Supplementary Material Available: A listing of atomic coordinates for crystals I and II (6 pages). Ordering information is given on any current masthead page.

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Paramagnetic Carbon Monoxide Adducts of Reduced Iron–Sulfur Tetramers: Relevance to the Mechanism of Action of Hydrogenase

Sir:

The reversible oxidation of molecular hydrogen and reduction of protons catalyzed by the bacterial enzyme hydrogenase is conceptually the simplest reaction in biochemistry. Despite the apparent simplicity of the reaction, the detailed mechanism of action has yet to be elucidated. Studies on the enzyme-catalyzed equilibration of H_2 and D_2O demonstrated that molecular hydrogen was cleaved heterolytically,[1] suggesting that the enzyme induces an inequivalence of the two hydrogen atoms of bound H_2. It has been shown[2,3] that a preparation of hydrogenase from *Clostridium pasteurianum*, containing 4Fe and $4S^{2-}$ per molecule, possesses as prosthetic group a single tetranuclear iron-sulfur cluster. (A different preparation with a higher Fe-S content has been shown to contain three tetrameric units per molecule[4].) In addition, Erbes et al.[2] demonstrated that the Fe_4S_4 cluster of hydrogenase could exist in three distinct oxidation states related by transfer of one electron, and that carbon monoxide, a competitive inhibitor of H_2,[5] could bind directly to the iron-sulfur cluster in both the most oxidized and reduced states.

We anticipated that CO would also coordinate to nonprotein bound iron-sulfur clusters in the appropriate oxidation states. Salts of $[Fe_4S_4(SR)_4]^{1-}$, isoelectronic with the most oxidized state of hydrogenase, have not yet been prepared,[6,7] while $[Fe_4S_4(SR)_4]^{2-}$, corresponding to the diamagnetic intermediate oxidation state of hydrogenase, shows no evidence from optical spectra for reaction with CO. Consequently, we have investigated the interaction of CO with the reduced iron-sulfur species, $[Fe_4S_4(SPh)_4]^{3-}$, generated in solution by chemical reduction of the dianion with sodium acenaphthalenide.[8] Inasmuch as the intense absorption of acenaphthalene and its radical anion precluded absorbance measurements, we chose to monitor the reaction using EPR spectrometry. As shown in Figure 1, the reaction of CO with $[Fe_4S_4(SPh)_4]^{3-}$ produces

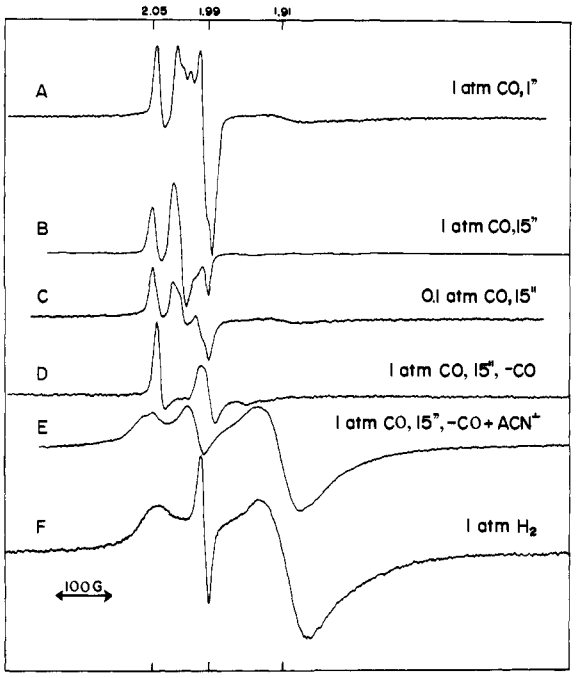

Figure 1. EPR spectra of 0.7–0.8 mM $(Et_4N)_2[Fe_4S_4(SPh)_4]$ in *N,N*-dimethylacetamide treated as follows: A, 1 atm of CO, frozen 1 min after adding 4 equiv of 1 M acenaphthalenide radical ion (ACN^-) in THF; B, same as A, but frozen 15 min after ACN^- addition; C, same as B, but under 0.1 atm of CO; D, same as B, but evacuated after 15 min and CO replaced with argon; E, same as D, but with addition of 4 equiv of ACN^-; F, 1 atm of H_2, frozen 15 min after addition of 4 equiv of ACN^-. The small $g = 2.00$ signal seen in Figure 1F (and also in Figure 2A) is of variable intensity and unknown origin (possibly ACN^-). It is always observed in $[Fe_4S_4(SPh)_4]^{3-}$ samples prepared in this way. The broader signal at $g \sim 2.01$ in Figure 1E may be due to some degradation of the cluster during the cycle. Conditions of EPR spectroscopy: microwave frequency, 9.1 kHz; microwave power, 30 μW; modulation amplitude, 10 G; magnetic field sweep rate, 500 G min^{-1}; time constant, 0.3 s; sample temperature, 10.2 K; instrument gain, 5000 (A), 200 (B), 1000 (C, E), 2000 (D), 3200 (F).

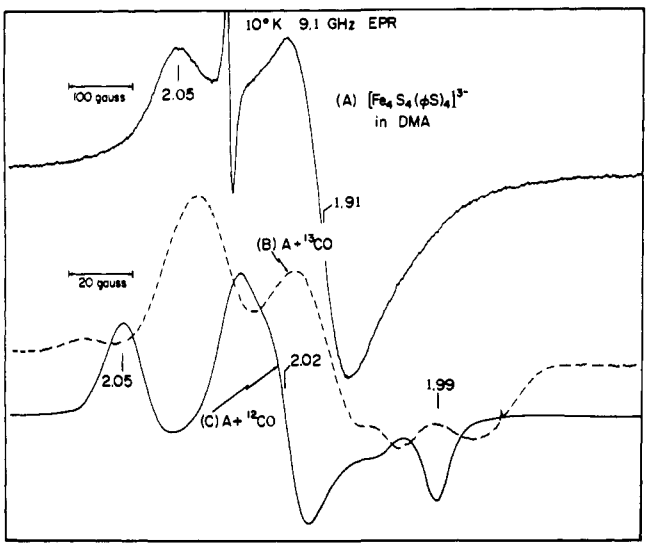

Figure 2. EPR spectra of A, $[Fe_4S_4(SPh)_4]^{3-}$; B, sample as in A, under 0.4 atm of ^{13}CO, frozen at 20 min after ACN^- addition; C, sample as in A, under 1 atm of ^{12}CO, frozen at 15 min after ACN^- addition. Conditions of EPR spectroscopy are as in Figure 1 except the following: magnetic field sweep rate, 100 G min^{-1} (B, C); instrument gain, 1600 (A), 400 (B), 320 (C).

complex EPR spectra which slowly (~15 min) evolve to reach what appears to be an equilibrium mixture, in which ~5–10%

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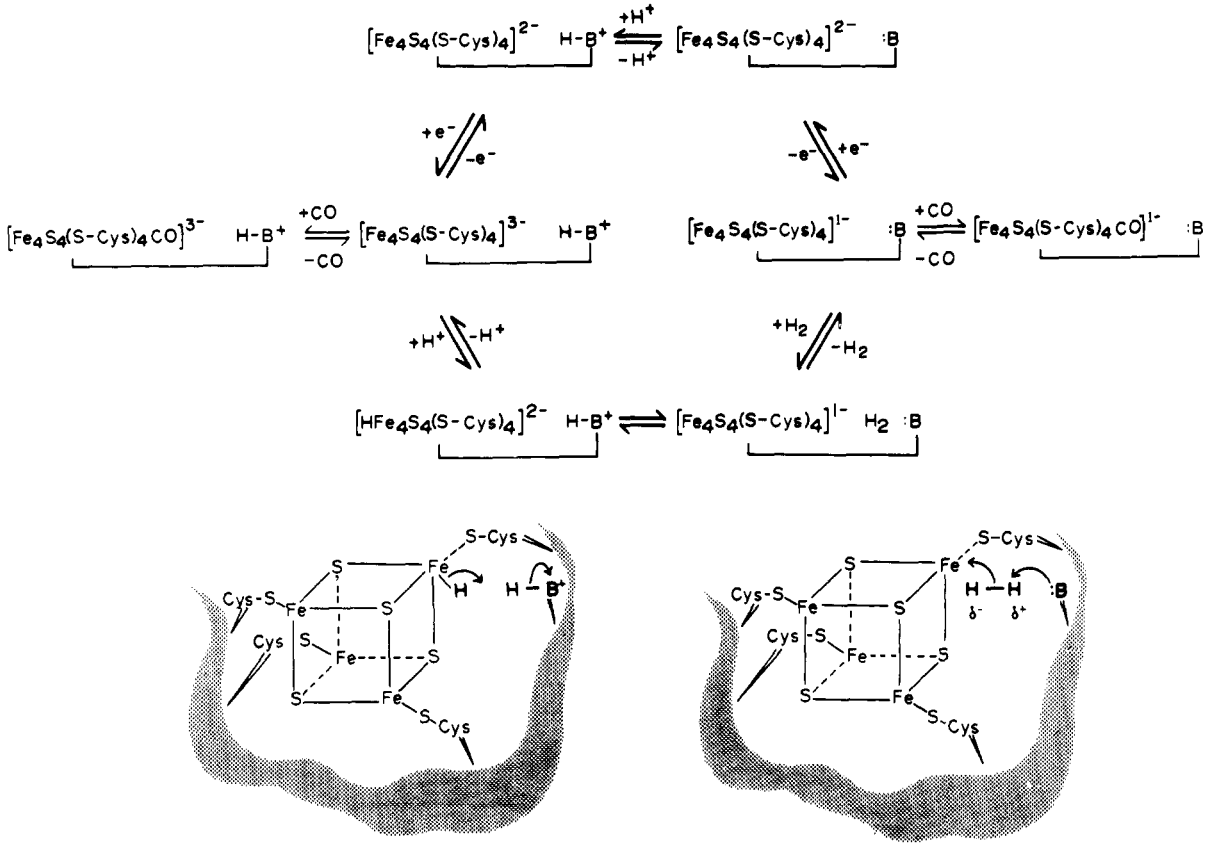

Figure 3. Proposed mechanism of action of hydrogenase. The catalytic groups required include a protein-bound 4Fe-4S cluster[5] and a nearby base B.[13] The protonation of the cluster in the trianion state leads to the formation of hydride which combines with the proton of HB. The microscopic reverse requires the binding of H_2 to the monoanion state of the cluster followed by metal hydride formation.[12] The two protons are thus inequivalent. Binding of CO renders the active site groups incapable of reduction of protons by competition for the hydride production site.

(by double integration) of the total iron-sulfur cluster content is paramagnetic. The final EPR spectrum (Figure 1B) is stable for hours in the absence of oxygen, but its rate of formation is highly dependent on the CO concentration (cf. Figure 1B,C). Evacuation and replacement of CO by argon results in the loss of >90% of the paramagnetic species (Figure 1D); subsequent addition of fresh acenaphthalenide[9] results in formation of $[Fe_4S_4(SPh)_4]^{3-}$ in amounts comparable with those obtained under argon (Figure 1E) (i.e., 30–50% of the total iron-sulfur cluster content), demonstrating the (at least partially) reversible nature of CO binding.[10] Finally, the EPR spectrum of $[Fe_4S_4(SPh)_4]^{3-}$ prepared under a hydrogen atmosphere (Figure 1F) shows no evidence for binding of H_2 to the reduced cluster.

To ascertain whether CO was directly bound to the iron-sulfur tetramer, we used ^{13}CO in a parallel experiment. The results are shown in Figure 2. Although the EPR spectrum with ^{12}CO is clearly a superposition of at least two species, each component is split by ~24 G when ^{13}CO is used. This is especially clear at the $g = 1.99$ and $g = 2.05$ features. This argues that both of the species present contain *one* ^{13}CO ($I_{^{13}C} = \frac{1}{2}$) interacting with the unpaired electron, i.e., bound to iron, and that, fortuitously, both have hyperfine coupling constants to the ^{13}C nucleus of the same magnitude. It must be noted that there are significant differences between these spectra and those reported for *reduced* hydrogenase treated with CO. First, the EPR of the latter has $g_{av} < 2$, as found for reduced iron-sulfur clusters, while the CO adducts of the synthetic species have $g_{av} > 2$. Second, the ^{13}C hyperfine interaction observed with the synthetic compounds is an order of magnitude greater than that observed for hydrogenase.[2] The origin of these differences is not obvious, but may be related to restricted ligation of the tetramer in the protein vs. the synthetic species in solution. Thus, for example, the two species observed by EPR may differ in only one ligand. Nonetheless, these results demonstrate that iron-sulfur tetramers are capable of binding ligands such as CO *both* in the presence and the absence of polypeptide chain ligands.

Together with the observation that CO is a competitive inhibitor of substrate (H_2) oxidation,[5] these data suggest that *H_2 also binds directly to the iron-sulfur prosthetic group.* A possible mechanism for hydrogenase incorporating this feature is shown in Figure 3. The key feature of this proposal is an iron hydride intermediate, produced either by protonation of cluster trianion or by H_2 reduction of the monoanion.[11] This is consistent with mechanistic studies of homogeneous hydrogenation by transition metal complexes, in which metal hydride intermediates are implicated.[12] The Krasna and Rittenberg H-D exchange data are accounted for if exchange of protons from the protonated base HB proposed to be in the active site[13] is fast compared with the overall reaction. Bruice and co-workers[14] have reported that the dianionic tetramers may be protonated to yield $[Fe_4S(SR)_4H]^-$ in a rapid equilibrium (pK_a = 3.9–7.4) preceding irreversible acid-catalyzed disruption of the Fe_4S_4 core; the actual site of protonation is not, however, clear.

Further experiments to test this proposed mechanism and detect a hydride intermediate are in progress in collaboration with Professors L. E. Mortenson and J.-S. Chen.

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(10) The solution when frozen under CO is distinctly red in color; removal of CO and addition of acenaphthalenide results in the green color characteristic of $[Fe_4S_4(SPh)_4]^{3-}$. Since the paramagnetic species observed by EPR is only a small fraction of the total iron–sulfur cluster content, it seems likely that the species responsible for the red color are diamagnetic CO adducts, probably containing more than one CO per tetramer.
(11) If, as is suggested by data relating the rate of hydrogen evolution to the solution redox potential, the H_2 evolving species is the tetraanion (L. E. Mortenson and J.-S. Chen, personal communication), the oxidation number of the clusters in our scheme should be decreased by one. The *paramagnetic* CO complexes reported by Erbes et al.[2] should, however, be as indicated.
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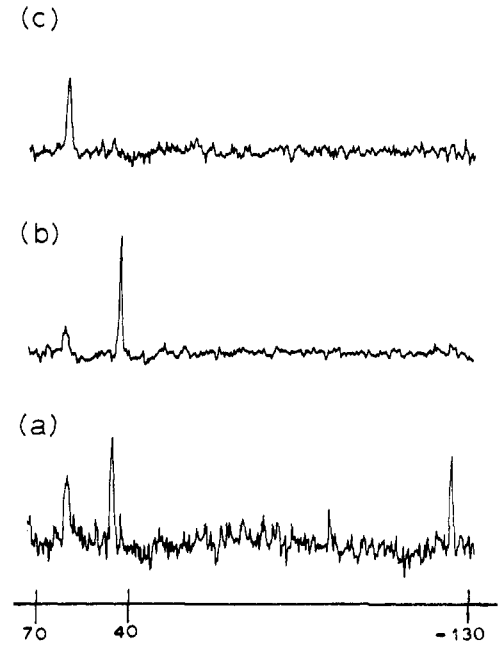

Figure 1. (a) ^{113}Cd NMR spectrum of concanavalin A to which 2 equiv of $^{113}Cd\ Cl_2$ per monomer have been added to the previously apoprotein. The same is 2.1 mM in Con A protomer. The buffer used in these experiments is 0.2 M NaCl, 50 mM NaAc, pH* 5.2, with D_2O added to provide an internal lock. The experimental conditions used to obtain all spectra shown follow: flip angle, 45°; recycle time, 0.6 s; spectral window, 10 000 Hz; number of data points collected, 8192. These spectra typically require 70 000 transients. The variations in signal-to-noise ratio within this figure denote differences in total accumulation times and the concentrations of the various samples. A 5-kHz enlargement of the spectra is displayed with 8 Hz of line broadening for sensitivity enhancement. Resonances occur at 68, 43, and −125 ppm. (b) ^{113}Cd NMR spectrum of Con A containing 2 equiv of $^{113}Cd(II)$ and an excess of Ca(II). The sample is 2.3 mM in Con A protomer. The resonance occurs at 41 ppm. (c) ^{113}Cd NMR spectrum of Con A containing 2 equiv of $^{113}Cd(II)$ and an excess of Zn(II). The sample is 1.6 mM in Con A protomer. The resonance is at 68 ppm.

Cadmium-113 Nuclear Magnetic Resonance Studies of Metalloproteins. 1. [^{113}Cd]Concanavalin A: A Preliminary Investigation

Sir:

Recent developments in multinuclear Fourier transform (FT) NMR techniques[2-5] have given researchers the capability of using a variety of NMR nuclei as probes to investigate chemical and biological systems. One application of interest is the use of metal nuclides as probes of metal–protein interactions in metalloproteins and enzymes. The native metals found in these proteins have, in general, poor high resolution NMR characteristics but may in many cases be replaced by metals with more favorable properties. One substitute nuclide with excellent NMR properties is ^{113}Cd. Several studies have been published that have investigated the ^{113}Cd NMR of a variety of inorganic and organometallic models systems.[6-10] More recently, Armitage and co-workers[11,13] have investigated the ^{113}Cd NMR of Cd(II) substituted alkaline phosphatases, human and bovine carbonic anhydrase B. Two of these metalloproteins are similar in that each has basically the same symmetry around the metal site (four coordinate or tetrahedral) and, for each protein, three of the ligands binding the metal are nitrogen; bovine carbonic anhydrase may be pentacoordinate. Despite these similarities, the reported ^{113}Cd chemical shifts of these proteins are in a range of over 160 ppm. Sudmeier[12] has also investigated the ^{113}Cd NMR of Cd(II) human carbonic anhydrase **B** with findings that differ considerably from the former work. The difference in the chemical shifts reported is 80 ppm. The origin of this chemical shift difference is unclear at the present time. In any case, the sensitivity of ^{113}Cd NMR as a probe of metal environment has been clearly demonstrated.

We wish to report here preliminary results of a ^{113}Cd FT NMR study of the protein concanavalin A (Con A). This lectin has been of great interest owing to its ability to agglutinate transformed cells selectively in regard to the normal parent cells.[14] In addition, it specifically binds saccharide moieties[15] and induces blastogenesis in lymphocytes.[16] Several reviews have appeared concerning the structure and function of Con A.[17] Con A requires two metals per monomer for saccharide binding activity. In the native protein this consists of Mn(II) occupying a site denoted S1 and Ca(II) occupying a site denoted S2.[18,19] Both of these metals may be removed and replaced with Cd(II) with saccharide binding activity retained.[20] In addition, there are other metal binding site(s) reported for Con A, although none of these site(s) have been shown to bind Cd(II).[21] ^{113}Cd NMR should be able to resolve the number and type of Cd(II) binding sites. Also, any changes in the metal environment upon binding of saccharide to Cd(II) Con A should be reflected in the ^{113}Cd NMR.

Figure 1a presents a ^{113}Cd NMR spectrum of [^{113}Cd]Con

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