[2Fe-2S] to [4Fe-4S] Cluster Conversion in *Escherichia coli* Biotin Synthase†

Evert C. Duin,‡ Meghan E. Lafferty,‡ Brian R. Crouse,‡ Ronda M. Allen,§ Indrajit Sanyal,§ Dennis H. Flint,§ and Michael K. Johnson*,‡

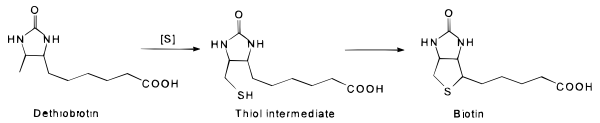
Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, and Central Research and Development, Experimental Station, E. I. du Pont de Nemours and Co., P.O. Box 80328, Wilmington, Delaware 19880

Received March 19, 1997; Revised Manuscript Received July 1, 1997⊗

ABSTRACT: The type and properties of the Fe−S cluster in recombinant *Escherichia coli* biotin synthase have been investigated in as-prepared and dithionite-reduced samples using the combination of UV−visible absorption and variable-temperature magnetic circular dichroism (VTMCD), EPR, and resonance Raman spectroscopies. The results confirm the presence of one $S = 0$ $[2Fe\text{-}2S]^{2+}$ cluster in each subunit of the homodimer in aerobically purified samples, and the Fe−S stretching frequencies suggest incomplete cysteinyl-S coordination. However, absorption and resonance Raman studies show that anaerobic reduction with dithionite in the presence of 60% (v/v) ethylene glycol or glycerol results in near-stoichiometric conversion of two $[2Fe\text{-}2S]^{2+}$ clusters to form one $S = 0$ $[4Fe\text{-}4S]^{2+}$ cluster with complete cysteinyl-S coordination. The stoichiometry and ability to effect reductive cluster conversion without the addition of iron or sulfide suggest that the $[4Fe\text{-}4S]^{2+}$ cluster is formed at the subunit interface via reductive dimerization of $[2Fe\text{-}2S]^{2+}$ clusters. EPR and VTMCD studies indicate that more than 50% of the Fe is present as $[4Fe\text{-}4S]^{+}$ clusters in samples treated with 60% (v/v) glycerol after prolonged dithionite reduction. The $[4Fe\text{-}4S]^{+}$ cluster exists as a mixed spin system with $S = {}^1/_2$ ($g = 2.044, 1.944, 1.914$) and $S = {}^3/_2$ ($g = 5.6$ resonance) ground states. Subunit-bridging $[4Fe\text{-}4S]^{2+,+}$ clusters, that can undergo oxidative degradation to $[2Fe\text{-}2S]^{2+}$ clusters during purification, are proposed to be a common feature of Fe-S enzymes that require *S*-adenosylmethionine and function by radical mechanisms involving the homolytic cleavage of C−H or C−C bonds, i.e., biotin synthase, anaerobic ribonucleotide reductase, pyruvate formate lyase, lysine 2,3-aminomutase, and lipoic acid synthase. The most likely role for the $[4Fe\text{-}4S]^{2+,+}$ cluster lies in initiating the radical mechanism by directly or indirectly facilitating reductive one-electron cleavage of *S*-adenosylmethionine to form methionine and the 5′-deoxyadenosyl radical. It is further suggested that oxidative cluster conversion to $[2Fe\text{-}2S]^{2+}$ clusters may play a physiological role in these radical enzymes, by providing a method of regulating enzyme activity in response to oxidative stress, without irreversible cluster degradation.

Biotin is an essential vitamin that is synthesized by microorganisms (Eisenberg, 1987) and plants (Baldet et al., 1993). As a mobile carrier of activated CO_2, it is the critical cofactor for carboxylase-catalyzed reactions and hence plays a crucial role in gluconeogenesis and maintaining the level of citric acid cycle intermediates. The final step of the biosynthetic pathway is catalyzed by biotin synthase and involves the insertion of sulfur into dethiobiotin via a thiol intermediate or some derivative thereof (Baxter et al., 1992; Marquet et al., 1993) (see Scheme 1). Little is known about the catalytic mechanism, and although there is accumulating evidence that the sulfur for this reaction is ultimately derived from cysteine (Birch et al., 1995; Florentin et al., 1994), the nature of the direct sulfur donor has yet to be determined (Sanyal et al., 1996). However, stereochemical results indicate that initial formation of the C−S bond and possibly the subsequent cyclization to form the thiophene ring proceed via radical mechanisms with homolytic cleavage of a C−H bond as the initial step (Marti, 1983; Jestin et al., 1996). Similar enzyme-catalyzed sulfur insertion reactions occur in the synthesis of isopenicillin *N* (Borovok et al., 1996), lipoic acid (Eisenberg, 1987; Reed & Cronan, 1993), and thiamine (DeMoll & Shive, 1995).

Scheme 1



Biotin synthases, or more specifically the *bioB* gene product, have recently been purified to homogeneity from recombinant strains of *Escherichia coli* (Sanyal et al., 1994) and *Bacillus sphaericus* (Méjean et al., 1995). The *E. coli* enzyme is a homodimer of 39-kDa subunits and, surprisingly, aerobically purified samples were found to contain one $[2Fe\text{-}2S]^{2+}$ cluster per monomer (Sanyal et al., 1994). The presence of $[2Fe\text{-}2S]^{2+}$ clusters were subsequently reported in aerobically purified samples of *B. sphaericus* biotin synthase, although the enzyme was deficient in clusters and the aggregation state of the native protein was not reported (Méjean et al., 1995). However, the role of the cluster and a fully-optimized *in vitro* assay system have yet to be found.

† This work was supported by a grant from the National Institutes of Health (GM51962 to M.K.J.) and by a National Science Foundation Research Training Group Award to the Center for Metalloenzyme Studies (BIR-9413236).

* Corresponding author. Telephone: 706-542-9378. Fax: 706-542-2353. E-mail: johnson@sunchem.chem.uga.edu.

‡ University of Georgia.

§ E. I. du Pont de Nemours and Co.

⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1997.

S0006-2960(97)00643-0 CCC: $14.00

A redox role for the $[2Fe\text{-}2S]^{2+}$ cluster seemed unlikely, since dithionite reduction appeared to be accompanied by cluster degradation and <10% reduction to the $S = {}^1/_2$ $[2Fe\text{-}2S]^{+}$ state (Sanyal et al., 1994). *In vitro* activity requires *S*-adenosyl-L-methionine (Ado-Met),[1] NADPH, Fe^{2+} or Fe^{3+}, an electron transport system [which appears to involve flavodoxin and flavodoxin reductase *in vivo* (Ifuku et al., 1994; Sanyal et al., 1996)], and other proteins and/or cofactors in cell-free extracts of *bioB*$^-$ strains of *E. coli* (Sanyal et al., 1994, 1996; Birch et al., 1995) or wild-type strains of *B. sphaericus* (Florentin et al., 1994; Méjean et al., 1995). Well-defined reaction mixtures involving *E. coli* biotin synthase, flavodoxin, flavodoxin reductase, NADPH, Ado-Met, Fe^{2+}, fructose 1,6-bisphosphate, cysteine, and dithiothreitol are maximally capable of generating 2 mol of biotin per biotin synthase monomer, indicating that one or more hitherto unidentified factors are also required (Sanyal et al., 1996).

The obligate requirement for Ado-Met and the presence of an Fe-S cluster has led to speculation (Florentin et al., 1994; Méjean et al., 1995; Sanyal et al., 1994, 1996) that biotin synthase is a member of a family of Fe-S enzymes with radical-based mechanisms that includes anaerobic ribonucleotide reductase (ARR) (Mulliez et al., 1993; Reichard, 1993; Harder et al., 1992), pyruvate formate-lyase (PFL) (Knappe et al., 1984; Knappe & Sawers, 1990; Broderick et al., 1997), and lysine 2,3-aminomutase (LAM) (Petrovich et al., 1992; Frey & Reed, 1993). Each of these three enzymes catalyzes the homolytic cleavage of C−H or C−C bonds and uses Ado-Met as the source of the 5′-deoxyadenosyl radical to initiate the radical reaction. In the case of ARR and PFL, the Fe-S cluster is associated with a dimeric activating enzyme (PFL-AE and ARR-AE) (Ollangnier et al., 1996; Knappe & Sawers, 1990; Broderick et al., 1997), and, in common with biotin synthase, these enzymes also require flavodoxin, flavodoxin reductase, and NADPH (Knappe & Sawers, 1990; Bianchi et al., 1993). However, in contrast to biotin synthase, the original EPR studies of ARR and LAM indicate that the Fe-S clusters are cubane-type [4Fe-4S] or [3Fe-4S] centers (Mulliez et al., 1993; Petrovich et al., 1992). For ARR-AE and PFL-AE, the most recent spectroscopic and analytical data have been interpreted in terms of a labile [4Fe-4S] cluster that bridges the subunits (Ollangnier et al., 1996; Broderick et al., 1997).

In this work, we have used the combination of UV−visible absorption and variable-temperature magnetic circular dichroism (VTMCD), EPR, and resonance Raman (RR) spectroscopies to investigate the properties of the Fe-S center in biotin synthase. The results indicate one $[2Fe\text{-}2S]^{2+}$ cluster with incomplete cysteinyl cluster ligation in each subunit of the aerobically purified recombinant biotin synthase. However, they also demonstrate that the $[2Fe\text{-}2S]^{2+}$ clusters can be converted under anaerobic reducing conditions to form a $[4Fe\text{-}4S]^{2+,+}$ cluster at the subunit interface of the homodimer. This raises the possibility that subunit-bridging $[4Fe\text{-}4S]^{2+,+}$ clusters are present in active forms of biotin synthase as well as other enzymes in this family. Possible roles for the cluster in generating the 5′-deoxyadenosyl radical from Ado-Met and/or regulating enzyme activity in response to O_2 exposure are discussed.

MATERIALS AND METHODS

Biotin synthase was purified aerobically and assayed as described previously (Sanyal et al., 1994, 1996). Samples were in 50 mM Tris/HCl buffer, pH 7.8, and protein determinations were determined by the Bradford method (Bradford, 1976) standardized against bovine serum albumin and multiplied by a correction factor of 1.1 (Nozaki, 1986). All sample concentrations and molar extinction coefficients are expressed per 39-kDa monomer.

UV−visible absorption spectra were recorded under anaerobic conditions in septum-sealed 1-mm cuvettes using a Shimadzu UV-3101PC spectrophotometer. Variable-temperature MCD measurements were made on samples containing 60% (v/v) ethylene glycol in 1-mm cuvettes using a Jasco J-715 spectropolarimeter mated to an Oxford Instruments SM4000 split-coil superconducting magnet. The experimental protocols for measuring MCD spectra of oxygen-sensitive samples at fixed temperatures over the range 1.5−300 K with magnetic fields up to 6 T have been described elsewhere (Johnson, 1988; Thomson et al., 1993). X-band (~9.5 GHz) EPR spectra were recorded on a Bruker ESP-300E EPR spectrometer equipped with a ER-4116 dual-mode cavity and an Oxford Instruments ESR-9 flow cryostat. Spin quantitations were carried out under nonsaturating conditions using 1 mM CuEDTA as the standard. Resonance Raman spectra were recorded using an Instruments SA Ramanor U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube with 90° scattering geometry. Spectra were recorded digitally using photon counting electronics, and improvements in signal-to-noise were achieved by signal-averaging multiple scans. Band positions were calibrated using the excitation frequency and CCl_4 and are accurate to ± 1 cm^{-1}. Lines from a Coherent Innova 100 10-W Argon Ion Laser or Coherent Innova 200-K2 Krypton Ion Laser were used for excitation, and plasma lines were removed using a Pellin Broca Prism premonochromator. Using a custom-designed sample cell (Drozdzewski & Johnson, 1988), samples under an Ar atmosphere were placed on the end of a cold finger of an Air Products Displex Model CSA-202E closed cycle refrigerator. Scattering was collected from the surface of a frozen 10-μL droplet. This enables the samples to be cooled down to 17 K, which facilitates improved spectral resolution and prevents laser-induced sample degradation.

RESULTS

UV−Visible Absorption. Evidence for a $[2Fe\text{-}2S]^{2+}$ cluster in each subunit of aerobically purified recombinant biotin synthase has thus far rested largely on the UV−visible absorption spectrum and non-heme iron and acid-labile sulfide analyses (Sanyal et al., 1994). As shown in Figure 1A, the samples used in this work exhibited identical absorption spectra with extinction coefficients indicative of a single $[2Fe\text{-}2S]^{2+}$ cluster in each 39-kDa subunit (Dailey et al., 1994), and RR studies (see below) have confirmed the presence of $[2Fe\text{-}2S]^{2+}$ clusters. During the course of anaerobic reduction with excess sodium dithionite, different changes in the absorption spectrum were observed depending on whether or not the samples contained 60% (v/v) ethylene

[1] Abbreviations: biotin synthase, *E. coli BioB* gene product; Ado-Met, *S*-adenosyl-L-methionine; ARR, anaerobic ribonucleotide reductase; ARR-AE, anaerobic ribonucleotide activating enzyme; PFL, pyruvate formate-lyase; PFL-AE, pyruvate formate-lyase activating enzyme; LAM, lysine 2,3-aminomutase; LAS, lipoic acid synthase; Fd(s), ferredoxin(s); (VT)MCD, (variable-temperature) magnetic circular dichroism; RR, resonance Raman; S^b, bridging or inorganic S; S^t, terminal or cysteinyl-S.

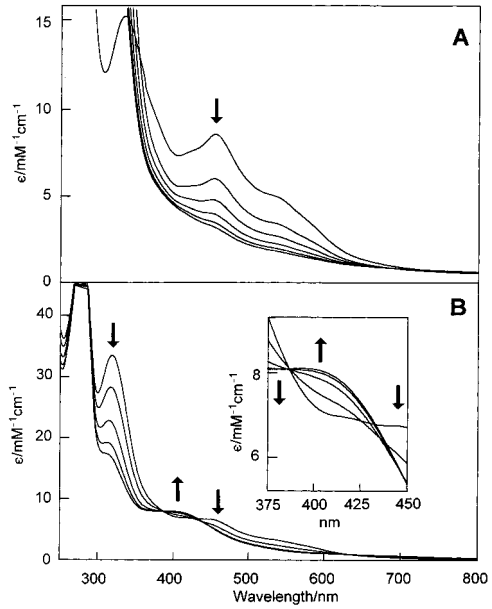


FIGURE 1: Dithionite reduction of biotin synthase monitored by UV−visible absorption. (A) Spectra for biotin synthase (1.3 mM in anaerobic 50 mM Tris/HCl buffer, pH 7.8) recorded prior to dithionite addition and at 5, 30, 60, 90, and 120 min after making the solution 3.6 mM in sodium dithionite. (B) Spectra for biotin synthase [0.5 mM in anaerobic 50 mM Tris/HCl buffer, pH 7.8, with 60% (v/v) ethylene glycol] recorded at 5 , 30, 90, 180, and 270 min after making the solution 3.0 mM in sodium dithionite. The inset shows an expansion of the 375−450 nm region. The arrows indicate the direction of change in absorption at selected wavelengths as the dithionite incubation time increases, and the intense band centered at 314 nm arises from excess dithionite.

glycol or glycerol. This is illustrated for ethylene glycol in Figure 1B, and the same results were obtained with glycerol.

In the absence of ethylene glycol or glycerol, dithionite reduction results in a gradual decrease in the visible absorption at all wavelengths >350 nm over a period of several hours (Figure 1A). In the presence of ethylene glycol or glycerol, dithionite reduction also proceeds slowly over a period of several hours, but the absorbance decreases in the region between 430 and 620 nm and increases in the regions between 390−430 and 630−800 nm (Figure 1B). The resulting spectrum has a pronounced shoulder at 410 nm and is characteristic of proteins containing $[4Fe\text{-}4S]^{2+}$ clusters (Johnson et al., 1982). Moreover, on the basis of the visible molar extinction coefficients for well-characterized $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters [$\epsilon_{460} \approx 8000\ M^{-1}\ cm^{-1}$ per $[2Fe\text{-}2S]^{2+}$ cluster (Dailey et al., 1994) and $\epsilon_{410} \approx 15\,000\ M^{-1}\ cm^{-1}$ per $[4Fe\text{-}4S]^{2+}$ cluster (Johnson et al., 1982)], the observed extinction coefficients per 39-kDa monomer [$\epsilon_{460} \approx 8000\ M^{-1}\ cm^{-1}$ prior to reduction and $\epsilon_{410} \approx 8000\ M^{-1}\ cm^{-1}$ after reduction in the presence of 60% (v/v) ethylene glycol or glycerol] are consistent with stoichiometric cluster conversion of two $[2Fe\text{-}2S]^{2+}$ clusters (one in each monomer) to one $[4Fe\text{-}4S]^{2+}$ cluster per homodimer, i.e.:

$$2[2Fe\text{-}2S]^{2+} + 2e^- \rightarrow [4Fe\text{-}4S]^{2+}$$

Subsequent addition of a 10-fold excess of dithionite had no effect on the absorption intensity at 410 nm. Additional spectroscopic evidence for the formation of [4Fe-4S] clusters under reducing conditions has come from EPR, VTMCD, and RR studies (see below).

In an attempt to assess the reversibility of this reductive cluster conversion, the absorption spectrum of the $[4Fe\text{-}4S]^{2+}$ form of biotin synthase was monitored after exposing the sample to air (Figure 2). This resulted in gradual bleaching of the visible absorption over a period of 10−12 h, indicating cluster degradation. However, by comparing spectra taken during the course of the process with that of the $[2Fe\text{-}2S]^{2+}$ form of biotin synthase (Figure 2), it is evident that this cluster degradation occurs with some formation of $[2Fe\text{-}2S]^{2+}$ clusters. Hence, the cluster conversion *in vitro* is at least partially reversible on exposure to air, and it seems likely that the presence of $[2Fe\text{-}2S]^{2+}$ clusters in purified preparations of biotin synthase is a direct consequence of aerobic isolation.

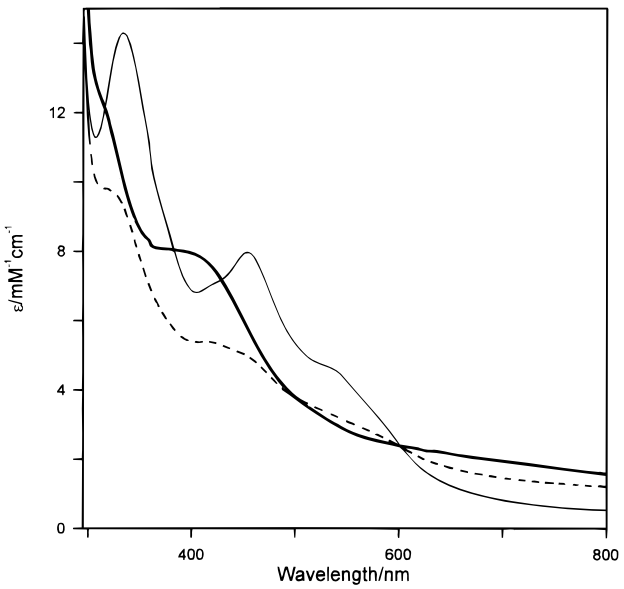


FIGURE 2: Effect of air exposure on the UV−visible absorption spectrum of the $[4Fe\text{-}4S]^{2+}$ form of biotin synthase. The $[4Fe\text{-}4S]^{2+}$ form of biotin synthase was prepared by anaerobic dithionite reduction in the presence of 60% (v/v) ethylene glycol (solid line) and was subsequently exposed to air for 2 h at room temperature (dashed line). The spectrum of the as-prepared $[2Fe\text{-}2S]^{2+}$ form of biotin synthase is shown to facilitate comparison (thin line).

EPR. EPR spectra were recorded for four distinct types of biotin synthase samples: as-prepared, dithionite-reduced, dithionite-reduced followed by addition of 60% (v/v) glycerol, and dithionite-reduced in the presence of 60% (v/v) ethylene glycol. In each case, dithionite reduction was monitored by UV−visible absorption (see Figure 1), and samples were frozen for EPR only when no further changes were apparent over a period of 30 min. Two of these samples, as-prepared and dithionite-reduced in the presence of 60% (v/v) ethylene glycol, did not exhibit any significant X-band EPR signals (parallel- or perpendicular-mode) over the temperature range 4−50 K (data not shown), consistent with the presence of Fe-S clusters with $S = 0$ ground states, i.e., $[2Fe\text{-}2S]^{2+}$ or $[4Fe\text{-}4S]^{2+}$ clusters.

Perpendicular-mode, X-band EPR spectra were recorded at temperatures in the range 4.2−100 K with microwave powers between 0.002 and 200 mW for dithionite-reduced samples with and without the subsequent addition of 60% (v/v) glycerol. A less extensive series of experiments has been conducted with ethylene glycol in place of the glycerol, and the results were the same. Selected spectra that facilitate direct comparisons and illustrate the resonances observed for each sample are shown in Figure 3. In the absence of glycerol, the dithionite-reduced sample exhibited two resonances that are readily resolved based on differences in relaxation behavior, i.e., a slow relaxing isotropic resonance

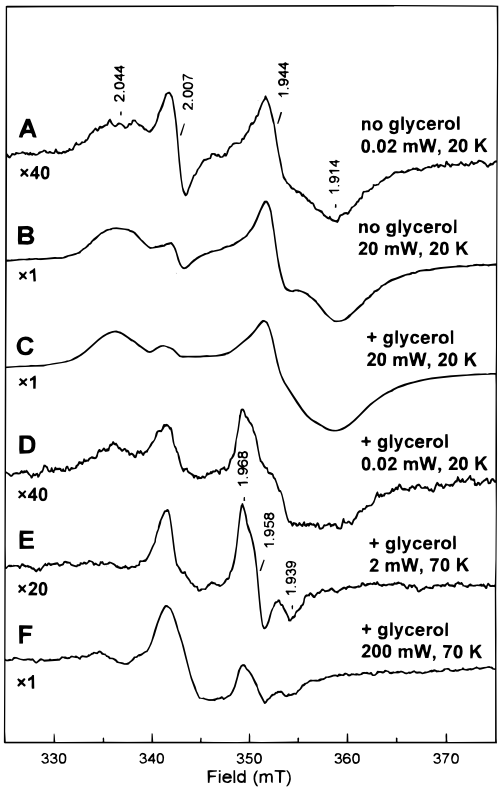


FIGURE 3: X-band EPR spectra of dithionite-reduced samples of biotin synthase. (A and B) Biotin synthase, 0.90 mM, in 50 mM Tris/HCl buffer, pH 7.8, anaerobically reduced with 5 mM sodium dithionite for 120 min. (C−F). The sample is as described for (A) and (B) except that 60% (v/v) glycerol was added anaerobically and the sample (0.36 mM in biotin synthase) was incubated for an additional 15 min prior to freezing. Conditions of measurement: temperature and microwave powers, as indicated; microwave frequency, 9.612 GHz; modulation amplitude, 0.63 mT. The multiplication factors indicate the relative spectrometer gains.

centered at $g = 2.004$ ("$g = 2.00$" resonance) and a faster relaxing rhombic resonance with $g = 2.044$, 1.944, 1.914 ("$g = 2.04$" resonance) (see Figure 3A,B). No other resonances were observed for this sample. The $g = 2.00$ resonance is still observable at 100 K and hence is attributed to a minor organic radical species. The $g = 2.04$ resonance accounts for 0.1 spin/monomer, and a similar resonance with analogous principal *g*-values and spin quantitation was reported in previous studies (Sanyal et al., 1994). On the basis of the g_{av} value and the *g*-value anisotropy, it can be attributed to either an $S = {}^1/_2$ $[2Fe\text{-}2S]^+$ or $[4Fe\text{-}4S]^+$ cluster. The cluster type in this case is usually distinguished based on relaxation behavior, with $[4Fe\text{-}4S]^+$ clusters having faster relaxation such that they are only generally observable below 30 K and $[2Fe\text{-}2S]^+$ clusters having slower relaxation such that they can be observed without significant broadening at 70 K (Johnson, 1994). However, the $g = 2.04$ resonance has relaxation behavior intermediate between these two extremes; readily saturating at low temperatures (starts to saturate at microwave powers >0.1 mW at 10 K, >10 mW at 20 K, and >100 mW at 35 K) and observable at 50 K but not 70 K. As discussed below, parallel EPR and VTMCD studies of a dithionite-reduced sample containing glycerol indicate that the $g = 2.04$ resonance arises from a $S = {}^1/_2$ $[4Fe\text{-}4S]^+$ cluster.

The $g = 2.04$ and 2.00 resonances were also observed in samples reduced with dithionite and treated anaerobically with 60% (v/v) glycerol prior to freezing (see Figure 3C−F). However, these samples exhibit an additional near-axial

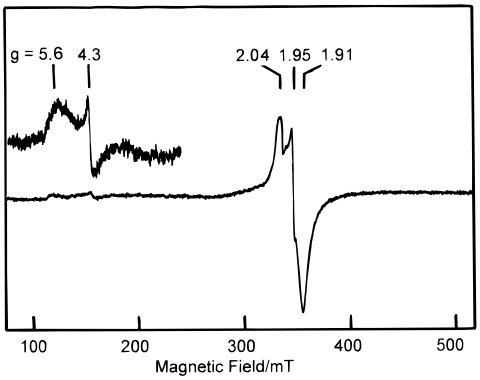


FIGURE 4: X-band EPR spectrum of dithionite-reduced biotin synthase. The sample is as used in Figure 3C−F. Conditions of measurement: temperature, 10 K; microwave power, 50 mW; microwave frequency, 9.612 GHz; modulation amplitude, 0.63 mT. The expanded low-field region was recorded at 4.2 K with a microwave power of 10 mW.

resonance from a slower relaxing $S = {}^1/_2$ species, $g = 1.967$, 1.958, 1.939 ("$g = 1.97$" resonance). The $g = 1.97$ resonance starts to saturate at microwave powers >0.01 mW at 20 K and is barely observable at 20 mW (cf. Figure 3C,D). The line shape of the $g = 1.97$ resonance is most clearly apparent at 70 K, when the $g = 2.04$ resonance is too broad to be observed, and the power dependence differentiates the contributions from $g = 2.00$ and 1.97 resonances (cf. Figure 3E,F). Spin quantitations at 35 K under nonsaturating conditions indicate the $g = 2.04$ and 1.97 resonances together account for 0.4 spin/monomer. By simulating the resonance under nonsaturating conditions as the sum of two overlapping resonances and quantifying the simulated spectra individually, we estimate that the $g = 2.04$ resonance accounts for 0.3 spin/monomer which translates to 0.6 $S = {}^1/_2$ $[4Fe\text{-}4S]^+$ cluster/homodimer and the $g = 1.97$ resonance accounts for 0.1 spin/monomer. The $g = 1.97$ EPR signal is unique among Fe-S proteins in terms of both *g*-values and relaxation behavior.

Additional evidence for the presence of $[4Fe\text{-}4S]^+$ clusters in the dithionite-reduced sample containing 60% (v/v) glycerol comes from the observation of a low-field resonance attributable to a component of $[4Fe\text{-}4S]^+$ clusters with an $S = {}^3/_2$ ground state (see Figure 4). Under conditions at which the resonances in the $S = {}^1/_2$ region are strongly power saturated, 10 K and 50 mW, a weak low-field resonance is clearly discernible at $g = 5.6$, in addition to the isotropic $g = 4.3$ resonance that is characteristic of adventitiously bound Fe(III) ion. Following the initial discovery in nitrogenase Fe-protein (Lindahl et al., 1985; Hagen et al., 1985; Watt & McDonald, 1985), there are now numerous examples of biological $[4Fe\text{-}4S]^+$ clusters that exist in frozen solution as mixed spin species with $S = {}^1/_2$ and ${}^3/_2$ components (Onate et al., 1989; Zambrano et al., 1989; Conover et al., 1990; Flint et al., 1993). Although it is difficult to estimate the spin quantitation of the $S = {}^3/_2$ species (the resonance is very broad, spanning at least 500 mT), comparison of the relative intensities of the $S = {}^1/_2$ and $S = {}^3/_2$ signals with other well-characterized systems (Lindahl et al., 1985; Onate et al., 1993) and the VTMCD data presented below indicates that this is likely to be a minor component accounting for <0.1 spin/monomer.

VTMCD. EPR and VTMCD data together provide a discriminating method for identifying cluster type in paramagnetic Fe-S proteins (Johnson et al., 1982), and the initial

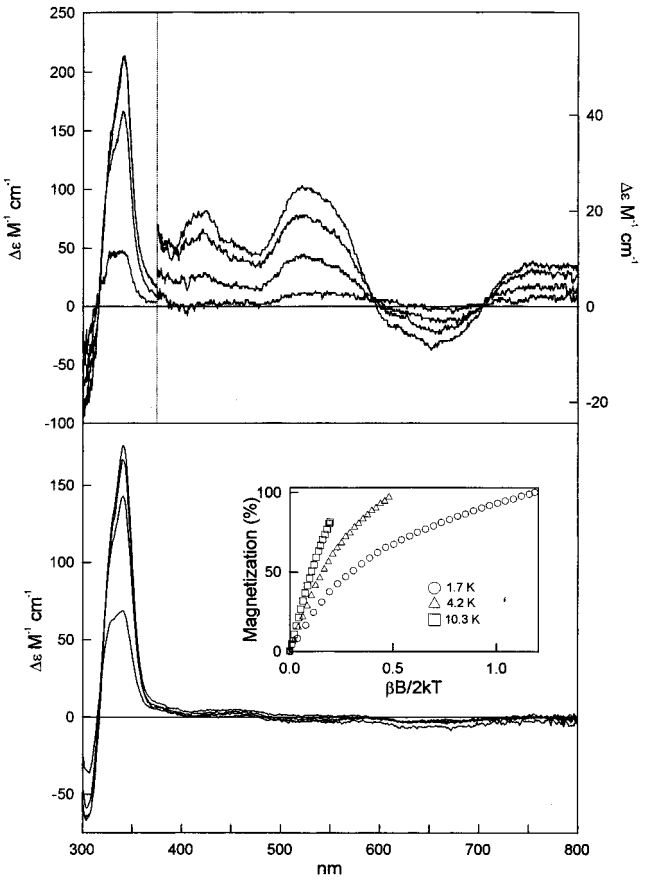


FIGURE 5: VTMCD spectra for dithionite-reduced samples of biotin synthase. (Upper panel) Sample incubated with dithionite anaerobically for 2 h followed by addition of 60% (v/v) glycerol. The sample is as described in Figure 3C−F. MCD spectra were recorded with a magnetic field of 4.5 T at 1.97, 4.22, 9.1, and 50 K. All bands increase in intensity with decreasing temperature. (Lower panel) Sample incubated with dithionite anaerobically for 3 h in the presence of 60% (v/v) ethylene glycol. The sample is described in Figure 1B. MCD spectra were recorded with a magnetic field of 6 T at 1.70, 4.22, 10.3, and 35 K. The bands below 400 nm increase in intensity with decreasing temperature. The inset shows MCD magnetization for the band at 340 nm. The MCD intensity was monitored as a function of increasing field, 0−6 T, at 1.70 K (○), 4.22 K (△) , and 10.3 K (□) and plotted as percent magnetization *vs* $\beta B/2kT$ where β is the Bohr-magneton, B is the magnetic flux, k is Boltzmann's constant, and T is the absolute temperature.

rationale for adding 60% (v/v) ethylene glycol or glycerol to biotin synthase samples was the need to obtain optical quality glasses on freezing samples for VTMCD measurements. In accord with the presence of $S = 0$ $[2Fe\text{-}2S]^{2+}$ clusters, no temperature-dependent MCD bands were observed in the wavelength region 300−800 nm for biotin synthase as purified over the temperature range 1.7−100 K (data not shown). VTMCD data for dithionite-reduced samples in which ethylene glycol was present during the reduction (identical to the resultant sample of the absorption studies in Figure 1B) or glycerol was added subsequent to anaerobic incubation with dithionite (identical to the sample used for EPR studies in Figures 3C-F and 4) are shown in Figure 5, lower and upper panels, respectively. In the former case, no temperature-dependent MCD bands were observed in the visible region above 400 nm, which is in accord with the assignment of the chromophore responsible for the broad 410-nm absorption feature as a $S = 0$ $[4Fe\text{-}4S]^{2+}$ cluster. In the latter case, temperature-dependent MCD bands are observed throughout the 400−800 nm region, and the pattern of bands is uniquely indicative of a $S = {}^1/_2$ $[4Fe\text{-}4S]^{+}$ cluster (Johnson et al., 1982; Onate et al., 1993; Conover et al., 1990, 1991), and quite distinct from that expected for $S = {}^1/_2$ $[2Fe\text{-}2S]^{+}$ clusters (Fu et al., 1992). The saturation behavior as a function of temperature (1.9−50 K at 4.5 T) is consistent with the transitions arising for the most part (>80%) from a $S = {}^1/_2$ ground state (data not shown).

The intensities of the low-temperature MCD spectra of paramagnetic Fe-S clusters, compared under equivalent conditions after normalizing for concentration and path length and correcting for sample depolarization (Johnson, 1988; Thomson et al., 1993), provide a crude estimation of cluster concentration. Synthetic and biological $S = {}^1/_2$ $[4Fe\text{-}4S]^{+}$ clusters have $\Delta\epsilon$ values for the positive band at ~520 nm in the range 60−90 M^{-1} cm^{-1} per cluster at 4.5 T and 2 K (Johnson et al., 1982; Onate et al., 1993). Hence, the intensity of the low-temperature MCD spectrum of the $S = {}^1/_2$ $[4Fe\text{-}4S]^{+}$ cluster in biotin synthase, $\Delta\epsilon = 25$ M^{-1} cm^{-1} per monomer under analogous conditions, translates to 0.27−0.42 $[4Fe\text{-}4S]^{+}$ clusters per monomer or 0.54−0.84 $[4Fe\text{-}4S]^{+}$ cluster per homodimer, which is in good agreement with the $S = {}^1/_2$ EPR spin quantitation for the $g = 2.04$ resonance (0.6 spin/homodimer).

Both samples of dithionite-reduced biotin synthase used for MCD measurements exhibited intense temperature-dependent bands below 380 nm, i.e., a positive band at 340 nm and a negative band at 310 nm, with very different temperature-dependent behavior compared to the bands above 380 nm (see Figure 5). Very similar VTMCD bands have been reported for reduced rubredoxin (Johnson et al. 1982; Werth & Johnson, 1989), and they are attributed to charge transfer transitions involving a $S = 2$ Fe(II)−thiolate center. This assignment is supported by MCD magnetization studies at 340 nm (inset in Figure 5), which are nested and characteristic of a $S = 2$ ground state with significant zero field splitting, $D > +5$ cm^{-1}, leaving a $M_s = 0$ singlet state lowest in energy (Werth & Johnson, 1989; Thomson & Johnson, 1980). Since the VTMCD and EPR data for as-purified biotin synthase show no evidence for a thiolate-coordinated mononuclear Fe(II) or Fe(III) center and the VTMCD intensity is an order of magnitude less than for reduced rubredoxins under comparable conditions, we conclude that this is a minor species resulting from cluster degradation under reducing conditions.

Resonance Raman. Resonance Raman spectra in the Fe-S stretching region, 200−450 cm^{-1}, provide a means of identifying Fe-S cluster type. In particular, RR provides a method for discriminating between clusters with diamagnetic ground states, such as $[4Fe\text{-}4S]^{2+}$ and $[2Fe\text{-}2S]^{2+}$ clusters (Spiro et al., 1982, 1988), and assessing the possibility of partial noncysteinyl ligation for both $[2Fe\text{-}2S]^{2+,+}$ clusters (Meyer et al., 1994; Crouse et al., 1996; Kuila et al., 1992) and $[4Fe\text{-}4S]^{+}$ clusters (Conover et al., 1990). Resonance Raman spectra for biotin synthase as purified obtained with 406.7-nm, 457.9-nm, 488.0-nm, and 514.5-nm laser excitation are shown in Figure 6. The spectra identify the diamagnetic Fe-S cluster as a $[2Fe\text{-}2S]^{2+}$ cluster, and the vibrational modes can be readily assigned under idealized D_{2h} symmetry by direct analogy with well-characterized [2Fe-2S] ferredoxins which have been assigned via isotope shift and analog studies (Han et al., 1989a,b; Fu et al., 1992) (see Table 1).

The RR spectra for biotin synthase are most similar to those recently reported for the $[2Fe\text{-}2S]^{2+}$ center in human ferrochelatase (see Table 1), which was tentatively interpreted

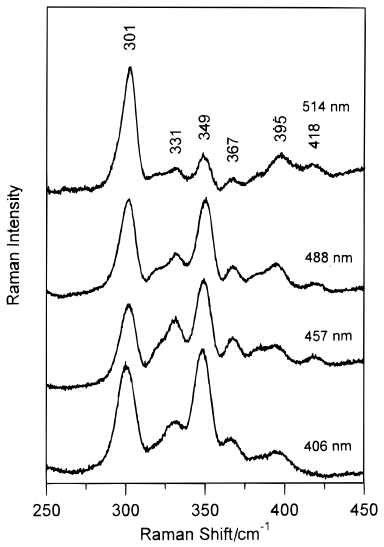


FIGURE 6: Low-temperature resonance Raman spectra of biotin synthase as prepared. The protein concentration was ~2 mM, and the buffering medium was 100 mM Tris/HCl, pH 7.8. The spectra were obtained at 17 K using 406.7-nm, 457.9-nm, 488.0-nm, and 514.5-nm laser excitation, and each is the sum of 20 scans. Each scan involved advancing the spectrometer in 0.2 cm^{-1} increments and photon counting for 1 s/point with 6 cm^{-1} spectral resolution. Bands arising from the frozen buffer solution have been subtracted from each spectrum after normalizing the intensities of the ice lattice modes at 230 cm^{-1}.

Table 1: Low-Temperature Resonance Raman Fe−S Stretching Frequencies (cm^{-1}) and Assignments for the [2Fe-2S]$^{2+}$ Centers in *S. oleracea* Fd, *P. putida* Fd, *C. pasteurianum* 2Fe−Fd, Human Ferrochelatase, and *E. coli* Biotin Synthase

mode,[a] D_{2h}	*S. oleracea* Fd[b]	*P. putida* Fd[b]	*C. pasteurianum* Fd[b]	human ferro-chelatase[c]	*E. coli* biotin synthase
$B_{2u}{}^b$	427	426	404	420	418
$A_g{}^b$	395	400	387	398	395
$B_{3u}{}^b$	367	350	366	367	367
$B_{1u}{}^t$,$B_{2g}{}^t$	357	344	353		
$A_g{}^t$	338	338	335	350	349
$B_{1g}{}^b$	329	320	313	320	331
$B_{3u}{}^t$	283	291	290	295	301

[a] Symmetry labels under idealized D_{2h} symmetry for a $Fe_2S_2{}^bS_4{}^t$ unit. [b] Assignments taken from Fu et al. (1992). [c] Assignments taken from Crouse et al. (1996).

in terms of one oxygenic and three cysteinyl-S ligands for the cluster (Crouse et al., 1996). This interpretation was based largely on the observation that the frequencies of the vibrational modes with dominant Fe−S^t stretching character, $B_{3u}{}^t$ and $A_g{}^t$, show a substantial upshift in mutant [2Fe-2S] ferredoxins in which one of the four coordinating cysteinyl residues has been mutated to a serine. The upshift is expected based on the S *versus* O mass difference, whereas a downshift is expected and observed for histidyl-N coordination due to the large effective mass of the imidazole ring (Kuila et al., 1992). For [2Fe-2S]$^{2+}$ clusters with complete cysteinyl coordination, the $B_{3u}{}^t$ and $A_g{}^t$ modes occur in the range 281−291 and 326−340 cm^{-1}, respectively, compared to 289−302 and 332−351 cm^{-1}, respectively, for clusters in which one of the coordinating cysteines is mutated to serine (Crouse et al., 1996). As in the case of the human ferrochelatase, the assignment of the $A_g{}^t$ mode in as-prepared biotin synthase to the band at 349 cm^{-1} must be viewed as tentative in the absence of $^{34}S^t$ isotope shift data, but the assignment of the $B_{3u}{}^t$ mode at 301 cm^{-1} is unambiguous.

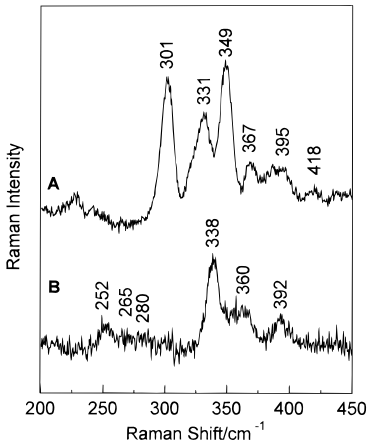


FIGURE 7: Comparison of the low-temperature resonance Raman spectra (457-nm excitation) of the Fe-S clusters in biotin synthase as-purified (A) and after dithionite reduction in the presence of 60% (v/v) ethylene glycol (B). The samples are as described in Figure 1, i.e., prior to addition of dithionite and 270 min after addition of dithionite to a sample containing 60% (v/v) ethylene glycol. The conditions of measurement are as described in Figure 6, and spectra A and B are the sum of 20 and 90 scans, respectively. Bands originating from ethylene glycol have been subtracted from (B) after normalizing the intensities of the ethylene glycol band at 482 nm.

An intense RR band below 305 cm^{-1} corresponding to this out-of-phase symmetric Fe−S^t stretching mode (due to incomplete cancellation of two large polarization changes as a result of cluster asymmetry) is the hallmark of biological [2Fe-2S]$^{2+}$ clusters. Therefore, the anomalously high frequency of this mode in as-prepared biotin synthase is consistent with cluster coordination by one O or N (not histidine) ligand.

RR spectra were also recorded for samples reduced with dithionite in the absence and presence of 60% (v/v) ethylene glycol (identical to the resultant samples of the absorption studies in Figure 1A,B). No Fe−S stretching modes were observed above background with excitation wavelengths in the range 406−568 nm for samples that were reduced with dithionite in the absence of ethylene glycol. This argues against the presence of significant amounts of [4Fe-4S]$^{2+}$, [2Fe-2S]$^{2+}$, or [2Fe-2S]$^{+}$ clusters since all three type of clusters would be expected to exhibit measurable enhancement of Fe−S stretching modes under these conditions (Spiro et al., 1988; Fu et al., 1992). However, [4Fe-4S]$^{+}$ clusters exhibit negligible RR spectra in the Fe−S stretching region (Spiro et al., 1988), and the same is likely to be the case for the hypothetical all-ferrous clusters, [4Fe-4S]^0 and [2Fe-2S]^0, since the enhancement of the Fe−S stretching mode with visible excitation requires S→Fe(III) charge transfer bands and hence correlates approximately with the percentage of Fe(III) character for the Fe in the cluster.

For the biotin synthase sample reduced with dithionite in the presence of ethylene glycol, Raman bands due to ethylene glycol were subtracted by recording a spectrum of the equivalent ethylene glycol/buffer solution and subtracting after normalization of the intensities of the band at 483 cm^{-1} that is exclusively due to ethylene glycol. The resulting RR spectrum in the Fe−S stretching region is compared to that of the [2Fe-2S]$^{2+}$ center in biotin synthase as-purified in Figure 7. The complete loss of the 301-cm^{-1} band indicates the absence of [2Fe-2S]$^{2+}$ clusters, and the resulting RR spectrum is characteristic of a [4Fe-4S]$^{2+}$ cluster with complete cysteinyl coordination. For example, RR spectra

Table 2: Low-Temperature Resonance Raman Fe−S Stretching Frequencies (cm^{-1}) and Assignments for the $[4Fe\text{-}4S]^{2+}$ Centers in $[Fe_4S_4(SCH_2Ph)_4]^{2-}$, *C. pasteurianum* Nitrogenase Fe Protein, and *E. coli* Biotin Synthase

mode, T_d[a]	$[Fe_4S_4(SCH_2Ph)_4]^{2-}$ [b]	Fe protein[c]	biotin synthase
A_1^t	389	391	392
T_2^b	389	391	392
T_2^t	360	356	360
A_1^b	336	335	338
E^b	273	281	280
T_1^b	273	265	265
T_2^b	244	248	252

[a] Symmetry labels under idealized T_d symmetry for the $Fe_4S_4^bS_4^t$ unit. [b] Frozen 90/5/5 water/DMA/Triton X-100 solution at 77 K. Taken from Czernuszewicz et al., 1987. [c] Taken from Fu et al., 1991.

with very similar relative band intensities and frequencies are observed for the $[4Fe\text{-}4S]^{2+}$ clusters in nitrogenase Fe protein (Fu et al., 1991) and the synthetic analog complexes with benzyl thiolate ligands (Czernuszewicz et al., 1987). The vibrational modes of the synthetic analog complex have been rigorously assigned under idealized T_d symmetry via ^{34}S and ^{54}Fe isotope shifts, IR studies, and normal mode calculations (Czernuszewicz et al., 1987), and the spectra for the $[4Fe\text{-}4S]^{2+}$ clusters in reduced biotin synthase and nitrogenase Fe protein can be assigned by direct analogy (see Table 2). The frequency of the most intense band, which corresponds to the symmetric breathing mode of the Fe_4S_4 cubane, has been found to be a useful indicator of non-cysteinyl coordination at a specific Fe (Conover et al., 1990). This band occurs at 338 cm^{-1} for the $[4Fe\text{-}4S]^{2+}$ cluster in biotin synthase, which is in the range established for clusters with complete cysteinyl coordination [333−339 cm^{-1} (Conover et al., 1990)]. The decreased signal-to-noise level compared to the as-purified sample of biotin synthase is a consequence of several factors: the lower sample concentration and glass formation that result from dilution with ethylene glycol; the subtraction of an ethylene glycol spectrum; and the inherently lower (∼5-fold less) resonant enhancement of Fe−S stretching modes for $[4Fe\text{-}4S]^{2+}$ centers compared to $[2Fe\text{-}2S]^{2+}$ centers with 457-nm excitation (Spiro et al., 1982, 1988; Fu et al., 1991). Nevertheless, these RR data, combined with the absorption data presented above, leave little doubt that dithionite reduction of biotin synthase in the presence of ethylene glycol results in near-stoichiometric conversion of two $[2Fe\text{-}2S]^{2+}$ clusters to yield one $[4Fe\text{-}4S]^{2+}$ cluster.

Activity Assays. Since the spectroscopic studies reported above show that biotin synthase samples can be prepared with $[2Fe\text{-}2S]^{2+}$, $[4Fe\text{-}4S]^{2+}$, or $[4Fe\text{-}4S]^{+}$ centers as the major cluster species, it was clearly of interest to determine the effects of cluster type on the enzymatic activity. *In vitro* activity studies were carried out under anaerobic conditions as described previously (Sanyal et al., 1996) using a defined assay mixture involving biotin synthase, dethiobiotin, KCl, NADPH, $Fe(NH_4)_2(SO_4)_2$, fructose 1,6-biphosphate, dithiothreitol, L-cysteine, Ado-Met, flavodoxin, and flavodoxin reductase. Three different preparations of biotin synthase were used, i.e., as-prepared under aerobic conditions ($[2Fe\text{-}2S]^{2+}$-containing biotin synthase), reduced with dithionite in the presence of 60% (v/v) ethylene glycol ($[4Fe\text{-}4S]^{2+}$-containing biotin synthase), and reduced with dithionite followed by addition of 60% (v/v) glycerol ($[4Fe\text{-}4S]^{+}$-containing biotin synthase). However, the type of biotin synthase preparation used in the assay did not affect the activity within the experimental error of the measurement (±20%).

DISCUSSION

The combination of absorption, VTMCD, EPR, and RR studies on biotin synthase has revealed the first example of a reductive $2\times[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ cluster conversion. In accord with previous analytical and absorption data (Sanyal et al., 1994), the absorption and RR spectra reported here indicate that recombinant biotin synthase as-purified has one $S = 0$ $[2Fe\text{-}2S]^{2+}$ cluster in each of the 39-kDa subunits of the homodimer. However, the Fe−S stretching frequencies are indicative of incomplete cysteinyl ligation. Moreover, in contrast to all known biological $[2Fe\text{-}2S]^{+}$ clusters, there is no spectroscopic evidence for the formation of a $[2Fe\text{-}2S]^{+}$ cluster on reduction by dithionite. Rather, dithionite reduction proceeds slowly over a period of several hours and leads to the medium-dependent formation of $[4Fe\text{-}4S]^{2+}$ or $[4Fe\text{-}4S]^{+}$ clusters.

The absorption and RR studies of the product of dithionite reduction in the presence of ethylene glycol or glycerol indicate near-stoichiometric conversion of two $[2Fe\text{-}2S]^{2+}$ clusters to yield one $[4Fe\text{-}4S]^{2+}$ cluster with complete cysteinyl-S ligation. However, further addition of dithionite does not effect reduction to the $[4Fe\text{-}4S]^{+}$ form, as evidenced by EPR, VTMCD, and absorption studies. In contrast, EPR and VTMCD results show substantial formation (>0.5 spin/homodimer) of a $S = {}^1/_2$ $[4Fe\text{-}4S]^{+}$ cluster ($g = 2.044, 1.944, 1.914$) on addition of glycerol after prolonged incubation with dithionite. The $S = {}^1/_2$ spin quantitation is likely to be an underestimation of the $[4Fe\text{-}4S]^{+}$ cluster concentration, since a weak $g = 5.6$ feature in the low field EPR spectra indicates a mixture of $S = {}^1/_2$ and ${}^3/_2$ ground states in frozen solutions. In addition, there is a slow relaxing $S = {}^1/_2$ resonance, $g = 1.968, 1.958, 1.939$, accounting for 0.2 spin/homodimer. The origin of this resonance is unknown at present, but it is tentatively attributed to a paramagnetic cluster with anomalous properties or a radical species in close proximity to the paramagnetic $[4Fe\text{-}4S]^{+}$ cluster. We do not as yet have a satisfactory explanation for the observation that the redox properties of the [4Fe-4S] cluster in biotin synthase are dependent on the method of formation. The implication is that the dimerization process is critically dependent on medium effects.

The only logical rationalization for the near-stoichiometric, reductive cluster conversion in biotin synthase is that a [4Fe-4S] cluster is formed at the interface of the two subunits via dimerization of two [2Fe-2S] clusters. Moreover, the only other well-documented example of cluster conversion involving [4Fe-4S] and [2Fe-2S] clusters is provided by the nitrogenase Fe protein (Anderson & Howard, 1984; Fu et al. 1991), which has a [4Fe-4S] cluster that bridges identical subunits with two equivalent cysteines in each subunit providing the cluster ligands (Georgiadis et al., 1992). As purified under anaerobic conditions in the presence of dithionite, the Fe-protein contains a $[4Fe\text{-}4S]^{+}$ cluster. However, absorption, analytical, and RR studies have shown that the cluster can be converted to a single $[2Fe\text{-}2S]^{2+}$ cluster upon oxidation in the presence of MgATP and an Fe-chelator (Anderson & Howard, 1984; Fu et al., 1991), and RR results indicate that this cluster conversion occurs to a limited extent even in the absence of the Fe-chelator. Although not considered previously, reductive $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ cluster conversion could account for the anomalous reduction

properties of the $[2Fe\text{-}2S]^{2+}$ form of the Fe protein (Anderson & Howard, 1984; Ryle et al., 1996), and this possibility is discussed in more detail below.

One puzzling aspect of the dithionite reduction results is the low spin quantitation for the $S = {}^1/_2$ $[4Fe\text{-}4S]^+$ resonance (~0.2 spin/homodimer) prior to addition of glycerol. This result is essentially the same as that obtained in previous work, although in the absence of any spectroscopic evidence for reductive [2Fe-2S] to [4Fe-4S] cluster conversion, the EPR signal was erroneously attributed to a $S = {}^1/_2$ $[2Fe\text{-}2S]^+$ cluster, albeit with anomalous relaxation properties (Sanyal et al., 1994). Absorption and RR studies clearly demonstrate that the low spin quantitation is not a consequence of the majority of the Fe being present as $S = 0$ $[4Fe\text{-}4S]^{2+}$ clusters, as is the case for reduction in the presence of ethylene glycol. In the previous studies, this EPR and absorption behavior was attributed to reductive cluster degradation since 60% of the total Fe could be removed by anaerobic gel filtration following dithionite reduction (Sanyal et al., 1994). Although some cluster degradation may also be occurring in this work during the course of dithionite reduction, absorption and EPR studies indicate that at least 50% of the Fe is still present in the form of reduced [4Fe-4S] clusters. First, air-oxidation results in an initial 20% increase in the $[4Fe\text{-}4S]^{2+}$ absorption at 410 nm, which subsequently decays as a result of O_2-induced cluster degradation. Second, the total concentration of EPR-detectable $[4Fe\text{-}4S]^+$ clusters increases at least 3-fold on addition of glycerol, suggesting that intact clusters are present or at least can be rapidly re-formed on glycerol addition.

We have considered two explanations both with precedents in nitrogenase Fe protein for the presence of reduced but "EPR-silent" clusters. The first is that the bulk of the Fe is present as $S = {}^3/_2$ $[4Fe\text{-}4S]^+$ clusters in the absence of glycerol. The $[4Fe\text{-}4S]^+$ cluster in the nitrogenase Fe protein exists in frozen solution as a medium-dependent $S = {}^1/_2/S = {}^3/_2$ spin mixture, with the addition of 50% (v/v) glycerol increasing the $S = {}^1/_2$ component at least 3-fold so that it accounts for 80−90% of the total Fe (Lindahl et al., 1985; Hagen et al., 1985; Watt & McDonald, 1985; Onate et al., 1993). Thus far, we have been unable to detect a significant $S = {}^3/_2$ resonance on dithionite reduction in the absence of glycerol or ethylene glycol. However, heterogeneous $S = {}^3/_2$ species could exhibit resonances that are too broad to be detected. Hence, a medium-dependent mixture of $S = {}^3/_2$ and $S = {}^1/_2$ $[4Fe\text{-}4S]^+$ clusters is currently our best explanation for the low $S = {}^1/_2$ spin concentration in the absence of glycerol.

The second explanation is that all-ferrous $[2Fe\text{-}2S]^0$ and/or $[4Fe\text{-}4S]^0$ clusters are present in dithionite-reduced samples in the absence of glycerol. Both types of all-ferrous cluster have been reported in the nitrogenase Fe protein (Anderson & Howard, 1984; Ryle et al., 1996; Watt & Reddy, 1994). A $S = {}^1/_2$ EPR signal ($g = 2.00, 1.94, 1.92$) with relaxation properties indicative of a $[2Fe\text{-}2S]^+$ cluster was observed on reduction of the [2Fe-2S]-containing form of the Fe protein with substoichiometric amounts of dithionite (Anderson & Howard, 1984). The observation that this resonance is lost on addition of excess dithionite, coupled with reversible changes in the absorption and CD spectra (Ryle et al., 1996), has led to speculation that the $[2Fe\text{-}2S]^{2+}$ cluster in this case can be reduced by two electrons to an all-ferrous $[2Fe\text{-}2S]^0$ cluster (Anderson & Howard, 1984; Ryle et al., 1996). Recently, reduction of the $[2Fe\text{-}2S]^{2+}$ cluster in a ferredoxin using a Cr(II) macrocycle has also been interpreted in terms of two-electron reduction to a $[2Fe\text{-}2S]^0$ state (Im et al., 1995). However, in light of the changes in visible absorption on reduction of the $[2Fe\text{-}2S]^{2+}$-containing Fe-protein to an "EPR-silent" state (Ryle et al., 1996), it seems more likely that a reductive $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ cluster conversion of the type reported herein is responsible for the anomalous reduction properties in this instance. Hence, there is no well-documented precedent for invoking $[2Fe\text{-}2S]^0$ clusters in dithionite-reduced samples of biotin synthase. The evidence for a $[4Fe\text{-}4S]^0$ cluster in samples of Fe protein at biologically relevant potentials is more persuasive (Watt & Reddy, 1994), but clearly requires confirmation by Mössbauer spectroscopy. Although the presence of such clusters with medium-dependent midpoint potentials would provide an alternative explanation for the increase in the concentration of $S = {}^1/_2$ $[4Fe\text{-}4S]^+$ clusters on addition of glycerol, we view this as a remote possibility at the present time.

Biotin synthase	· K · G · **C** · E d **C** · · **C** · Q s
LAS	· L G · · **C** T R · **C** · F **C** · V ·
ARR-AE	L F V · G **C** · H · **C** · G **C** Y N ·
PFL-AE	· F · Q G **C** · · R **C** · Y **C** H N ·

FIGURE 8: Comparison of consensus sequences for biotin synthase, lipoic acid synthase, anaerobic ribonucleotide reductase activating enzyme, and pyruvate formate-lyase activating enzyme in the region of conserved cysteine residues. Nonconserved residues are indicated by dots, and the lower case letters in the biotin synthase consensus sequence indicate that the residue is conserved in all but 1 of the 12 known sequences.

The finding that biotin synthase can exist in a form with a [4Fe-4S] cluster at the subunit interface of the homodimer suggests a correspondence to ARR-AE and PFL-AE (Ollangnier et al., 1996; Broderick et al., 1997) and other Ado-Met-dependent enzymes that are known, or at least suspected, to contain a Fe-S cluster, i.e., LAS (Sanyal et al., 1994) and LAM (Petrovich et al., 1992; Frey & Reed, 1993). Moreover, primary sequences are available for two or more representatives of each type of enzyme, except for LAM, and it is interesting to note that the consensus sequences each contain a $C\text{-}X_3\text{-}C\text{-}X_2\text{-}C$ motif (see Figure 8). Although no other residues are conserved in the region of these three closely spaced conserved cysteines, it may be important to note that the third cysteine always has an adjacent aromatic residue, since Tyr or Phe always precedes this cysteine in the biotin synthase sequences. There are now 12 biotin synthase sequences in the SWISS-PROT data base, representing organisms as diverse as plants, yeast, bacteria, and archaea, and 4 cysteines are conserved in all cases, Cys53, Cys57, Cys60, and Cys188, using the *E. coli* numbering system. In view of the RR results which are best interpreted in terms of three cysteine ligands for the [2Fe-2S] cluster and the X-ray structures for plant-type [2Fe-2S] ferredoxins which show that the cluster is coordinated by a $C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_{29}\text{-}C$ arrangement of cysteines (Johnson, 1994), it seems likely that all three of the cysteines in the $C\text{-}X_3\text{-}C\text{-}X_2\text{-}C$ motif of biotin synthase are involved with coordinating the [2Fe-2S] cluster. The fourth ligand is presumably water or some other protein ligand. Unless a major rearrangement involving cluster breakdown and reassembly occurs on formation of the [4Fe-4S] cluster at the subunit interface, which seems unlikely in view of the stoichiometry, two of these cysteines are likely to be involved in coordinating the [4Fe-4S] cluster. The consensus sequences for LAS, ARR-

AE, and PFL-AE are based on more limited data (2, 3, and 3 sequences, respectively, in the SWISS-PROT data base), but the observation that each has a C-X_3-C-X_2-C motif suggests that a [4Fe-4S] cluster at the subunit interface that is capable of [4Fe-4S]/[2Fe-2S] cluster conversion may be common to all three enzymes.

This hypothesis was subsequently tested in the case of PFL-AE using the combination of UV−visible absorption, EPR, and resonance Raman spectroscopies (Broderick et al., 1997). As prepared, the enzyme was found to contain a mixture of $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters. Dithionite reduction resulted in a homogeneous form containing only $[4Fe\text{-}4S]^{2+}$ clusters, and reduction to the $[4Fe\text{-}4S]^{+}$ state ($S = {}^1/_2$, $g = 2.01, 1.89, 1.88$) was only possible in the presence Ado-Met. The presence of $[2Fe\text{-}2S]^{2+}$ clusters was considered to be a consequence of incomplete anaerobicity during purification, and the analytical data were consistent with approximately one $[4Fe\text{-}4S]^{2+,+}$ cluster per homodimer in reduced samples. While it has yet to be determined if Ado-Met is required for the reduction of the $[4Fe\text{-}4S]^{2+}$ in biotin synthase, these results add strong support for [4Fe-4S]/[2Fe-2S] cluster conversion being a unifying property among this class of Fe-S enzymes.

Thus far, the Fe-S clusters in LAM and ARR-AE have only been investigated by absorption and EPR spectroscopies (Petrovich et al., 1992; Ollangnier et al., 1996). While this leaves some uncertainty concerning cluster type, it is clearly of interest to compare these results with those reported herein for biotin synthase. LAM is a hexameric enzyme, and since the analytical and EPR data, taken together, are consistent with one [4Fe-4S] cluster per dimeric unit, it seems likely that the cluster bridges subunits (Petrovich et al., 1992). However, the EPR properties of the cluster are quite different from those of biotin synthase. The anaerobically purified enzyme exhibits a resonance from variable amounts of a stable radical species ($g = 2.006$) and a fast-relaxing Fe-S resonance, $g = 2.027, 2.007, 1.99$, accounting for <0.1 spin/[4Fe-4S] cluster. Hence, the majority of the clusters appear to be in a diamagnetic state (presumably $[4Fe\text{-}4S]^{2+}$ clusters), and the EPR signal has been attributed to a trace of $S = {}^1/_2$ $[4Fe\text{-}4S]^{3+}$ clusters which are reduced to an EPR-silent form by dithionite. The main evidence for the presence of [4Fe-4S] clusters comes from the observation that the cluster can be almost stoichiometrically converted to a species that gives an EPR signal ($g = 2.032, 2.015, 2.0125$) suggestive of a $S = {}^1/_2$ $[3Fe\text{-}4S]^{+}$ cluster, on oxidation with air or ferricyanide. Neither of the Fe-S EPR signals observed in LAM have been observed in EPR studies of biotin synthase, and EPR or VTMCD evidence for oxidative degradation to yield a $[3Fe\text{-}4S]^{+}$ cluster has not been forthcoming. However, more recent EPR studies of LAM have shown that the $[4Fe\text{-}4S]^{2+}$ cluster can be reduced to $[4Fe\text{-}4S]^{+}$ in the presence of Ado-Met,[2] and it was this observation that prompted Ado-Met mediated reduction of PFL-AE.

Recent absorption and EPR studies of the fully reconstituted ARR-AE (i.e., 2Fe and 2S per17.5-kDa monomer) were interpreted in terms of one $[4Fe\text{-}4S]^{2+,+}$ cluster per homodimer (Ollangnier et al., 1996). The assignment of the dithionite-reduced EPR signal ($g = 2.02, 1.92$, and 1.88) to a $[4Fe\text{-}4S]^{+}$ cluster was based largely on relaxation properties. However, the relaxation properties and g-values were medium-dependent, and reduction in the presence of 10% (v/v) glycerol resulted in the appearance of a slower-relaxing more axial resonance ($g_{\|} = 2.03$, $g_{\perp} = 1.92$). Although the authors interpret both resonances in terms of $[4Fe\text{-}4S]^{+}$ clusters, the line shape and relaxation properties of the 10% glycerol sample could equally well be interpreted in terms of a mixture of $[2Fe\text{-}2S]^{+}$ and $[4Fe\text{-}4S]^{+}$ clusters. Moreover, the absorption spectrum of the protein as-prepared and prior to reconstitution clearly resembles that of a $[2Fe\text{-}2S]^{2+}$ center, as opposed to a $[4Fe\text{-}4S]^{2+}$ center, or a mixture thereof. While more detailed spectroscopic studies are required with a wider range of techniques, the available evidence suggests that the $[4Fe\text{-}4S]^{2+,+}$ cluster bridges the subunits of ARR-AE and that this protein is also susceptible to [2Fe-2S]/[4Fe-4S] cluster conversions.

By comparison with the other Fe-S enzymes requiring Ado-Met, the results presented here suggest that the [4Fe-4S]-containing form of biotin synthase is responsible for catalytic activity. All indications are that the [2Fe-2S]-containing form is a direct consequence of aerobic isolation and that conversion to the [4Fe-4S] form occurs under reducing conditions. Since the enzymatic assays are carried out anaerobically in the presence of physiological reductants (i.e., NADPH, flavodoxin, and flavodoxin reductase), reductive cluster conversion may well be occurring under assay conditions, and experiments are in progress to address this question. This would provide an explanation for the absence of a significant difference in the *in vitro* catalytic activities for biotin synthase samples containing $[2Fe\text{-}2S]^{2+}$, $[4Fe\text{-}4S]^{2+}$, and $[4Fe\text{-}4S]^{+}$ clusters as their major species. However, such comparisons should be viewed with caution in light of the very low turnover numbers observed in the *in vitro* assay. This implication of the low turnover numbers is that a physiologically significant factor is not present in the assay mixture (Sanyal et al., 1996). Until a fully optimized *in vitro* assay is available, it is difficult to draw meaningful conclusions concerning the relationship between cluster type and activity in biotin synthase.

The above discussion indicates the presence of a subunit-bridging $[4Fe\text{-}4S]^{2+,+}$ cluster that is capable of undergoing reversible oxidative conversion to $[2Fe\text{-}2S]^{2+}$ clusters in biotin synthase, PFL-AE, ARR-AE, and possibly LAM. In the latter case, there is as yet no evidence for [4Fe-4S]/[2Fe-2S] cluster conversion. Although the present work does not address the catalytic role of the [4Fe-4S] cluster in biotin synthase, studies of PFL-AE, ARR-AE, and LAM implicate a role for the $[4Fe\text{-}4S]^{2+,+}$ couple in effecting the one electron of Ado-Met to form methionine and the 5′-deoxyadenosyl radical (Broderick et al., 1997; Ollangnier et al., 1996; Frey & Perry, 1993). The mechanism of this novel reaction is clearly of great interest and is under active investigation by several groups including our own.

Finally we turn to the question of whether or not the [2Fe-2S]/[4Fe-4S] cluster conversion in this class of enzymes has any physiological significance. While we are still in the process of optimizing conditions for $[4Fe\text{-}4S]^{2+}$ to $[2Fe\text{-}2S]^{2+}$ cluster conversion in biotin synthase, the absorption studies presented herein demonstrate that some $[2Fe\text{-}2S]^{2+}$ clusters are re-formed during O_2-induced degradation of the $[4Fe\text{-}4S]^{2+}$ clusters. Coupled with the observation of $[2Fe\text{-}2S]^{2+}$ clusters in aerobic or semi-anaerobic preparations of biotin synthase and PFL-AE (Broderick et al., 1997), this indicates the likelihood of $[4Fe\text{-}4S]^{2+} \leftrightarrow [2Fe\text{-}2S]^{2+}$ cluster interconversions and raises the possibility that

[2] P. A. Frey, personal communication.

this process may be used *in vivo* to regulate enzyme activity in response to oxidative stress. A specific mechanism to turn off enzyme activity on exposure to O_2 in order to avoid the formation of superoxide radicals, without irreversible degradation of the active site cluster, would clearly be particularly advantageous for Fe-S enzymes that function by radical mechanisms. It remains to be determined if the $[2Fe\text{-}2S]^{2+}$ clusters in these enzymes are oxidative artifacts or integral components of a novel O_2-protection mechanism.

NOTE ADDED IN PROOF

Since submission of this paper, O_2-induced degradation of the $[4Fe\text{-}4S]^{2+}$ cluster in the *E. coli* transcription factor FNR (fumarate nitrate reduction) has been shown to occur via a $[2Fe\text{-}2S]^{2+}$ cluster (Khoroshilova et al., 1997). This raises the possibility that [4Fe-4S] ↔ [2Fe-2S] cluster interconversion is involved in the oxygen sensing mechanism of FNR.

REFERENCES

Anderson, G. L., & Howard, J. B. (1984) *Biochemistry 23*, 2118−2122.

Baldet, P., Gerbling, H., Axiotis, S., & Douce, R. (1993) *Eur. J. Biochem. 217*, 479−485.

Baxter, R. L., Camp, D. J., Coutts, A., & Shaw, N. (1992) *J. Chem. Soc., Perkin Trans. I*, 255−258.

Bianchi, V., Eliasson, R., Fontecave, M., Mulliez, E., Hoover, D. M., Matthews, R. G., & Reichard, P. (1993) *Biochem. Biophys. Res. Commun. 197*, 792−797.

Birch, O. M., Fuhrmann, M., & Shaw, N. M. (1995) *J. Biol. Chem. 270*, 19158−19165.

Borovok, I., Landman, O., Kreisberg-Zakarin, R., Aharonowitz, Y., & Cohen, G. (1996) *Biochemistry 35*, 1981−1987.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Broderick, J. B., Duderstadt, R. E., Fernandez, D. C., Wojtuszewski, K., Henshaw, T. F., & Johnson, M. K. (1997) *J. Am. Chem. Soc. 119*, 77396−77397.

Conover, R. C., Kowal, A. T., Fu, W., Park, J.-B., Aono, S., Adams, M. W. W., & Johnson, M. K. (1990) *J. Biol. Chem. 265*, 8533−8541.

Conover, R. C., Park, J.-B., Adams, M. W. W., & Johnson, M. K. (1991) *J. Am. Chem. Soc. 113*, 2799−2800.

Crouse, B. R., Sellers, V. M., Finnegan, M. G., Dailey, H. A., & Johnson, M. K. (1996) *Biochemistry 35*, 16222−16229.

Czernuszewicz, R. S., Macor, K. A., Johnson, M. K., Gewirth, A., & Spiro, T. G. (1987) *J. Am. Chem. Soc. 109*, 7178−7187.

Dailey, H. A., Finnegan, M. G., & Johnson, M. K. (1994) *Biochemistry 33*, 403−407.

DeMoll, E., & Shive, W. (1995) *Biochem. Biophys. Res. Commun. 132*, 217−222.

Drozdzewski, P. M., & Johnson, M. K. (1988) *Appl. Spectrosc. 42*, 1575−1577.

Eisenberg, M. (1987) in *Escherichia coli and Salmonella typhimurium—Cellular and Molecular Biology* (Neidhardt, F. C., Ed.) pp 544−550, American Society for Microbiology, Washington, D.C.

Flint, D. H., Emptage, M. H., Finnegan, M. G., Fu, W., & Johnson, M. K. (1993) *J. Biol. Chem. 268*, 14732−14742.

Florentin, D., Bui, B. T. S., Marquet, A., Ohshiro, T., & Izumi, Y. (1994) *C. R. Acad. Sci. Paris*, 485−488.

Frey, P. A., & Reed, G. H. (1993) *Adv. Enzymol. Relat. Areas Mol. Biol. 66*, 1−39.

Fu, W., Morgan, T. V., Mortenson, L. E., & Johnson, M. K. (1991) *FEBS Lett. 284*, 165−168.

Fu, W., Drozdzewski, P. M., Davies, M. D., Sligar, S. G., & Johnson, M. K. (1992) *J. Biol. Chem. 267*, 15502−15510.

Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J., & Rees, D. C. (1992) *Science 257*, 1653−1659.

Hagen, W. R., Eady, R. R., Dunham, W. R., & Haaker, H. (1985) *FEBS Lett. 189*, 250−254.

Han, S., Czernuszewicz, R. S., Kimura, T., Adams, M. W. W., & Spiro, T. G. (1989a) *J. Am. Chem. Soc. 111*, 3505−3511.

Han, S., Czernuszewicz, R. S., & Spiro, T. G. (1989b) *J. Am. Chem. Soc. 111*, 3496−3504.

Harder, J., Eliasson, R., Pontis, E., Ballinger, M. D., & Reichard, P. (1992) *J. Biol. Chem. 267*, 25548−25552.

Ifuku, O., Koga, N., Haze, S.-I., Kishimoto, J., & Wachi, Y. (1994) *Eur. J. Biochem. 224*, 173−178.

Im, S.-C., Lam, K.-Y., Lim, M.-C., Ooi, B.-L., & Sykes, A. G. (1995) *J. Am. Chem. Soc. 117*, 3635−3636.

Jestin, E., Moreau, F., Florentin, D., & Marquet, A. (1996) *Bioorg. Med. Chem. 4*, 1065−1075.

Johnson, M. K. (1988) in *Metal Clusters in Proteins* (Que, L., Jr., Ed.) pp 326−342, ACS, Washington, D.C.

Johnson, M. K. (1994) in *Encyclopedia of Inorganic Chemistry* (King, R. B., Ed.) pp 1896−1915, John Wiley & Sons, Chichester.

Johnson, M. K., Robinson, A. E., & Thomson, A. J. (1982) in *Iron−sulfur Proteins* (Spiro, T. G., Ed.) pp 367−406, Wiley-Interscience, New York.

Khoroshilova, N., Popescu, C., Münck, E., Beinert, H., & Kiley, P. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 6087−6092.

Kim, J., & Rees, D. C. (1992) *Science 257*, 1677−1682.

Knappe, J., & Sawers, G. (1990) *FEMS Microbiol. Rev. 75*, 383−398.

Knappe, J., Neugebauer, F. A., Blaschkowski, H. P., & Gänzler, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1332−1335.

Kuila, D., Schoonover, J. R., Dyer, R. B., Batie, C. J., Ballou, D. P., Fee, J. A., & Woodruff, W. H. (1992) *Biochim. Biophys. Acta 1140*, 175−183.

Lindahl, P. A., Day, E. P., Kent, T. A., Orme-Johnson, W. H., & Münck, E. (1985) *J. Biol. Chem. 260*, 11160−11173.

Marquet, A., Frappier, F., Guillerm, G., Azoulay, M., Florentin, D., & Tabet, J.-C. (1993) *J. Am. Chem. Soc. 115*, 2139−2145.

Marti, F. B. (1983) Diss. E. T. H. Zürich, Nr 7236.

Méjean, A., Bui, B. T. S., Florentin, D., Ploux, O., Izumi, Y., & Marquet, A. (1995) *Biochem. Biophys. Res. Commun. 217*, 1231−1237.

Meyer, J., Fujinaga, J., Gaillard, J., & Lutz, M. (1994) *Biochemistry 33*, 13642−13650.

Mulliez, E., Fontecave, M., Gaillard, J., & Reichard, P. (1993) *J. Biol. Chem. 268*, 2296−2299.

Nozaki, Y. (1986) *Arch. Biochem. Biophys. 249*, 437−446.

Ollangnier, S., Mulliez, E., Gaillard, J., Eliasson, R., Fontecave, M., & Reichard, P. (1996) *J. Biol. Chem. 271*, 9410−9416.

Onate, Y. A., Switzer, R. L., Vollmer, R. A., & Johnson, M. K. (1989) *J. Biol. Chem. 264*, 18386−18391.

Onate, Y. A., Finnegan, M. G., Hales, B. J., & Johnson, M. K. (1993) *Biochim. Biophys. Acta 1164*, 113−123.

Petrovich, R. M., Ruzicka, F. J., Reed, G. H., & Frey, P. A. (1992) *Biochemistry 31*, 10774−10781.

Reed, K. E., & Cronan, J. E. (1993) *J. Bacteriol. 175*, 1325−1336.

Reichard, P. (1993) *J. Biol. Chem. 268*, 8383−8386.

Ryle, M. J., Lanzilotta, W. N., Seefeldt, L. C., Scarrow, R. C., & Jensen, G. M. (1996) *J. Biol. Chem 271*, 1551−1557.

Sanyal, I., Cohen, G., & Flint, D. H. (1994) *Biochemistry 33*, 3625−3631.

Sanyal, I., Gibson, K. J., & Flint, D. H. (1996) *Arch. Biochem. Biophys. 326*, 48−56.

Spiro, T. G., Hare, J., Yachandra, V., Gewirth, A., Johnson, M. K., & Remsen, E. (1982) in *Iron-sulfur Proteins* (Spiro, T. G., Ed.) pp 407−423, Wiley-Interscience, New York.

Spiro, T. G., Czernuszewicz, R. S., & Han, S. (1988) in *Resonance Raman Spectra of Heme and Metalloproteins* (Spiro, T. G., Ed.) pp 523−554, John Wiley & Sons, New York.

Thomson, A. J., & Johnson, M. K. (1980) *Biochem. J. 191*, 411−420.

Thomson, A. J., Cheeseman, M. R., & George, S. J. (1993) *Methods Enzymol. 226*, 199−232.

Watt, G. D., & McDonald, J. W. (1985) *Biochemistry 24*, 7226−7231.

Watt, G. D., & Reddy, K. R. N. (1994) *J. Inorg. Biochem. 53*, 281−294.

Werth, M. T., & Johnson, M. K. (1989) *Biochemistry 28*, 3982−3988.

Zambrano, I. C., Kowal, A. T., Adams, M. W. W., Mortenson, L. E., & Johnson, M. K. (1989) *J. Biol. Chem 264*, 20974−20983.

BI9706430