Interaction of Cobalt(II) Bovine Carbonic Anhydrase with Aniline, Benzoate, and Anthranilate

I. Bertini,* C. Luchinat, and A. Scozzafava

Contribution from the Cattedra di Chimica Generale ed Inorganica, Facolta' di Farmacia dell 'Universita' di Firenze, and Laboratorio per lo Studio dei Composti di Coordinazione del CNR, Firenze, Italy. *Received May 10, 1976*

Abstract: Interaction of cobalt(II) bovine carbonic anhydrase with benzoate, aniline, and anthranilate has been investigated through electronic and ^{13}C NMR spectroscopy. The apparent equilibrium constants have been determined from the electronic spectral data through a curve fitting program. The spectroscopic data indicate that the aniline adduct is predominantly tetracoordinated and tetrahedral, whereas the system containing the benzoate also shows evidence for five-coordinated species. The experimental data for the enzyme–anthranilate system are consistent with a monodentate behavior of the ligand and a predominant tetrahedral geometry.

Carbonic anhydrase is a metalloenzyme containing Zn(II) with molecular weight of ca. 30 000, which catalyzes hydration of CO_2 and dehydration of HCO_3^- as well as hydrolysis of esters.[1-3] The metal, which is involved in the biological active site, can be removed and substituted with other bivalent metal ions; when cobalt(II) substitutes the native zinc(II), the enzyme activity is substantially preserved.[4]

X-ray studies[5] on the crystalline native enzyme have shown that the zinc ion is bound to three histidine nitrogens of the protein and to a fourth small ligand group whose nature has been controversial,[6-8] although it often has been suggested to be either H_2O or OH^-.[9,10] The cobalt(II) enzyme in solution shows an electronic spectrum which is strongly pH dependent and indicative of an equilibrium between two forms.[3,4] The form predominant at low pH values (5-6) is assumed to be tetrahedral probably with H_2O as the fourth ligand, whereas the structure of the form predominant at higher pH values (8-9) (probably containing OH^-) is not yet established, since both a tetracoordinated tetrahedral[4,6] and a five-coordinated coordination[6] could be consistent with the spectral data.

Several ligands are capable of binding to the metal of the enzyme, either native[11] or cobalt(II) substituted,[12-14] with formation constants which do not parallel the usual stability constants of metal-ligand systems of inorganic models. For example, pyridine and acetylacetone have a very low affinity for the native enzyme, whereas the cyanate ion and especially the sulfonamides are very strong inhibitors.[14]

In this framework we have investigated the donor ability of the benzoate, anthranilate, and aniline against cobalt(II) bovine carbonic anhydrase (CoBCA hereafter) through the analysis of the electronic and ^{13}C NMR spectra. A goal of the present research is the investigation of the geometry of the adduct species, including an attempt to establish the factors determining the affinity of the inhibitor for the cobalt(II) ion.

Experimental Section

Bovine carbonic anhydrase (carbonate hydrolyase, E.C. 4.2.1.1.) was obtained as a lyophilized material from Sigma Chemical Co. and used without further purification. Aniline, benzoic acid, anthranilic acid, and all other materials were of analytical grade. The concentration of bovine carbonic anhydrase was determined from the absorbance at 280 nm, using a molar absorbance of 5.7×10^4 M^{-1} cm^{-1}.[15]

Enzymatic activity was assayed using *p*-nitrophenyl acetate as substrate, at 25 °C.[16] The specific activity compared with the value reported by Cockle[16] showed the enzyme to be greater than 94% active.

Apocarbonic anhydrase was prepared from the commercial enzyme by dialysis against 0.01 M 1,10-phenanthroline in 0.1 M acetate buffer, pH 5.2, to a residual activity of less than 5%.[4] The cobalt enzyme was obtained by dialysis of apocarbonic anhydrase against cobalt sulfate, pH 7.9, followed by exhaustive dialysis against phosphate or tris(hydroxymethyl)aminomethane–H_2SO_4 buffers at pH 6.75, 7.5, or 8.0. Esterase activity assayed on CoBCA showed no significant change with respect to native enzyme.

CoBCA concentration was determined from the absorbance at both 280 and 550 nm (ϵ_{550} 315, 360, 375 M^{-1} cm^{-1} at pH 6.75, 7.5, and 8.0, respectively). Concentration values obtained from the measurements at the two wavelengths were the same within 10% error, indicating that the protein was almost entirely in the cobalt enzyme form. The optical spectra of the CoBCA were strictly similar to those already reported.[3]

NMR Measurements. ^{13}C NMR spectra were recorded on a Varian CFT-20 spectrometer at 20 °C, using ca. 5×10^{-2} M acetone as an internal reference.

Spectrophotometric Measurements. Optical spectra were recorded on a Cary 17 spectrophotometer, on the 0–0.1 absorbance range, using 10^{-3}–10^{-4} M solutions of the enzyme at 22 °C.

Calculations. Spectrophotometric data were treated with a least-squares program described in ref. 17. The experimental spectra were always reproduced by the computer within an accuracy of $\pm 1 \times 10^{-3}$ in optical density assuming the equilibrium: enzyme + inhibitor $\rightleftharpoons$ enzyme·inhibitor. No fitting is obtained if a 1:2 enzyme to inhibitor stoichiometric ratio is assumed for the above equilibrium.

Results

In every case addition of varying amounts of inhibitor to the same amount of CoBCA greatly affects the shape of the electronic spectra and for each inhibitor at least two neat isosbestic points have been observed. As an example, the curves of the system CoBCA-anthranilate at pH 7.5 are reported (Figure 1). From these spectral data both the least-squares apparent constants relative to the equilibrium

$$\text{enzyme} \cdot \text{inhibitor} \rightleftharpoons \text{enzyme} + \text{inhibitor}$$

and the limit spectrum corresponding to the enzyme completely bound have been determined (Figure 2 and Table I). Since the cobalt(II) enzyme by itself displays an equilibrium between an acidic and a basic form, by assuming that the equilibrium is of the type:[4]

$$\text{adduct species} \underset{+1}{\overset{K}{\rightleftharpoons}} EH^+ \overset{K_a}{\rightleftharpoons} E + H^+$$

the actual instability constants may be determined through the relation

$$K = K_{app}(1 + K_a/[H^+])^{-1} \quad (1)$$

where K_a is the equilibrium constant between the two forms of the enzyme. For the system CoBCA-anthranilate the apparent equilibrium constants have been determined at pH 6.75,

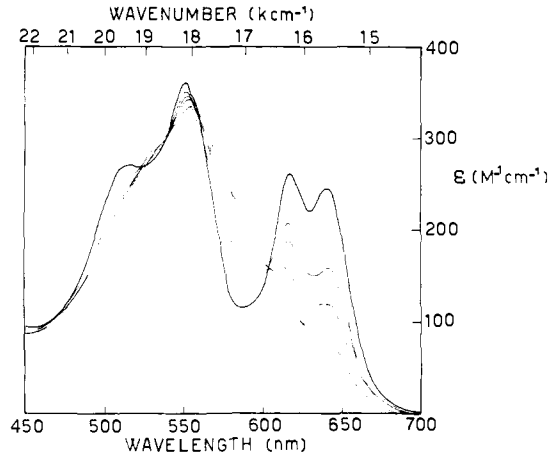


Figure 1. Electronic spectra of CoBCA 2.3×10^{-4} M with anthranilate-cobalt ratios 0, 14, 28, 56, and 112 in order of decreasing ϵ_{640}. All the spectra were recorded in 0.1 M phosphate buffer at pH 7.5.

Table I. Apparent Instability Constants of CoBCA with Anthranilate, Benzoate, and Aniline

Inhibitor	pH	K_{app}, M
Anthranilate	6.75	$(1.83 \pm 0.07) \times 10^{-3}$
	7.5	$(7.40 \pm 0.10) \times 10^{-3}$
	8.0	$(2.08 \pm 0.08) \times 10^{-2}$
Aniline	7.5	$(6.66 \pm 0.06) \times 10^{-2}$
Benzoate	7.5	$(1.49 \pm 0.05) \times 10^{-2}$

7.5, and 8.0 (Table I). The limit curves are essentially the same within the experimental error, indicating either that a single adduct species is formed or that the distribution over more than one species is essentially not pH dependent. The equilibrium constants determined through eq 1 assuming a pK_a value of 6.6[3] are 7.6×10^{-4}, 8.2×10^{-4}, and 8.0×10^{-4} M at the three pH values. The slight discrepancy among the three values might well be due to the presence of a further equilibrium with the buffering species other than to experimental errors. These data, however, seem to confirm that the choice of the equilibria is essentially correct and that the acidic species of the enzyme is the sole species binding to the inhibitor.

^{13}C NMR spectra for solutions containing a molar ratio CoBCA-inhibitor ca. $1/100$ have been recorded in order to gain structural information on the inhibited enzyme. The observed ^{13}C isotropic shifts of the inhibitors, relative to the free inhibitor at the same concentration, are as large as 1 ppm (Table II) and the line widths range between 3 and 30 Hz. The shifts for aniline are both upfield and downfield with a pattern similar to that observed for the aniline adducts of bis(acetylacetonate)nickel(II)[18] and for the hexakis(pyridine *N*-oxide) complexes of cobalt(II) and nickel(II).[19] The most shifted signals are the C-N and *p*-C, indicating that the isotropic shifts are essentially contact in origin, and that dipolar shifts, which are expected to decrease with the distance from the metal,[20,21] if any, are negligible. The detection of a carbon I·S contact term is the final evidence of direct metal to inhibitor bond.

The isotropic shift values for benzoate ion are sizable for C(1) and COO^- and then attenuate for the other ring positions. The fact that C(1) shows a shift larger than COO^- confirms that the shift pattern cannot be governed by a dipolar mechanism.

The isotropic shift pattern of the anthranilate ion with respect to the shift alternancy and signal width is similar to that of aniline. However, the observed pattern is not simply proportional to the shifts of aniline, but there is a sensible bias of

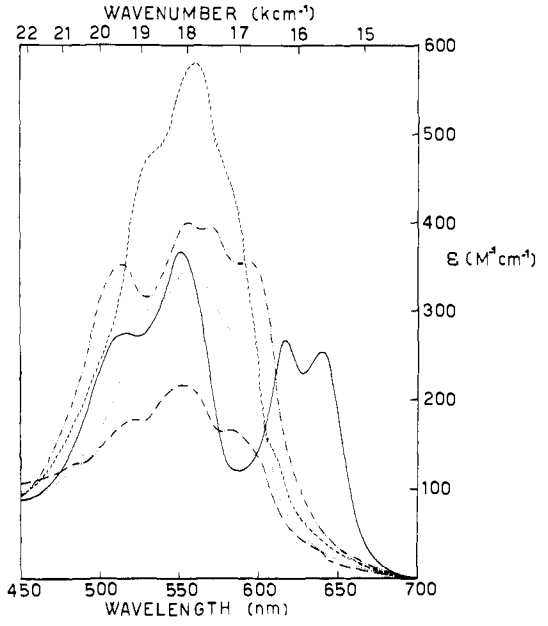


Figure 2. Calculated limit spectra of CoBCA at pH 7.5 in 0.1 M phosphate buffer with the following inhibitors: aniline (- - - - -); *p*-toluenesulfonamide (- · - · - ·); anthranilate (· · · · ·); benzoate (- · · - · · -). The full line corresponds to the pure CoBCA.

downfield shift, especially at the ring positions 1, 2, and 6, recalling the shift pattern of benzoate. ^{13}C NMR spectra were recorded at pH values of 6.75, 7.5, and 8.0; the isotropic shifts are found to be essentially pH independent.

In every case the isotropic shifts have been titrated by adding *p*-toluenesulfonamide[22] to the NMR sample solution. This allowed us to check the resonance signal dependence on the molar fraction of the bound inhibitor in order to ascertain that the ligands exchange rapidly on the NMR time scale and to rule out the possibility of irreversible reactions with the protein part of the enzyme.

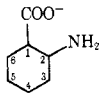
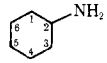
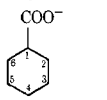
Discussion

X-ray studies[23] on the crystalline isoenzyme C of human carbonic anhydrase inhibited with a sulfonamide derivative showed that the zinc ion is tetrahedrically coordinated with three histidine nitrogens and a donor atom, presumably the nitrogen, of the inhibitor. Sulfonamide adducts of cobalt carbonic anhydrase are also believed to be tetrahedral with the same donor set.[24] We report in Figure 2 the electronic spectrum of CoBCA with *p*-toluenesulfonamide as a typical spectrum, although several sulfonamides have larger molar absorbances.

The calculated limit spectrum of the system CoBCA-aniline is also strongly indicative of a tetrahedral coordination owing to the large molar absorbance and to the position and shape of the absorption bands.[25,26]

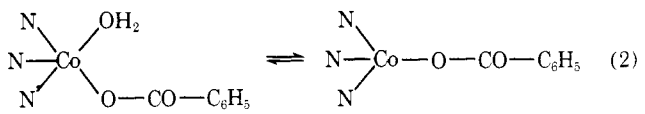
The CoBCA-benzoate spectrum shows a molar absorbance of 220 M^{-1} cm^{-1}, corresponding to one-third of that of the aniline containing system. Such a value of absorbance is relatively low with respect to many CoBCA-inhibitor systems and to tetrahedral or pseudotetrahedral cobalt(II) complexes.[7,25] The electronic spectrum of a solution 1.9×10^{-3} M in enzyme and 1.12 M in benzoate at pH 7.5 extended down to 7000 cm^{-1} shows several well-defined bands (Figure 3): among them the band at ca. 13 200 cm^{-1} has the features of a spin-allowed d-d transition and is reasonably assigned to the highest F-F transition.[27] Although the absorption ranges of low-symmetry chromophores depend on the type and extent of distortions, tetracoordinated pseudotetrahedral or six-

Table II. ^{13}C Isotropic Shifts (ppm) and Line Widths (Hz)[a] of Anthranilate, Aniline, and Benzoate in Presence of CoBCA[b]

	pH	C=O	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)
	7.5	−0.20 (5)	−0.90 (18)	0.25 (5)	−0.65[c] (16)	0.50 (13)	−0.70[c] (16)	0.20 (7)
	7.5		−0.10 (17)	0.15 (7)	−0.10 (17)	0.10 (17)	−0.15 (31)	0.10 (17)
	7.5	−0.65 (7)	−1.20 (7)	−0.70 (4)	−0.10 (3)	−0.05 (3)	−0.10 (3)	−0.70 (4)

[a] Measured from the free ligand at the same concentration and pH; estimated errors for isotropic shifts are ±0.05 ppm. [b] CoBCA 1.84×10^{-3}, 2.83×10^{-3}, 2.42×10^{-3} M, with 1.29×10^{-1} M aniline, 2.70×10^{-1} M benzoate, and 2.43×10^{-1} M anthranilate, respectively. [c] Accidentally degenerate in the adduct.

coordinated species do not generally show F-F transitions that high in energy. This band, on the other hand, could be consistent with the presence of a five-coordinated species;[28] its low molar absorbance, as well as the resulting intensity of the absorptions in the visible region and the complexity of the spectrum in the infrared region are consistent with the following equilibrium (eq 2). An equilibrium of this type was already



(2)

suggested by Williams[6] for other anionic inhibitors on the basis of the spectral features of the adducts in the visible region.

The ^{13}C signal width deserves a comment with respect to the possibility that five coordination is reached through a bidentate behavior of the benzoate. The line width of $^{13}COO^-$ (and consistently of the other carbon atoms) are one order of magnitude smaller than those of the CoBCA-acetate system under the same conditions, whereas the shifts are comparable.[29] If it is assumed as determinant for the line width the exchange rate[20,22] and if such a rate depends on the type of ligation of the carboxylate group it may be concluded that the exchange rate is quite fast and that the benzoate behaves as monodentate.

The assignment of the structure to the anthranilate adduct of CoBCA is complicated by the presence of two groups capable of binding to the metal. If the ligand behaved as bidentate a coordination number larger than four would have been expected. However, the electronic spectrum for a solution 1.6×10^{-3} M in enzyme and 8.7×10^{-1} M in anthranilate extended in the near infrared region does not show any observable band between 12 and 14 μm^{-1}. Molar absorbances (ϵ_{550} 340 M^{-1} cm^{-1}) as well as band positions are essentially consistent with a tetrahedral structure. If it is assumed that the ligand does not cause the detachment of any histidine nitrogen of the protein, a tetrahedral geometry can be obtained only through a monodentate behavior of the anthranilate. On the other hand, if the anthranilate group behaved as bidentate, major changes in the electronic spectra would have been expected and the ^{13}C NMR signal width would have been drastically affected, for bidentate ligands of this type do not exchange rapidly on the NMR time scale in inorganic models.[30] Therefore we feel that it is reasonable to assume that the anthranilate ion behaves as monodentate. The second uncoordinate donor group could be involved in hydrogen bonding with the surface of the active site cavity. Actually it is worth mentioning that the carboxylate group of the acetate ion has been found to bind to two different sites of zinc, manganese,[31,32] and cobalt[29] enzymes, i.e., to the metal and to the cavity, with larger affinity for the latter site. This second site probably plays an important role in the enzymatic catalysis and in the dissociation rates of CoBCA-inhibitor adducts.[22] Therefore the amino group could bind to the metal and the carboxylate group to the surface of the cavity. However, if the bias of downfield shift in the aromatic ring is taken as evidence that the latter group also binds to the metal, the conclusion is that the anthranilate ligand behaves as monodentate with only one donor group coordinated at a time.

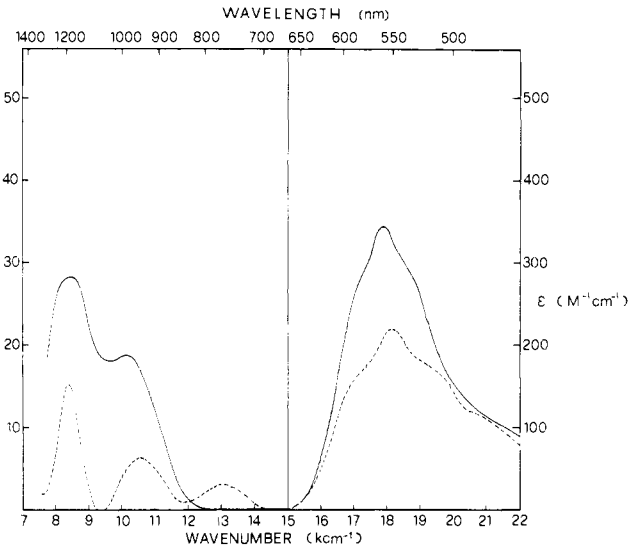


Figure 3. Electronic spectra of CoBCA 1.9×10^{-3} M in the presence of 1.12 M benzoate in D_2O (- - - - -) and of CoBCA 1.6×10^{-3} M in the presence of 8.7×10^{-1} M anthranilate in D_2O (—) at uncorrected pH 7.5 and 0.1 M phosphate buffer.

Acknowledgment. Thanks are expressed to Professor L. Sacconi for helpful discussions and encouragement.

References and Notes

(1) J. E. Coleman, "Inorganic Biochemistry", Vol. 1, G. I. Eichhorn, Ed., Elsevier, London, 1973, p 488.
(2) S. Lindskog, L. E. Enderson, K. K. Kannan, A. Liljas, P. O. Nyman, and B. Strandberg, *Enzymes, 3rd Ed.*, **5**, 587 (1971).
(3) S. Lindskog, *Struct. Bonding (Berlin)*, **8**, 153 (1970).
(4) S. Lindskog and B. G. Malmström, *J. Biol. Chem.*, **237**, 1129 (1962).
(5) K. K. Kannan, B. Notstrand, K. Fridborg, S. Lövgren, A. Ohlsson, and M. Petef, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 51 (1975).
(6) A. E. Dennard and R. P. J. Williams, *Transition Met. Chem.*, **2**, 115 (1966).
(7) S. Lindskog, *J. Biol. Chem.*, **238**, 945 (1963).
(8) S. H. Koenig and R. D. Brown III., *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2422 (1972).
(9) M. E. Riepe and J. M. Wang, *J. Biol. Chem.*, **243**, 2779 (1968).
(10) D. N. Silverman and C. K. Tu, *J. Am. Chem. Soc.*, **98**, 978 (1976).
(11) J. T. Stone and Y. Pocker, *Biochemistry*, **7**, 2936 (1968).
(12) P. W. Taylor, J. Feeney, and A. S. V. Burgen, *Biochemistry*, **10**, 3866 (1971).
(13) E. Grell and R. C. Bray, *Biochim. Biophys. Acta*, **236**, 503 (1971).
(14) S. A. Cockle, S. Lindskog, and E. Grell, *Biochem. J.*, **143**, 703 (1974).
(15) P. O. Nyman and S. Lindskog, *Biochim. Biophys. Acta*, **85**, 141 (1954).

(16) S. A. Cockle, *Biochem. J.*, **137**, 587 (1974).
(17) R. Barbucci, L. Fabbrizzi, and P. Paoletti, *J. Chem. Soc., Dalton Trans.*, 1099 (1972).
(18) I. Morishima, T. Yonezawa, and K. Goto, *J. Am. Chem. Soc.*, **92**, 6651 (1970).
(19) I. Bertini, C. Luchinat, and A. Scozzafava, *Inorg. Chim. Acta*, **19**, 201 (1976).
(20) G. N. La Mar, W. De W. Horrocks Jr., and R. H. Holm, "NMR of Paramagnetic Molecules", Academic Press, New York, N.Y., 1973.
(21) B. R. Mc Garvey, *J. Chem. Phys.*, **53**, 86 (1970).
(22) P. L. Yeagle, C. H. Lochmuller, and R. W. Henkens, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 454 (1975).
(23) K. Fridborg, K. K. Kannan, A. Liljas, J. Lunden, et al., *J. Mol. Biol.*, **25**, 505 (1967).
(24) J. E. Coleman and R. V. Coleman, *J. Biol. Chem.*, **247**, 4718 (1972).
(25) R. L. Carlin, *Transition Met. Chem.*, **1**, 1 (1966).
(26) S. Lindskog and A. Ehrenberg, *J. Mol. Biol.*, **24**, 133 (1967).
(27) R. Morassi, I. Bertini, and L. Sacconi, *Coord. Chem. Rev.*, **11**, 343 (1973).
(28) I. Bertini, D. Gatteschi, and A. Scozzafava, *Inorg. Chem.*, **14**, 812 (1975).
(29) I. Bertini, C. Luchinat, and A. Scozzafava, *Biochim. Biophys. Acta*, **452**, 239 (1976).
(30) I. Bertini and L. Sacconi, *J. Mol. Struct.*, **19**, 371 (1973).
(31) A. Lanir and G. Navon, *Biochim. Biophys. Acta*, **341**, 65 (1974).
(32) A. Lanir and G. Navon, *Biochim. Biophys. Acta*, **341**, 75 (1974).

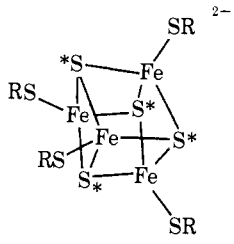
Quantitative Extrusions of the Fe_4S_4* Cores of the Active Sites of Ferredoxins and the Hydrogenase of *Clostridium pasteurianum*

W. O. Gillum,[1a] L. E. Mortenson,[1b,c] J.-S. Chen,[1b] and R. H. Holm*[1a]

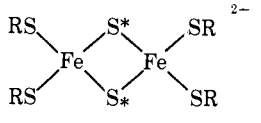
Contribution from the Department of Chemistry, Stanford University, Stanford, California 94305, and the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. *Received May 17, 1976*

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

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



1



2

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

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

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

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

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