Table I. Relative Extinction Coefficients[a] for Ag_2 and Ag_3

	peak height	peak area
$\epsilon_1^{315}/\epsilon_2^{260}$ (Ar)	0.8 ± 0.2	0.40 ± 0.05
$\epsilon_1^{315}/\epsilon_3^{245}$ (Ar)	1.2 ± 0.5	0.6 ± 0.3
$\epsilon_1^{323}/\epsilon_2^{270}$ (Kr)		0.43 ± 0.05

[a] The corresponding wavelengths (nm) in Ar and Kr matrices are indicated as superscripts. The uncertainty limits represent estimated upper and lower bounds.

to increases in diatomic and triatomic absorptions in terms of the appropriate extinction coefficients:

$$(A_1' - A_1'') = 2\epsilon_1/\epsilon_2(A_2'' - A_2') + 3\epsilon_1/\epsilon_3(A_3'' - A_3') \quad (1)$$

It is prearranged in these experiments that Ag_4 and higher clusters are not produced in significant quantities. A_n' represents the absorbance due to Ag_n at time t', A_n'' is the absorbance due to Ag_n at time t'', and ϵ_n represents the molar extinction coefficients for Ag_n.

For very dilute conditions and short irradiation times, we can arrange that only negligible quantities of Ag_3 are formed so that eq 1 may be solved directly for ϵ_1/ϵ_2. For longer irradiation times the complete eq 1 may be used to obtain a value for ϵ_1/ϵ_3. We note here that a similar procedure, using multiple depositions at different concentrations, may also be used. However, the advantage of the photoaggregation method is that only one deposition is required for each ϵ_1/ϵ_2 or ϵ_1/ϵ_3 determination, so that the method is much more convenient and considerably more accurate since mass balance of total metal is maintained after each irradiation, thus eliminating the need for multiple quantitative depositions.

The extinction coefficient results are summarized in Table I where ϵ_1/ϵ_2 and ϵ_1/ϵ_3 values for Ag/Ar matrices are given for both peak-height and peak-area measurements. The value of ϵ_1/ϵ_2 was determined by both the photoaggregation and deposition procedures and the results were in satisfactory agreement. The results in Table I show that ϵ_1/ϵ_2 is essentially invariant, within experimental error, to the change from Ar to Kr matrices. It is appropriate to note here that the final cluster size distribution is quite different in Ar compared with Kr matrices. Thus, in Ar matrices Ag_3 forms readily on irradiation, but only to a very small extent in Kr matrices. The possibility of exploiting this matrix dependence to produce very narrow cluster size distributions will be discussed elsewhere.

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Steven A. Mitchell, Geoffrey A. Ozin*

Lash Miller Chemical Laboratories and Erindale College
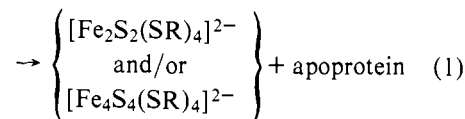*University of Toronto, Toronto, Ontario, Canada M5S 1A1*

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Structural Identification of the Extruded Cores of the Active Centers of Iron–Sulfur Proteins by Fluorine-19 Nuclear Magnetic Resonance Spectroscopy. Application to Milk Xanthine Oxidase

Sir:

The core extrusion method for identification of active centers in iron–sulfur proteins, as developed in this[1-4] and another laboratory,[5-7] is based on the ligand exchange reaction (eq 1)

holoprotein + RSH

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

conducted with excess thiol in a medium capable of unfolding protein tertiary structure. In usual practice the reaction solution after extrusion is complete is examined spectrophotometrically at 400–700 nm in order to determine the type and number (n_d, Fe_2S_2; n_t, Fe_4S_4) of Fe–S centers removed from a protein molecule. Spectrophotometric assay of extrusion products is unsatisfactory for proteins containing visible chromophores (e.g., flavin) or components (e.g., Mo) possibly capable of forming such chromophores upon reaction with thiol, unless suitable blanks are available[3] or recourse is taken to separation procedures. To circumvent this difficulty[7b] we have developed a ^{19}F FT NMR method of identification of extruded protein core structure which is based on the paramagnetism of $[Fe_2S_2(SR)_4]^{2-}$ and $[Fe_4S_4(SR)_4]^{2-}$ complexes and the attendant sensitivity of their contact-shifted resonances[8-10] to differences in these structures.

p-Trifluoromethylbenzenethiol[11] (δ 9.5), $(Et_4N)_2$-$[Fe_2S_2(SR_F)_4]$[12,13] (δ 3.7, ϵ_M^{476} 11 200), and $(Et_4N)_2$-$[Fe_4S_4(SR_F)_4]$[14] (δ 6.4, ϵ_M^{452} 18 000) were prepared by published procedures; band maxima (nanometers), extinction coefficients, and ^{19}F chemical shifts (parts per million at -15 °C relative to $PhCFCl_2$) as determined in the extrusion medium (4:1 v/v $HMPA/H_2O$ (50 mM TrisCl, pH 8.5), R_FSH/Fe mol ratio $\sim100/1$) used throughout this work are indicated. The efficacy of R_FSH as an extrusion reagent was first investigated using the spectrophotometric method[2] as applied to *Clostridium pasteurianum* Fd_{ox}[15] (A_{390}/A_{285} 0.80, mol wt 6200, 2 Fe_4S_4) and spinach Fd_{ox}[16] (A_{420}/A_{285} 0.44, mol wt 10 660, 1 Fe_2S_2) preparations.[17] The following data reveal R_FSH to be as effective as $PhSH$[1,2] under comparable conditions: *C. pasteurianum* Fd_{ox}, seven determinations (9–25 μM), R_FSH/Fe 55–150/1, $\bar{n}_t = 1.94 \pm 0.02$; spinach Fd_{ox}, six determinations (25–44 μM), R_FSH/Fe 100–400/1, $\bar{n}_d = 1.00 \pm 0.09$.

The ^{19}F FT NMR method of extruded protein core structure identification is illustrated with *C. pasteurianum* Fd_{ox} and spinach Fd_{ox} in Figure 1, where experimental conditions are defined. As in the spectrophotometric procedure, aqueous protein buffer solutions are diluted 5-fold with HMPA containing R_FSH, the extrusion reaction is allowed to proceed to completion at 25 °C, and the ^{19}F spectrum is then acquired at -15 °C, at which temperature the limit of slow exchange between free R_FSH and coordinated thiolate is attained or very closely approached. Chemical shifts and line widths are the same as those of $[Fe_2S_2(SR_F)_4]^{2-}$ and $[Fe_4S_4(SR_F)_4]^{2-}$ measured separately under the same conditions. Complete signal resolution of these complexes and of the large excess of R_FSH is apparent; contact shifts of -5.8 ($[Fe_2S_2(SR_F)_4]^{2-}$ and -3.1 ppm ($[Fe_4S_4(SR_F)_4]^{2-}$) are obtained from the chemical shifts. Note that the presence of 4 CF_3 groups affords a factor of 12 in equivalent ^{19}F concentration over M (complex). Quantitation is achieved by addition of a fixed amount
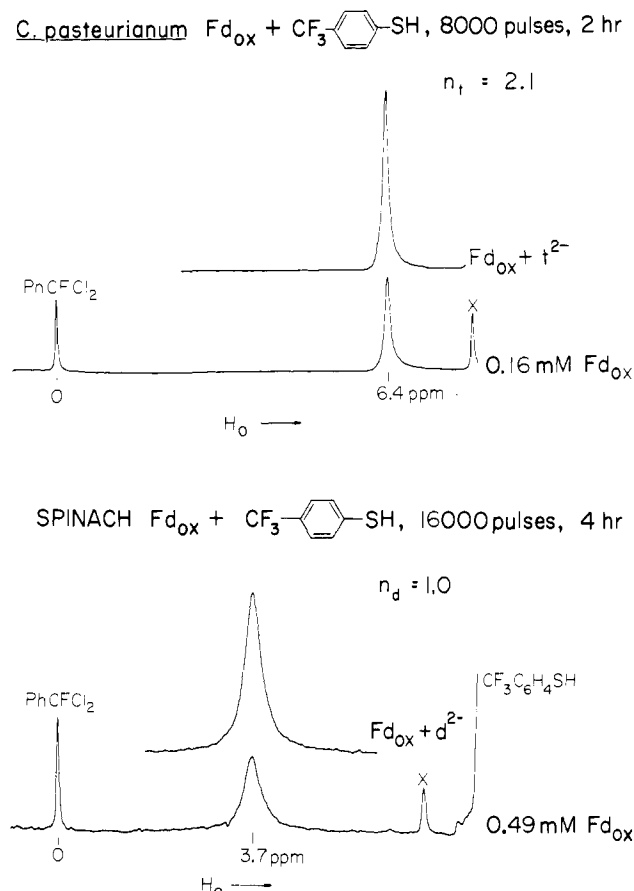
<u>C. pasteurianum</u> Fd$_{ox}$ + CF$_3$—⟨◯⟩—SH, 8000 pulses, 2 hr

XANTHINE OXIDASE + CF$_3$—⟨◯⟩—SH, 16000 pulses, 4 hr

SPINACH Fd$_{ox}$ + CF$_3$—⟨◯⟩—SH, 16000 pulses, 4 hr

Figure 2. ^{19}F FT NMR spectra (94.1 MHz) of 4:1 v/v HMPA/H$_2$O (50 mM TrisCl, pH 8.5) xanthine oxidase solutions at -15 °C after completion of active site core extrusion reaction and addition of standard. Lower spectrum: initial protein concentration 0.12 mM (based on flavin), R$_F$SH/Fe mol ratio 100/1, after 45-min reaction time at 25 °C. Upper spectrum: after addition of 2.4 μL of 46.5 mM [Fe$_2$S$_2$(SR$_F$)$_4$]$^{2-}$ (d^{2-}) solution in DMF to 450 μL of reaction solution. Both spectra were acquired with 16 000 pulses (4 hr); n_d = 2.1/flavin.

Figure 1. ^{19}F FT NMR spectra (94.1 MHz) of 4:1 v/v HMPA/H$_2$O (50 mM TrisCl, pH 8.5) protein solutions at -15 °C after completion of active site core extrusion reactions and addition of standard. Top: *C. pasteurianum* Fd$_{ox}$, initial concentration 0.16 mM, R$_F$SH/Fe mol ratio 100/1; lower spectrum, after 30-min reaction time at 25 °C; upper spectrum, after addition of 7.0 μL of 23.4 mM [Fe$_4$S$_4$(SR$_F$)$_4$]$^{2-}$ (t^{2-}) solution in DMF to 500 μL of reaction solution. Both spectra were acquired with 8000 pulses (2 h); n_t = 2.1. Bottom: spinach Fd$_{ox}$, initial concentration, 0.49 mM, R$_F$SH/Fe mol ratio 52/1; lower spectrum, after 45-min reaction time at 25 °C; upper spectrum, after addition of 3.0 μL of 29.5 mM [Fe$_2$S$_2$(SR$_F$)$_4$]$^{2-}$ (d^{2-}) solution in DMF to 500 μL of reaction solution. Both spectra were acquired with 16000 pulses (4 h); n_d = 1.0. The signal marked with x here and in Figure 2 is associated with R$_F$SH.

of the appropriate complex, a second spectral acquisition, and determination of the ratio of integrated signal intensities. The following results have been obtained (R$_F$SH/Fe 50–100/1): *C. pasteurianum* Fd$_{ox}$, seven determinations (100–170 μM), n_t = 2.0 ± 0.1; spinach Fd$_{ox}$, five determinations (190–490 μM), n_d = 1.0 ± 0.1. Control experiments with [Fe$_2$S$_2$(SR$_F$)$_4$]$^{2-}$ and [Fe$_4$S$_4$(SR$_F$)$_4$]$^{2-}$ under the same conditions have shown the two species to be stable to dimer ↔ tetramer conversion.

With satisfactory core extrusion quantitation achieved for small proteins, the NMR method has been applied to enzymes, among them the exhaustively studied xanthine oxidase of milk[18,19] (2 subunits, 8 Fe, 8 S, 2 Mo, 2 flavin; ~280 000 mol wt) whose Fe–S centers have not been fully clarified. XO was purified from raw, unpasteurized buttermilk according to Massey et al.[20] (A_{280}/A_{450} 5.3–5.5; lit.[20] 5.4[21]). The results of one extrusion experiment with oxidized XO[22] are shown in Figure 2; the only paramagnetically shifted signal observed is from [Fe$_2$S$_2$(SR$_F$)$_4$]$^{2-}$. Using spectral acquisition times of 4–5 h and ~100/1 R$_F$SH/Fe mole ratios at the specified protein concentrations these n_d/flavin results were obtained: 93 μM, 1.9; 117 μM, 2.1; 136 μM, 2.1. Thus we conclude that the XO 2-subunit complex contains 8 Fe atoms organized into 4 Fe$_2$S$_2$ centers, consistent with (but not proven by) the ab-
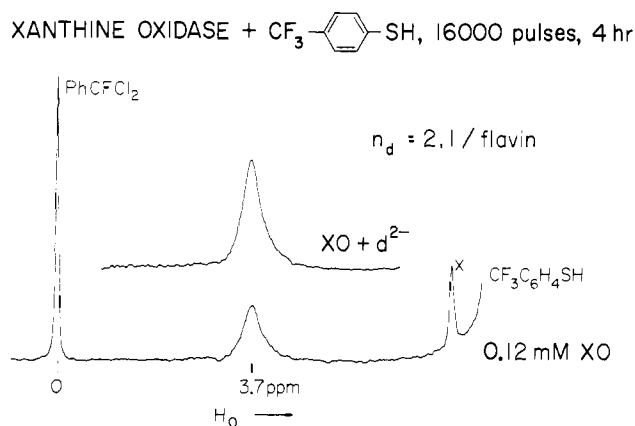
sorption spectrum of the deflavo enzyme,[23] the EPR spectrum of the reduced enzyme in 4/1 v/v Me$_2$SO/H$_2$O,[24] and the 2e$^-$ uptake/half XO molecule in the fully reduced enzyme.[25] These results should assist in further interpretation of the spectroscopic properties of XO$_{ox,red}$,[18,26] for which collective evidence[18,19] suggests an electron storage and transfer role of its Fe$_2$S$_2$ centers.

Full details of the ^{19}F NMR extrusion technique and its application to xanthine oxidase and other Fe–S enzymes will be reported subsequently.[27]

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Donald M. Kurtz, Jr.,[28] **Geoffrey B. Wong, R. H. Holm***

Department of Chemistry, Stanford University
Stanford, California 94305
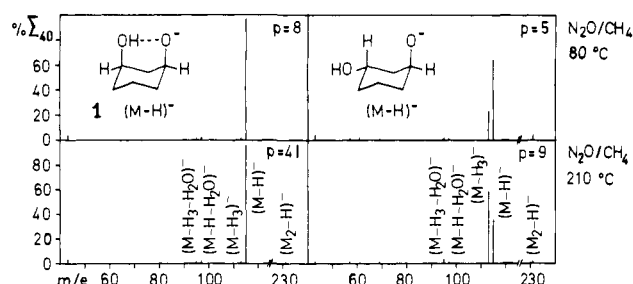*Received May 11, 1978*

Figure 1. OH$^-$ NCI mass spectra of *cis*- and *trans*-1,3-cyclohexanediols.[12] Intensities in percentage of substrate ions, %Σ_{40}. The C-13 isotope peaks are omitted. The "*p*" values represent the percentage of substrate ions relative to total ionization, used as a sample pressure indication.
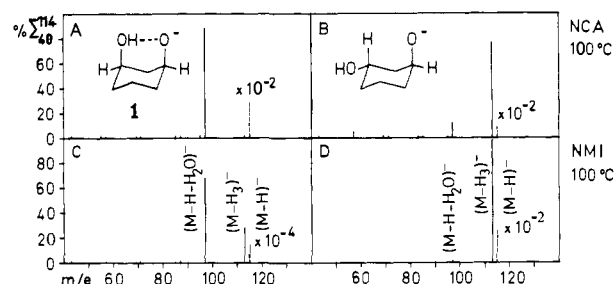

Figure 2. NCA and NMI spectra of (M $-$ H)$^-$ ions from *cis*- and *trans*-1,3-cyclohexanediols.[12] Intensities in percentage of product ions, %Σ_{40}^{114}. The NCA spectra, corrected for NMI contributions, were obtained at a helium pressure which reduced the precursor ion intensity to one third of its original value.

Stereochemical Effects on Anion Mass Spectra of Cyclic Diols. Negative Chemical Ionization, Collisional Activation, and Metastable Ion Spectra

Sir:

Gaseous alkoxide anions, (M $-$ H)$^-$, can be formed under a variety of ionizing conditions and provide conclusive mass spectrometric molecular weight information in the alcohol series.[1-3] In contrast to the importance of stereochemistry in positive ion mass spectrometry,[4] there are no detailed and systematic investigations on the stereochemistry of gas phase anions.[5] This also applies to the class of cyclic alcohols, especially diols, which are model compounds of natural products. The observations on dimeric (M$_2$ $-$ H)$^-$ alkoxide ions[2,6] indicated to us that the stereochemical studies, based on intramolecular hydrogen bridge effects in diol type mass spectra,[7-11] might be extended from the cationic species MH$^+$ and M$^+$· to the anionic species (M $-$ H)$^-$.

We used OH$^-$ negative chemical ionization (NCI)[3] to produce (M $-$ H)$^-$ parent ions for the stereochemical investigations.[12] The NCI mass spectra of the cis and trans isomers of 1,3- and 1,4-cyclohexanediol and 1,2-cyclopentanediol are shown in Figure 1 and Table I. The differences in the spectra of the configurational isomers are substantial and depend strongly on the geometry of the (M $-$ H)$^-$ ions. The only large peaks in the spectra of the cis isomers are the (M $-$ H)$^-$ parent ions; fragment ions are generally below 5% of the total substrate ion current. All of the trans isomers form (M $-$ H$_3$)$^-$ ion products with high intensity by loss of H$_2$ from (M $-$ H)$^-$,

Table I. Partial OH$^-$ NCI Spectra of Cyclic Diols (% Σ_{40})^a

| | 1,4-cyclohexanediol | | | | 1,2-cyclopentanediol | |
| | 140 °C | | 230 °C | | 240 °C | |
ion	p 0.8 cis	p 0.2 trans	p 26 cis	p 43 trans	p 51 cis	p 65 trans
(M $-$ H $-$ H$_2$O)$^-$	1.7	12	4.0	20	2.2	1.4
(M $-$ H$_3$)$^-$	7	6	3.7	11	3.1	56
(M $-$ H)$^-$	81	61	82	50	82	37

^{a} See Figure 1

a fragmentation known from anion mass spectra of primary and secondary monoalcohols.[1-3] Different from the 1,2- and 1,3-diol constitution, the trans-1,4 derivative preferentially loses H$_2$O to form (M $-$ H $-$ H$_2$O)$^-$ ions.

The stabilization of the (M $-$ H)$^-$ ions with cis geometry should arise from the existence of an intramolecular hydrogen bridge as shown for the various cis conformations **1–3**. The
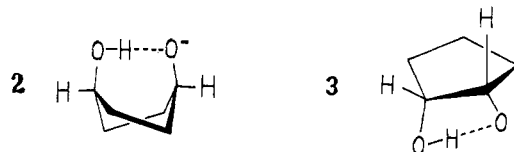
trans configuration of the diols discussed here does not permit any conformation suitable for intramolecular hydrogen bridging. A similar hydrogen bridge stabilization effect is well known for the MH$^+$ species in positive CI spectra of cyclic cis diols[9,10] and cis amino alcohols.[8] Dimeric (M$_2$ $-$ H)$^-$ peaks in the NCI spectra of the diols (Figure 1) are due to intermolecular H bonding.[2]

The temperature dependence of the NCI spectra was checked for 1,3- and 1,4-cyclohexanediols. High ion source temperatures around 220 °C are more favorable than lower temperatures for assignment of the diol configuration. As NCI generally gives low energy (M $-$ H)$^-$ sample ions,[13] additional thermal energy will increase the intensity of the stereospecific fragmentations. In positive CI spectra appropriate energy conditions are also needed for maximizing stereochemical effects.[9]

Fragmentation of unreactive anions can also be achieved by collisional activation[14] as reported by Bowie.[15] Figure 2A,**B** gives the negative collisional activation (NCA) spectra of the (M $-$ H)$^-$ ions from *cis*- and *trans*-1,3-cyclohexanediols. The difference for the configurational isomers is increased dramatically regarding the ratio of the (M $-$ H$_3$)$^-$ product peaks, even compared with the high temperature NCI spectra. Additional structural information is shown by intense (M $-$ H