Iron-Sulfur Center of Biotin Synthase and Lipoate Synthase

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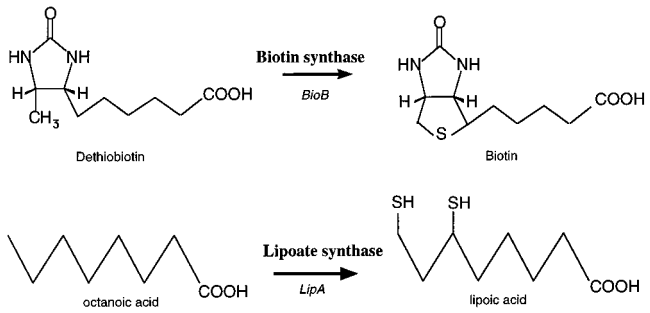
ABSTRACT: Biotin synthase and lipoate synthase are homodimers that are required for the C−S bond formation at nonactivated carbon in the biosynthesis of biotin and lipoic acid, respectively. Aerobically isolated monomers were previously shown to contain a (2Fe-2S) cluster, however, after incubation with dithionite one (4Fe-4S) cluster per dimer was obtained, suggesting that two (2Fe-2S) clusters had combined at the interface of the subunits to form the (4Fe-4S) cluster. Here we report Mössbauer studies of ^{57}Fe-reconstituted biotin synthase showing that anaerobically prepared enzyme can accommodate two (4Fe-4S) clusters per dimer. The (4Fe-4S) cluster is quantitatively converted into a $(2Fe\text{-}2S)^{2+}$ cluster upon exposure to air. Reduction of the air-exposed enzyme with dithionite or photoreduced deazaflavin yields again (4Fe-4S) clusters. The (4Fe-4S) cluster is stable in both the 2+ and 1+ oxidation states. The Mössbauer and EPR parameters were $\Delta E_q = 1.13$ mm/s and $\delta = 0.44$ mm/s for the diamagnetic $(4Fe\text{-}4S)^{2+}$ and $\Delta E_q = 0.51$ mm/s, $\delta = 0.85$ mm/s, $g_{par} = 2.035$, and $g_{perp} = 1.93$ for the $S = {}^1/_2$ state of $(4Fe\text{-}4S)^{1+}$. Considering that we find two (4Fe-4S) clusters per dimer, our studies argue against the early proposal that the enzyme contains one (4Fe-4S) cluster bridging the two subunits. Our study of lipoate synthase gave results similar to those obtained for BS: under strict anaerobiosis, lipoate synthase can accommodate a (4Fe-4S) cluster per subunit [$\Delta E_q = 1.20$ mm/s and $\delta = 0.44$ mm/s for the diamagnetic $(4Fe\text{-}4S)^{2+}$ and $g_{par} = 2.039$ and $g_{perp} = 1.93$ for the $S = {}^1/_2$ state of $(4Fe\text{-}4S)^{1+}$], which reacts with oxygen to generate a $(2Fe\text{-}2S)^{2+}$ center.

Iron−sulfur enzymes display a broad range of functions, including electron transfer, catalysis in redox and nonredox reactions, regulation of gene transcription or translation (*1*, *2*). Recently, a new function has been suggested since enzymes such as biotin synthase and lipoate synthase from *Escherichia coli* are assumed to utilize an iron−sulfur center to catalyze the conversion of an unactivated C−H bond into a C−S bond (*3*−*6*).

The last step of biotin biosynthesis (Scheme 1) has been shown, from genetic studies, to require the product of the *bioB* gene (*7*, *8*). This protein, by simplification, is often named biotin synthase (BS)[1] even though other proteins might be also involved in the reaction. Here as well, BS designates the product of the *bioB* gene. In vitro assays with well-defined mixtures containing BS, flavodoxin, flavodoxin reductase, NADPH, *S*-adenosylmethionine (AdoMet), cysteine, and dithiothreitol are not capable to generate more than

Scheme 1



1−3 mol of biotin/mol of BS (*3*, *9*). This indicates either that extra important factors are missing or that BS rather works as a substrate, with the (Fe−S) cluster providing the sulfur atoms for incorporation into biotin, as recently suggested (*10*). This question of whether the designation "enzyme" is appropriate for biotin synthase requires further investigation.

Little is known about the mechanism of the reaction. The requirement for *S*-adenosylmethionine and NADPH/flavodoxin reductase/flavodoxin as a reducing agent (*9*, *11*) has led to the suggestion that biotin synthase belongs to the family of Fe−S enzymes with radical-based mechanisms, such as the anaerobic pyruvate formate lyase (PFL) and ribonucleotide reductase (RNR) systems and the lysine aminomutase (*12*−*14*). In the proposed mechanism, the

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[1] Abbreviations: LS, lipoate synthase; BS, biotin synthase; PFL, pyruvate formate lyase; RNR, ribonucleotide reductase; 5-DAF, 5-deaza-7,8-demethylisoalloxazine; DTT, dithiothreitol; (Fe−S), iron−sulfur center; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide.

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iron−sulfur center transfers a single electron from reduced flavodoxin for reductive cleavage of AdoMet to produce methionine and the 5′-deoxyadenosyl radical. The latter then abstracts a hydrogen atom of a specific C−H bond of the dethiobiotin substrate with the resulting carbon radical supposed to be reactive to incorporate a sulfur atom (*15*). The direct source of the sulfur atom is still a matter of investigation. Recently, it has been assigned to the cluster itself, as noted above (*10*).

Lipoate synthase (LS) catalyzes one or both of the carbon−sulfur bond formations during the conversion of octanoic acid into lipoic acid (Scheme 1) (*16*). It is the product of the *lipA* gene, and cells with mutations in *lipA* do not produce lipoic acid (*17*). Purification of lipoate synthase has been reported by two groups only recently, which both demonstrated that it is an iron−sulfur enzyme (*5*, *6*). Lipoate synthase shares with biotin synthase, PFL activating enzyme and the anaerobic RNR some amino acid sequences, in particular, the CXXXCXXC motif probably involved in Fe chelation (*12*, *18*). Even though there is still no evidence for the requirement for AdoMet and the flavodoxin system, it is tempting to suggest that lipoate synthase belongs to the same family of Fe−S enzymes and that lipoic acid formation proceeds through the formation of an octanoate radical.

Both BS and LS have been characterized as homodimers (*3*, *6*). In both cases, each monomer has been reported to contain a maximal amount of two Fe and two S atoms, in the form of a $(2Fe\text{-}2S)^{2+}$ center, deduced on the basis of spectroscopic studies (*3*−*6*). In a different report, the cluster of LS was suggested to be a (4Fe-4S) cluster bridging the two subunits (*6*). During anaerobic reduction, the (2Fe-2S) clusters were converted into one (4Fe-4S) cluster (*4*, *5*). A similar behavior has been reported with the anaerobic RNR (*19*). However, in the case of the anaerobic RNR, we have recently shown that, with strict adherence to anaerobiosis, each polypeptide chain of the small component of the system can bind four Fe and four S in the form of a $(4Fe\text{-}4S)^{2+}$ center which degrades to a $(2Fe\text{-}2S)^{2+}$ center during exposure to air (*20*). We suspected that the limited amount of Fe and S atoms and the presence of (2Fe-2S) clusters in previously reported preparations of BS and LS were a direct consequence of aerobic isolation. Here we report that, indeed, under strict anaerobiosis, biotin synthase and most likely lipoate synthase also can bind one (4Fe-4S) cluster per subunit. The clusters of both enzymes are air sensitive and are decomposed into (2Fe-2S) centers upon reaction with oxygen. Our data are thus inconsistent with the previous interpretations that BS and LS are homodimers containing one subunit-bridging (4Fe-4S) cluster.

EXPERIMENTAL PROCEDURES

^{57}Fe was converted into its ferric chloride form by dissolving it in a hot concentrated (35%) hydrochloric acid of analytical grade (Carlo Erba) and, repetitively, concentrated in water. $Fe(NH_4)_2(SO_4)_2$ and dithiothreitol were from Aldrich. Sodium dithionite, titrated under anaerobic conditions with $K_3Fe(CN)_6$, was from Fluka. Ethylenediaminetetraacetic acid was from Sigma. 5-Deaza-7,8-demethyl-10-methyl-isoalloxazine (5-DAF) was available in our laboratory. Dr. Y. Jouanneau and J.M Moulis, respectively (CEA, Grenoble, France), provided ferredoxin IV from *Rhodobacter capsulatus* and Pyruvate ferredoxin oxidoreductase from *Clostridium pasteurianum*. Lipoate synthase was purified as previously reported (*5*). Biotin synthase purification was as previously described (*3*) with some modifications and is thus described below.

Protein concentration was determined by the method of Bradford (*21*) standardized against bovine serum albumin and multiplied by a correction factor of 1.1 for the biotin synthase (*22*). Protein-bound iron was determined under reducing conditions with bathophenantroline disulfonate after acid denaturation of the protein (*23*) and labile sulfide by Beinert's method (*24*).

Purification of the Biotin Synthase. *E. coli bioB* was amplified by PCR and subcloned into pET24d(+) to give the plasmid pKH200. pKH200 was transformed into *E. coli* BL21(DE3) and grown aerobically at 37 °C in 100 mL of 2TY until the OD_{600} reached 1.0. The temperature was reduced to 30 °C, and the cells induced with 0.5 mM isopropyl thiogalactoside. The cells, which express the enzyme to ∼10−15% of soluble protein, were harvested after 2.5 h and stored at −80 °C. pKH200/BL21(DE3) cells (100 g) were thawed and resuspended in lysis buffer (300 mL, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 1% Triton X-100) and lysed by sonication on ice (20−30 s bursts). Polyethyleneimine (5% v/v, pH 8.0 with HCl) was added to a final concentration of 0.2% v/v. The lysate was centrifuged (Beckman JA14 rotor at 12 000 rpm) for 30 min. $(NH_4)_2SO_4$ was then added to the supernatant to a final concentration of 35% saturation, stirred for 10 min and centrifuged (12 000 rpm) for 30 min. The resulting pellet was resuspended in buffer A (50 mM Tris-HCl, pH 7.5) and subsequently an equal volume of buffer B was added [50 mM Tris-HCl, pH 7.5, and 0.5 M $(NH_4)_2SO_4$]. This was loaded onto a Phenyl Resource column (150 mL) previously equilibrated with buffer B, and the protein eluted with a 0 to 100% gradient of buffer A. The purest samples, as judged by SDS−PAGE analysis, were combined, concentrated to 50 mg/mL and chromatographed on a Superdex-S200 column (700 mL) equilibrated with buffer A. The resultant fractions containing biotin synthase were loaded onto a MonoQ 16/10 column equilibrated with buffer A. The protein was eluted with a 0 to 60% gradient of buffer C (50 mM Tris-HCl, pH 7.5, and 1.0 M NaCl). The purest fractions, >95% pure by SDS−PAGE analysis, were combined and concentrated to approximately 50 mg/mL before being stored at −80 °C.

Preparation of the Apoprotein Forms of Biotin and Lipoate Synthase. Isolated BS and LS contain around 0.9 and 0.5 iron/polypeptide chain, respectively (*5*). Protein-bound iron was removed by chelation during irradiation of proteins in the presence of 10 mM EDTA and 50 μM 5-DAF in buffer D (0.1 M Tris-HCl, pH 8) over 1 h at 18 °C, under anaerobic conditions. The light was provided by a slide projector 20 cm away from a tube containing 0.5 mL of the protein solution (20 mg/mL). The solutions became colorless after 30 min of irradiation. Then, potassium cyanide (5-fold M excess) was added and incubation was maintained during 30 min, in the dark. Proteins were loaded onto a Sephadex G-25 column (50 mL) equilibrated and eluted with buffer D containing 30 mM KCl and concentrated to approximately 10 mg/mL over a YM30 Diaflo membrane (Amicon) (80% recovery).

Reconstitution of the Iron Center of Biotin and Lipoate Synthase. All the steps of the reconstitution procedure were made anaerobically inside a glovebox [Jacomex B553 (NMT)] in an N_2 atm. containing less than 2 ppm O_2. The apoproteins were incubated with a 6-fold molar excess of Na_2S and either $Fe(NH_4)_2(SO_4)_2$ or $^{57}FeCl_3$ in 3 mL of buffer D containing 5 mM DTT, for 3 h at 18 °C. Then a deaerated solution of EDTA (2 mM final concentration) was added, and incubation lasted for 30 min. After chromatography on a Sephadex G-25 column (equilibrated and eluted with buffer D) the dark-brown fractions were collected and concentrated to approximately 10 mg/mL over a YM30 Diaflo membrane (75−80% recovery). This preparation was then analyzed for its iron and sulfide content and by spectroscopies (EPR, Mössbauer, UV−vis).

Oxidation of Reconstituted Proteins. The reaction was monitored by UV−vis spectroscopy. The ^{57}Fe-reconstituted protein (10 mg/mL) in 0.5 mL of buffer D was exposed to air on ice and analyzed by light absorption spectrophotometry. The incubation was stopped when the absorbance at 420 nm remained stable. The reaction mixture was then loaded onto a Sephadex G-25 column (20 mL) equilibrated and eluted with buffer D. Protein fractions were concentrated to approximately 10 mg/mL.

Reduction of Biotin Synthase and Lipoate Synthase. Reduction of the iron−sulfur centers of the reconstituted and oxidized forms of lipoate and biotin synthase were performed and monitored by light absorption spectroscopy inside the anaerobic glovebox. Reducing agents were 5-DAF, prepared in DMSO, dissolved in water (560 μM final concentration), and stored in the dark inside the box, and dithionite prepared in buffer D (150 mM final concentration) just before reduction. Proteins (200−250 μM) were prepared in 0.2 or 0.3 mL of buffer D and either irradiated with a slide projector in the presence of 5-DAF (50 μM) over 60 min or reduced with a 6-fold M excess of dithionite over 40 min. Reduction was monitored from the decay of the light absorption band at 420 nm. Reduced proteins were then divided into two portions, one being transferred in EPR tubes (200 μL) and the other to Mössbauer cups (300 μL). All samples were frozen inside the box, in a well filled with isopentane cooled outside the box by liquid nitrogen.

Light Absorption Spectroscopy. UV−vis spectra were recorded with a Cary 1 Bio (Varian) spectrophotometer. Spectra could be also recorded inside the anaerobic glovebox using a Hewlett-Packard 8453 diode array spectrophotometer equipped with optical fibers connected to a sample holder fixed inside the glovebox.

EPR Spectroscopy. EPR first derivative spectra were recorded on a Bruker EMX (9.5 GHz) EPR spectrometer equipped with an ESR 900 Helium flow cryostat (Oxford Instruments). Double integrals of the EPR signals were evaluated by using a computer on-line with the spectrometer. Spin concentration in the protein samples was determined by calibrating double integration of the EPR spectra with a standard sample of a (2Fe-2S) protein (62 μM of ferredoxin IV of *Rhodobacter capsulatus*) recorded under non saturating conditions.

Mössbauer Spectroscopy. ^{57}Fe-Mössbauer spectra were recorded on 300 μL cups containing the protein (200−250 μM). The spectra were recorded on either a strong-field or a weak-field spectrometer operating in constant acceleration mode using Janis Research Inc. cryostats that allow for a variation in temperature from 1.5 to 300 K. One of the dewars housed a superconducting magnet that allowed for the application of magnetic fields up to 8 T parallel to the γ-irradiation. Isomer shifts are quoted relative to Fe metal at room temperature.

Assay of Biotin Synthase Activity. Biotin synthase was assayed according to published procedures (*9*). After 1 h of reaction at 37 °C, the Fe-reconstituted form (sample A) generated 1 mol of biotin/mol of protein, whereas the oxidized form (sample C) generated 0.5 mol of biotin/mol of protein.

RESULTS

Reconstitution of the Iron Center of BS and LS. After purification, both proteins were slightly brownish, with an absorption band at 420 nm, indicating the presence of a (Fe−S) center. However, from one preparation to another, variable amounts of iron and sulfide were determined, from 0.5 to 0.9 atoms/polypeptide chain, probably reflecting a loss of the iron center during purification. In general, lower amounts of iron were found in LS than in BS.

To evaluate the exact amount of Fe and S that the proteins can bind, the purified enzymes were converted into apoproteins by reaction with EDTA in the presence of a reducing agent. Reconstitution was achieved by incubation with an excess of ferrous iron and sodium sulfide in the presence of dithiothreitol (DTT) for 3 h and then desalted on Sephadex G-25. For Mössbauer spectroscopy analysis, reconstitution was carried out with $^{57}FeCl_3$. All manipulations were performed within an anaerobic glovebox equipped with optical fibers and a sample holder coupled to a spectrophotometer, so that the light absorption spectrum of the anaerobic solutions could be monitored without contact to air. Aliquots of ^{57}Fe-reconstituted proteins were frozen for EPR analysis. Furthermore, reconstituted proteins were analyzed for their Fe and S content and, in the case of biotin synthase, for its enzyme activity.

Both BS and LS were thus found to contain 3.7−4.2 Fe atoms/polypeptide chain and a comparable amount of sulfide. This is in marked contrast with previous reports in which monomers of BS and LS were shown to contain only two Fe and two S atoms per polypeptide chain (*3*−*6*). Figure 1 shows the UV−vis spectra of reconstituted EPR-silent (see below) preparation of BS and LS recorded in the anaerobic glovebox. Both spectra are rather similar with a broad band at 420 nm (ϵ = 4000 M^{-1} cm^{-1} and 4500 M^{-1} cm^{-1} respectively), characteristic for sulfide to iron charge transfers within (Fe−S) centers.

Oxidation and Reduction of Reconstituted Biotin Synthase and Lipoate Synthase. For sake of clarity, both reconstituted enzymes are denoted A, and Scheme 2 is shown with the various samples derived from sample A. Scheme 2 applied for both LS and BS, since the enzymes behaved remarkably similarly.

Sample B was obtained from A during exposure to air for 40−60 min. The reaction of the Fe center with oxygen is characterized by a decrease of the intensity of the light absorption spectrum with a half-life of 20−25 min at 10 °C. During the reaction, the dark brownish solutions turned red.

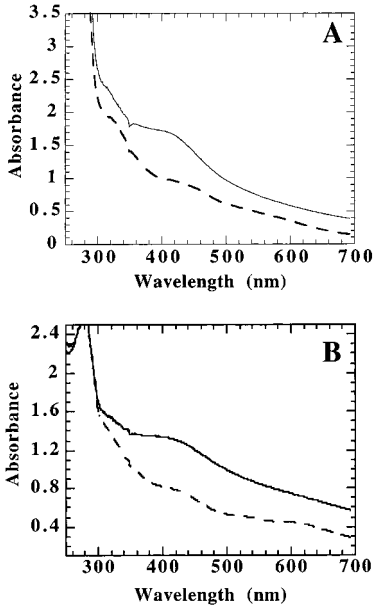
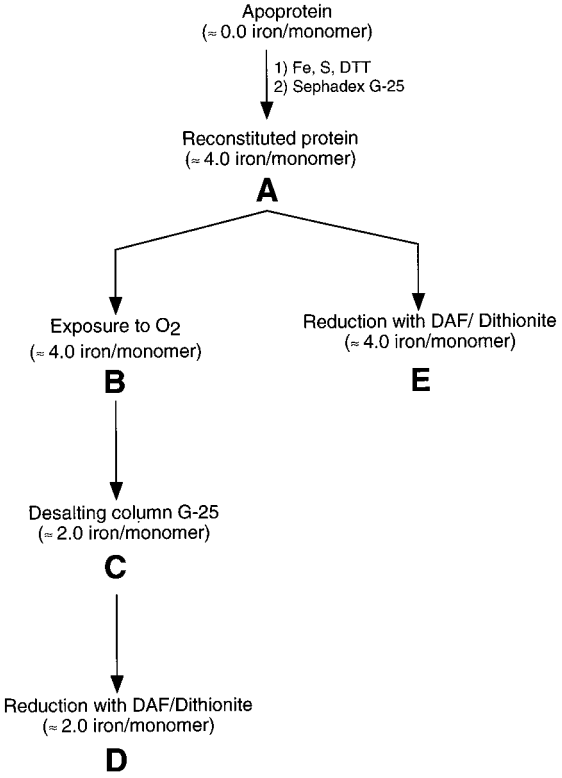


FIGURE 1: UV-vis light absorption spectra of the reconstituted BS (90 μM) (A) and LS (70 μM) (B) proteins in 50 mM Tris-HCl, pH 8, before (solid line) and after exposure to air (dashed line).

Scheme 2



The resulting proteins displayed a slightly different UV−vis spectrum, with a decreased intensity at 420 nm, a shoulder at 460 nm, and a more or less pronounced band at 590 nm (Figure 1). Further exposure to air did not change the spectra, indicating that the resulting proteins were air-stable.

Sample C was obtained by desalting sample B by chromatography on a Sephadex G-25 column. It had a UV−vis spectrum similar to that of sample B, but was found to contain only 1.9−2.1 Fe and S/subunit.

Finally, anaerobic reduction of samples A and C with a chemical reagent (either dithionite or photoreduced deazaflavin) generated samples E and D, respectively (Scheme 2).

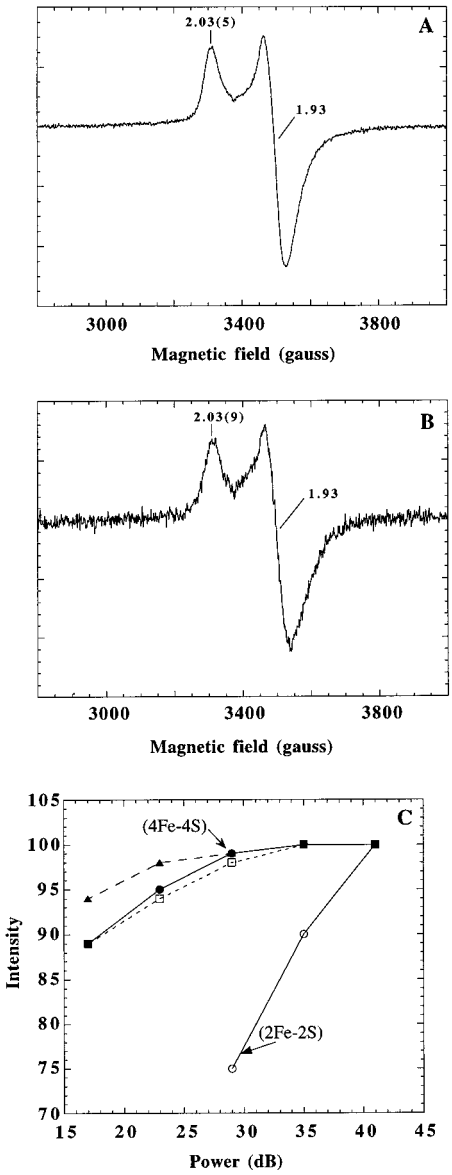


FIGURE 2: X-band EPR spectra of reconstituted BS and LS proteins in 30 mM Tris-HCl, pH 8. (A) Reconstituted BS (200 μM). Temperature, 10 K; microwave power, 0.160 mW; modulation amplitude, 10 G, receiver gain, 2×10^5; frequency, 9.447 GHz. (B) Reconstituted LS (200 μM) after irradiation in the presence of 50 μM deazaflavin during 60 min at 18 °C. Temperature, 10 K; microwave power, 0.1 mW; modulation amplitude, 10 G; receiver gain, 2×10^5. (C) Microwave power saturation curves at 10 K for the BS [200 μM (▲)] and LS [200 μM (□)]. The EPR signal amplitudes were normalized to the maximum value. Standard samples were ferredoxin IV (2Fe-2S) from *R. capsulatus* (○) and pyruvate ferredoxin: oxidoreductase (4Fe-4S) from *C. pasteurianum* (●).

EPR Spectroscopy Studies. Reconstituted BS, sample A, contained variable amounts of a paramagnetic $S = {}^1/_2$ species, characterized by an axial EPR signal with g values at 2.035 and 1.93 (Figure 2A). The temperature dependence and the microwave power saturation properties of this signal were consistent with a $(4Fe\text{-}4S)^{1+}$ center (Figure 2C). This assignment was confirmed by Mössbauer spectroscopy (see below). The proportion of total iron in the form of a $(4Fe\text{-}4S)^{1+}$ cluster varied from 0 to 70%. We attribute this to a variable amount of contamination of the atmosphere with

Table 1: Proportion of Total Iron within the Different Iron Species in Biotin Synthase

					adventitious iron	
sample[a]	iron content[b]	$(4Fe\text{-}4S)^{2+}$	$(4Fe\text{-}4S)^{1+}$	$(2Fe\text{-}2S)^{2+}$	$Fe^{2+}(S{=}2)$[c]	species Y[d]
Batch 1						
1. A	3.7	≈72	8		20	
2. C	1.9			≈40		≈60
3. D(DAF)	1.9	78		15	7	
Batch 2						
4. A	3.8	20	70		10	
5. B	3.8			≈40	−	≈60
6. C	2.1			≈40	−	≈60
7. D(DAF)	2.1		80	10	10	
8. D(dithionite)	2.1		70	10	20	
9. E(DAF)	3.8		80		20	
10. E(dithionite)	3.8		53		47	

[a] For details on the preparation of the samples see text and Scheme 2. [b] In Fe atoms per protein monomer. [c] Sum of $\delta = 0.72$ mm/s and $\delta = 1.28$ mm/s contributions. [d] Species Y refers to the unidentified broad magnetic background observed in the 4.2 K spectra (Figure 5).

traces of oxygen due to the frequent utilization of the glovebox and by the extreme air sensitivity of the cluster (see below). Accordingly, the intensity of the EPR signal increased during anaerobic reduction with phoreduced deazaflavin (data not shown).

$(4Fe\text{-}4S)^{1+}$ clusters occur frequently in physical mixtures of $S = {}^1/_2$ and $S = {}^3/_2$ forms. The EPR spectra of BS indicate similar mixtures. For instance, very weak and broad features observed at $g = 5.25$, 4.58, 2.71, and 1.34 can be assigned to a $S = {}^3/_2$ species (data not shown). Since the amplitude of these features was quite small, the spin concentrations of $S = {}^3/_2$ species were difficult to determine, but probably did not account for more than 5−10% of total iron.

Compared to BS, reconstituted LS, sample A, in general contained much smaller amounts of a $S = {}^1/_2$ species (accounting for less than 10% of total iron), characterized by an axial EPR signal with g values at 2.039 and 1.93, which was very similar to that of BS, and assigned to a $(4Fe\text{-}4S)^{1+}$ cluster from its temperature dependence and microwave power saturation properties (Figure 2C). Anaerobic reduction with photoreduced deazaflavin increased the intensity of this signal. Figure 2B shows an EPR spectrum, recorded under nonsaturating conditions, of a reduced sample of LS. The total spin concentration accounted for about 0.3 spin/monomer. No $S = {}^3/_2$ EPR signal was observed.

For both reconstituted BS and LS, the EPR signal disappeared during exposure to air and, with no exception, samples B and C (Scheme 2) were EPR-silent. Anaerobic reduction of sample C with either photoreduced 5-DAF or dithionite regenerated the $S = {}^1/_2$ EPR signal (as well as the $S = {}^3/_2$ signal in the case of BS). The yield of the reaction varied from one experiment to another, again probably depending on the quality of the anaerobic environment achieved inside the glovebox. Thus, in general, sample D is EPR-active and displays the same spectrum as samples A and E (Scheme 2).

Mössbauer Studies of Biotin Synthase. Using ^{57}Fe-reconstituted enzyme, we have studied two preparations of the protein, referred to as batch 1 and 2, with Mössbauer spectroscopy. The two batches differed from each other in terms of their content in the $S = {}^1/_2$ $(4Fe\text{-}4S)^{1+}$ species. In the following, we present spectra from both preparations. The fractions of iron associated with each detected species are listed in Table 1. The Mössbauer and EPR parameters characterizing the different clusters are given in Table 2.

Table 2: Parameters for the Iron−Sulfur Centers from Biotin Synthase (BS) and Lipoate Synthase (LS)

	cluster	ΔE_Q (mm/s)	δ (mm/s)	η	g_{par}	g_{perp}
BS	$[4Fe\text{-}4S]^{2+}$	1.13	0.44	0.6		
	$[2Fe\text{-}2S]^{2+}$	0.55	0.27	n.d.[a]		
	$[4Fe\text{-}4S]^{1+}$	0.51[b]	0.85[b]	n.d.[a]	2.035[c]	1.93[c]
LS	$[4Fe\text{-}4S]^{2+}$	1.20	0.44	0.6		
	$[2Fe\text{-}4S]^{2+}$	0.60	0.30	0.4		
	$[4Fe\text{-}4S]^{1+}$				2.039[c]	1.93[c]

[a] Not determined. [b] At 150 K. [c] In the $S = {}^1/_2$ state.

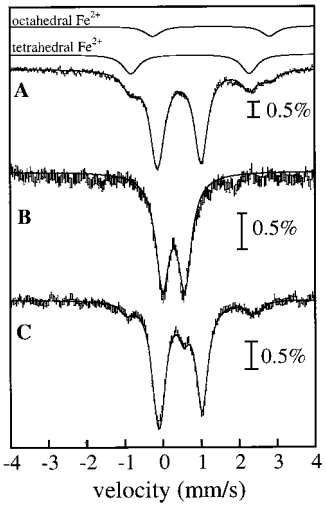


FIGURE 3: Mössbauer spectra of the ^{57}Fe-reconstituted biotin synthase (200 μM in 0.1 M Tris-HCl, pH 8) from batch 1 recorded at 4.2 K. (A) Spectrum of the reconstituted enzyme. The major doublet represents a $(4Fe\text{-}4S)^{2+}$ cluster. The features of the two minority species, almost certainly contaminants, are outlined above the data. (B) Sample exposed for 1 h to air and desalted. The doublet belongs to a $(2Fe\text{-}2S)^{2+}$ cluster. A spectrum recorded on a larger velocity sweep is shown in Figure 5A. (C) Spectrum of reconstituted biotin synthase obtained after deazaflavin-reduction of an air-exposed sample. The major doublet belongs to a $(4Fe\text{-}4S)^{2+}$ cluster. The small peak at 0.9 mm/s belongs to the high-energy line of remnant $(2Fe\text{-}2S)^{2+}$ cluster.

Figure 3A shows a 4.2 K Mössbauer spectrum of ^{57}Fe-reconstituted enzyme, batch 1 (sample A, batch 1). The spectrum can be represented as a superposition of three quadrupole doublets. The major species has quadrupole splitting, $\Delta E_q = 1.13(3)$ mm/s, and isomer shift, $\delta = 0.44(2)$ mm/s, and represents about 72% of Fe (Table 1, entry 1). Spectra recorded in an 8.0 T applied field (not shown)

revealed that this component is diamagnetic. The quoted parameters together with the observation of diamagnetism leaves little doubt that this spectral component represents a (4Fe-4S) cluster in the 2+ core oxidation state. The remainder of the absorption belongs to two doublets with $\Delta E_q = 3.12$ mm/s and $\delta = 0.72$ mm/s (14% of Fe; labeled "tetrahedral Fe^{2+}" in Figure 3) and $\Delta E_q = 3.06$ mm/s and $\delta = 1.28$ mm/s (6%; labeled "octahedral Fe^{2+}"). The value $\delta = 0.72$ mm/s strongly suggested a ferrous site with tetrahedral thiolate coordination, whereas the value $\delta = 1.28$ mm/s indicates a penta- or hexacoordinate ferrous contaminant with N/O ligation. Adventitious ferrous iron thus accounts for 20% of iron (Table 1, entry 1). The sample of Figure 3A contained 3.6 Fe/monomer, and thus, our results show that about 70−75% of the monomers contain a $(4Fe\text{-}4S)^{2+}$ cluster. From a detailed consideration of spectra taken at various temperatures, it is inferred that sample A also contains a half integer spin paramagnetic species (~8%), which is assigned to a $(4Fe\text{-}4S)^{1+}$ cluster (Table 1, entry 1). EPR spectra from an aliquot from this sample show a weak $(4Fe\text{-}4S)^{1+}$, $S = {}^1/_2$ signal.

Figure 3B shows a 4.2 K spectrum of the sample C, batch 1 (Scheme 2), obtained after exposure of sample A, batch 1 to air for 60 min and passage through an anaerobic Sephadex column. This sample contains 1.9 Fe/monomer. It can be seen that the central doublet present in the spectrum of Figure 3A has disappeared and a new doublet with $\Delta E_q = 0.55$ mm/s and $\delta = 0.27$ mm/s has appeared (Table 1, entry 2). Studies in strong applied fields show that this doublet belongs to a diamagnetic species. The Mössbauer parameters, in particular the isomer shift of 0.27 mm/s, strongly suggested that this diamagnetic species represents a $(2Fe\text{-}2S)^{2+}$ cluster. A spectrum recorded over a larger velocity range (Figure 5A) shows that the doublet is superimposed on a broad and unresolved magnetic component. The latter represents iron released upon the 4Fe to 2Fe cluster conversion. We do not know the chemical nature of the iron in the magnetic component, but it may contain colloidal sulfides (noted species Y in Table 1).

A Mössbauer spectrum of sample D, batch 1 (deazaflavin-reduced) is shown in Figure 3C. It can be seen that a spectrum characteristic of the $(4Fe\text{-}4S)^{2+}$ cluster (now 78% of Fe) has reappeared. About 15% of the iron still belongs to the $(2Fe\text{-}2S)^{2+}$ clusters, with the remainder (7%) distributed among the $\delta = 0.72$ mm/s and $\delta = 1.28$ mm/s species (Table 1, entry 3). It is noteworthy that the iron contained in the broad magnetic component of Figure 5A has been recruited for reconstitution of the $(4Fe\text{-}4S)^{2+}$ clusters.

Figure 4A shows a 4.2 K spectrum of a reconstituted sample A from batch 2, and Figure 4B shows a spectrum of a parallel sample that was photoreduced, sample E, batch 2 (entry 4 and 9 of Table 1, respectively). The spectrum of the reduced sample has features similar to those observed for $(4Fe\text{-}4S)^{1+}$ clusters; however, these features are quite broad and the contributions of the $Fe^{2+}Fe^{3+}$ and $Fe^{2+}Fe^{2+}$ pairs, well resolved in some $(4Fe\text{-}4S)^{1+}$ ferredoxins (*25*), are not resolved. With the aid of the spectrum of Figure 4B, we recognize that the sample of Figure 4A contains a mixture of $(4Fe\text{-}4S)^{2+}$ (20%) and $(4Fe\text{-}4S)^{1+}$ (70%) clusters; approximately 10% of the Fe in the sample belongs to $\delta = 0.72$ mm/s and $\delta = 1.28$ mm/s type iron. We thus conclude that about 90% of the polypeptide chain of the anaerobically

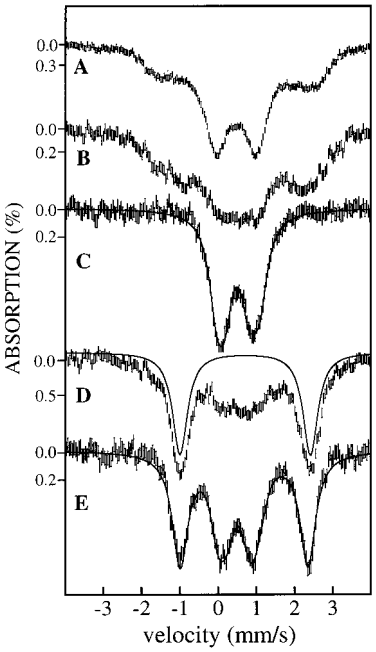


FIGURE 4: Mössbauer spectra of biotin synthase samples from batch 2 (200 μM in 0.1 M Tris-HCl, pH 8). All spectra were recorded in a parallel applied field of 0.05 T. (A) Spectrum of ^{57}Fe-reconstituted enzyme at 4.2 K. 70% of the iron in the sample belongs to $(4Fe\text{-}4S)^{1+}$ and about 20% to $(4Fe\text{-}4S)^{2+}$ clusters. (B) Spectrum of a reconstituted sample after deazaflavin reduction (50 μM final concentration) recorded at 4.2 K. (C) Spectrum of 150 K of the deazaflavin-reduced protein. [Footnote]. (D) Spectrum at 4.2 K of a dithionite-reduced sample. The prominent doublet outlined by the solid line belongs to an integer spin paramagnet, most likely mononuclear Fe^{2+} with tetraedral $Fe(RS)_4$ or $Fe(RS)_3X$ coordination. Spectrum E shows a 150 K spectrum of the sample of spectrum D.

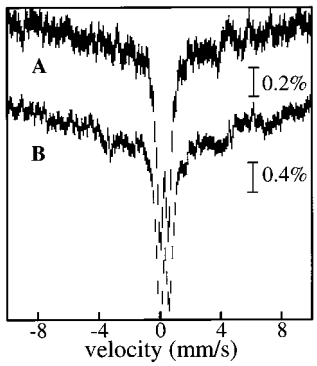


FIGURE 5: Mössbauer spectra at 4.2 K of air-exposed desalted biotin synthase (200 μM in 0.1 M Tris-HCl, pH 8) samples recorded in a parallel field of 0.05 T. (A) Same sample from batch 1 as in Figure 3B. (B) Air-exposed sample B from batch 2. The broad features in both spectra reflect heterogeneous magnetic components that may contain iron sulfides. This component reflects iron released by the 4Fe → 2Fe cluster conversion.

reconstituted sample contained a (4Fe-4S) cluster.

Mössbauer studies of a variety of proteins have shown that the four iron sites of the $S = {}^1/_2$ form belong to a "ferrous" pair and a valence delocalized $Fe^{2+}Fe^{3+}$ pair. The two equivalent ^{57}Fe magnetic hyperfine tensors of the ferrous pair have positive components while those of the two irons of the delocalized pair are negative. These signs can be determined by application of strong applied fields. An 8.0 T spectrum of the sample E, batch 2, revealed a pair with positive *A*-values, but the resolution was insufficient to

attempt detailed simulations. Further, for some proteins, the quadrupole splitting of the pairs differs substantially so that the two pairs can be resolved at temperatures above 100 K under conditions of fast relaxation of the electronic spin (*25*). $S = {}^3/_2$ forms, on the other hand, typically exhibit one doublet at higher temperature. The 150 K spectrum of sample E, batch 2 (Figure 4C), shows one, broad, doublet with average $\delta_{av} \approx 0.51$ mm/s and $\Delta E_q \approx 0.85$ mm/s. The doublet shown should contain at least three subspectra, namely a doublet of the $S = {}^3/_2$ form plus two doublets associated with the $S = {}^1/_2$ state. The spectrum of Figure 4C shows that all three doublets have rather similar parameters. It is particularly noteworthy that the ferrous pair of the $S = {}^1/_2$ form has an unusual small value, namely $\Delta E_q < 1.0$ mm/s.[2]

A 4.2 K spectrum of a dithionite-reduced sample A, batch 2, is shown in Figure 4D (entry 10, Table 1). This spectrum contains a doublet (species X; 47% of Fe) with $\Delta E_q = 3.35$ mm/s and $\delta = 0.7$ mm/s superimposed on a spectral component like that shown in Figure 4B. The 150 K spectrum of the sample, shown in Figure 4E, exhibits the doublet of Figure 4C in addition to the doublet of species X. Spectra taken in strong applied fields show that species X is an integer spin paramagnet (data not shown). Its isomer shift suggests an Fe^{2+} site in a tetrahedral environment of (predominantly) thiolates. We have considered the possibility that species X might belong to an all-ferrous (4Fe-4S) cluster, but have rejected this possibility for two reasons. First, when studied in strong applied magnetic field, species X exhibits splitting similar to those observed for ferrous rubredoxin, suggesting a mononuclear site. Second, we have observed this species also in a deazaflavin-reduced sample of batch 1 together with a (4Fe-4S) cluster in the 2+ state (Figure 3). Most likely, species X reflects iron released by cluster destruction. The idea that about 47% of the iron in our sample would exist as tetrathiolate-coordinated mononuclear Fe^{2+} raises the question of the origin of the thiolate ligands. With nearly 53% of the active sites occupied by a $(4Fe\text{-}4S)^{1+}$, cluster there are not enough cysteine ligands to accommodate four times as many mononuclear sites. The most likely source of some, or all, thiolates might be remnant DTT not removed by the column steps.

We have also studied a sample B, batch 2, obtained during exposure of sample A, batch 2, to air for 1 h and then frozen without a filtration column step; a Mössbauer spectrum is shown in Figure 5B. A total of 35−40% of the Fe in this sample was found to belong to $(2Fe\text{-}2S)^{2+}$ clusters. Thus, approximately 80−90% of the (4Fe-4S) clusters initially present has been converted to $(2Fe\text{-}2S)^{2+}$ clusters. The iron released upon 4Fe → 2Fe cluster conversion and the $\delta = 0.72$ mm/s and $\delta = 1.28$ mm/s components originally present are contained in the broad magnetic component. An attempt to selectively remove the iron of the broad component by passing the sample down a Sephadex column (thus, generating sample C, batch 2) was unsuccessful. While the column step removed about 40% of the iron, the loss was equally distributed among both spectral components, i.e., the resultant Mössbauer spectrum was quite similar to that shown in Figure 5B (Table 1, entry 6).

[2] The 150 K spectrum reveals a doublet with parameters similar to "tetrahedral Fe^{2+}" species. Its contribution (∼20%) has been removed from the spectrum shown in Figure 4C but it is included in Table 1 (entry 9).

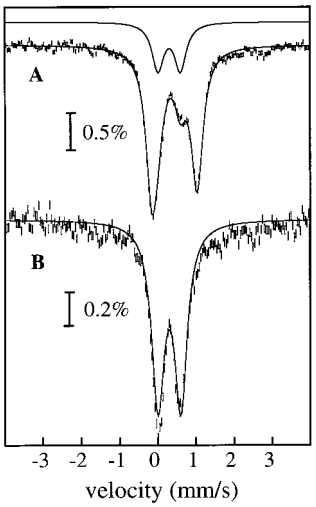


FIGURE 6: Mössbauer spectra of lipoate synthase samples (200 μM in 0.1 M Tris-HCl, pH 8). All spectra were recorded at 4.2 K and in zero-field. (A) Spectrum of ^{57}Fe-reconstituted enzyme. 50% of the iron in the sample belongs to $(4Fe\text{-}4S)^{2+}$, about 10−15% to $(2Fe\text{-}2S)^{2+}$. The iron (35−40%) belongs to paramagnetic form not resolved in this spectrum. (B) Spectrum of a reconstituted sample after oxidation which is characteristic of $(2Fe\text{-}2S)^{2+}$ clusters formed by 4Fe → 2Fe conversion.

Reduction of the column-treated sample with either dithionite or photoreduced 5-DAF regenerated $(4Fe\text{-}4S)^{1+}$ clusters in high yield (70 and 80%, respectively), in agreement with EPR analysis (Table 1, entry 7 and 8).

Mössbauer Studies of Lipoate Synthase. Figure 6A shows a 4.2 K Mössbauer spectrum of a ^{57}Fe-reconstituted protein sample (3.9 Fe/monomer). We have studied this sample in zero-field (shown) and in applied fields of 50 mT, 0.2, 5.0, and 8.0 T. Moreover, we have studied the sample at 77 and 150 K. The majority component, accounting for about 50% of the iron, is a diamagnetic species with $\Delta E_q = 1.20$ mm/s and $\delta = 0.44$ mm/s, i.e., a $(4Fe\text{-}4S)^{2+}$ with parameters similar to those of biotin synthase (Table 2). The sample contains a minority component accounting to 10−15% of Fe. This component is diamagnetic and has $\Delta E_q \approx 0.6$ mm/s and $\delta \approx 0.3$ mm/s; on account of these properties, we assign it to a $(2Fe\text{-}2S)^{2+}$ cluster. Spectra recorded over a larger velocity range at various magnetic fields show that the diamagnetic doublets are superimposed on a broad background containing one or, perhaps, two paramagnetic species. Neither of these species yields well-resolved spectra for any of the experimental conditions employed, and therefore, we cannot make a definite assignment. A fraction of the paramagnetic absorption, at most 20% of Fe, seems to be similar to the broad component observed in the spectra of Figure 4, and some of the magnetic features (less than 15%) confined to velocities -3 mm/s $< \nu < +3$ mm/s could belong to a $(4Fe\text{-}4S)^{+1}$ cluster.

Figure 6B shows a spectrum for a reconstituted protein sample that has been exposed to air for 1 h. The spectrum contains a doublet with $\Delta E_q = 0.60$ mm/s and $\delta = 0.30$ mm/s superimposed on a broad magnetic component. As discussed for BS, the doublet represents $(2Fe\text{-}2S)^{2+}$ clusters formed by 4Fe → 2Fe cluster transformation.

Enzyme Activity of the Fe-Reconstituted BS. Biotin synthase activity is routinely measured during incubation of the pure enzyme with dethiobiotin, bacterial extracts, cysteine,

NADPH, and *S*-adenosylmethionine (*9*). The extracts are supposed to provide electron transfer enzymes, various required cofactors, and the machinery to convert cysteine into the direct sulfur donor. Because of the complexity of the assay mixture and since certain key components of the enzyme have not been identified yet, very low activity (less than one turnover) is usually detected. We speculated that such a low activity could be due to the fact that all previous enzyme assays were made with an enzyme preparation that contained oxidized clusters. However, an assay of Fe-reconstituted BS (sample A), containing a (4Fe-4S) center per monomer, only showed a 2-fold larger activity with respect to an oxidized sample C, containing a (2Fe-2S) center per monomer. This simply showed a correlation between the activity and the amount of protein-bound Fe and sulfide. The comparison is not possible yet in the case of LS, for which an assay is not available.

DISCUSSION

Conversion of a C−H bond to a C−S bond as observed during the transformation of dethiobiotin to biotin and octanoic acid to lipoic acid is an intriguing chemical problem. Very little is known on how biotin synthase and lipoate synthase function; however, it is quite likely that both enzymes use a similar mechanism.

The study of the redox centers and the chemistry of these enzymes requires a sufficient amount of pure protein. Some years ago, some researchers had already obtained biotin synthase in a rather pure form (*3*, *11*, *26*); our preparation described above provides substantially larger amounts of the highly pure enzyme than previously reported. For lipoate synthase, the availability of a pure enzyme is much more recent (*5*, *6*); we recently described a method for obtaining LS in sufficient amount for the characterization of the metal center (*5*).

Both BS and LS have been shown to contain an iron−sulfur center that is thought to participate in the activation of the C−H bond of the substrate (*3*−*6*). In previous reports, *aerobically* purified enzymes were studied, and shown to contain 2 Fe and 2 S per monomer in the form of a $(2Fe\text{-}2S)^{2+}$ cluster. Our studies show that in fact, under strict anaerobiosis, BS and most likely LS also accommodate one (4Fe-4S) cluster per polypeptide chain. This cluster is stable in either the 2+ or 1+ state.

^{57}Fe-reconstituted biotin synthase, when kept under reasonably anaerobic conditions, contains (4Fe-4S) clusters as shown by Mössbauer spectroscopy. While we obtained from both batches samples that contained most of the iron (∼80% and ∼90% for batch 1 and 2, respectively) incorporated into (4Fe-4S) cluster, the oxidation state of the clusters was either predominantly 2+ or 1+, suggesting that our procedures are not always as anaerobic as desired, but sufficiently anaerobic to prevent conversion to (2Fe-2S) clusters. The $(4Fe\text{-}4S)^{1+}$ cluster is easily oxidized by oxygen, and the presence of traces of air leads to the formation of larger proportions of the $(4Fe\text{-}4S)^{2+}$ cluster form. Counting both the 2+ and 1+ forms, our data thus show that reconstituted biotin synthase can accommodate as much as 0.9 (4Fe-4S) clusters per monomer.

Duin and co-workers have described *aerobically* purified biotin synthase preparations that contained about 2Fe/monomer (*4*). Using optical and resonance Raman spectroscopy, these workers showed that the enzyme thus isolated contains (2Fe-2S) clusters. This conclusion agrees with our results on air-exposed biotin synthase (samples B and C), which show that the (4Fe-4S) cluster of the anaerobically reconstituted protein is quantitatively degraded into a (2Fe-2S) center during exposure to air. Duin et al. obtained (4Fe-4S) clusters after addition of dithionite to their samples and concluded that their observation suggests a conversion of two $(2Fe\text{-}2S)^{2+}$ clusters (one in each monomer) to one $(4Fe\text{-}4S)^{2+}$ cluster per homodimer according to

$$2(2Fe\text{-}2S)^{2+} + 2e^- \rightarrow (4Fe\text{-}4S)^{2+}$$

and, moreover, they suggested that this conversion takes place at the interface of two subunits via dimerization of two $(2Fe\text{-}2S)^{2+}$ clusters. We have shown here that addition of a suitable reductant to the air-exposed samples leads to reformation of (4Fe-4S) clusters, in a process that utilized all iron available in the sample, i.e., that belonging to $(2Fe\text{-}2S)^{2+}$ clusters as well as the adventitiously bound iron contained in the broad magnetic components of Figure 5. This process, in our view most likely involves destruction of (2Fe-2S) clusters, followed de novo cluster reconstitution. Moreover, our observation of nearly one (4Fe-4S) cluster per monomer in reconstituted enzyme argues forcefully against the idea of a dimeric enzyme carrying one (4Fe-4S) cluster at the dimer interface. Instead, it suggests that during reduction of the two (2Fe-2S) clusters of the oxidized BS dimer, mobilization of iron and sulfide leads to the formation of a (4Fe-4S) cluster in half of the polypeptide chains. Since the enzyme assay provides the reductive conditions for such a cluster conversion, it is not surprising that oxidized BS, containing one (2Fe-2S) cluster per monomer, displays just half of the activity of the anaerobically Fe-reconstituted preparations containing one (4Fe-4S) per monomer.

Our preliminary studies of lipoate synthase show that this enzyme can also accommodate a (4Fe-4S) cluster. The reconstituted enzyme studied contained around 4.0 Fe/monomer, 50% of which belonged to a $(4Fe\text{-}4S)^{2+}$ cluster, 10−15% to a $(2Fe\text{-}2S)^{2+}$ cluster, and, perhaps, as much as 15% to a $(4Fe\text{-}4S)^{1+}$ clusters. The percentages quoted for $(4Fe\text{-}4S)^{2+}$ and $(2Fe\text{-}2S)^{2+}$ are lower limits. The (4Fe-4S) cluster of LS, as the BS cluster, is sensitive to oxygen and loses iron and sulfide to yield a (2Fe-2S) center upon exposure to air. If we make the plausible assumption that the (2Fe-2S) cluster observed in the reconstituted enzyme occupies a site that would contain a (4Fe-4S) cluster if the sample would have been prepared under strictly anaerobic conditions, we can conclude that at least 70−80% of the active sites of the monomers are occupied by a cluster. If we include a very tentative $(4Fe\text{-}4S)^{1+}$ fraction (all preparations studied so far exhibit the $g_{par} = 2.039$ and $g_{perp} = 1.93$ EPR signal, ∼0.1 spin/monomer), 85−95% of the monomers appear to contain an Fe−S cluster. This suggests that fully reconstituted lipoate synthase may accommodate one (4Fe-4S) cluster per monomer. In parallel work, Busby and co-workers have studied a preparation of LS containing ∼1.8 Fe and ∼2.2 sulfides/monomer (*6*). On the basis of visible absorption and resonance Raman spectra, these authors concluded that the enzyme contained one $(4Fe\text{-}4S)^{2+}$ cluster per dimer. In addition, these authors reported that a mono-

meric fraction of the protein carried a $(2Fe\text{-}2S)^{2+}$ cluster. On the basis of these observations, Busby et al. have concluded that LS may form a (4Fe-4S) cluster at its dimeric interface, referring to the biotin synthase precedent suggested by Duin et al. (*4*). As indicated above, our data on biotin synthase do not support the proposal of a subunit-bridging (4Fe-4S) cluster in biotin synthase, and our preliminary data on LS suggest that each monomer can support one (4Fe-4S) cluster.

The iron−sulfur centers of BS and LS are remarkably similar. They are mainly characterized by an extreme sensitivity to oxygen. In fact, in vitro, they can exist in three different forms: $(4Fe\text{-}4S)^{1+}$, $(4Fe\text{-}4S)^{2+}$, and $(2Fe\text{-}2S)^{2+}$, in relative amounts depending on the amount of air present in protein solutions. Under aerobic conditions, the stable $(2Fe\text{-}2S)^{2+}$ form accumulates due to the oxidative conversion of the (4Fe-4S) cluster into the (2Fe-2S) cluster. Such a reaction has also been observed in the case of the anaerobic RNR (*20*), the activating enzyme of the PFL (*27*), and the FNR transcription factor (*28*). The sensitivity of the clusters to oxygen is intriguing in the case of the aerobic enzymes BS and LS. Further studies are needed to investigate whether such redox-dependent cluster interconversions take place in vivo as well and whether they participate to a physiological function. In particular, it would be interesting to see whether the (4Fe-4S) to (2Fe-2S) conversion occurs during biotin synthesis, thus providing a mechanism that makes S atoms available for incorporation into biotin, in agreement with the recent proposal that the iron−sulfur cluster is the direct sulfur source (*10*).

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