function of the environment of the heme (ligand structure, electron density, acid-base properties, etc.). Therefore, it is not surprising that both pathways operate exclusively or simultaneously in a variety of hemeproteins.

Acknowledgment. This project was supported by a research grant from the National Institutes of Health (CA 47479). We are indebted to Jan Crowley for conducting some of the epoxidation experiments. NMR and mass spectra were obtained in the Central Instrument Facility of the Chemistry Department at Wayne State University. We thank John Groves, Alan Balch, and Teddy Traylor for communication of unpublished results.

^1H NMR Spectra and Electron-Transfer Properties of Oxidized and Reduced $[Fe_4Se_4]$ Derivatives of *Clostridium vinosum* High-Potential Iron Protein

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Abstract: The high-potential iron protein (HiPIP) from *Clostridium vinosum* has been reconstituted with a $[Fe_4Se_4]$ cluster. The modified protein has a reduction potential of 321 (7) mV vs NHE [μ 0.1 M phosphate; pH 7.0; 23 °C]. The upfield and downfield isotropically shifted resonances in the ^1H NMR spectra of both the oxidized and reduced proteins are assigned to β-CH_2 protons of coordinating cysteine residues and to neighboring aromatics. The variable-temperature behavior of each of these resonances is reported. Unlike the $2[Fe_4Se_4]$Fd's from *C. pasteurianum* and *C. acidi-urici*, no evidence was found for the involvement of higher spin states. HiPIP self-exchange electron-transfer rate constants have been estimated from T_1 measurements: native, 1.7 (4) $\times$ 10^4; Se-modified, 7 (2) $\times$ 10^4 M^{-1} s^{-1} [μ 0.1 M phosphate; pH 7.0 (D_2O); 25 °C].

The EPR, Mossbauer, and NMR spectral features of the selenium-substituted ferredoxins ($2[Fe_4Se_4]$Fd's) from *C. pasteurianum* and *C. acidi-urici* have been interpreted in terms of a mixture of spin states ($S = 1/2$, $S = 3/2$, $7/2$) for each cluster, both in frozen solution and at ambient temperature.[1,2] Since only the $S = 1/2$ state is observed for the modified $[Fe_4Se_4]$Fd from *Bacillus stearothermophilus*,[1] it has been inferred that the available spin states following substitution of sulfur by selenium are determined by the interaction between the cluster(s) and the polypeptide chain. This conclusion is generally supported by the close similarity of the EPR and NMR parameters for the synthetic analogues $[Fe_4X_4(SR)_4]^{2-,3-}$ (X = Se, S),[3,4] although recent work[5] has identified a $S = 3/2$ ground state for $[Fe_4Se_4(SPh)_4]^{3-}$. It has been suggested that a similar type of cluster-polypeptide interaction is responsible for the different redox properties of $[Fe_4S_4]$ low-potential and high-potential ferredoxins.[6-8] The substitution of selenium for sulfur might, therefore, be useful in determining the structural basis for differences in the physical properties of the cluster center between these two classes of proteins. In this paper, we report the ^1H NMR spectra and certain electron-transfer properties of oxidized and reduced $[Fe_4Se_4]$ derivatives of *C. vinosum* high-potential iron protein (HiPIP). A study of the resonance Raman spectra of these Se derivatives already has appeared.[9]

Experimental Section

The preparation of the apoprotein and reconstitution with $[Fe_4Se_4]$ were carried out using a modification of standard procedures.[10,11] A sample of HiPIP (20 mg) from *C. vinosum* [ATCC No. 17899 (strain D)] in μ 0.1 M Tris (pH 8, 7 mL) was added to a similar volume of trichloroacetic acid (32%) and stirred at 0 °C for 2 h under aerobic conditions. The precipitate was centrifuged (13 000 rpm, 10 min) and washed with trichloroacetic acid (5%). After dissolving in μ 0.1 M Tris (pH 9, 4 mL), the solution was dialyzed overnight against μ 0.1 M Tris (pH 8). Subsequent manipulations were carried out under an argon atmosphere. The solution of apoprotein (4 mL) was treated with a 50-fold excess of dithiothreitol (DTT) and left stirring for 30 min at room temperature. A 20-fold excess of Fe^{3+} (as a solution of $FeCl_3$) and Se^{2-} (prepared immediately before use)[11] was then added. After 1 h at ambient temperature, the solution was eluted through a DE-52 column equilibrated with μ 0.1 M Tris (pH 8), and the protein was eluted with the same buffer. A black band remained at the top of the column, while the reconstituted protein was eluted immediately following an orange band of inorganic reagents. Minor residual traces of these reagents were removed by three cycles of dilution/ultrafiltration with μ 0.1 M phosphate buffer (pH 7.0).

The electronic absorption spectrum of the Se-HiPIP is very similar to that of the native protein[12] in both the oxidized and reduced states [A_{red}(native) 283 and 388 nm, A_{red}(Se) 283 and 384 nm; A_{ox}(native) 283 nm, A_{ox}(Se) 283 nm]. In comparison with the selenium derivative of Fd from *C. pasteurianum*,[11] the Se-HiPIP is remarkably stable under aerobic conditions at ambient temperature. A half-life of 10 days was estimated by following the decrease in absorbance at 283 nm. The stability is greatly increased by maintaining the protein under an inert atmosphere (Ar) at 4 °C. Thus, the stability of the Se derivative does not differ significantly from that of the native species.

NMR samples were prepared by washing the protein several times with a μ 0.1 M phosphate buffer, made up in D_2O (pH 7.2, uncorrected

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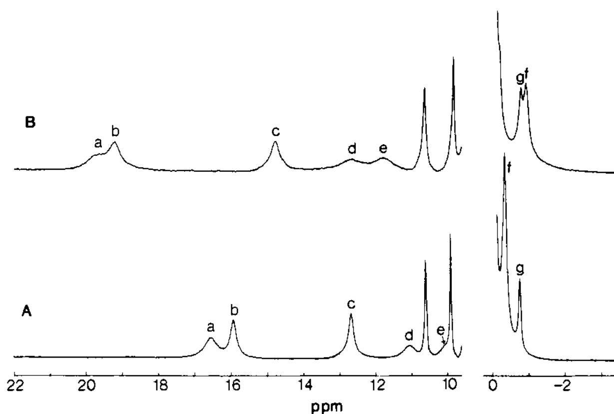

Figure 1. The 500-MHz ^{1}H NMR spectra of reduced HiPIP from *C. vinosum* in μ 0.1 M sodium phosphate buffer (pH 7.2, D_2O), T = 25 °C: (A) native protein; (B) Se derivative. The diamagnetic region has been omitted.

for the deuterium isotope effect), until complete isotopic exchange had occurred (ca. 5 cycles). Spectra were recorded on a Bruker AM 500 instrument operating at 500.13 MHz. Generally, 1000–3000 transients were accumulated by using a 90° pulse (6.5 μs) with a delay time of 0.5 s between pulses. Solvent suppression was achieved with a presaturation pulse from the decoupler. Chemical shifts were internally referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). T_1 relaxation times were determined by the usual inversion–recovery method, with τ values in the range 0.1–200 ms, and by fitting the signal intensities to the equation $M_z = M_0[1 - 2\exp(-\tau/T_1)]$. Electron-transfer self-exchange rates were obtained from T_1 measurements on 1:1 mixtures of oxidized:reduced species.[13]

The reduction potential of the selenium derivative was determined by a spectroelectrochemical titration procedure. The initial absorbance of the reduced protein solution was measured at 500 nm (neither ferri- nor ferrocyanide absorbs significantly in this region). Ferricyanide was added from a 0.1 M stock solution (giving a final ferricyanide concentration of 0.1 mM) to produce fully oxidized protein, and the absorbance was again measured. An equivalent amount of ferrocyanide was added (from a 0.1 M stock solution) to give an ambient potential of ~400 mV vs NHE (measured with a Pt wire and Ag/AgCl reference, connected to a Keithley 177 voltmeter), and additional aliquots of ferrocyanide were then introduced to alter the measured potential. The volumes of ferri/ferrocyanide were minimal, and changes in concentration of the protein solution were neglected. The ratio of oxidized to reduced protein was calculated from the optical absorbance at 500 nm. The reduction potential was obtained from a Nernst plot of the data.

Results and Discussion

The hyperfine-shifted signals of the reduced form of native and Se-modified HiPIP (pH 7.2) are shown in Figure 1, and the NMR parameters are reported in Table I. The native protein is diamagnetic in frozen solution (200 K),[14] due to the antiferromagnetic coupling of the iron atoms of the cluster. The increased population of magnetic states at room temperature is therefore responsible for the observation of the isotropically shifted resonances. Previously, four resonances had been observed in the downfield region of the spectrum of the native protein.[15] We have now detected a fifth signal (e) that partially lies under a resonance attributable to a slowly exchanging proton; this signal can be clearly observed only after several hours of D_2O exchange. An additional hyperfine-shifted signal (f), corresponding to two protons, was detected in the upfield region. Signal g, having no temperature dependence, and a T_1 value of 270 ms, is not isotropically shifted. The downfield set of signals shows anti-Curie behavior, while signal f shows Curie behavior. The temperature dependence of the isotropically shifted resonances and the close similarity with the behavior shown by synthetic analogues[4] indicate that the electron–nucleus coupling is predominantly contact in origin. The same spectral features were recently observed for the reduced form of HiPIP from *C. gracile*.[16] This protein has a 74% sequence homology with that from *C. vinosum* and both show similar physicochemical properties. We therefore assume the same assignments for the *C. vinosum* protein that we have previously determined for the former:[16] in particular, signals b, c, d, and e are assigned to four β-CH_2 protons from the cysteine ligands (each belongs to a different cysteine residue, due to the angular dependence of the contact coupling constant[15]), signal a is assigned to a proton on Tyr-19, and signal f is attributed to protons from another aromatic residue that is near the cluster.

The Se derivative displays the same signal pattern observed with native HiPIP, although the slightly increased hyperfine shifts (Table I) allow the clear detection of signal e. The increase in chemical shift values ranges between 0.6 ppm for signal f to 3.3 ppm for signal b. The T_1 values are 40–60% lower than those of the native species. The variable-temperature behavior of the

Table I. NMR Parameters for the Hyperfine-Shifted Signals in the Reduced and Oxidized Forms of Native HiPIP (*C. vinosum*) and Its Se Derivative[a]

signal	ppm^N	ppm^{Se}	T_1^N	T_1^{Se}	T_{dep}^N	T_{dep}^{Se}
			Reduced Form			
a	16.53	19.65	2.5	1.7	A	A
b	15.91	19.20	5.9	2.9	A	A
c	12.67	14.76	7.2	4.2	A	A
d	11.08	12.65	2.8	1.1	A	A
e	10.10	11.79	2.9	1.3	A	A
f	-0.33	-0.91	9.4	6.4	C	C
			Oxidized Form			
a	108.61	112.9	5.5	2.6	C	C
b	39.51	40.93	10.8	5.8	C	C
c	36.92	39.67	4.1	1.7	C	C
d	30.13	29.92	5.7	3.4	C	C
e	27.22	27.42	42.4	25.3	C	C
f	24.33	29.92	7.8	3.4	A	A
g	23.87	29.29	6.7	3.3	A	A
h	-35.54	-36.64	21.0	21.2	C	C
i	-35.93	-40.97	6.0	4.3	C	C

[a] μ 0.1 M sodium phosphate buffer (pH 7.2, D_2O), T = 25 °C; T_1 values are in milliseconds. N, native; Se, selenium derivative; C, Curie behavior; A, anti-Curie behavior; T_{dep}, temperature dependence.

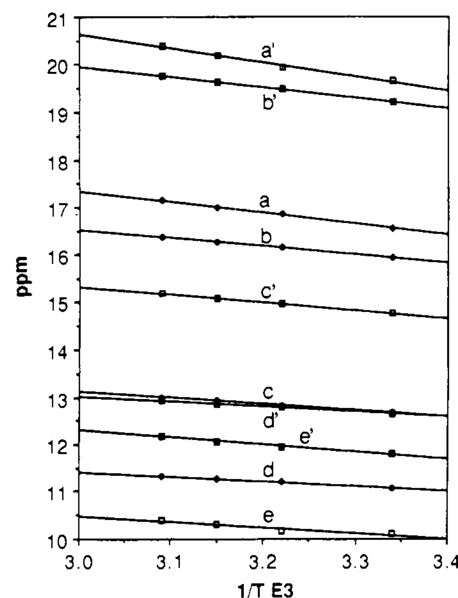

Figure 2. Curie plot for the hyperfine-shifted signals of reduced native HiPIP and the Se derivative [μ 0.1 M sodium phosphate buffer (pH 7.2, D_2O)]. Signals from the Se derivatives are indicated by primes.

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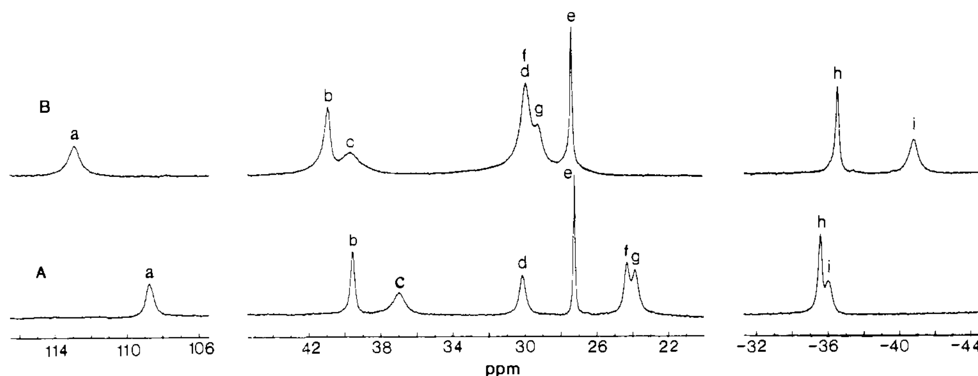

Figure 3. The 500-MHz ^{1}H NMR spectra of oxidized HiPIP from *C. vinosum* in μ 0.1 M sodium phosphate buffer (pH 7.2, D_2O), T = 25 °C: (A) native protein; (B) Se derivative. The diamagnetic region has been omitted.

signals is qualitatively similar to that of the native protein, although the Se derivative shows slightly larger slopes in the Curie plots. These observations indicate increased paramagnetism for the Se derivative and parallel the results obtained for the oxidized form of 2[Fe_4Se_4]Fd from *C. pasteurianum* (*Cp* Fd). The increase in the dimensions of the [Fe_4Se_4] cluster[4] leads to reduced antiferromagnetic coupling among the Fe atoms,[3,17] thereby accounting for the increased paramagnetism and larger contact shifts in both synthetic analogues[17,18] and proteins.

The effects of Se substitution on the NMR parameters of *Cp* Fd and HiPIP in their "paramagnetic states" (reduced for the former and oxidized for the latter) are quite different. Relative to the native protein, reduced 2[Fe_4Se_4]*Cp* Fd displays both an expansion of the chemical shift range of the hyperfine-shifted resonances (−40 to +160 ppm vs 0 to +60 ppm, respectively) as well as differences in the temperature dependences of some signals.[2] In comparison with the native species (Figures 3 and 4, Table I), oxidized Se–HiPIP has a higher oxidation level relative to the reduced Fd and lower T_1 values but exhibits only slightly increased paramagnetic shifts and almost identical temperature dependences. The dramatic spectral differences observed for reduced *Cp* Fd have been attributed to the occurrence of higher spin states ($S = {}^3/_2, {}^7/_2$), as indicated by magnetic susceptibility, EPR, and Mossbauer data.[2] The behavior of the high-potential protein resembles that of synthetic analogues,[18,19] which is generally rationalized in terms of an increase of spin delocalization onto the ligands by Se (for S) substitution in the $S = {}^1/_2$ state. The absence of higher spin states has, however, also been noted in an EPR study of the one-cluster [Fe_4Se_4]Fd from *B. Stearothermophilus*,[1] although no NMR data have been reported for either this or other one-cluster Se-substituted Fd's.

The selenium derivative of *C. vinosum* HiPIP was found to be useful for assignment purposes. In a previous NMR study of the homologous HiPIP from *C. gracile*,[16] the resonances in the oxidized form, corresponding to signals a, c, f, and g, in the spectrum shown in Figure 3a, were assigned to β-CH_2 cysteine protons (one from each residue), those corresponding to signals b and i to protons from the aromatic residue closest to the cluster (Tyr-19),[20] h to an α-CH proton, e to a proton on the polypeptide backbone, and signal d to a proton on either Tyr-19 or to an additional β-CH_2 cysteine proton. The observation that these resonances experience the largest shifts after substitution of Se for S strongly supports the proposed assignments for both the *C. vinosum* and *C. gracile* proteins.

A reduction potential of 321 (7) mV [vs NHE, μ 0.1 M phosphate, pH 7.0, 23 °C] was obtained for Se-modified HiPIP; thus the Se for S substitution in the *C. vinosum* protein does not lead to substantial changes in the redox properties of the cluster [$E°$(native) ~ 350 mV].[12] It is apparent, however, that the relative redox and magnetic properties of the [Fe_4Se_4] centers in high- and low-potential iron proteins are affected to a considerable

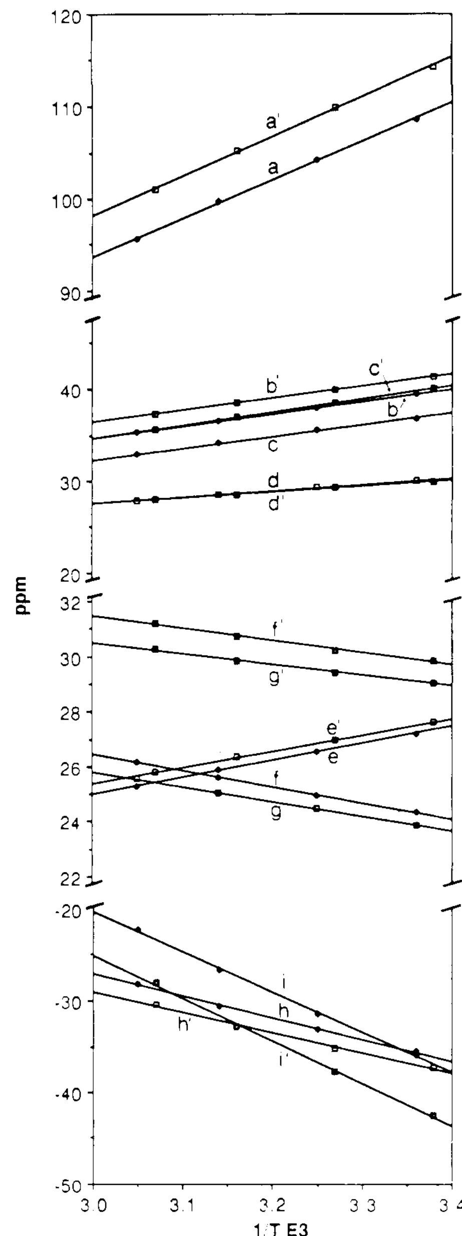

Figure 4. Curie plots for the hyperfine-shifted signals of oxidized native and Se-HiPIP [μ 0.1 M sodium phosphate buffer (pH 7.2, D_2O)]. Signals from the Se derivatives are indicated by primes.

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extent by the immediate polypeptide environment of the cluster. Furthermore, the absence of any correlation between redox and magnetic properties for low- and high-potential [Fe_4Se_4] proteins, in particular the absence of high-spin states in the selenium derivative of the low-potential Fd from *B. stearothermophilus*, strongly suggests that the factors controlling the reduction potentials of the clusters are different from those influencing the magnetic properties. Although the protein–cluster interactions responsible for these differences are not known, it is interesting to note that in those cases where higher spin states are observed (*C. pasteurianum* and *C. acidi-urici*), the Se-modified Fd's each contain two [Fe_4Se_4] clusters.

Self-exchange ET rates were determined from T_1 measurements on approximately 1:1 mixtures of the oxidized:reduced species.[21] The similarity in the rate constants for native and Se-modified HiPIP ($k_{ex} \sim 1.7\ (4) \times 10^4$ and $7\ (2) \times 10^4\ M^{-1}\ s^{-1}$, respectively) is fully consistent with studies of the electron self-exchange rates of synthetic analogues,[3,4,17,18] thereby suggesting that the presence of Se does not significantly increase the electronic coupling of the cluster to its immediate protein environment (for instance, to the neighboring Tyr-19, a possible residue along the natural ET pathway). The rate constant for native protein is in accord with that previously estimated from certain cross-reaction rates with inorganic reagents. For the $Co(phen)_3^{3+}$ reaction, a self-exchange rate constant for HiPIP of $1.8 \times 10^4\ M^{-1}\ s^{-1}$ was calculated.[22] The finding that the HiPIP rate constants are lower than those obtained for inorganic [Fe_4S_4] complexes (10^6–$10^7\ M^{-1}\ s^{-1}$) can be explained[18] in terms of steric influences of protein structure on the ET reaction.

Acknowledgment. We are grateful to Dr. R. G. Bartsch for a generous gift of *C. vinosum* HiPIP. J.A.C. thanks the Science and Engineering Research Council (United Kingdom) for a NATO Postdoctoral Fellowship. This research was supported by the National Institutes of Health (DK-19038).

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A Fundamental Structural Study of Inclusion Chemistry:† The Mutual Effects of Host and Guest in the Transition-Metal Complexes of Superstructured Cyclidene Macrobicycles

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Abstract: Detailed structural analysis is reported on the cavities of lacunar cyclidene complexes having polymethylene chains as bridging groups. Bridge lengths ranged from trimethylene to dodecamethylene, and studies were based on 14 crystal structure determinations and conformational analysis including molecular mechanics calculations using MM2 and MMP2. Variations in cavity dimensions and geometry arise from changes in the saddle-shaped parent cyclidene macrocycle and in the conformations of the polymethylene chain. Three-carbon and four-carbon chains present small cavities that are relatively inaccessible. The pentamethylene bridge is also somewhat inflexible, adopting a single conformation in both the presence and absence of coordinated ligands (in the cavity). In contrast, the hexamethylene-bridged complex demonstrates a fundamental mode for accommodating a guest molecule; it adopts alternative low-energy conformations depending on the presence or absence of the guest ligand. The "vacant" conformation is typical of the tendency of a flexible ring to fill the volume it encircles, while the "filled" conformation presents a more extensive void. For chain lengths through eight carbons, vacant cavities are associated with "lid-off" conformations having relatively broad and low shapes. The 12-membered chain produces a tall narrow cavity having a "lid-on" conformation. Eight new crystal structures are reported.

Molecular inclusion chemistry[1] is among the most promising interfaces between synthetically based chemistry and molecular biology. It is providing foundations for molecular recognition and for the understanding of how molecules can be organized, subjects of broad-ranging implications as the focus of chemistry passes from the individual molecule to systems of molecules.[2] A particularly high expectation of this field is the genesis of new molecules that have powerful performance capabilities.

Families of cyclidene ligands[3] (Figure 1) have been designed to produce species with remarkable capabilities. The lacunar iron(II) cyclidenes[4] are the only well-established non-porphyrin complexes of iron(II) that bind O_2 reversibly. The lacunar co-

† Part 1 of this series is ref 8.
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