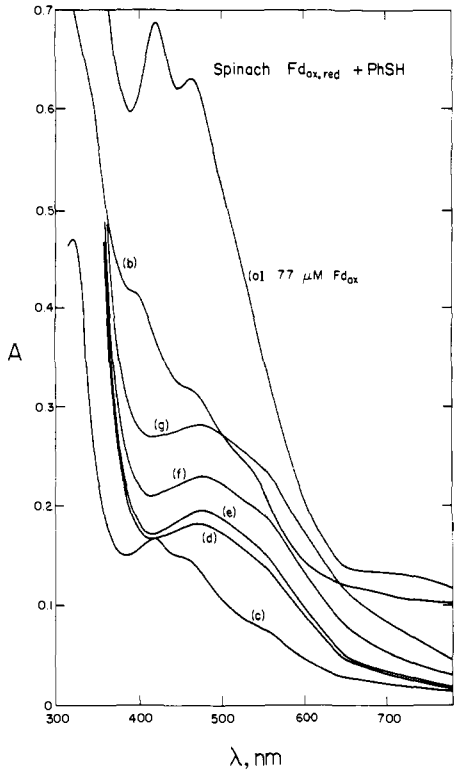

Figure 9. Active site core extrusion of spinach Fd_{ox} + Fd_{red}, l = 1 cm: (a) 77 μM Fd_{ox} in 50 mM TrisCl, pH 8.5; (b) solution a + 20 mM $Na_2S_2O_4$ sufficient to form ~85% Fd_{red}; (c) 0.50 ml of solution b + 1.15 ml of HMPA; (d) solution c + 4 μl of PhSH 20 min after mixing, λ_{max} 472 nm, A_{458}/A_{550} 1.27; (e) solution d after 70 min, λ_{max} 474 nm, A_{458}/A_{550} 1.24; (f) solution e + 0.5 μl of 50 mM $Na_3Fe(CN)_6$ immediately after mixing, λ_{max} 478 nm, A_{458}/A_{550} 1.20; (g) solution f + 1.5 μl of 50 mM $Na_3Fe(CN)_6$ 8 min after mixing, λ_{max} 478 nm, A_{458}/A_{550} 1.17. Further ferricyanide addition caused small absorbance increases and slight precipitate formation.

of as yet unidentified types of sites. The Fe–Mo protein of N_2ase[24b] and succinate dehydrogenase[25] appear to be leading candidates for mixed sites. Bearing in mind the possibility of coextrusion of oxidized 2-Fe and 4-Fe cores from one protein molecule, pairs of arylthiolate dimer and tetramer dianions were synthesized, among them $[Fe_2S_2(SPh)_4]^{2-}$/$[Fe_4S_4(SPh)_4]^{2-}$, and their spectral properties found to be additive and sufficiently different to allow analysis of certain mixtures.[12] In order to examine cases of mixed site extrusion and provide additional support of the foregoing assertion that H_2ase contains only 4-Fe sites, mixtures of *C. pasteurianum* Fd_{ox} and spinach Fd_{ox} were treated with benzenethiol in 80% HMPA. Under similar experimental conditions the extrusion of spinach Fd_{ox} separately was found to be slower than that of the clostridial protein, and was essentially complete after 90 min. Spectra of mixed protein solutions, initially 18–29 μM in total ferredoxin and containing Fe_4S_4*/Fe_2S_2* core mole ratios of 0.25 to 2.2, were recorded at various times up to 90 min after thiol addition. The latter spectra are shown in Figure 10 and experimental conditions and data are given in Table IV. As the proportion of spinach Fd_{ox} in the mixtures is increased, band shapes in the 450–700-nm region broaden, absorption maxima are red-shifted, a shoulder develops near 540 nm, and the A_{458}/A_{550} ratio steadily decreases. As may be seen upon comparison with Figure 11, the spectrum resulting from extrusion of spinach Fd_{ox} alone is that of $[Fe_2S_2(SPh)_4]^{2-}$. As is the case in aprotic solvents,[12] the spectrum of the dimer dianion is red-shifted compared to that of $[Fe_4S_4(SPh)_4]^{2-}$ and in the extrusion medium consists of a maximum at 482 nm (ϵ 11 900) and a pronounced shoulder near 540 nm. The additive nature of the 90-min mixture spectra was demonstrated by

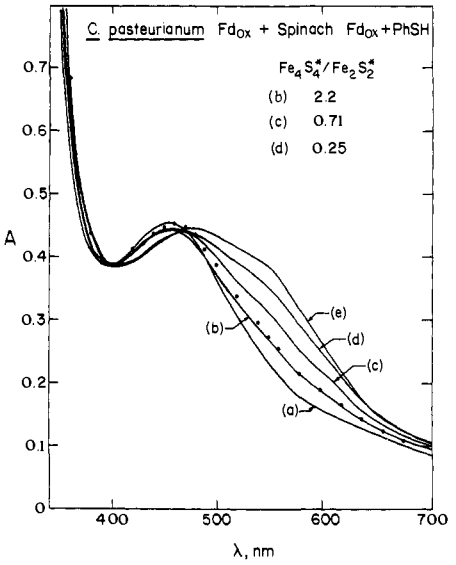

Figure 10. Active site core extrusions of mixtures of *C. pasteurianum* Fd_{ox} (8-Fe Fd_{ox}) and spinach Fd_{ox} (2-Fe Fd_{ox}); experimental conditions are described in Table IV. Spectra of the following initial protein solutions 90 min after addition of benzenethiol are shown: (a) 13 μM 8-Fe Fd_{ox}; (b) 9.6 μM 8-Fe Fd_{ox} + 8.7 μM 2-Fe Fd_{ox}; (c) 6.4 μM 8-Fe Fd_{ox} + 18 μM 2-Fe Fd_{ox}; (d) 3.2 μM 8-Fe Fd_{ox} + 26 μM 2-Fe Fd_{ox}; (e) 35 μM 2-Fe Fd_{ox}. The solid points represent the reconstructed curve for solution b [0.75(a) + 0.25(e)]. Initial core mole ratios are indicated.

Table IV. Active Site Core Extrusion of Mixtures of *C. pasteurianum* Fd_{ox} and Spinach Fd_{ox}[c]

Fd_{ox}, μM					
C. pasteurianum	Spinach	Fe_4S_4*/ Fe_2S_2*	A_{458}/A_{550}	λ_{max}, nm	n[a]
13	0		1.92	458	2.0
9.6	8.7	2.2	1.64	460	
6.4	18	0.71	1.46	463	
3.2	26	0.25	1.23	470	
0	35	0	1.12[b]	482	1.0

[a] Mol liberated analogue/mol protein. [b] For $(Et_4N)_2[Fe_2S_2(SPh)_4]$ in the extrusion medium (mole ratio PhSH/Fe ~100:1), this ratio is 1.15 ± 0.01 (five determinations). [c] Conditions: 4:1 v/v HMPA/H_2O (50 mM TrisCl, aqueous pH 8.5); 2 μl of PhSH added to 1 ml of solution and final spectra recorded 90 min after mixing.

normalizing extrusion spectra of separate proteins to their mixture concentrations and summing the resultant curves. Results for one mixture are shown in Figure 10. The agreement of reconstructed and observed spectra for all three mixtures is satisfactory.

These results demonstrate that oxidized 4-Fe and 2-Fe sites in different proteins (and, by inference, in the same protein) can be quantitatively coextruded, and provide further evidence of the structural integrity of oxidized ($[Fe_4S_4*]^{2+}$, $[Fe_2S_2*]^{2+}$) cores. Additionally, the results do not allow a 4-Fe/2-Fe *extruded* analogue ratio from H_2ase of less than two. If appreciable dimer → tetramer core conversion (vide infra) is absent under extrusion conditions and the Fe content of ~12 g-atom/mol is accepted, this preparation of H_2ase contains only 4-Fe sites.

(b) Site Oxidation Levels and Dimer → Tetramer Core Conversion. In order that the extrusion technique yield reliable results, core units of analogue chromophores elicited from proteins must correspond in number and type to those in protein sites. In our investigations of synthetic analogues two reactions have been observed which result in spontaneous dimer → tetramer core conversion. These are the reactions

$$2[Fe_2S_2(SPh)_4]^{2-} \rightarrow [Fe_4S_4(SPh)_4]^{2-} + PhSSPh + 2PhS^- \quad (6)$$

$$2[Fe_2S_2(SPh)_4]^{3-} \rightarrow [Fe_4S_4(SPh)_4]^{2-} + 4PhS^- \quad (7)$$

with the indicated apparent stoichiometries. We have yet to observe spontaneous tetramer → dimer conversion under anaerobic conditions in any solvent. Reaction 6 was first encountered in the course of extrusion studies of the 2-Fe Fd_{ox} protein from *Spirulina maxima* in 80% Me_2SO/H_2O solutions.[20] In this medium the conversion rate can be rendered negligible compared to the rate of core extrusion by use of sufficiently large PhSH/Fe mole ratios and aqueous component pH ≳ 8.[20,59] Here the stability of $[Fe_2S_2(SPh)_4]^{2-}$, previously isolated and characterized as its Et_4N^+ salt,[12] was investigated in the 80% HMPA extrusion medium containing benzenethiol (Figure 11). The constancy of the spectrum over a 44-h period demonstrates the stability of the dimer dianion. Further, spinach Fd_{ox}, and oxidized adrenodoxin[22] as well, have been successfully extruded with benzenethiol in 80% HMPA. Hence, conversion of a $[Fe_2S_2*]^{2+}$ core to a 4-Fe analogue during extrusion, and therewith incorrect identification of the protein site, is improbable under the conditions employed here.

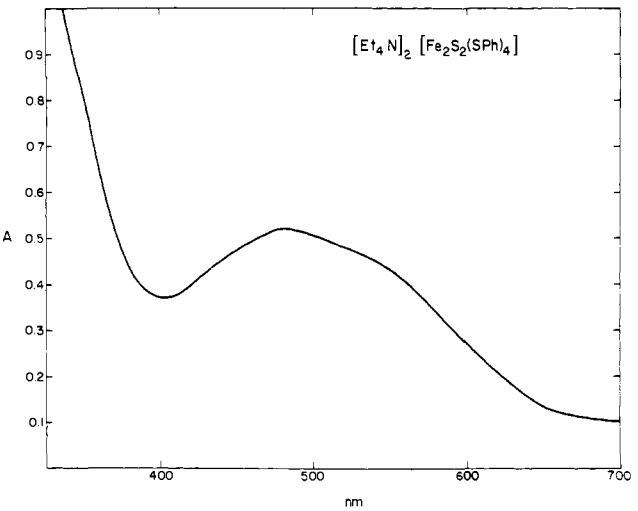

Figure 11. Visible spectrum of ~2 mM $(Et_4N)_2[Fe_2S_2(SPh)_4]$ in 4:1 v/v HMPA/H_2O (50 mM TrisCl, pH 8.5) with a PhSH/Fe mole ratio of ~100:1. Spectra recorded on a freshly prepared solution and after 44 h under anaerobic conditions were the same.

Formation of $[Fe_4S_4(SPh)_4]^{2-}$ from $[Fe_2S_2(SPh)_4]^{2-}$ under electrochemical[12] or strongly reducing chemical conditions[59] has been observed. These processes presumably proceed by reaction 7, in which the dimer trianion is initially formed and converts to the tetramer dianion without change in core oxidation level. This reaction suggests the possibility that, if a reduced 2-Fe site is extruded as $[Fe_2S_2(SPh)_4]^{3-}$, it could spontaneously convert to $[Fe_4S_4(SPh)_4]^{2-}$, resulting in incorrect site identification. Direct examination of reaction 7 is not possible because the dimer trianion has not been isolated nor has a means for unambiguously generating it in solution been attained. A less direct approach has been taken in which, as in the H_2ase extrusions, reduced protein was placed in aqueous HMPA and then treated with benzenethiol. Spectra and experimental details are given in Figure 9. Spinach Fd_{ox} was ≳80% reduced in aqueous solution,[60a] diluted with HMPA to give a 70% v/v HMPA/H_2O solution,[60b] and treated with benzenethiol. Comparison of spectra a–c suggests a small amount of oxidation upon introduction of HMPA. A PhSH/Fe

mole ratio of ~500:1 was used; as with the oxidized protein extrusion was rather slow. The first recognizable spectrum after thiol addition is d, which has the features of $[Fe_2S_2(SPh)_4]^{2-}$; spectra recorded at shorter times after thiol addition appear to correspond to admixtures of c and the dimer dianion spectra. The final spectrum (g) after ferricyanide addition corresponds closely to that of $[Fe_2S_2(SPh)_4]^{2-}$, but with a very small blue shift of the maximum compared to the 80% HMPA spectrum. If this spectrum is assigned to the dimer dianion only, n is calculated to be 1.0. A related set of experiments was conducted in which 30 μM spinach Fd_{ox} in the 80% HMPA extrusion medium was spectrophotometrically titrated with 20 mM dithionite to $\gtrsim$70% Fd_{red}[60a] and then treated with benzenethiol in PhSH/Fe mole ratios varying from 160 to 820:1. The observed behavior in these systems was similar to that in Figure 9. Extrusion proceeded slowly and spectral changes ceased after ca. 90 min. At this point the spectra closely resembled that of $[Fe_2S_2(SPh)_4]^{2-}$, having λ_{max} 474–476 nm and A_{458}/A_{550} 1.12–1.17. Ferricyanide addition afforded absorbance increases close to the values corresponding to n = 1.0, provided the final spectrum is that of the dimer dianion alone. Owing to slight precipitate formation at this stage the extent of extrusion could be determined only semiquantitatively.

These experiments show that under the experimental conditions employed here extrusion reactions of solutions initially containing spinach Fd_{red} yield $[Fe_2S_2(SPh)_4]^{2-}$ as the principal product. The formation of a small amount of $[Fe_4S_4(SPh)_4]^{2-}$ is allowed, but not proven, by the observation of λ_{max} 474–476 nm after 90 min in solutions containing thiol compared to λ_{max} 482 nm found in the extrusion of spinach Fd_{ox} (Table IV). The reduced 2-Fe sites may be extruded as oxidatively unstable $[Fe_2S_2(SPh)_4]^{3-}$ ($E_{1/2}(2-,3-) = -1.09$ V in DMF vs. SCE[12]) which, as $[Fe_4S_4(SPh)_4]^{3-}$, may be autoxidizable in the extrusion medium. We cannot totally eliminate the possibility that some proportion of reduced 2-Fe sites are oxidized[54] prior to extrusion. However, because the same conditions were applied in both the spinach Fd and H_2ase experiments, it is not unreasonable to assume that any 2-Fe sites in the latter would be largely oxidized and hence be extruded as $[Fe_2S_2(SPh)_4]^{2-}$. We interpret the collective results described above as showing that the sole detectable extrusion product of H_2ase, $[Fe_4S_4(SPh)_4]^{2-}$, conveys the correct nature of the active sites in this enzyme preparation. In offering this conclusion we note, however, that no small 2-Fe Fd protein provides a suitable control of certain conceivable extrusion situations. If, for example, in a poly-Fe protein molecule the cores of two closely juxtaposed reduced 2-Fe sites are displaced as dimer trianions, reaction 7 could follow, resulting in incorrect site identification. Another possibility is spontaneous reaction of two such centers to form a Fe_4S_4* entity prior to extrusion. Reactions 6 and 7 are currently under further study.[59] When extruding known or suspected 2-Fe sites it is clearly desirable to adjust these sites to the oxidized level prior to extrusion and to employ large excesses of thiol at an appropriate pH in order to suppress reactions 6 and 7.

(c) **Extraneous Iron.** During the course of the extrusion studies of proteins containing only 4-Fe sites, some instances were encountered in which band shapes and relative intensities of final spectra in the 500–700-nm region did not correspond to those of pure $[Fe_4S_4(SPh)_4]^{2-}$ in 80% HMPA containing excess thiol. This behavior was traced to lack of maintenance of adequately anaerobic conditions and/or the use of stock protein preparations which had been subjected to a number of freeze–thaw cycles. Inasmuch as low-level contamination of iron protein preparations by extraneous or adventitious iron is not uncommon, the following experiments were performed. Dilute solutions of *C. pasteurianum* Fd_{ox} and ferric chloride were treated with excess benzenethiol under extrusion conditions. Red- or blue-violet colors, similar to those in the foregoing instances and readily perceptible by eye, were developed and the solutions showed absorption maxima at 550–560 nm whose intensity decreased with time. Similarly colored and unstable chromophores have been detected in Fe(III)/thiol systems.[61] These observations have led to the use of the absorbance ratio A_{458}/A_{550} as a criterion for an acceptable extrusion reaction of a 4-Fe site; experimental values are included in Tables II and III. Despite considerable care we have found it difficult to achieve the value of 2.18 ± 0.16 observed for pure salts of $[Fe_4S_4(SPh)_4]^{2-}$, but in nearly all cases the lower limit of 2.02 is approached. The largest deviations were found in the more dilute Fd_{ox} solutions, but in such cases n was never less than 1.9. Values below that of the tetramer dianion may be due to extraneous Fe(III) present initially or to slight decomposition of Fe_4S_4* cores before or during extrusion. As an empirical control we have rejected as unsuitable for quantitation of $n(Fe_4S_4*)$ all extrusion experiments with final $A_{458}/A_{550} \lesssim 1.8$. The close similarity among absorbance ratios for extrusions of highly purified ferredoxins and H_2ase indicates that the latter contains little if any iron not organized in 4-Fe sites.

These observations require addition of two other experimental conditions to the foregoing list: (v) adjustment of protein oxidation level, aqueous component pH, and thiol concentration so as to prevent spontaneous dimer → tetramer conversion; (vi) minimization of extraneous iron content in protein preparations. The latter is particularly important if 2-Fe and 4-Fe sites separately or as mixtures are to be quantitated.

Fe Protein of Nitrogenase. As a final check on the extrusion method, the cold-labile, extremely oxygen-sensitive Fe protein (azoferredoxin) of the *C. pasteurianum* N_2ase complex[24a,c,33] was extruded under anaerobic conditions. Data are given in Table I. The preparation employed contained, by prior analysis, 3.4 g-atom Fe/55 000 g. The final spectrum under extrusion conditions is that of $[Fe_4S_4(SPh)_4]^{2-}$, corresponding to a total iron concentration of 143 μM compared to the calculated initial value of 153 μM. The value n = 0.93 was obtained and is within experimental error of 1.0. These results confirm a prior extrusion experiment[24c] in which the presence of a 4-Fe site was demonstrated. Conceivably the single Fe_4S_4* core could act as a bridge between the two apparently identical subunits of ~27 500 daltons. In this connection it is noted that a recent sequence determination of this protein[62] has established that the six Cys residues per subunit are randomly distributed rather than systematically spaced, as is the case for the lower molecular weight clostridial ferredoxins containing eight Cys residues.[63]

Summary

Our previous[20] and present results, together with those of Orme-Johnson and co-workers,[21,22,24c] demonstrate the feasibility of the extrusion technique for detecting and quantitating Fe_2S_2* and Fe_4S_4* active site core structures in lower molecular weight redox proteins and in more complex, higher molecular weight enzymes as well. Results at this stage are sufficient to encourage further extension of the method to enzymes. A number of these may be amenable to the general approach as represented by experimental conditions i–vi, but not necessarily the specific methodology described here. One evident problem with the use of benzenethiol as the extruding agent is interference of its analogue chromophores by those in enzymes, e.g., in succinate dehydrogenase[25] (flavin) and spinach nitrite reductase[64] (siroheme). Development of analogue chromophores in order to circumvent this problem is in progress. The extrusion technique is complementary to other methods of probing active site structures. Among these the temperature dependencies of EPR signal intensities, as indi-

cated in a number of earlier publications, appear to be characteristic of 2-Fe and 4-Fe sites in the smaller proteins and a recent application has been made to the problem of site identification in succinate dehydrogenase.[65] For further details the recent report by Cammack[44] may be consulted.

The principal conclusion from this investigation is that this preparation of *C. pasteurianum* H_2ase most probably contains three $[Fe_4S_4(S\text{-}Cys)_4]$ sites. The presence of fine structure in the EPR spectrum of the reduced enzyme[34] provides additional evidence for the presence of more than one magnetic site. Spectra of this type have been found for a number of 8-Fe Fd_{red} proteins, including *P. aerogenes* Fd_{red},[66] whose 4-Fe sites are separated by ~12 Å,[6,9] and has been interpreted in terms of spin–spin coupling between sites.[45] The composition of this preparation marks it as currently unusual compared to H_2ases from the same[22] and different organisms. The present results reinforce a recent report[27] that a more extensively purified clostridial enzyme preparation contains a higher Fe and S* g-atom content than that (~4Fe, ~4S*) found earlier.[67] The H_2ase from *Chromatium* has been reported to have been purified to homogeneity and to contain ~4Fe and ~4S* per molecular weight of ~100 000 daltons.[68] Different preparations of H_2ase from *Desulfovibrio vulgaris* with variant iron content (~1–8 g-atom) have been described.[69–71] Most recently iron and sulfide contents of 7–9 g-atom have been reported.[72] The spectra of the oxidized forms of these enzymes are similar to that of *C. pasteurianum* H_2ase, suggesting that all may contain 4-Fe sites. The establishment of such sites in the clostridial enzyme represents the necessary first step in a study of the mechanism of the hydrogenase reaction,

$$H_2 + C_{ox} \overset{H_2ase}{\rightleftharpoons} 2H^+ + C_{red} \qquad (8)$$

in which C is a two-electron carrier. We subscribe to the generally held view[26] that dihydrogen activation and heterolytic cleavage, evidenced by H/D isotope exchange in the absence of carrier, must occur at or near an iron center, which is now recognized as incorporated into a 4-Fe site. Isolation[4,11] of the analogues $[Fe_4S_4(SR)_4]^{2-,3-}$ should permit an assessment of activation and catalytic properties of individual 4-Fe clusters in those oxidation levels which we currently consider most likely to be present in the functioning enzyme. These studies may also be useful in assessing the roles of multiple 4-Fe sites in the H_2ase studied here. At present we conjecture that one of these is the catalytic site and remaining clusters constitute an intramolecular electron transfer conduit coupling the former to the endogenous ferredoxin electron carrier. Synthetic hydrogenase systems are currently under investigation in these laboratories.

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(8) Abbreviations: Rd, rubredoxin; Fd, ferredoxin; HP, "high-potential" protein; S*, sulfide (used as necessary for clarity); H_2ase, hydrogenase; S_2-*o*-xyl, *o*-xylyl-α,α'-dithiolate; N_2ase, nitrogenase.
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(55) The points raised here are readily appreciated on examination of the spectra of various Fe–S protein chromophores assembled by Hall et al.[5b]
(56) Owing to limited amounts of H_2ase, no detailed attempts have been made to examine certain potential variables of the extrusion reaction, including the minimal amounts of ferricyanide required to produce the final A_{458} values obtained by addition of the quantities stated in Table III.
(57) We have been unable to bind ligands simulating other types of peptide side-chain coordination to $Fe_4S_4^*$ cores. Consequently, it has not been established whether groups other than thiolate undergo ligand substitution with thiols.
(58) If, e.g., the true n values for solution A were 2.0 or 4.0, the stated protein concentration would have to be in error by ca. ±40%. The close agreement between initial calculated and independently determined final Fe molar concentrations makes an error of this size improbable.
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Alkylation of a Tripeptide by a Carcinogen: The Crystal Structures of Sarcosylglycylglycine, 9-Methyl-10-chloromethylanthracene, and Their Reaction Product

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Abstract: The effect of alkylation of a peptide by a polycyclic aromatic hydrocarbon has been investigated as a model for protein–carcinogen interactions. The tripeptide sarcosylglycylglycine (I) reacts with 9-methyl-10-chloromethylanthracene (II) with the elimination of HCl to form the alkylated tripeptide (III). The crystal data are (I) $a = 20.847$ (3), $b = 10.252$ (1), and $c = 8.719$ (1) Å, space group $Pbca$, $Z = 8$; (II) $a = 14.379$ (1), $b = 16.359$ (1), $c = 5.0993$ (4) Å, space group $P2_12_12_1$, $Z = 4$; and (III) $a = 19.022$ (4), $b = 10.727$ (2), $c = 10.728$ (2) Å, $\beta = 94.24$ (2)°, space group $P2_1/c$, $Z = 4$. The crystal structures were determined by direct methods using MULTAN and refined by least-squares techniques. The tripeptide and the alkylated tripeptide both occur as zwitterions. In the crystalline alkylated peptide, III, the hydrophobic polycyclic groups stack together, in a manner similar to that found in the simple alkylating agent, II. The structure of III consists of layers of aromatic side chains, peptide residues, and water of crystallization. In addition, one of the peptide groups in the alkylated tripeptide is nonplanar (torsion angle 159°), possibly a result of packing forces in the crystal. The simple peptide I does not crystallize with water of crystallization. The crystal structure involves extensive hydrogen bonding, although a pleated sheet structure is not formed. The zwitterion folds so that part of the structure of the peptide I resembles the conformation found in an α helix. This helicity is lost on alkylation.

When polycyclic aromatic hydrocarbons interact with living matter they are generally metabolized, by enzyme systems, to compounds containing hydroxy or keto groups. These metabolites, now more soluble in water than the parent hydrocarbon, generally are then excreted. However some hydrocarbon molecules are found to become covalently bound to proteins and to nucleic acids, possibly through the interaction of these macromolecules with the metabolites of the polycyclic aromatic hydrocarbons.[1-5] Carcinogenesis may result from the interaction of certain (but not all) polycyclic aromatic hydrocarbons with living cells, but it is not yet clear whether the critical target is a protein or a nucleic acid.[6,7]

In order to investigate the molecular basis of chemical carcinogenesis by polycyclic aromatic hydrocarbons we are