A ^{19}F NMR Method for Identification of Iron–Sulfur Cores Extruded from Active Centers of Proteins, with Applications to Milk Xanthine Oxidase and the Iron–Molybdenum Proteins of Nitrogenase

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Abstract: The core extrusion technique has recently been developed as a chemical method of identification of the type and number (n_d, Fe_2S_2; n_t, Fe_4S_4) of Fe-S sites in proteins. The method is based on removal of Fe_nS_n core structure (n = 2, 4) with excess thiol in a medium capable of unfolding protein structure, a reaction followed in usual practice by spectrophotometric assay of the extrusion products $[Fe_2S_2(SR)_4]^{2-}$ and/or $[Fe_4S_4(SR)_4]^{2-}$ in the visible region. To circumvent possible interference by protein visible chromophores such as flavins, a Fourier transform (FT) ^{19}F NMR method has been developed using p-$CF_3C_6H_4SH$ (R_FSH) as the extrusion reagent. The corresponding extrusion products $[Fe_2S_2(SR_F)_4]^{2-}$ and $[Fe_4S_4(SR_F)_4]^{2-}$ exhibit contact-shifted ^{19}F resonances which are separated by ~3 ppm and are well resolved from the R_FSH resonance. Control experiments with small ferredoxin proteins have demonstrated that in 4:1 v/v hexamethylphosphoramide/H_2O solutions R_FSH effects quantitative removal of Fe_nS_n cores within the estimated accuracy of the spectrophotometric (±10%) and NMR (±15%) methods. Quantitation in the latter method is achieved by integration of signal intensities after completion of the extrusion reaction and after addition of standard solutions of extrusion products. Application of the method to milk xanthine oxidase (8Fe, 2Mo, 2FAD per α_2 subunit structure) yielded n_d = 1.9–2.1/FAD, showing that all Fe in this enzyme is organized into 4 Fe_2S_2 centers. Spectrophotometric extrusion of deflavoxanthine oxidase afforded n_d = 4.3 based on total Fe, in good agreement with the results of the NMR method. Extrusion reactions of FeMo proteins (23–30 Fe, 1.8–2.0 Mo per $\alpha_2\beta_2$ subunit structure) of the nitrogenases from *Clostridium pasteurianum* and *Azotobacter vinelandii* yielded n_t = 1.2–1.4 for the reduced forms and n_t = 1.2–1.6, n_d = 0.8–1.5 for the thionin-oxidized forms. When normalized to $32Fe/\alpha_2\beta_2$ subunit structure, n_t = 1.4–2.0 for the reduced forms and n_t = 1.4–2.0, n_d = 1.0–1.6 for the oxidized forms. Results for the clostridial protein indicated the presence of at least two Fe_4S_4 sites in both oxidation levels and one Fe_2S_2 site in the oxidized form. Removal of all Fe not included in the FeMo cofactor was ca. 40–60% complete. The lack of a higher extent of extrusion of noncofactor Fe is attributed to insufficient unfolding of FeMo protein structure by the 4:1 v/v hexamethylphosphoramide/H_2O medium.

Introduction

The facile thiolate ligand substitution reactions with aryl thiols exhibited by the complexes $[Fe_2S_2(SR)_4]^{2-}$ [2] and $[Fe_4S_4(SR)_4]^{2-}$ [3] (R = alkyl) have led to the development of a new technique for the structural identification in iron–sulfur proteins of 2-Fe and 4-Fe sites, for which these complexes serve as synthetic analogues.[4,5] This technique is based on reaction 1 carried out anaerobically in aqueous–nonaqueous solvent

$$\text{holoprotein} + \text{RSH} \rightarrow \left\{\begin{matrix}[Fe_2S_2(SR)_4]^{2-} \\ \text{and/or} \\ [Fe_4S_4(SR)_4]^{2-}\end{matrix}\right\} + \text{apoprotein} \quad (1)$$

media capable of unfolding protein tertiary structure and containing a large molar excess of thiol to Fe (typically ≳100/1) over the stoichiometric ratio. In this reaction, Fe_2S_2 and/or Fe_4S_4 cores[4] of the protein sites, which in ferredoxins (Fd) have the essential compositions $[Fe_2S_2(S\text{-}Cys)_4]$ and $[Fe_4S_4(S\text{-}Cys)_4]$, are extruded[6,7] (in an alternative description, displaced[8]) in the form of their synthetic analogues. In conventional practice, the concentrations of the latter species are assayed spectrophotometrically in the reaction system at 400–700 nm. If there are no interfering chromophores, dimer–tetramer core interconversion[8,9] is absent, and extrusion is complete, this procedure affords determination of total extrudable Fe content and its organization in terms of the number of 2-Fe and 4-Fe sites (n_d, Fe_2S_2; n_t, Fe_4S_4) in a protein molecule.

Previous research with a variety of low molecular weight Fd proteins (6000–25 000) having known 2-Fe and 4-Fe sites has amply demonstrated that their Fe_2S_2 and Fe_4S_4 cores can be extruded and quantitated spectrophotometrically.[6-8,10-12] Useful experimental conditions include 4:1 v/v hexamethylphosphoramide (HMPA)/H_2O as a solvent medium and benzenethiol as an extrusion reagent. Values of n_d and n_t within ±10% of those expected on the basis of protein composition and concentration are typically obtained. The core extrusion method has also been applied to larger proteins whose Fe-S sites were not necessarily well defined beforehand. Thus the Fe protein (58 000 daltons) of *Clostridium pasteurianum* nitrogenase has been found to contain one Fe_4S_4 cluster,[7,11] different preparations of the hydrogenase of *C. pasteurianum* (60 000 daltons) contain three[7] or less[10] Fe_4S_4 clusters, and the hydrogenase of *Desulfovibrio gigas* (90 000 daltons) possesses three such clusters.[13] The only chromophores present in all of these proteins are extrudable Fe-S sites.

Examination of other proteins and enzymes whose Fe-S centers might profitably be investigated by the core extrusion technique[14] reveals that the large majority contain visible chromophores (e.g., flavin, heme) or other components (e.g., Mo), which might form such chromophores in the presence of added thiol. In such cases accurate spectrophotometric determination of extruded analogues would be impossible unless separation procedures are employed or suitable blanks are available for use in difference spectrophotometry.[15] Possible alternative means of distinguishing and quantitating extruded analogues directly in the reaction system have been outlined.[14] One of these, a ^{19}F NMR method using a fluorinated thiol as the extruding reagent and requiring resolvable chemical-shift differences among this reagent and the two extrusion products in reaction 1, has been investigated in this research and communicated recently.[16] The purpose of this paper is twofold: to report full experimental details which provide the foundation

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Table I. Properties of $[Fe_2S_2(SR)_4]^{2-}$, $[Fe_4S_4(SR)_4]^{2-}$, RSH, and RSSR (R = *m*- and *p*-$C_6H_4CF_3$)

species	δ, ppm		λ_{max}, nm (ϵ_M)	*E*, V,[i,j] 2−/3−, 3−/4−
	^{19}F[a]	^{1}H[g]		
$[Fe_2S_2(S\text{-}m\text{-}C_6H_4CF_3)_4]^{2-}$	10.4[b]	−5.23 (*o*-H), −9.32 (*m*-H), −3.78 (*p*-H)	277 (27 000), 314 (21 700) 475 (11 100), 540 (sh, 9070)[i]	−1.16,[k] −1.47[k]
$[Fe_4S_4(S\text{-}m\text{-}C_6H_4CF_3)_4]^{2-}$	10.1[b]	−6.31 (*o*-H), −8.31 (*m*-H), −5.63 (*p*-H)	300 (sh, 29 600), 360 (sh, 19 700) 444 (17 000)[i]	−0.88,[l] − 1.73[k]
$m\text{-}CF_3C_6H_4SH$	10.2,[b] 9.6[c]	−7.4 (m)		
$[Fe_2S_2(S\text{-}p\text{-}C_6H_4CF_3)_4]^{2-}$	2.8,[b] 3.7[d]	−5.14 (*o*-H), −9.40 (*m*-H)	476 (11 200)[d,h] 290 (30 100), 340 (sh, 20 400) 475 (11 100), 540 (sh, 9300)[i]	−1.05,[k] −1.43[k]
$[Fe_4S_4(S\text{-}p\text{-}C_6H_4CF_3)_4]^{2-}$	6.3[b,d]	−6.10 (*o*-H), −8.38 (*m*-H)	452 (18 000)[d,h] 278 (40 200), 300 (sh, 37 200), 360 (sh, 22 500), 447 (17 900)[i]	−0.83,[l] −1.56[l]
$p\text{-}CF_3C_6H_4SH$	9.6,[b] 9.5[e] 8.0[d,f]	−7.4 (m)		
$p\text{-}CF_3C_6H_4\text{-}S\text{-}S\text{-}C_6H_4\text{-}p\text{-}CF_3$	9.8[b,e]	−7.60		

[a] Shifts upfield from $PhCFCl_2$ internal standard. [b] Me_2SO (35 °C). [c] Anion in 80% Me_2SO/H_2O (35 °C). [d] 80% HMPA/H_2O (50 mM Tris-Cl, pH 8.5), RSH/Fe mol ratio ~100:1, NMR at −15 °C, spectra at 25 °C. [e] 80% HMPA/H_2O (−15 °C). [f] Anion. [g] Shifts downfield from Me_4Si internal standard in CD_3CN (35 °C), disulfide in $CDCl_3$. [h] Higher energy bands obscured by thiol. [i] DMF (25 °C). [j] Versus SCE. [k] $E_{p(c)}$, irreversible step. [l] $E_{1/2} = (1/2)(E_{p(c)} + E_{p(a)})$.

for present and future application of the method, and to describe the results of its application to two enzymes of continuing intense interest, milk xanthine oxidase[17] and the FeMo proteins of the nitrogenases[1t,18] from *C. pasteurianum* and *Azotobacter vinelandii*.

Experimental Section

All operations and manipulations in the preparation of compounds and their physical measurements were performed under a purified dry dinitrogen atmosphere.

Preparation of Compounds. Physical properties of compounds prepared by the following procedures are collected in Table I.

***p*-Trifluoromethylbenzenethiol.** This compound has been reported previously only in the patent literature;[19] the following procedure is based in part on that report and the synthesis of $o\text{-}CF_3C_6H_4SH$.[20] After distillation (70 °C (10 Torr)) 29.9 g (186 mmol) of 4-aminobenzotrifluoride (Aldrich) was dissolved in 200 mL of boiling 6 M hydrochloric acid plus 100 mL of water. Cooling to <5 °C caused separation of the white, crystalline amine hydrochloride. To this mixture was added in small portions a solution of 13.1 g (190 mmol) of $NaNO_2$ in 30 mL of water, with the temperature maintained below 10 °C. The diazonium salt solution was added dropwise over the course of 1 h to a solution of potassium ethylxanthate[21] (13.6 g, 83.7 mmol) in water at 40–50 °C. The reaction mixture was kept basic by the addition of 40% NaOH (150 mL total), and 27.1 g (169 mmol) of solid potassium ethylxanthate was added in portions in order to maintain it in excess. After addition was complete, the reaction mixture was stirred for an additional hour and extracted with ether. The extracts were washed with 10% NaOH and saturated aqueous NaCl and dried over $MgSO_4$. The red oil (~30 mL) remaining after solvent removal, 120 mL of absolute ethanol, and 50 g of KOH pellets were combined, degassed, and refluxed for 20 h under nitrogen. The crude potassium thiolate remaining after solvent removal was dissolved under anaerobic conditions in 200 mL of water, and this solution was adjusted to pH <2 with 3 M H_2SO_4. The acidified mixture was extracted with ether, the ether solution extracted anerobically with 10% NaOH, the aqueous layer acidified as previously, and the thiol extracted into ether. After we dried the ether solution over $MgSO_4$, it was concentrated to a red oil containing a pale orange solid. This mixture was distilled at 60 °C (10–15 Torr) to afford 10.5 g (32%) of the pure thiol as a colorless liquid: d_{25} = 1.29 g/mL. Anal. Calcd for $C_7H_5F_3S$: C, 47.19; H, 2.83; S, 18.00. Found: C, 47.18; H, 2.73; S, 18.22.

***m*-Trifluoromethylbenzenethiol.** This compound was prepared in 35% yield by the reaction of diazotized *m*-aminobenzotrifluoride (Aldrich) with potassium ethylxanthate followed by base hydrolysis, acidification, and purification by vacuum distillation. The procedure was similar to that described for $p\text{-}CF_3C_6H_4SH$; identity and purity of the product were established by NMR (Table I). This compound has also been prepared by a Grignard reaction of $m\text{-}CF_3C_6H_4I$.[22]

Bis(*p*-trifluoromethylbenzene) Disulfide. *p*-Trifluoromethylbenzenethiol (0.25 mL, 1.8 mmol) was dissolved in a solution of 0.5 g of potassium hydroxide in 5 mL of water. To this solution was added dropwise 7.0 mL of a 0.56 M solution of $K_3Fe(CN)_6$ (3.9 mmol), resulting in the formation of a white solid and a yellow supernatant. The solid was collected by filtration, washed with water, and dried. Purification was effected by recrystallization from 5 mL of hot ethanol to which water was added until the solution became turbid. The solution was heated to dissolve any oil which formed, seeded after it had cooled to 50 °C, and then slowly cooled to −20 °C; 0.31 g (97%) of colorless needles was obtained after washing with water and drying in vacuo. This compound was also prepared by the oxidation of the sodium salt of the thiol in methanol with iodine. After purification the products from both oxidation reactions have identical melting points, 52.5–54 °C,[23] which is also the melting point of their mixture. Anal. Calcd for $C_{14}H_8F_6S_2$: C, 47.46; H, 2.28; S, 18.10. Found: C, 47.28; H, 2.40; S, 18.26.

α,α-Dichloro-α-fluorotoluene. This compound was desired as a ^{19}F NMR chemical-shift standard and was prepared according to a published method[24] by refluxing $C_6H_5CCl_3$ with Na_2SiF_6 in the presence of iron powder. The mixture of $C_6H_5CCl_3$, $C_6H_5CCl_2F$, $C_6H_5CClF_2$, and $C_6H_5CF_3$ was separated by spinning band distillation. Center cuts of the fraction with bp 96 °C (~50 Torr[25]) contained only $C_6H_5CCl_2F$ by GLC and ^{19}F NMR analysis.

$(Et_4N)_2[Fe_4S_4(SC_6H_4\text{-}p\text{-}CF_3)_4]$. To a solution of 1.71 g (1.76 mmol) of $(Et_4N)_2[Fe_4S_4(S\text{-}t\text{-}Bu)_4]$[26] in 125 mL of acetonitrile at 35 °C was added 1.02 mL (7.24 mmol) of $p\text{-}CF_3C_6H_4SH$. A rapid color change from yellow-brown to orange-brown occurred. After we stirred the reaction mixture for 2 h at room temperature, it was evaporated to dryness in vacuo. The residue was dissolved in 20 mL of acetonitrile, filtered, and warmed to 50 °C. Addition of 80–100 mL of 2-propanol followed by cooling to room temperature and partial solvent removal in vacuo initiated crystallization. The solution was warmed to 40–50 °C to dissolve most of the solid and then allowed to cool slowly to −20 °C. The product was collected by filtration, washed with 2-propanol (3 × 40 mL), and dried in vacuo; 1.85 g (80%) of large black rhom-

bohedral flakes was obtained. Using this procedure, the product was purified by recrystallization from acetonitrile/2-propanol. Anal. Calcd for $C_{44}H_{56}F_{12}Fe_4N_2S_8$: C, 40.01; H, 4.27; Fe, 16.91; N, 2.12; S, 19.42. Found: C, 40.08; H, 4.10; Fe, 16.69; N, 2.32; S, 19.13.

$(Et_4N)_2[Fe_2S_2(SC_6H_4\text{-}p\text{-}CF_3)_4]$. To a stirred slurry of 1.41 g (1.82 mmol) of $(Et_4N)_2[Fe_2S_2(S_2\text{-}o\text{-}xyl)_2]$[2] ($S_2$-*o*-xyl = *o*-xylyl-$\alpha,\alpha'$-dithiolate) in 50 mL of acetonitrile was added 2.5 mL (18 mmol) of *p*-$CF_3C_6H_4SH$. The color of the reaction mixture became purple-red as the solid dissolved. The reaction was hastened by warming the solution to 40-50 °C until no solid was visible (~10 min). After filtration of the solution, diethyl ether was slowly added to precipitate fine purple-black crystals. (Addition of ether should be stopped when the supernatant appears brownish.) The product was collected by filtration, washed with 2 × 50 mL of ether, and dried in vacuo. This material was recrystallized by dissolving it in a minimum volume of acetonitrile (~50 mL), filtering, and adding 2-3 vol of ether; 1.54 g (74%) of purple-black crystals was obtained. Anal. Calcd for $C_{44}H_{56}F_{12}Fe_2N_2S_6$: C, 45.95; H, 4.93; Fe, 9.75; N, 2.45; S, 16.80. Found: C, 45.94; H, 4.75; Fe, 9.89; N, 2.32; S, 16.52.

$(Et_4N)_2[Fe_4S_4(SC_6H_4\text{-}m\text{-}CF_3)_4]$. To a solution of 1.23 g (1.24 mmol) of $(Et_4N)_2[Fe_4S_4(S\text{-}t\text{-}Bu)_4]$[26] in 125 mL of acetonitrile at 40-50 °C was added 1 mL (~6 mmol) of *m*-$CF_3C_6H_4SH$. The stirred mixture was allowed to react overnight at room temperature. Solvent removal in vacuo left an oily solid which was taken up in 50 mL of methanol. After we heated the solution to 40-50 °C, it was filtered and allowed to cool. Small black crystals were isolated by filtration, washed with methanol, and dried in vacuo. The product (1.12 g, 67%) was recrystallized from warm methanol and obtained in 34% yield. Anal. Found: C, 39.76; H, 4.35; N, 1.98.

$(Et_4N)_2[Fe_2S_2(SC_6H_4\text{-}m\text{-}CF_3)_4]$. To a stirred slurry of 0.73 g (0.95 mmol) of $(Et_4N)_2[Fe_2S_2(S_2\text{-}o\text{-}xyl)_2]$[2] in 35 mL of acetonitrile was added 3 mL (~20 mmol) of *m*-$CF_3C_6H_4SH$, whereupon the color of the reaction mixture changed immediately to purple-red. After we stirred the solution for 1 h, during which time all solid dissolved, the volume of the solution (maintained at 60 °C) was reduced to 15 mL, and 50 mL of THF was added. Slow cooling to −20 °C caused separation of crystals, which were collected by filtration, washed with THF, and dried in vacuo. The product (0.73 g, 68%) was twice recrystallized from acetonitrile-THF containing excess thiol and obtained in 23% yield. Anal. Found: C, 46.00; H, 4.87; N, 2.35.

Proteins. *C. pasteurianum* 8-Fe Fd_{ox} (A_{390}/A_{285} = 0.80) was obtained by the procedure of Mortenson.[27] Spinach Fd_{ox} (A_{420}/A_{285} = 0.44) was prepared according to Buchanan and Arnon[28] or was a commercial sample (Sigma Chemical Co.) purified to the indicated absorbance ratio. *C. pasteurianum* 2-Fe Fd_{ox} was isolated from crude cell extracts by DEAE column chromatography essentially as described.[29] Passage through a second DEAE column followed by Sephadex G-100 chromatography gave protein which exhibited a single band upon $NaDodSO_4$ disc gel electrophoresis. *Chromatium* high-potential protein[30] (HP, A_{283}/A_{385} = 2.57) was a gift from Dr. R. G. Bartsch.

Xanthine oxidase was purified by the procedure of Massey et al.[31] from raw unpasteurized buttermilk (Altadena Dairy, Industry, CA) to A_{280}/A_{450} = 5.3-5.9 (lit.[31] 5.4). Activity:flavin ratios of 90-140 were obtained in multiple preparations (average value ~120). Deflavoxanthine oxidase was prepared from the native form by the KI procedure of Kanda et al.:[32] A_{450}/A_{550} = 1.95 (lit.[32,33] 1.8-1.9).

The FeMo protein of nitrogenase from *C. pasteurianum* W5 was isolated and purified essentially as described by Zumft and Mortenson,[34] except that the Sephadex G-100 chromatography step was eliminated and the crude extract was immediately subjected to repetitive DEAE-cellulose column chromatography. In one preparation (no. 2), the 2-12% protamine sulfate treatment was also eliminated and the crude extract was chromatographed on DEAE-cellulose using a 0.1 → 1 M NaCl gradient in 0.5 M Tris-Cl pH 8 buffer followed by the repetitive DEAE column chromatography and finally by Sephadex G-200 column chromatography. The FeMo protein of nitrogenase from *Azotobacter vinelandii* OP was prepared by a modification of the procedure outlined by Shah and Brill.[35] Suspensions of thawed cell paste were disrupted in a Model 15M Gaulin homogenizer (Gaulin Corporation, Everett, Mass.). A second modification involved the use of a salt gradient as opposed to batch elution during the second DEAE column chromatography. Protein molybdenum was determined by the method of Clark and Akley,[36] total protein iron by the method of Lovenberg et al.,[37] acid-labile sulfur by the method of Brumby et al.,[38] and protein concentrations by the procedures of Gornall et al.[39] and Lowry et al.[40] The following analytical data (g-atoms, based on the indicated $\alpha_2\beta_2$ molecular weight) and activities (nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$) were obtained. *C. pasteurianum* FeMo protein (220 000 daltons[41a]): preparation 1, 1.78 Mo, 23.4 Fe, 23.2 S, 1583; preparation 2, 1.88 Mo, 28.4 Fe, 1466. *A. vinelandii* FeMo protein (245 000 daltons[41b]): 2.01 Mo, 29.8 Fe, 30.4 S, 1807. EPR measurements of these protein preparations at 4.2 K gave g = 4.26, 3.76, and 2.01 and g = 4.32, 3.64, and 2.01 for the principal signals of the *C. pasteurianum* and *A. vinelandii* proteins, respectively. Signal shapes and g values are in good agreement with previously reported results.[11,18,42]

Solvents and Reagents. Acetonitrile was distilled from calcium hydride. DMF (~40 °C (20 Torr)) and Me_2SO (~70 °C (10 Torr)) were freshly distilled from barium oxide and stored under argon. HMPA (Aldrich) was distilled under reduced pressure at ~60 °C from barium oxide and stored under argon at −20 °C in the dark. Not more than 3 days prior to use, HMPA was redistilled from P_2O_5 and handled anaerobically to minimize peroxide formation.[43] Trizma base (Tris, Sigma), thionin (Baker), and sodium dithionite (85%, BDH Chemicals, Ltd.) were commercial samples used as received.

Physical Measurements. Electronic absorption spectra were recorded on a Cary Model 14, 17, or 219 spectrophotometer. Cyclic voltametric measurements were performed with previously described equipment[9] using a Pt disk working electrode, a SCE reference electrode, and DMF solutions containing 1 mM complex and 0.1 M $[(n\text{-}Pr)_4N](PF_6)$ supporting electrolyte. Magnetic susceptibilities were determined with a Superconducting Technology, Inc. SQUID-type susceptometer as described elsewhere.[44] EPR spectra were measured at X-band frequencies using a Varian E-12 spectrometer equipped with an Air Products Model LT-3-110 Helitran refrigerator and a Model APD-B temperature controller. ^1H and ^{19}F NMR spectra were obtained in the Fourier transform mode with either a Varian XL-100 spectrophotometer equipped with an XL-100 variable-temperature accessory or a modified Bruker HXS-360 high-resolution spectrometer utilizing an Oxford 84.6-kG magnet. The latter instrument is located in the Stanford Magnetic Resonance Laboratory, whose staff fabricated a probe for observation of ^{19}F NMR at 338.7 MHz; the corresponding XL-100 spectrometer frequency is 94.1 MHz. A Nicolet Instrument Corporation Model 1180 data system was used for acquiring and processing data. All spectra were acquired using a quadrature phase detection system (sweep width ±1200 Hz (XL-100), ±5000 Hz (HXS-360); 4K computer memory; 2 Hz exponential multiplication). Solutions whose spectra were obtained on the XL-100 spectrometer contained 20% v/v HMPA-d_{18} (Aldrich) as an internal deuterium lock; no deuterium lock was required on the HXS-360 instrument. ^1H and ^{19}F chemical shifts are referenced to the internal standards Me_4Si and $PhCFCl_2$, respectively.

Active-Site Core Extrusions. The equipment employed, preparation of solutions, and provisions for maintenance of anaerobic conditions have been described earlier.[7] All extrusion reactions were conducted in 4:1 v/v (80%) HMPA/H_2O solutions using *p*-$CF_3C_6H_4SH$ (7.24 μmol/μL) as the extrusion reagent (vide infra). Aqueous protein solutions prior to dilution with HMPA contained Tris buffer and other additives specified in Tables II-V and appropriate figure legends. In these solutions Fd_{ox} protein concentrations[7] and xanthine oxidase flavin[31] concentrations were determined spectrophotometrically; FeMo protein concentrations (mg/mL) were obtained by the procedures above. Except for several cases in which it was desired to examine the protein absorption spectrum in the extrusion medium before reaction, the thiol reagent was present in the HMPA used in dilution of the protein solutions. In the ^{19}F NMR method the aqueous protein solution and HMPA (containing thiol and $PhCFCl_2$ internal standard) were mixed at ice temperature in an argon filled 5-mm o.d. NMR tube equipped with a latex septum (Wilmad Glass Co.). With aqueous protein concentrations ≳75 mg/mL, dropwise addition of the HMPA component to the protein solution at ice temperature in a septum-capped vial followed by thorough mixing was necessary to ensure complete dissolution of the protein. These solutions were then transferred anaerobically to NMR tubes by syringe; the total volume of solutions used in the NMR method was ~0.5 mL. The semireduced FeMo protein solutions contained ≲1 mM $Na_2S_2O_4$ prior to dilution with HMPA. Solutions containing oxidized FeMo proteins were prepared by titrations of solutions of the semireduced forms with 30-50 mM thionine containing 50 mM Tris-Cl (pH 8.5). Thionine concentrations were determined spectrophotometrically.[45] The re-

actions were monitored by observing at 4.2 K diminution of the g = 4.3 signal characteristic of the semireduced form.[11,18,42] The oxidized forms of the *C. pasteurianum* and *A. vinelandii* FeMo proteins produced by this procedure exhibited <5 and <15%, respectively, of their original signal intensities.

After completion of the extrusion reaction, each of which was conducted at 25 °C, concentrations of the extrusion products $[Fe_2S_2(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ (d^{2-}) and/or $[Fe_4S_4(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ (t^{2-}) were determined by a spectrophotometric[7] or NMR procedure. In the former, n values were calculated from eq 2:

$$n = A/\epsilon l C_{\text{protein}} \tag{2}$$

using absorbances of final (limiting) spectra and the extinction coefficients in Table I. In the NMR method a known volume of a standard DMF solution, 10-60 mM in d^{2-} or t^{2-}, was added to a fixed volume of the extrusion solution after the ^{19}F spectrum of this solution had been obtained at −15 °C. The spectrum of this solution was then acquired under identical conditions and usually for the same period of time as for the initial solution. Amounts of added standard were chosen such that a ca. 2:1 ratio of final (f):initial (i) signal intensities was obtained. These intensities were determined by planimetric integration of appropriately expanded spectra. Equation 3 was used to calculate n values from concentrations, C_i, of d^{2-} or t^{2-} prior to addition of a known volume, V_s, of standard solution of concentration C_s.

$$n = C_i/C_{\text{protein}} = \frac{V_s C_s}{\left[V_f\left(\frac{I_f}{I_i}\right) - V_i\right] C_{\text{protein}}} \tag{3}$$

Further experimental details concerning the extrusion reactions may be found in the tables and figure legends. In the latter, information enclosed in parentheses refers to the aqueous solution prior to dilution with HMPA; all other data pertain to the 80% HMPA/H_2O solutions.

Results and Discussion

To illustrate certain of the difficulties associated with a spectrophotometric determination of the extrusion products of an Fe-S protein having a visible chromophore, attention is directed to the results of the reaction of oxidized milk xanthine oxidase (XO) with $p\text{-}CF_3C_6H_4SH$ in 80% HMPA/H_2O shown in Figure 1. Native XO in its most highly purified form[17a] is composed of two ca. 140 000-dalton subunits, each of which contains 1 flavin (FAD), 1 g-atom of molybdenum, and 4 g-atoms each of iron and sulfide. The intense visible band in aqueous solution (ϵ_M^{450}/FAD = 36 000[17a]) arises from the combined absorbance of the FAD and Fe-S chromophores. In the 80% HMPA/H_2O solution, 12 μM in flavin, the band shape is only slightly altered and the intensity at λ_{max} 450 nm is decreased by 20% compared to the aqueous solution spectrum. Addition of sufficient thiol to afford a thiol/Fe mole ratio of 300:1 per subunit results in spectral changes which at ≳540 nm are complete after ~45 min but occur over a 4-h period in the region of flavin absorbance. Limiting spectrum e indicates bleaching of the flavin chromophore in the presence of thiol and is highly suggestive of the formation of $[Fe_2S_2(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ (vide infra). If the observed A_{476} value is assumed to be due only to this species, n_d is calculated to be 2.2/FAD. However, without suitable controls it is difficult to justify the assumptions that in the ca. 450-600-nm region flavin bleaching is complete and that absorbance by any Mo-thiol chromophores which may possibly form is negligible. Furthermore, a number of Fe-S enzymes contain heme groups[14] whose intense visible absorbance is likely to be little altered under extrusion conditions. These and related observations have led to the development in this laboratory of an alternative method of detecting and quantitating extrusion products of 2-Fe and 4-Fe sites.

Preparation and Properties of 2-Fe and 4-Fe Fluorinated Complexes. The new method of detection and quantitation of protein extrusion products, which has been briefly described earlier,[16] utilizes ^{19}F FT NMR spectroscopy. Basic require-

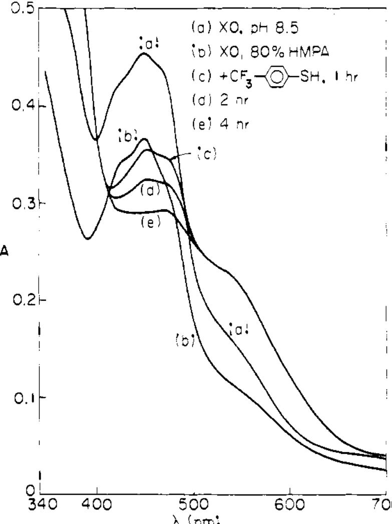


Figure 1. Reaction of native XO with $p\text{-}CF_3C_6H_4SH$ in 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 8.5), l = 1 cm, 25 °C: (a) XO in aqueous buffer, 12 μM in flavin; (b) 0.2 mL of XO solution in aqueous buffer (60 μM in flavin) + 0.8 mL of HMPA; solution b + 2 μL of thiol (c) 1 h, (d) 2 h, and (e) 4 h after addition. The limiting spectrum (λ_{max} 470, 540 (sh) nm) is e.

ments of the method include (i) complete resolution of the ^{19}F signals of the 2-Fe and 4-Fe complexes of the fluorinated extrusion reagent and of the large excess of this reagent present under extrusion conditions; (ii) the inclusion of as many equivalent ^{19}F nuclei as possible in the complexes in order to produce suitable signal/noise ratios when normalized against the stringencies of protein concentration and available spectrometer time. Two fluorinated thiols and their complexes were investigated. The dimeric (**3**) and tetrameric (**4**) complexes were prepared from m- and $p\text{-}CF_3C_6H_4SH$ and the appropriate precursors **1** and **2** by the ligand substitution reactions (4), which form the basis of the core extrusion method. Ana-

$$\left.\begin{array}{c}[Fe_2S_2(S_2\text{-}o\text{-xyl})_2]^{2-}\\ \mathbf{1}\\ [Fe_4S_4(S\text{-}t\text{-Bu})_4]^{2-}\\ \mathbf{2}\end{array}\right\} \xrightarrow[CH_3CN]{CF_3C_6H_4SH} \left\{\begin{array}{c}[Fe_2S_2(SC_6H_4CF_3)_4]^{2-}\\ \mathbf{3},\ d^{2-}\\ [Fe_4S_4(SC_6H_4CF_3)_4]^{2-}\\ \mathbf{4},\ t^{2-}\end{array}\right. \tag{4}$$

lytical data and the spectroscopic and electrochemical properties in Table I are in agreement[2,3a,4,46] with formulations **3** and **4**. In DMF solution, the usual medium in which properties of $[Fe_2S_2(SR)_4]^{2-}$ and $[Fe_4S_4(SR)_4]^{2-}$ species have been examined in past work, the presence of a $m\text{-}CF_3$ or $p\text{-}CF_3$ substituent induces small blue shifts in the electronic spectra of Figure 2 and positive shifts in redox potentials compared to the corresponding R = Ph complexes.[47] These differences are qualitatively consistent with the expected effects of electron-withdrawing substituents. Based upon X-ray structural results for $[Fe_2S_2(SC_6H_4\text{-}p\text{-}CH_3)_4]^{2-}$ [2] and $[Fe_4S_4(SPh)_4]^{2-}$ [3a] and the close correspondence of their properties to those of **3** and **4**, the latter are assigned the structures illustrated in Figure 3 for the R = $p\text{-}C_6H_4CF_3$ complexes.

NMR Spectral Properties. The ^{19}F spectra of mixtures of $[Fe_2S_2(SR)_4]^{2-}$ and $[Fe_4S_4(SR)_4]^{2-}$ (R = m- and $p\text{-}C_6H_4CF_3$) with the corresponding thiols in DMF solution at −15 °C shown in Figure 4 demonstrate that requirement i is met by the R = $p\text{-}C_6H_4CF_3$ system. Signals of the three species are mutually fully resolved and span a range of 6 ppm. Furthermore, the limit of slow ligand exchange between the two complexes and between each and the free thiol is achieved or very closely approached, a situation which also holds at this temperature in 80% HMPA/H_2O solvent in the presence of

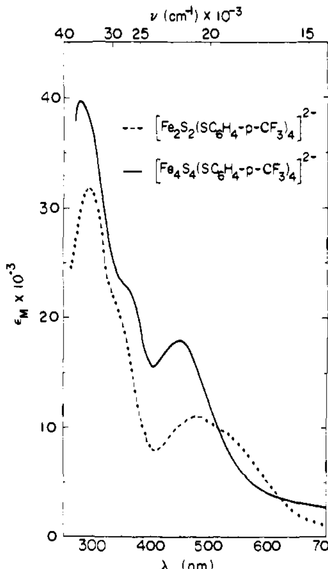


Figure 2. Electronic spectra of the Et_4N^+ salts of $[Fe_2S_2(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ and $[Fe_4S_4(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ in DMF solution at 25 °C.

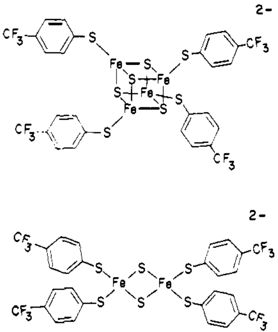


Figure 3. Schematic structures of $[Fe_4S_4(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ and $[Fe_2S_2(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ based on the known structures of $[Fe_4S_4(SPh)_4]^{2-}$ [3a] and $[Fe_2S_2(SC_6H_4\text{-}p\text{-}CH_3)_4]^{2-}$,[2] respectively.

the much larger thiol concentrations required in extrusion experiments. Higher temperatures are unsatisfactory owing to the onset of exchange processes and attendant line broadening which renders the signal integrations required for quantitation of extrusion products (vide infra) less reliable. In contrast, the ^{19}F shifts in the R = $m\text{-}C_6H_4CF_3$ system under the same conditions span an interval of only 0.3 ppm, and those of the two complexes cannot be accurately integrated in the presence of high thiol concentrations. The presence of four CF_3 groups in each complex gives a factor of 12 in equivalent ^{19}F nuclei over M(complex), thereby providing an acceptable solution to requirement ii. These observations have led to the selection of $p\text{-}CF_3C_6H_4SH$ as the extrusion reagent and −15 °C as the temperature at which ^{19}F NMR data were acquired subsequent to termination of the extrusion process in 80% HMPA/H_2O at ambient temperature.

Requirement i is satisfied by the R = $p\text{-}C_6H_4CF_3$ system mainly because of the much larger displacement of ^{19}F signals in the two complexes from the thiol resonance compared to the R = $m\text{-}C_6H_4CF_3$ system. This difference can be rationalized in terms of known electronic properties of $[Fe_2S_2(SR)_4]^{2-}$ and $[Fe_4S_4(SR)_4]^{2-}$ species, both of which exhibit contact-shifted ^1H NMR spectra[48-50] and antiferromagnetic properties[44,48] that are responsible for the contact shifts ($(\Delta H/H_0)_{con} = (\Delta H/H_0)_{obsd} - (\Delta H/H_0)_{dia}$). The complexes $[Fe_2S_2(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ and $[Fe_4S_4(SC_6H_4\text{-}p\text{-}CF_3)_4]^{2-}$ also behave as intramolecular antiferromagnets.[51] For complexes **3** and **4**, ^1H and ^{19}F contact shifts (ppm), obtained from the data in Table I and Figure 4, respectively, with the free

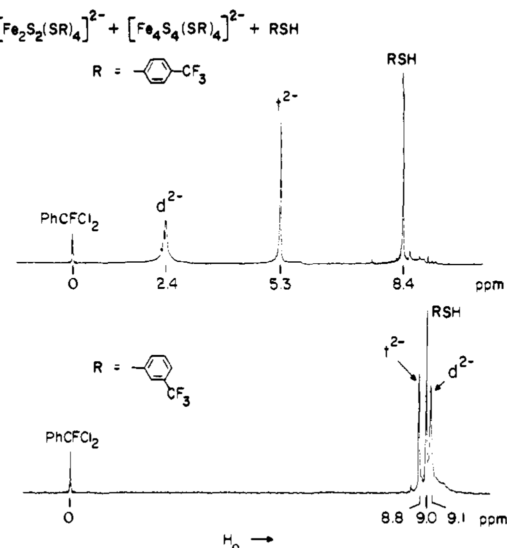


Figure 4. ^{19}F NMR spectra (338 MHz) of DMF solutions at −15 °C containing mixtures of $[Fe_2S_2(SR)_4]^{2-}$ (d^{2-}), $[Fe_4S_4(SR)_4]^{2-}$ (t^{2-}), and RSH (R = m-, $p\text{-}C_6H_4CF_3$), ca. 10^{-3} M in each component. Top: R = $p\text{-}C_6H_4CF_3$. Bottom: R = $m\text{-}C_6H_4CF_3$.

thiol as the diamagnetic reference are set out in **5–8**. The alternating signs of the ^1H shifts, previously observed in a variety of $[Fe_4S_4(SR)_4]^{2-}$ complexes,[49,50] are clear indicators of

CF_3 (+0.1); (+3.6); (+2.2) (−1.9) — **5**, d^{2-}

CF_3 (−0.2); (+1.8); (+1.1) (−0.9) — **6**, t^{2-}

(−6.0) CF_3; (+2.3) (−2.0) — **7**, d^{2-}

(−3.3) CF_3; (+1.3) (−1.0) — **8**, t^{2-}

contact shifts, which in these systems arise from ligand → Fe(core) antiparallel spin transfer.[49] From contact shift theory,[49] the ratio of $p\text{-}CF_3$ to $m\text{-}CF_3$ shifts in dimeric or tetrameric complexes at fixed temperature is given by eq 5:

$$\frac{(\Delta H/H_0)_{p\text{-}CF_3}}{(\Delta H/H_0)_{m\text{-}CF_3}} = \frac{Q_{p\text{-}CCF_3}\rho_{p\text{-}C}\chi_p}{Q_{m\text{-}CCF_3}\rho_{m\text{-}C}\chi_m} \quad (5)$$

in which $Q\rho_C = A$ (hyperfine coupling constant) and χ is the molar susceptibility. ^1H shifts indicate little differences in individual spin densities ρ_C or total spin delocalized ($\Sigma\rho_C$) in the pairs **5**/**7** and **6**/**8** (as expected from $\chi_p \simeq \chi_m$). Assuming ρ_C values are not largely perturbed by CF_3 substitution and employing the ^1H shifts in **5–8** and the values of $Q_{p\text{-}CCF_3} \simeq +38$ G and $Q_{m\text{-}CCF_3} \simeq +9$ G from Eaton et al.,[52] the ^{19}F shift ratios in eq 5 are estimated as −8.0 and −6.9 for dimeric and tetrameric complexes, respectively. The observed values[53] are larger, emphasizing that a substantially smaller fraction of unpaired spin is delocalized to a $m\text{-}CF_3$ than to a $p\text{-}CF_3$ group at parity of core structure. The ^{19}F shift ratios expected for CF_3 groups at the same ring position but in complexes with different core structures (pairs **5**/**6**, **7**/**8**) are more difficult to estimate. However, the smaller value of $Q_{m\text{-}CCF_3}$ vs. $Q_{p\text{-}CCF_3}$ will reduce the contact shifts in **5** and **6** with the attendant probability of less favorable chemical-shift resolution, as observed. Considerations of the sort outlined here are essential to any further elaboration of an NMR method for detecting extrusion products.

Table II. Summary of Control Experiments for Fe_2S_2 Core Extrusions Utilizing Spinach Fd, *C. pasteurianum* 2-Fe Fd, and $[Fe_2S_2(SR_F)_4]^{2-}$ (d^{2-})

species	initial concn, mM[a]	conditions[j] mol ratio R_FSH/Fe	solvent	$S_2O_4^{2-}$, mM[a]	method	detd. concn d^{2-}, mM	n_d	A_{476}/A_{540}	error, %[f]
2-Fe Fd_{ox}	0.028[b]	100	A		AS[c]	0.0288	1.01	1.20	+3
	0.029	150	A		AS	0.0275	0.95	1.21	−5
	0.040	300	A		AS	0.0371	0.93	1.21	−7
	0.042[g]	100	A		AS	0.0434	1.03	1.19	+3
	0.19	100	A		NMR	0.213	1.1		+12
	0.32	100	A		NMR	0.332	1.0		+4
	0.49	150	A		NMR	0.475	0.97		−3
	0.49	50	A		NMR	0.493	1.0		0
	0.026	150	B	0.012[d]	AS	0.0263	1.01	1.21	+1
2-Fe Fd_{red}	0.019	180	A	0.12	AS	0.0171	0.90	1.22	−10
	0.019	180	A	0.18	AS	0.0163	0.86	1.22	−14
	0.019	200	A	0.35	AS	0.0131	0.69	1.21	−31
	0.021[b]	320	A	0.14	AS	0.0201	0.96	1.23	−5
	0.051[b,h]	200	A	0.14	AS	0.0503	0.99	1.21	−1
$[Fe_2S_2(SR_F)_4]^{2-}$	0.0501	160	A		NMR	0.0456	0.91		−9
	0.277	100	A		NMR	0.294	1.06		+6
	0.484	100	A		NMR	0.439	0.91		−9
	0.811[i]	100	A		NMR	0.830	1.02		+2
	0.0193–0.741	50–190	A		AS			1.20 (0.01)[e]	
	0.0150–0.197	50–240	B		AS			1.20 (0.02)[e]	
	0.149	100	B	0.20	AS			1.18	

[a] Concentration in extrusion solution before reaction; dithionite and thionine were added to the aqueous solution before dilution with four parts (v/v) of HMPA. [b] *C. pasteurianum* Fd, all other entries refer to spinach Fd. [c] Absorption spectra. [d] Additional component 0.041 mM thionine. [e] Average of six–nine experiments, standard deviation in parentheses. [f] Based on initial and final (detd.) concentrations. [g] Figure 5. [h] Figure 6. [i] Figure 8. [j] Conditions: 4:1 v/v HMPA/H_2O, aqueous component 50 mM Tris-Cl. (A) pH 8.5. (B) pH 7.4 + 0.25 M NaCl.

Control Experiments: Extrusion of Fd Proteins. Prior to the application of the ^{19}F NMR method to extrusion of proteins with incompletely defined Fe–S sites, an extensive series of control experiments was performed with small Fd proteins in 80% HMPA/H_2O under the same or closely similar conditions employed with the former proteins. The purposes of these experiments were to establish the effectiveness of p-$CF_3C_6H_5SH$ (R_FSH) as an extrusion reagent, the stability of its complexes $[Fe_2S_2(SR_F)_4]^{2-}$ and $[Fe_4S_4(SR_F)_4]^{2-}$ under extrusion conditions, and, thereafter, the method and accuracy of quantitation of extrusion products by the NMR procedure. The following Fd proteins with the indicated molecular weights and types of sites were utilized: spinach Fd (10 660, 1 Fe_2S_2), *C. pasteurianum* 2-Fe Fd (25 000, 1 Fe_2S_2), and *C. pasteurianum* 8-Fe Fd (6200, 2 Fe_4S_4). Site identification in the spinach and 8-Fe Fd_{ox} proteins rests on a large body of physicochemical data;[54] in addition, the sites in all three proteins have been established by the extrusion technique.[6-8,10-12] Experimental conditions in the 80% HMPA/H_2O solvent medium which were used are designated as A and B in Tables II and III; the latter contain a representative sampling of results from a substantially larger number of experiments.[55]

(a) Spectrophotometric Results. Treatment of 80% HMPA/H_2O solutions containing 8-Fe Fd_{ox} (9–25 μM) and the two 2-Fe Fd_{ox} proteins (28–42 μM) with varying excesses of R_FSH affords $[Fe_4S_4(SR_F)_4]^{2-}$ (λ_{max} 452 nm) and $[Fe_2S_2(SR_F)_4]^{2-}$ (λ_{max} 476, 540 (sh) nm), respectively. A typical experiment under condition A is shown in Figure 5; here $n_t = 2.01$ and $n_d = 1.03$.

Measured concentrations of extrusion products under conditions A and B fall within the 10% error limit mentioned earlier. The integrity of the two species under extrusion conditions is further indicated by absorbance decreases of <5% over at least 2–3 h and the satisfactory agreement of absorbance ratios with those of synthetic complexes measured under identical conditions except for the presence of protein (Tables II and III).

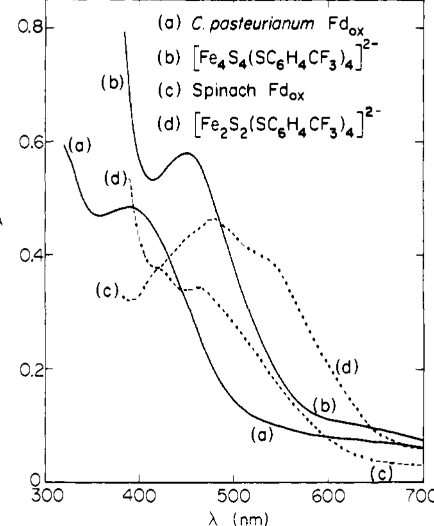


Figure 5. Active-site core extrusions of *C. pasteurianum* 8-Fe Fd_{ox} and spinach Fd_{ox} with p-$CF_3C_6H_4SH$ in 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 8.5), l = 1 cm, 25 °C: (a) 16 μM *C. pasteurianum* Fd_{ox} in buffer; (b) 0.2 mL of 80 μM Fd_{ox} + 0.8 mL of HMPA containing 2.7 μL of thiol 10 min after mixing: $n_t = 2.01$; (c) 42 μM spinach Fd_{ox} in buffer; (d) 0.2 mL of 210 μM spinach Fd_{ox} + 0.8 mL of HMPA containing 1.2 μL of thiol 45 min after mixing: $n_d = 1.03$.

We[7,14] and others[8] have emphasized the desirability of conducting extrusion reactions on oxidized proteins (i.e., those whose sites have the core oxidation levels $[Fe_2S_2]^{2+}$ and $[Fe_4S_4]^{2+}$) in order to avoid the spontaneous dimer → tetramer conversion,[56] eq 6, observed upon reduction of dimeric dianions:[9]

$$2[Fe_2S_2(SR)_4]^{3-} \rightarrow [Fe_4S_4(SR)_4]^{2-} + 4RS^- \quad (6)$$

In an attempt to examine the consequence of extrusion reactions of reduced 2-Fe and 4-Fe sites such as may be encoun-

Table III. Summary of Control Experiments for Fe_4S_4 Core Extrusions Utilizing *C. pasteurianum* 8-Fe Fd and $[Fe_4S_4(SR_F)_4]^{2-}$ (t^{2-})

		conditions[h]							
species	initial concn, mM[a]	mol ratio R_FSH/Fe	solvent	$S_2O_4^{2-}$ (thionine), mM	method	detd. concn t^{2-}, mM	n_t	A_{452}/A_{540}	error, %[d]
C. p. 8-Fe Fd_{ox}	0.0091	100	A		AS[b]	0.0178	1.96	2.56	−2
	0.014	500	B		AS	0.0278	1.99	2.54	−1
	0.016[e]	150	A		AS	0.0322	2.01	2.61	+1
	0.020	200	B	0.016 (0.052)	AS	0.0390	1.95	2.23	−3
	0.021	100	B		AS	0.0379	1.80	2.44	−9
	0.025	90	A		AS	0.0486	1.94	2.55	−3
	0.060	400	A		NMR	0.112	1.9		−7
	0.11	100	A		NMR	0.231	2.1		+5
	0.16	75	A		NMR	0.312	2.0		−3
	0.32	100	A		NMR	0.580	1.8		−9
C. p. 8-Fe Fd_{red}	0.0048	150	B	0.057	AS	0.00928	1.93	2.48	−3
	0.013	560	B	0.090	AS	0.0247	1.90	2.32	−5
	0.013[f]	150	B	0.090	AS	0.0238	1.83	2.40	−8
$[Fe_4S_4(SR_F)_4]^{2-}$	0.0520	250	A		NMR	0.0529			+2
	0.104	100	A		NMR	0.0994			−4
	0.249[g]	100	A		NMR	0.261			+5
	0.489	100	A		NMR	0.477			−2
	0.703	100	A		NMR	0.671			−5
	0.0425	200	B	(0.25)	AS			2.19	
	0.0199–0.487	40–100	A		AS			2.40 (0.07)[c]	
	0.0116–0.206	50–160	B		AS			2.36 (0.11)[c]	
	0.149	100	B	0.20	AS			2.49	

[a] Concentration in final extrusion solution; dithionite and/or thionine were added to the aqueous solution before dilution with four parts (v/v) of HMPA. [b] Absorption spectra. [c] Average of seven experiments, standard deviation in parentheses. [d] Based on initial and final (detd.) concentrations. [e] Figure 5. [f] Figure 7. [g] Figure 8. [h] Conditions: 4:1 v/v HMPA/H_2O, aqueous component 50 mM Tris-Cl, (A) pH 8.5, (B) pH 7.4 + 0.25 M NaCl.

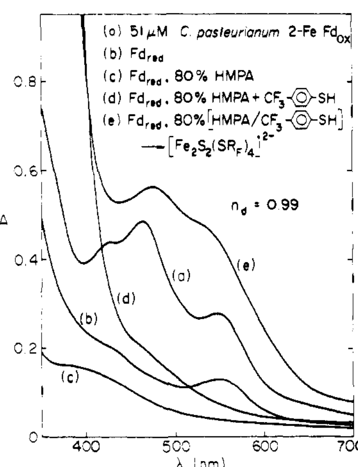


Figure 6. Active-site core extrusion of *C. pasteurianum* 2-Fe Fd_{red} with *p*-$CF_3C_6H_4SH$ in 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 8.5). l = 1 cm, 25 °C: (a) 51 μM Fd_{ox} in buffer; (b) Fd_{red}, from 1 mL of solution a + 7 μL of ~20 mM $Na_2S_2O_4$; (c) 0.2 mL of 260 μM Fd_{red} + 0.8 mL of HMPA; (d) 1 mL of solution c + 2.8 μL of thiol 15 min after mixing; (e) 0.2 mL of 260 μM Fd_{red} + 0.8 mL of HMPA containing 2.8 μL of thiol 30 min after mixing; n_d = 0.99. All Fd_{red} solutions contain a $S_2O_4^{2-}$/Fe mol ratio of 1.4:1.

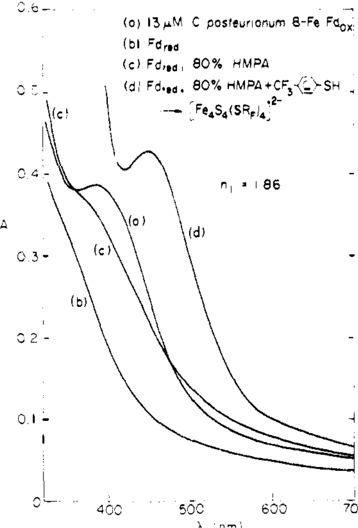
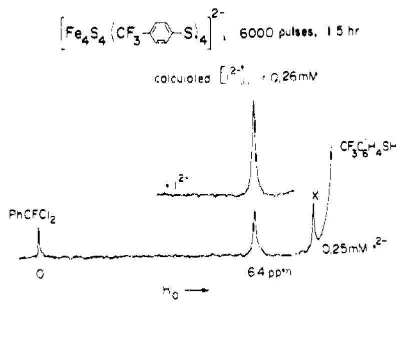


Figure 7. Active-site core extrusion of *C. pasteurianum* 8-Fe Fd_{red} with *p*-$CF_3C_6H_4SH$ in 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 7.4, 0.25 M NaCl). l = 1 cm, 25 °C: (a) 13 μM Fd_{ox} in buffer; (b) Fd_{red}, from 1 mL of solution a + 4 μL of ~23 mM $Na_2S_2O_4$; (c) 0.2 mL of 63 μM Fd_{red} ($S_2O_4^{2-}$/Fe mol ratio 0.9:1) + 0.8 mL of HMPA; (d) 1 mL of solution c + 2.1 μL of thiol 10 min after mixing; n_t = 1.83. A similar experiment but using HMPA containing thiol to prepare solution c gave a spectrum virtually identical with d; n_t = 1.85.

tered in this or future work, the reactions of the two 2-Fe Fd_{red} proteins and the clostridial 8-Fe Fd_{red} protein under conditions A and B, respectively, have been investigated. In all experiments the oxidized proteins in buffer solutions were spectrophotometrically titrated with various excesses of dithionite to their reduced forms[29,57] prior to dilution with HMPA. Results are included in Tables II and III and experiments utilizing small excesses of reductant can be followed in Figures 6 and 7. In the extrusion of *C. pasteurianum* 2-Fe Fd_{red} (Figure 6, initial $S_2O_4^{2-}$/Fe mol ratio, 1.4:1), dilution of the Fd_{red} solution with HMPA resulted in no appreciable oxidation. Subsequent addition of R_FSH afforded a nearly featureless



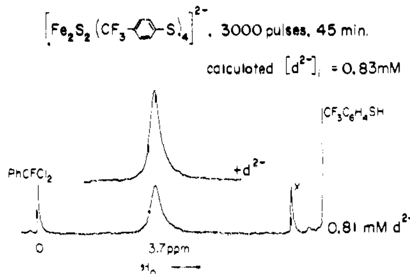


Figure 8. ^{19}F FT NMR spectra (94.1 MHz) of 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 8.5) solutions of $[Fe_4S_4(SR_F)_4]^{2-}$ (t^{2-}) and $[Fe_2S_2(SR_F)_4]^{2-}$ (d^{2-}) at −15 °C, before and after the addition of standard. Top—lower spectrum: 0.249 mM t^{2-}, R_FSH/Fe mol ratio 100:1; upper spectrum: after addition of 3.4 μL of 37.3 mM t^{2-} solution in DMF to 510 μL of initial solution, I_f/I_i = 1.94. Both spectra were acquired with 6000 pulses (1.5 h). Bottom—lower spectrum: 0.811 mM d^{2-}, R_FSH/Fe mol ratio 100:1; upper spectrum: after addition of 7.5 μL of 56.0 mM d^{2-} solution in DMF to 518 μL of initial solution, I_f/I_i = 1.95. Both spectra were acquired with 3000 pulses (45 min). Initial concentrations calculated from intensity ratios are given. The signal "X" in these and other spectra is associated with the thiol.

spectrum (d) from unidentified species. However, dilution with HMPA containing R_FSH gave as the limiting spectrum e that of $[Fe_2S_2(SR_F)_4]^{2-}$ from which n_d = 0.99 was calculated. Extrusion of spinach Fd_{red} (initial $S_2O_4^{2-}$/Fe mol ratio, 3.2:1) using the latter procedure was also satisfactory (n_d = 0.90). However, larger initial $S_2O_4^{2-}$/Fe mol ratios (up to 9.2:1) resulted in substantially reduced recovery of the spinach protein Fe_2S_2 cores as $[Fe_2S_2(SR_F)_4]^{2-}$ (Table II). These findings are consistent with the observation that treatment of the latter complex in the extrusion medium lacking protein with similar amounts of dithionite reduces the intensity of its spectrum without significant changes in band shape. A comparable extrusion experiment with *C. pasteurianum* 8-Fe Fd_{red} (initial $S_2O_4^{2-}$/Fe mol ratio, 0.9:1) is depicted in Figure 7. Here spectrum c indicates a small amount of oxidation upon dilution of the Fd_{red} solution with HMPA: limiting spectrum d is that of $[Fe_4S_4(SR_F)_4]^{2-}$. The extent of core extrusion (Table III) is insignificantly affected by procedures involving subsequent addition of R_FSH (n_t = 1.86), its inclusion in the HMPA diluent (n_t = 1.88), or by the presence of $S_2O_4^{2-}$/Fe mol ratios up to 1.5:1 (n_t = 1.93).

The results in Tables II and III, obtained by a now well-tested spectrophotometric procedure,[7,8] demonstrate that R_FSH, as benzenethiol, is a quantitative core extrusion reagent for $Fd_{ox,red}$ proteins in 80% HMPA/H_2O. Unlike previous experimentation,[7] it has not proven necessary in this work to add ferricyanide in order to obtain essentially quantitative core removal from Fd_{red} proteins. The means by which reduced protein cores ($[Fe_2S_2]^+$, $[Fe_4S_4]^+$) emerge as oxidized (2+) cores in their extrusion products is still not clear, but is possibly a consequence of reducible matter in the medium and the strongly reducing potentials of the $[Fe_2S_2(SR_F)_4]^{2-,3-}$ and $[Fe_4S_4(SR_F)_4]^{2-,3-}$ couples[58] (Table I).

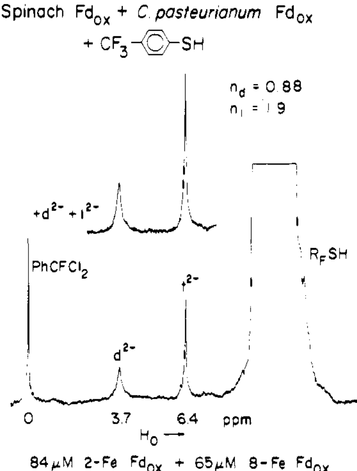


Figure 9. ^{19}F FT NMR spectra (338 MHz) of a 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 8.5) solution of spinach Fd_{ox} and *C. pasteurianum* 8-Fe Fd_{ox} at −15 °C after completion of the active-site core extrusion reactions and after addition of standards. Lower spectrum: 84 μM spinach Fd_{ox} + 65 μM 8-Fe Fd_{ox}, R_FSH/Fe mol ratio 400:1, after 30 min reaction time at 25 °C. Upper spectrum: after addition of $[Fe_2S_2(SR_F)_4]^{2-}$ (d^{2-}) and $[Fe_4S_4(SR_F)_4]^{2-}$ (t^{2-}) standard solutions in DMF: n_d = 0.88, n_t = 1.9. Spectra were acquired with 12 000 pulses (40 min).

(b) ^{19}F NMR Results. The basis for identification and quantitation of extrusion products in 80% HMPA/H_2O is illustrated by the ^{19}F spectra in Figure 8 recorded at −15 °C in the presence of excess thiol. In this medium $[Fe_2S_2(SR_F)_4]^{2-}$ and $[Fe_4S_4(SR_F)_4]^{2-}$ are readily identified by their resonances at 3.7 and 6.4 ppm, respectively. The quantitation procedure is described in detail in the figure legend. In this example, which employs relatively high concentrations, the agreement between calculated and known initial concentrations is ≤4%. Results of experiments of this type conducted over a 0.05–0.8 mM range are collected in Tables II and III, as are additional control experiments with spinach Fd_{ox} (0.19–0.49 mM) and *C. pasteurianum* 8-Fe Fd_{ox} (0.06–0.32 mM) separately.[59] Quantitation of protein extrusion is shown in Figure 9 for a mixture of 84 μM spinach Fd_{ox} and 65 μM *C. pasteurianum* 8-Fe Fd_{ox}. The values n_t = 1.9 and n_d = 0.88 are considered satisfactory quantitation. Extrusions of Fd_{red} proteins were not investigated by the NMR method inasmuch as spectrophotometric results demonstrated (in the presence of low $S_2O_4^{2-}$/Fe mol ratios) essentially quantitative formation of the same extrusion products as with oxidized proteins. The principal observations from a larger body of experimentation[55] are the following: (i) the practical error limit for concentrations determined by the standard addition NMR method is ±15%, as opposed to ±10% by spectrophotometric assay; (ii) the lowest practical concentrations of protein 2-Fe and 4-Fe sites which could be investigated, consistent with available spectrometer time and satisfactory signal:noise spectral properties, were ca. 0.05 mM (at 338 MHz) and ca. 0.1 mM (at 94.1 MHz); (iii) an R_FSH/Fe mol ratio of ≳100:1 is a generally satisfactory condition for quantitative extrusion over the concentration ranges employed in the NMR control experiments.

Milk Xanthine Oxidase. This enzyme is a representative of a class of hydroxylases[17] containing flavin, Mo, and Fe/S groups and itself is one of the most extensively studied of all metalloenzymes. Despite this activity, the nature of the Fe/S sites has not been fully clarified. The following evidence is suggestive of, but does not necessarily prove, the existence of Fe_2S_2 centers: the presence in reduced XO of two EPR-detectable sites (Fe/S I, Fe/S II), the spectrum of one of which (Fe/S I, g_{av} = 1.95) is very similar to that of 2-Fe Fd_{red} proteins;[60,61] the uptake by these centers of one electron each per subunit;[60] the enhanced similarity of the reduced enzyme and

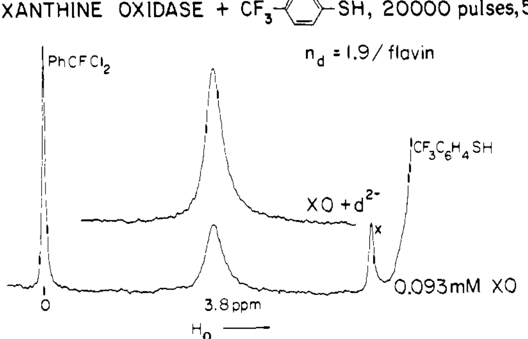


Figure 10. ^{19}F FT NMR spectra (94.1 MHz) of oxidized milk XO solutions in 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 8.5) at −15 °C after completion of the active-site core extrusion reaction and after addition of standard. Lower spectrum: XO solution 0.093 mM in flavin, R_FSH/Fe mol ratio 120:1, after 40 min reaction time at 25 °C. Upper spectrum: after addition of $[Fe_2S_2(SR_F)_4]^{2-}$ (d^{2-}) standard solution in DMF: n_d = 1.9/FAD. Both spectra were acquired with 20 000 pulses (5 h).

Table IV. Core Extrusions of Milk Xanthine Oxidase

initial concn, mM		conditions: mol ratio R_FSH/Fe	conditions: medium[a]	concn d^{2-}, mM	n_d
native,[b]	0.093[f]	120	A	0.180[d]	1.9/FAD
	0.12	100	A	0.246[d]	2.0/FAD
	0.14	110	A	0.288[d]	2.1/FAD
deflavo,[c]	0.077[g]	190	C	0.0414[e]	0.54/Fe

[a] 4:1 v/v HMPA/H_2O, aqueous component (A) 50 mM Tris-Cl, pH 8.5, (C) 100 mM Tris-acetate, pH 8.3, 1 mM EDTA, 0.5 M NaCl. [b] FAD concentration, extrusion reaction allowed to proceed 40 min at 25 °C. [c] Protein-bound Fe concentration, based on ϵ_{Fe}^{467} 6300;[17b] extrusion reaction allowed to proceed 90 min at 25 °C. [d] ^{19}F NMR. [e] Absorption spectra. [f] Figure 10. [g] Figure 11.

2-Fe Fd_{red} EPR spectra when observed in 80% Me_2SO/H_2O,[62] a medium capable of unfolding tertiary structure; the absorption spectrum of oxidized deflavo XO,[32,33] which resembles those of 2-Fe Fd_{ox} proteins; and the oxidized/reduced difference spectra of Fe/S I and Fe/S II which suggest that the two are similar chromophores.[63] Unusual features include the very broad EPR spectrum of Fe/S II with g_{av} = 2.01, the three- to fourfold greater absorbance of Fe/S I vs. Fe/S II in the difference spectral measurements of the deflavo form, and the average ϵ_{Fe}^{467} value of 6300.[17b] All 2-Fe Fd_{red} proteins have $g_{av} < 2$ and generally smaller g-value anisotropies, and 2-Fe Fd_{ox} proteins usually exhibit less intense (although variable) visible absorption spectra.

In order to provide a satisfactory definition of the Fe-S sites in native XO, the ^{19}F NMR extrusion method has been applied. Extrusion experiments in 80% HMPA/H_2O have been conducted with the oxidized enzyme under condition A (Table II), and with the ca. 40-min reaction times required to obtain limiting absorption spectra away from the flavin region (Figure 1). The similarity of spectra a and b in Figure 1 is taken as reasonable evidence that the extrusion medium alters the environments, but does not alter the type(s) of Fe-S centers present. Solutions with 0.093–0.14 mM flavin concentrations were employed. As shown by the representative spectrum in Figure 10, $[Fe_2S_2(SR_F)_4]^{2-}$ is the only extrusion product. The full set of results is given in Table IV; agreement among the three runs is quite satisfactory, with an average n_d = 2.0/FAD.

The availability of deflavo-XO[32,33] has permitted a check of the foregoing results, albeit with a nonnative form of the enzyme. The deflavo form retains the Mo, Fe, and S contents

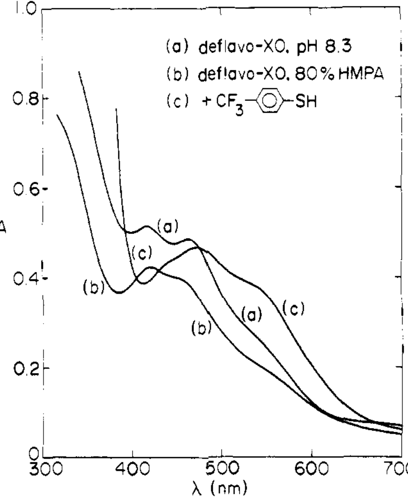


Figure 11. Active-site core extrusion of oxidized deflavo milk XO with p-$CF_3C_6H_4SH$ in 4:1 v/v HMPA/H_2O (100 mM Tris-acetate, pH 8.3, 1 mM EDTA, 0.5 M NaCl), l = 1 cm, 25 °C: (a) deflavo XO solution in buffer, 77 μM in Fe; (b) 0.2 mL of a deflavo XO solution in buffer, 385 μM in Fe, + 0.8 mL of HMPA; (c) solution b + 2 μL of thiol 90 min after addition (A_{476}/A_{540} = 1.23); n_d = 0.54/Fe.

and the dehydrogenase activity of the native enzyme, but lacks its oxidase activity.[33] The extrusion of oxidized deflavo-XO followed spectrophotometrically is depicted in Figure 11. The resemblance of spectra a and b to that of spinach Fd_{ox} (Figure 5) is evident. Spectrum c is that of $[Fe_2S_2(SR_F)_4]^{2-}$ and leads to n_d = 0.54/Fe (Table IV) or n_d = 4.3 based on total iron.

The core extrusion results provide convincing evidence that all iron in native and deflavo-XO is organized into four Fe_2S_2 sites per α_2 subunit structure. This finding, together with the results of the analysis of the Mo EXAFS spectrum,[64] which indicates $Mo(O)_2S_3$ coordination in the oxidized form, provides a highly increased definition of the metalloprosthetic groups in this enzyme. In a reduced form of the enzyme it appears that Fe/S I and Mo are separated by 20–25 Å.[65] Because the extrusion method identifies core structure only, it is uninformative with regard to the unusual Fe/S site properties noted above. These arise from environmental perturbations and structural effects peculiar to xanthine oxidase. Current mechanistic views of enzyme action[17,66] ascribe to Fe/S I and Fe/S II (now Fe_2S_2 I and Fe_2S_2 II) centers electron transfer and storage roles. Recent experiments have failed to detect interactions between these centers in the partially reduced enzyme.[67]

FeMo Proteins of Nitrogenase. Proteins from three organisms, *C. pasteurianum*, *A. vinelandii*, and *Klebsiella pneumoniae*, have been extensively purified and subjected to detailed enzymological and physicochemical characterization. Results obtained with the majority of the more recent preparations of these proteins are indicative of compositional similarities: molecular weights of ca. 210 000–250 000;[11,41] $\alpha_2\beta_2$ subunit structures;[41,68] ca. 1.5–2 g-atoms of Mo/mol and ca. 22–33 g-atoms of Fe/mol[11,68b,69-71] (and a somewhat smaller quantity of acid-labile sulfur); a relatively low molecular weight FeMo cofactor[69,72] (FeMo-co) dissociable from and possessing the entire Mo content of the native protein; the absence of other metals or prosthetic groups. Present evidence[11,18,70] conforms to the existence of at least three total oxidation levels of FeMo proteins. The reduced form is EPR silent and is generated in N_2-fixing systems which also contain the Fe protein, Mg·ATP, and a reductant such as Fd_{red}. The semireduced form is that in which the proteins are normally isolated and is EPR active (g values of 4.3, 3.6, and 2.0). The oxidized form is obtainable by $6e^-$ oxidation[70] of the semireduced form and is EPR silent. The following are the principal

Table V. Core Extrusions of Nitrogenase FeMo Proteins

species, initial concn, mM[a]	soln no.	conditions[j]: mol ratio R_FSH/Fe	conditions[j]: $S_2O_4^{2-}$, mM[a]	conditions[j]: thionine, mM[a]	detd. concn, mM[b]: d^{2-}	detd. concn, mM[b]: t^{2-}	detd. concn, mM[b]: total Fe	n_d	n_t	extruded Fe: g-atoms/mol	extruded Fe: % total
C. p., semireduced											
0.042[c,g]	1	100	0.1		0	0.0537	0.215	0	1.3	5.1	18
0.062[e]	2	100	0.04		trace	0.0734	0.294	<0.2	1.2	4.7	20
0.13	3	50	*f*		trace	0.162	0.648	<0.05	1.2	5.0	21
0.14	4	50			0	0.203	0.812	0	1.4	5.8	25
0.14	5	100			0	0.183	0.732	0	1.3	5.2	22
C. p., oxidized											
0.041[c,g]	6	100	0.1	0.40	0.0368	0.0654	0.335	0.90	1.6	8.2	29
0.063[e]	7	100	0.04	0.22	0.0562	0.0790	0.428	0.89	1.3	6.8	29
0.13	8	60	<0.1	0.40	0.101	0.153	0.814	0.77	1.2	6.3	27
0.14	9	50	<0.1	1.7[i]	0.230	0.201	1.034	1.6	1.4	9.0	39
A. v.,[d] semireduced											
0.051[h]	10	100	0.2		0.0275	0.0815	0.381	0.54	1.6	7.5	25
0.060	11	40	0.4		trace	0.0743	0.297	<0.2	1.2	5.0	17
A. V.,[d] oxidized											
0.050[h]	12	100	0.2	0.40	0.0750	0.0718	0.437	1.5	1.4	8.7	29
0.060	13	40	0.4	0.52	0.0924	0.0754	0.487	1.5	1.3	8.1	27

[a] Concentration in extrusion solution before reaction; dithionite, thionine, or ferricyanide was added to the aqueous solution before dilution with four parts (v/v) of HMPA. [b] After reaction times of 40–120 min. [c] Preparation 2 (28.4 g-atoms of Fe/mol); all other entries refer to preparation 1 (23.4 g-atoms of Fe/mol). [d] 29.8 g-atoms of Fe/mol. [e] Figure 14. [f] *C. pasteurianum* hydrogenase and 8-Fd_{ox} were added to aqueous component to discharge dithionite before HMPA dilution; the final concentration of the added proteins was ca. 4 μM, for which t^{2-} concentration was corrected. [g] Figure 12. [h] Figure 13. [i] Concentration of ferricyanide. [j] Conditions: 4:1 v/v HMPA/H_2O, aqueous component 50 mM Tris-Cl, pH 7.4, 0.25 M NaCl.

observations and conclusions concerning the current status of metal-containing sites and are drawn primarily from quantitative EPR and Mössbauer spectral investigations:[70,73-76] (i) there are 30 ± 2 g-atoms of Fe/mol in the most extensively purified proteins;[70] (ii) in the semireduced form ~16 Fe atoms are present in four Fe_4S_4 (P) clusters[70] having two distinct Fe environments (3:1 ratio) and a singlet ground state;[70,73,76] (iii) each of these clusters is oxidized by 1e$^-$ to a paramagnetic ($S \geq 3/2$) state in passing to the oxidized form;[70] (iv) the paramagnetic ($S = 3/2$) center resides in FeMo-co[74] (of which there are presumably two per $\alpha_2\beta_2$ structure) containing 5–7, and most probably 6, Fe atoms;[70,74,75] each center is oxidized by 1e$^-$ to an S = integer state in reaching the oxidized form;[70] (v) from EXAFS[77,78] and synthetic chemical studies[78] each Mo is apparently involved in a Mo–Fe–S cluster structure which constitutes part or all of the $S = 3/2$ center, leading to probable Fe/Mo atom ratios of ~6:1 (Mössbauer spectra) or 8:1 (chemical analysis[69]) in each center.

Because of the complexity and significance of FeMo proteins, determination of the number and structural types of their metal-containing sites should be pursued by all available means. Spectrophotometric core extrusion experiments conducted several years ago in this laboratory using 80% HMPA/H_2O and benzenethiol suggested that the preponderance of extruded Fe in a clostridial FeMo protein was removed in the form of $[Fe_4S_4(SPh)_4]^{2-}$. However, the spectra could not be satisfactorily analyzed in terms of this product alone or in combination with $[Fe_2S_2(SPh)_4]^{2-}$. Similar results have been noted by the Wisconsin group,[11,18] who further observed continuous spectral changes over 24 h. In view of these indeterminate observations, we have applied the ^{19}F NMR core extrusion method to preparations of the semireduced and oxidized FeMo proteins from *C. pasteurianum* and *A. vinelandii*, whose metal contents fall in the ranges noted above.

Core extrusion experiments have been performed under condition B using 80% HMPA solutions initially containing dithionite in most cases (semireduced form) and dithionite plus a larger quantity of thionin (oxidized form). Control experiments are summarized in Tables II and III, results of FeMo protein extrusion reactions are set out in Table V, and representative ^{19}F NMR spectra are presented in Figures 12–14.

The extrusion of *C. pasteurianum* semireduced FeMo protein is illustrated in Figure 12a; here $[Fe_4S_4(SR_F)_4]^{2-}$ is the only identifiable extrusion product. Experiments with the semireduced forms of the clostridial (solutions 1–5) and *A. vinelandii* (solution 11) proteins in the concentration range 42–140 μM have given consistent results. Values of n_t fall in the narrow interval 1.2–1.4 and are unaffected by varying initial concentrations of dithionite and R_FSH/Fe mol ratios of 40–100. These values correspond to 17–25% of *total* Fe in the different preparations. On the basis of the following evidence, we conclude that all Fe extruded under these conditions results from protein Fe_4S_4 clusters: no more than a trace ($n_d < 0.2$) of $[Fe_2S_2(SR_F)_4]^{2-}$ was detected in any of these solutions; minimization of dithionite concentration (in one case, solution 3, by the Fd/hydrogenase system) did not alter n_t within experimental uncertainty; control experiments (Tables II and III) have shown that at low dithionite levels Fe_2S_2 sites of reduced proteins are extruded as $[Fe_2S_2(SR_F)_4]^{2-}$ without appreciable dimer → tetramer conversion (reaction 6) or degradation.

Extrusion of the oxidized forms of the clostridial (solutions 6–8) and *A. vinelandii* (solutions 12 and 13) proteins, obtained by treatment with the 2e$^-$ oxidant thionine ($E_0' = +0.064$ V[79]), resulted in increases in n_t of ≤ 0.3 compared to the semireduced forms and the appearance of significant quantities of $[Fe_2S_2(SR_F)_4]^{2-}$. This behavior is illustrated in Figures 12 and 13 and has been further investigated by the experiment depicted in Figure 14, in which the *C. pasteurianum* protein was cycled from the semireduced to the oxidized to the semireduced state, using a quantity of thionine sufficient to remove ca. 6 e$^-$/protein molecule in the oxidation step. The value of n_t is nearly conserved through the cycle, whereas $n_d = 0.9$ after oxidation and did not appreciably decrease in the presence of 1 mM dithionite in the reduction step. These observations support Mössbauer spectral findings indicative of the absence of reversible reduction of P clusters with dithionite,[70] and together with the results for the *A. vinelandii* protein suggest that Fe_2S_2 centers are formed from Fe not extruded in the semi-

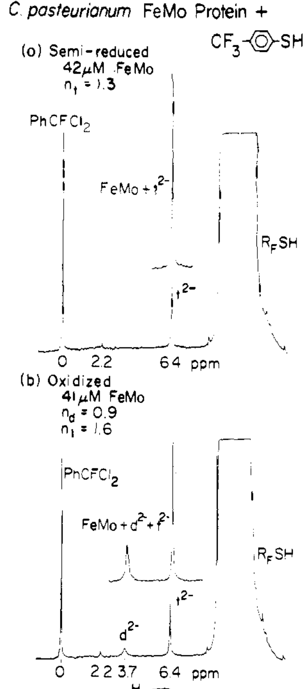


Figure 12. [19]FT NMR spectra (338 MHz) of 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 7.4, 0.25 M NaCl, ~0.5 mM $Na_2S_2O_4$) solution of *C. pasteurianum* FeMo protein at −15 °C after completion of the active-site core extrusion reactions and after addition of standards. (a) Semireduced form—lower spectrum: solution 1; upper spectrum: after addition of $[Fe_4S_4(SR_F)_4]^{2-}$ (t^{2-}) standard solution in DMF; n_t = 1.3. Both spectra were acquired with 8000 pulses (27 min). (b) Oxidized form-lower spectrum: solution 6; upper spectrum: after addition of t^{2-} and $[Fe_2S_2(SR_F)_4]^{2-}$ (d^{2-}) standard solutions in DMF; n_d = 0.9, n_t = 1.6. Both spectra were acquired with 10 000 pulses (34 min). The extrusion reaction time for both solutions was 1 h. In this and subsequent figures the signal at 2.2 ppm is unidentified and solution compositions are specified in Table V.

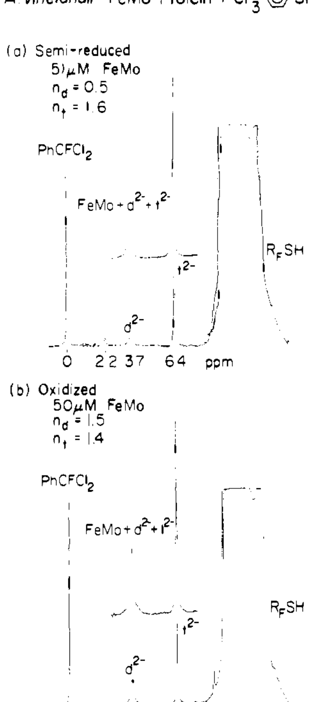


Figure 13. [19]FT NMR spectra (338 MHz) of 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 7.4, 0.25 M NaCl, ~1 mM $Na_2S_2O_4$) solutions of *A. vinelandii* FeMo protein at −15 °C after completion of active-site core extrusion reactions and after addition of standards. (a) Semireduced form—lower spectrum: solution 10; upper spectrum: after addition of $[Fe_2S_2(SR_F)_4]^{2-}$ (d^{2-}) and $[Fe_4S_4(SR_F)_4]^{2-}$ (t^{2-}) standard solutions in DMF; n_d = 0.5; n_t = 1.6. (b) Oxidized form—lower spectrum: solution 12; upper spectrum: after addition of d^{2-} and t^{2-} standard solutions in DMF; n_d = 1.5, n_t = 1.4. The extrusion reaction time for both solutions was 1 h; all spectra were acquired with 8000 pulses (27 min).

reduced forms. We regard the occurrence of any Fe_2S_2 centers in the semireduced forms as resulting from adventitious oxidation of the protein. For example, the preparation used for solution 10, which afforded n_d = 0.54, had been stored for a longer period and more extensively manipulated than had that used for solution 11 ($n_d < 0.2$). Additionally, the use of larger quantity of the stronger oxidant ferricyanide (solution 9) produced a significant increase in n_d compared with those from thionine oxidation.

Based on the Mössbauer interpretation of nearly all noncofactor Fe present as P clusters[70] and extrusion results from another laboratory (vide infra), several spectrophotometric experiments (Table III) were conducted in an attempt to discern the origin of Fe_2S_2 sites in the oxidized protein forms. Extrusion of *C. pasteurianum* Fd_{ox} in the presence of a thionine/protein mol ratio of 12:1 yielded ~30% dimer and ~70% tetramer products;[80] however, a solution having a thionine/$[Fe_4S_4(SR_F)_4]^{2-}$ mol ratio of 6:1 evidenced no significant change over a 3-h period (Table III). Furthermore, we have never observed any spontaneous $[Fe_4S_4(SR_F)_4]^{2-} \rightarrow [Fe_2S_2(SR_F)_4]^{2-}$ conversion in the extrusion medium, although this conversion has been found in several other aqueous/organic solvent mixtures. It is possible that the Fe_2S_2 centers were formed from Fe_4S_4 centers during, but not after, extrusion.

The quantitative extrusion data in the last four columns of Table V are number-average values derived from experiments with protein preparations having g-atom of Fe/mol contents slightly to appreciably below that (30 ± 2) currently believed to apply to the most highly purified preparations.[70] Recent values tend toward the high end of the range.[68-71] Selecting solutions with the larger percent total Fe extrusions and normalizing determined concentrations to 32 g-atoms of Fe/mol, solutions 3–5 give n_t = 1.7–2.0, and solutions 6–8 yield n_d = 1.0–1.2 and n_t = 1.6–1.8 for the semireduced and oxidized clostridial proteins, respectively. Based on the finding by Rawlings et al.[74] that FeMo-co contains no extrudable Fe_2S_2 and Fe_4S_4 clusters and assuming the analytical result of 8 Fe/Mo,[69] the normalized data indicate removal of 43–50% (semireduced) and 54–58% (oxidized) of potentially extrudable (noncofactor) Fe. Corresponding calculations give 33% (n_t = 1.3) for the semireduced (solution 11) and 55–58% (n_d = 1.6, n_t = 1.4–1.5) for the oxidized forms of the *A. vinelandii* protein.

Our quantitated normalized extrusion results are interpreted to indicate that the clostridial FeMo protein contains at least two Fe_4S_4 sites in the semireduced form and an equivalent minimal number in the thionine-oxidized form, together with ca. one-half as many Fe_2S_2 sites. While the corresponding types of sites surely exist in the semireduced and oxidized forms of the *A. vinelandii* protein, *n* values are decidedly nonintegral and the proportion of Fe_2S_2 sites in the oxidized forms is larger. An inescapable conclusion is that all noncofactor Fe has not been extruded. That all or nearly all such Fe is extrudable, albeit under different experimental conditions, is supported by the report that >12 Fe are removed from clostridial semireduced FeMo protein in 80% *N*-methylformamide (NMF)/H_2O as Fe_4S_4 complexes by benzenethiol,[70] and by displacement of ca. 4 Fe_4S_4 sites/32 Fe from this oxidation level by means of an interprotein cluster transfer reaction

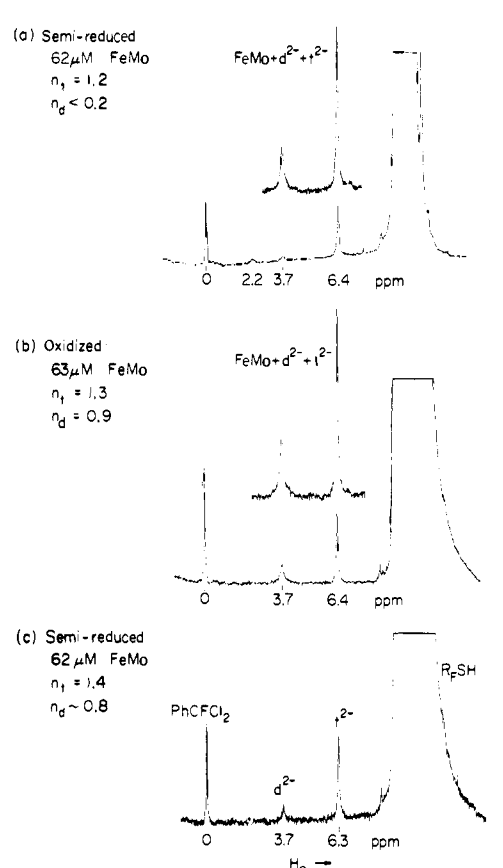


Figure 14. ^{19}FT NMR spectra (338 MHz) of 4:1 v/v HMPA/H_2O (50 mM Tris-Cl, pH 7.4, 0.25 M NaCl, ~0.2 mM $Na_2S_2O_4$) solutions of *C. pasteurianum* FeMo protein at −15 °C after completion of the active-site core extrusion reaction and after addition of standards. (a) Semireduced form—lower spectrum: solution 2; upper spectrum: after addition of $[Fe_2S_2(SR_F)_4]^{2-}$ (d^{2-}) and $[Fe_4S_4(SR_F)_4]^{2-}$ (t^{2-}) standard solutions in DMF: $n_d < 0.2$, $n_t = 1.2$. Both spectra were acquired with 12 000 pulses (41 min). (b) Oxidized form—lower spectrum: solution 7; upper spectrum: after addition of d^{2-} and t^{2-} standard solutions in DMF: $n_d = 0.9$, $n_t = 1.3$. Both spectra were acquired with 8000 pulses (27 min). (c) A separate portion of solution 7 was reduced with 1 mM $Na_2S_2O_4$: $n_d \simeq 0.8$, $n_t = 1.4$. The spectrum was acquired with 3000 pulses; that obtained after addition of standards is omitted. The extrusion reaction time in all cases was 1 h.

method.[81] This method, which also obviates the visible chromophore problem by EPR quantitation of extrusion products, has utilized for protein unfolding a ternary solvent mixture rich in Me_2SO.

We regard the principal cause of incomplete extrusion to be insufficient penetration of R_FSH to all potentially extrudable sites. Unfortunately, Me_2SO/H_2O and NMF/H_2O solvent mixtures (or combinations thereof) effected $[Fe_4S_4(SR_F)_4]^{2-} \rightarrow [Fe_2S_2(SR_F)_4]^{2-}$ conversion or degradation reactions of these complexes at rates sufficient to interfere with quantitation. Penetration of R_FSH to an extent sufficient for quantitative extrusion is potentially a separate problem for each protein. Molecular weight is not necessarily a criterion, as exemplified by the foregoing results with XO. The results for the FeMo proteins notwithstanding, 80% HMPA/H_2O is in our experience the most generally satisfactory solvent medium for extrusion work.

The ^{19}F NMR extrusion and cluster transfer[81] techniques concur in the identification of Fe_4S_4 sites in the semireduced FeMo protein forms,[86] essential conservation of the number of such sites in the oxidized form, and the appearance of a significant proportion of Fe_2S_2 sites only in the oxidized form. In addition to extrusion and Mössbauer spectral results, EPR observations of FeMo proteins in unfolding media,[18] under steady-state turnover conditions,[82] and in the presence of CO[11,83] all support the existence of Fe_4S_4 clusters. The extrusion technique has identified one such cluster in the Fe protein of clostridial nitrogenase[7,11,18] which serves as an electron donor to the FeMo protein. It is not yet known whether the multiple Fe_4S_4 clusters now known to be present in FeMo proteins serve only as electron-transfer and storage centers, or whether one or more of them binds substrate and participates directly in catalysis.

Lastly, it is noted that a weak signal is observed at 2.2 ppm in several extrusions of different preparations of FeMo proteins (Figures 12–14). This signal has never been observed in extrusions of $Fd_{ox,red}$ proteins nor in solutions of $[Fe_2S_2(SR_F)_4]^{2-}$ and $[Fe_4S_4(SR_F)_4]^{2-}$ free of protein, and is not due to the disulfide R_FSSR_F (Table I). It is also not found among the signals of the ligand substitution reaction products of $[Fe(S_2\text{-}o\text{-xyl})_2]^-$,[84] $[(MoFe_3S_4(SEt)_3)_2(\mu_2\text{-S})(\mu_2\text{-SEt})_2]^{3-}$,[78] and $[(MoFe_3S_4(SEt)_3)_2(\mu_2\text{-SEt})_3]^{3-}$ [85] with R_FSH in 80% HMPA/H_2O, the large majority of which occur downfield of $PhCFCl_2$ internal standard. The first of these complexes, being a rubredoxin analogue,[84] is a possible model for a 1-Fe protein site, and the two Fe-Mo-S cluster complexes contain Mo binding sites which resemble that in the proteins and FeMo-co.[78,85] It is also unlikely that this signal arises from reaction of R_FSH with any 4-Fe sites having the $[Fe_4S_4]^{3+}$ core oxidation level. Extrusion of HP_{ox} with R_FSH in 80% HMPA/H_2O, assayed spectrophotometrically, afforded high recovery of the core as $[Fe_4S_4(SR_F)_4]^{2-}$ ($n_t = 0.96$, $A_{452}/A_{540} = 2.34$); under the same conditions HP_{red} gave $n_t = 1.00$. Consequently, we are unable to identify the source of the 2.2-ppm signal, whose line width suggests that it derives from a paramagnetic species. If this species contains Fe, its intensity marks it as a minority species which cannot account for a significant proportion of total Fe extruded.

Acknowledgment. This research was supported by National Institutes of Health Grants GM-22352 at Stanford University and AI-04865 at Purdue University. Use of the facilities at the Stanford Magnetic Resonance Laboratory, supported by National Science Foundation Grant No. GR-23633 and National Institutes of Health Grant No. RR-00711, is acknowledged. We thank Dr. J. Cambray for early exploratory work in this research, R. Bare, T. D. Chung, and J. G. Reynolds for experimental assistance, and Drs. W. H. Orme-Johnson and E. Münck for useful discussions and access to experimental results before publication.

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