the hydrolysis of α-TAc stereoisomers is an *intrinsic* property of cholesterol esterases. The data for pure PCE (Table I, footnote *b*) show as much as a 60-fold and 14-fold range in *RRR/SRR* selectivity in the noncompetitive and competitive experiments, respectively. Interestingly, in the competitive experiments the rate of hydrolysis of *RRR*-α-TAc is significantly affected but that of *SRR*-α-TAc is little affected relative to the corresponding rates measured in the noncompetitive experiments.[18]

At 40 mM the bile salts will form mixed micelles[20] with the DMPC (2 mM) and α-TAc (0.1–0.2 mM). Although the initial rates of hydrolysis can be described by using classical Michaelis–Menten (MM) kinetics,[8] we are not, of course, dealing with a molecular solution of α-TAc but with micellized α-TAc which presumably reacts with the water-soluble BCE or PCE at the micelle–water interface.[21] The effect of the (chiral) bile salts on the relative (and absolute) rates of hydrolysis of *RRR*- and *SRR*-α-TAc could result from diastereoisomeric influences upon the epimeric acetates induced within the mixed micelle itself or, alternatively, may arise from direct bile salt–protein interactions.[19] The degree of chiral recognition that is achieved is genuinely dramatic in view of the fact that *the chiral center is separated by six bonds from the bond that is cleaved by the enzyme.*[23]

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(21) Such two-dimensional systems can appear to follow MM kinetics (even including product inhibition), but the derived kinetic parameters have more complicated meanings than for homogeneous enzyme reactions.[19,22]

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Exogenous Ligand Binding to the [Fe_4S_4] Cluster in *Pyrococcus furiosus* Ferredoxin

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Cubane-type [Fe_4S_4] clusters functionalized by non-cysteinyl and/or unique ligation of a specific Fe or by replacement of one Fe by another transition metal (e.g., Mo, V, or Ni) have been proposed as components of the active sites of a range of metalloenzymes. These include aconitase,[1] several (de)hydratases,[1] sulfite reductase,[2] nitrogenase,[3] hydrogenase,[4] and CO dehydrogenase.[5] Synthetic strategies to investigate subsite-specific ligand binding to such clusters in aprotic media have recently focused on [Fe_4S_4]$^{2+}$ clusters ligated by a semirigid trithiolate cavitand ligand.[6] Simple ferredoxins containing [Fe_4S_4] clusters offer the potential for investigating such ligand-binding interactions in a more physiologically relevant environment. However, of the more than 30 ferredoxins for which sequences are available,[7] only five appear to contain a [Fe_4S_4] cluster with incomplete cysteinyl ligation.[8] Four of these are 8Fe-ferredoxins in which only one of the two [Fe_4S_4] clusters has partial non-cysteinyl coordination. This leaves the ferredoxin (Fd) from the hyperthermophilic archaebacterium *Pyrococcus furiosus*[8] as the only example of a 4Fe-Fd with non-sulfur ligation to a specific Fe atom. This protein therefore provides an opportunity to study the subsite-specific ligand binding of a [Fe_4S_4] cluster in a biological environment. We report here the binding of cyanide, an inhibitor for numerous Fe–S containing enzymes and a substrate for nitrogenase, to the [Fe_4S_4]$^{1+}$ cluster in dithionite-reduced *P. furiosus* Fd. This is the first report of cyanide binding to a biological [Fe_4S_4] cluster, as well as the first example of exogenous ligand binding to a [Fe_4S_4]$^{1+}$ cluster in a bacterial ferredoxin.

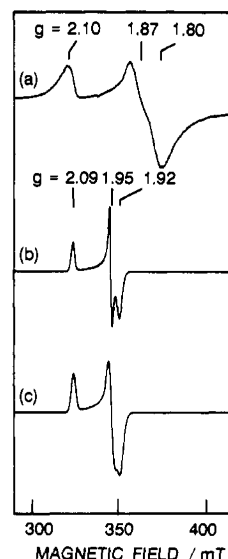

Figure 1. X-band EPR spectra of dithionite-reduced *P. furiosus* Fd, native (a), cyanide-treated (b), and ^{57}Fe-reconstituted cyanide-treated (c): (a) 0.85 mM Fd in 100 mM Tris-HCl buffer, pH 7.8; microwave power, 1 mW; temperature, 8 K; (b,c) 0.21 mM Fd in 100 mM Tris-HCl buffer with a 250-fold excess of potassium cyanide, pH 8.5; microwave power, 1 mW; temperature, 20 K. All spectra were recorded at 9.44 GHz with 0.63 mT modulation amplitude.

P. furiosus Fd, M_r = 7500, is a monomeric protein containing a single [Fe_4S_4] cluster.[8a] The arrangement of cysteine residues in the amino acid sequence is analogous to that of other bacterial ferredoxins except that the second cysteine in the traditional sequence is replaced by an aspartate residue. Non-cysteinyl coordination of a specific Fe is manifest by novel spectroscopic

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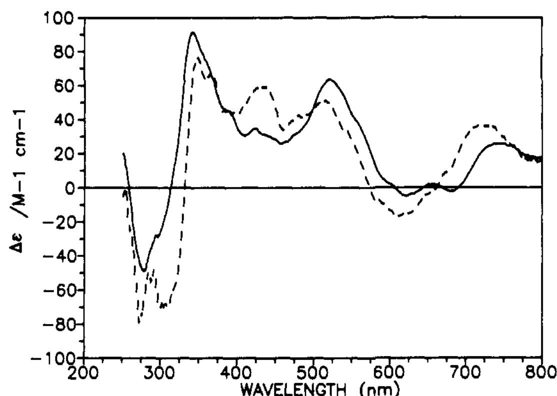

Figure 2. MCD spectra of dithionite-reduced native and cyanide-treated *P. furiosus* Fd. Solid line: 0.35 mM Fd in 100 mM Tris-HCl buffer with 50% (v/v) glycerol, pH 7.8. Dashed line: 0.25 mM Fd in 100 mM Tris-HCl buffer with 50% (v/v) glycerol and a 250-fold excess of potassium cyanide, pH 8.5. Both spectra were collected at 4.2 K and 4.5 T.

and magnetic properties for the $[Fe_4S_4]^{1+,2+}$ cluster and the facile and quantitative removal of one Fe atom upon oxidation with ferricyanide to generate a conventional $[Fe_3S_4]^{1+}$ cluster.[8a] Recent proton electron nuclear double resonance (ENDOR) studies of this Fd indicate that the unique Fe atom is ligated by OH^-.[8b]

The $[Fe_4S_4]^{1+}$ cluster in the dithionite-reduced Fd exists as a mixture of species with $S = {}^3/_2$ (80%) and $S = {}^1/_2$ (20%) ground states.[8] The $S = {}^1/_2$ form exhibits a broad, rapidly relaxing EPR signal, g = 2.10, 1.87, and 1.80, that is observable only below 15 K (Figure 1a). Incubation with a 250-fold excess of potassium cyanide at room temperature for 2 h at any pH in the range 7.5–10.5 results in the quantitative conversion of both the $S = {}^3/_2$ and $S = {}^1/_2$ EPR resonances to a new $S = {}^1/_2$ resonance, g = 2.09, 1.95, and 1.92, which accounts for 1.0 spin/molecule[9] (Figure 1b). This $S = {}^1/_2$ resonance is much slower relaxing, being observable without significant broadening up to 60 K, and is substantially broadened in samples reconstituted with ^{57}Fe ($I = {}^1/_2$) (Figure 1c). Clearly, this resonance originates from an Fe center, but the g values and relaxation properties are quite distinct from any known biological $[Fe_4S_4]^{1+}$ cluster. Rather they are more indicative of $[Fe_2S_2]^{1+}$ clusters in chloroplast ferredoxins (g = 2.09, 1.96, and 1.88–1.89; observable without significant broadening at 70 K).

The nature of the Fe–S cluster responsible for this EPR signal was assessed by low-temperature magnetic circular dichroism (MCD) spectroscopy, which provides a convenient and reliable method for identification of cluster type for paramagnetic Fe–S centers.[10] Figure 2 shows a comparison of the 4.2 K MCD spectra[11] of dithionite-reduced native and cyanide-treated *P. furiosus* Fd. The overall features and temperature dependence of both spectra are indicative of paramagnetic $[Fe_4S_4]^{1+}$ clusters. Moreover, the spectra of the native and cyanide-treated samples are characteristic of those observed for synthetic or biological $[Fe_4S_4]^{1+}$ clusters with predominantly $S = {}^3/_2$ and $S = {}^1/_2$ ground states, respectively.[8,12,13] MCD magnetization data collected at 512, 430, and 350 nm for the cyanide-treated sample are well fit at all temperatures by a theoretical curve constructed by using the EPR g values, confirming a $S = {}^1/_2$ ground state for this novel center.

Confirmation that the changes in the electronic and magnetic properties revealed by EPR and MCD spectroscopies originate from binding cyanide to the non-cysteinyl ligated Fe atom of the $[Fe_4S_4]^{1+}$ cluster comes from four additional pieces of evidence. First, parallel control experiments on the reduced 8Fe-Fd from *Clostridium pasteurianum* and the oxidized and reduced $[Fe_3S_4]$ form of *P. furiosus* Fd showed no cyanide-induced perturbation of the EPR or low-temperature MCD spectra. In contrast, cyanide does effect dramatic changes in the EPR and/or MCD characteristics of the $[NiFe_3S_4]^{1+}$ ($S = {}^3/_2$),[5b] $[ZnFe_3S_4]^{1+}$ ($S = {}^5/_2$),[14] and $[CoFe_3S_4]^{1+}$ (S = 1)[14] centers in *P. furiosus* Fd, indicating that cyanide only interacts with the unique, exchangeable metal site of this cubane cluster, whether it be Fe, Ni, Zn, or Co. Second, removal of cyanide from dithionite-reduced *P. furiosus* Fd by anaerobic gel filtration results in quantitative reversion to the native state, as evidenced by reappearance of the characteristic $S = {}^3/_2$ and $S = {}^1/_2$ EPR resonances. Third, air or thionine oxidation of the cyanide-treated Fd leads to quantitative loss of the unique Fe atom and the formation of a $[Fe_3S_4]^{1+}$ cluster, as demonstrated by EPR and MCD spectroscopies. This suggests that cyanide binds to the unique Fe site, since parallel oxidations of the $[Fe_4S_4]^{1+}$ cluster in the absence of cyanide yield the $[Fe_4S_4]^{2+}$ cluster almost exclusively.[8a] Fourth, the EPR spectra and the ratio of the $S = {}^1/_2$ and $S = {}^3/_2$ forms of the $[Fe_4S_4]^{1+}$ cluster in the native Fd are unaffected by pH over the range 6.0–10.5, ionic strengths up to 1 M NaCl, or the addition of denaturing agents such as 6 M guanidine hydrochloride or 1.0 M urea, making it extremely unlikely that the cyanide-induced changes in the EPR spectrum are a consequence of a protein conformational change.

The results presented herein are consistent with reversible binding of cyanide to the unique Fe site of the $[Fe_4S_4]^{1+}$ cluster in *P. furiosus* Fd and show that binding results in dramatic changes in the electronic and magnetic properties of the cluster. Spectroscopic studies to determine the mode of cyanide binding, the valence localization and magnetic interactions within the cyanide-bound cluster, and the binding of other ligands to the $[Fe_4S_4]^{1+}$ core are in progress.

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