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ARTICLE *in* BIOCHEMISTRY · AUGUST 2003

Impact Factor: 3.02 · DOI: 10.1021/bi034426c · Source: PubMed

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Fate of the $(2Fe\text{-}2S)^{2+}$ Cluster of *Escherichia coli* Biotin Synthase during Reaction: A Mössbauer Characterization†

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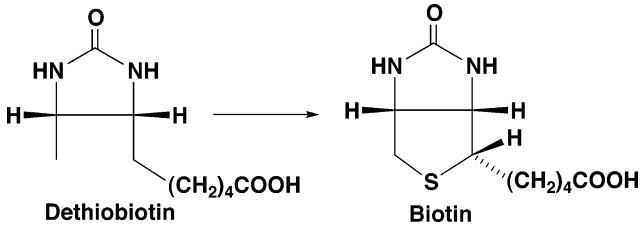
Received March 17, 2003; Revised Manuscript Received May 26, 2003

ABSTRACT: Biotin synthase, the enzyme which catalyzes the last step of the biosynthesis of biotin, contains only $(2Fe\text{-}2S)^{2+}$ clusters when isolated under aerobic conditions. Previous results showed that reduction by dithionite or photoreduced deazaflavin converts the $(2Fe\text{-}2S)^{2+}$ to $(4Fe\text{-}4S)^{2+,+}$. However, until now, no detailed investigation concerning the fate of the $(2Fe\text{-}2S)^{2+}$ during reduction under assay conditions (NADPH, flavodoxin, flavodoxin reductase) has been realized. Here, we show by Mössbauer spectroscopy on a partially purified fraction overexpressing the enzyme that, in the presence of a S^{2-} source and Fe^{2+}, there is conversion of the predominant $(2Fe\text{-}2S)^{2+}$ clusters into a 1:1 mixture of $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$. No change in this cluster composition was observed in the presence of the physiological reducing system. When the reaction was allowed to proceed by addition of the substrate dethiobiotin, the $(4Fe\text{-}4S)^{2+}$ was untouched whereas the $(2Fe\text{-}2S)^{2+}$ was degraded into a new species. This is consistent with the hypothesis that the reduced (4Fe-4S) cluster is involved in mediating the cleavage of AdoMet and that the $(2Fe\text{-}2S)^{2+}$ is the sulfur source for biotin.

Biotin synthase catalyzes the final step of the biotin biosynthetic pathway, namely, the insertion of a sulfur atom into dethiobiotin (DTB)[1] (Scheme 1) (*1*, *2*). It is a homodimeric Fe-S protein (*3*) which belongs to the family of "radical SAM" enzymes (*4*). In vitro activity of the enzyme requires the presence of *S*-adenosylmethionine (SAM or AdoMet) (*5*, *6*) and a reducing system [NADPH, flavodoxin (*7*), flavodoxin reductase (*8*)] whose role is to reduce AdoMet, via the reduced form of the Fe-S center of the enzyme into methionine and a deoxyadenosyl radical ($DOA^{\bullet}$) (*9*, *10*).

The mechanism we have proposed is depicted in Scheme 2 (*11*). The deoxyadenosyl radical abstracts a $H^{\bullet}$ at C-9 of dethiobiotin, generating $DTB^{\bullet}$. We have observed a direct transfer from the deuterated substrate DTB into deoxyadenosine (*12*), which indicates a similarity between biotin synthase and lysine 2,3-aminomutase (*13*) (the fact that this transfer was not quantitative is still under investigation in our group). Then the substrate radical $DTB^{\bullet}$ has to quench a sulfur atom to give an intermediate whose structure is not yet defined. A second $DOA^{\bullet}$ is generated from another AdoMet producing a radical at C-6 of this intermediate, ending in the ring closure. In a recent paper, Ollagnier de Choudens et al. (*14*) reported that, in their system, only 1 mol of AdoMet was needed to produce 1 mol of biotin. This is inconsistent with our conclusion which is based not only on the determination of the amount of methionine and deoxyadenosine produced but also on the deuterium transfer experiments mentioned above which reveal a transfer of deuterium from C-6 of DTB into $DOA^{\bullet}$ (*12*). Their findings are neither in accord with the picture very recently proposed by Ugulava et al., suggesting the binding of 2 AdoMet and 1 DTB per dimer (*15*). The origin of these discrepancies remains to be found.

Scheme 1: Reaction Catalyzed by Biotin Synthase



We postulated that the sulfur source for biotin was the Fe-S center of biotin synthase since [^{34}S]biotin was produced from $(2Fe\text{-}2[^{34}S])^{2+}$ biotin synthase obtained by reconstitution in vitro (*16*). The as-isolated enzyme was transformed into its apo form by treatment with sodium dithionite and EDTA and then reconstituted with $FeCl_3$ and $Na_2[^{34}S]$ in the

† This research has been supported by the French-German Cooperation Program, Procope (00373PJ), and by the Ministère de l'Education Nationale de la Recherche et de la Technologie (98C0326).

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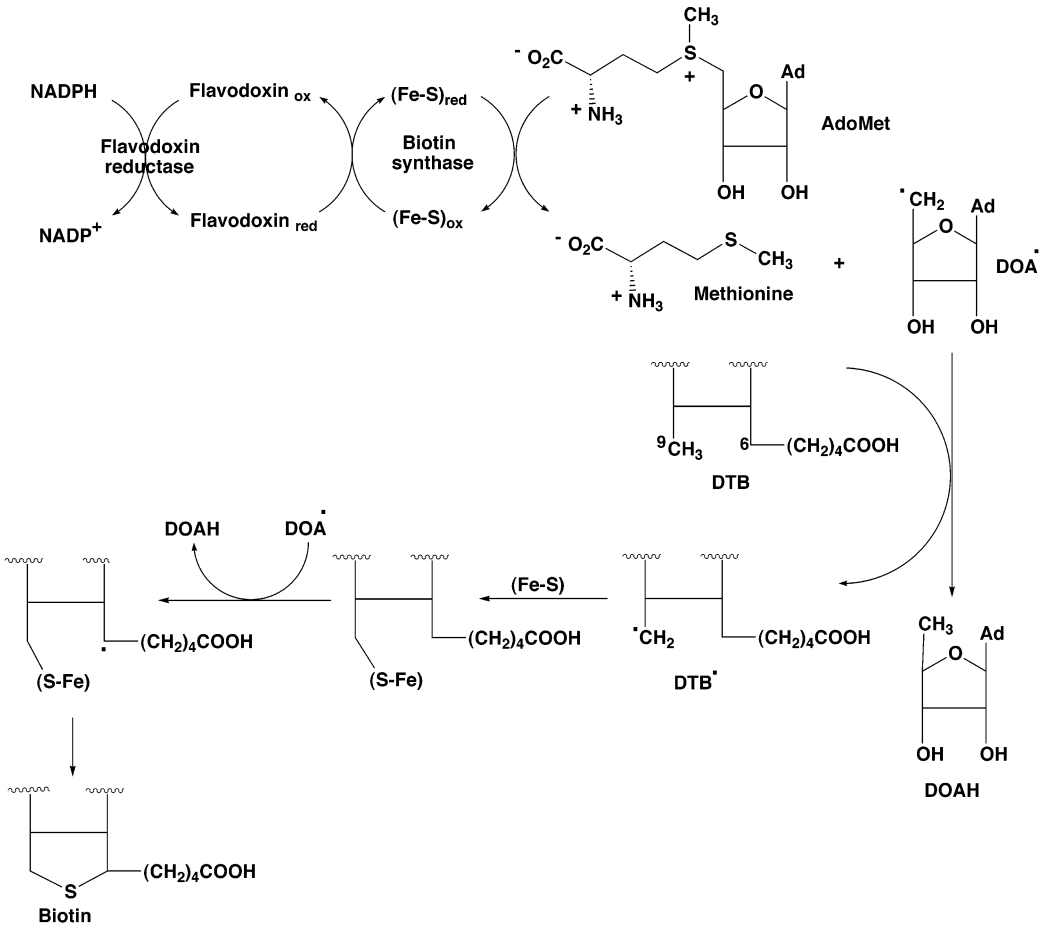
[1] Abbreviations: AdoMet, *S*-adenosylmethionine; $DOA^{\bullet}$, 5′-deoxyadenosyl radical; DTB, dethiobiotin; DTT, dithiothreitol; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; (Fe-S), iron−sulfur cluster; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; SAM, *S*-adenosylmethionine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

10.1021/bi034426c CCC: $25.00
Published on Web 07/04/2003

Scheme 2: Mechanism Postulated for Biotin Synthase



presence of 5 mM dithiothreitol (DTT). We concluded that we were labeling the Fe-S center since, under such reducing conditions, we could not generate a persulfide, a possible intermediate which we considered in our first relevant publication (*17*).

It had just been shown at that time by Duin et al. that dithionite reduction of $(2Fe\text{-}2S)^{2+}$ biotin synthase promoted its conversion into a $(4Fe\text{-}4S)^{2+,+}$ form (*18*). However, nothing was known about the transformation occurring with the physiological reducing system, and we could not discuss the precise nature of the Fe-S cluster used as the sulfur source. We did not claim that it was a $(4Fe\text{-}4S)^{2+}$ species, as mentioned by Fontecave and co-workers (*19*), nor a $(2Fe\text{-}2S)^{2+}$ one, as reported by Johnson and co-workers (*20*). Since that time, several papers concerning the nature and amount of clusters that biotin synthase could accommodate, in the nonreduced and reduced states, have been published (*21*−*24*), but the situation is still confusing. We shall analyze these data in the discussion before examining our own results.

Our new experimental results, obtained by Mössbauer spectroscopy, address two questions: (a) What is the fate of the $(2Fe\text{-}2S)^{2+}$ cluster present in the as-isolated enzyme in the assay conditions with the physiological reducing system? (b) Since we attributed the lack of turnover of the enzyme to the fact that a cluster is destroyed and not regenerated, is it possible to observe modifications of the cluster after reaction?

MATERIALS AND METHODS

Materials. All chemicals were purchased from either Sigma-Aldrich Chemical Co., Merck Eurolab, or Acros Organics unless otherwise stated.

Escherichia coli TK101pI$_3$BLS$_2$ overexpressing biotin synthase (*25*) was a generous gift from Prof. Y. Izumi (Tottori University, Japan). Obtention of cells and cell-free extracts enriched with ^{57}Fe has been described earlier (*26*).

Ammonium Sulfate Fractionation. The cell-free extract of *E. coli* TK101pI$_3$BLS$_2$ was treated with 1% (w/v) streptomycin sulfate added dropwise with stirring, and the mixture was held for 15 min in an ice bath. After centrifugation for 10 min at 35000*g*, the collected supernatant was fractionated with ammonium sulfate. Proteins precipitating between 25% and 45% saturation were suspended in 50 mM Tris-HCl, pH 7.5, and dialyzed thoroughly overnight through a dialysis membrane of molecular mass cutoff 6−8 kDa (Spectra/Por 1, Fisher Bioblock Scientific) against the same buffer at 4 °C. This enzyme fraction, termed F(25−45), was stored as aliquots of 1 mL at −80 °C.

Analytical Methods. Protein concentration was measured by the method of Bradford (*27*) using bovine serum albumin as a standard. Iron was assayed by the method of Fish (*28*). Gel densitometric analysis of Coomassie-stained gels was performed on a Syngene ChemiGenius gel analyzer (Syngene, Cambridge, U.K.). To determine expression levels, the relative band intensities corresponding to biotin synthase in

varying amounts of F(25−45) (10−26 μg) were compared with those of the purified enzyme (5−17 μg).

Anaerobic Test with Resazurin. This dye is known to be an oxidation−reduction indicator. One milliliter of 5% (w/v) sodium carbonate, 5 mg of cysteine, and 10 μL of 0.2% (w/v) resazurin were mixed together in a final volume of 10 mL of water. This solution was then left to stand inside the glovebox where it turned colorless. When just prepared, the dye solution was in its oxidized state and was blue. With time and due to the presence of cysteine, it was converted to the semireduced state, which was pink. In the absence of oxygen, the dye was completely reduced and turned colorless.

Enzymatic Assays. The standard reaction mixture (*25*) contained the appropriate amount of F(25−45), 0.1 mM DTB, 0.5 mM AdoMet, 2 mM NADPH, 5 mM DTT, 1 mM L-cysteine, and 2 mM FAD in 40 mM Tris-HCl buffer, pH 8, in a final volume of 100 μL. The reaction was done at 37 °C for 4 h.

The enzymatic assays for the Mössbauer analysis were performed inside a glovebox (Jacomex BS531 NMT) equipped with an oxymeter (ARELCO ARC), filled with an argon atmosphere containing less than 2 ppm O_2. Tris-HCl buffer (1 M), pH 8.0, the enzyme fraction F(25−45) at a concentration of 47 mg/mL, and a mixture containing 2.5 mM DTB, 500 mM DTT, 200 mM NADPH, 10 mM AdoMet, 200 mM FAD, and 100 mM L-Cys were placed, as aliquots not exceeding 200 μL, in separate 1.5 mL Eppendorf tubes and degassed under a continuous stream of moist argon for 30 min at ambient temperature before being introduced inside the glovebox. The Eppendorf tubes were opened and then left to stand overnight at 15 °C.

Before the assay was begun, the anaerobicity of the reagents was verified using the resazurin test. Generally, equal volumes (50 μL of reduced resazurin and 50 μL of Tris-HCl buffer that was left to stand overnight along with the other reagents) were added together. If the mixture stayed colorless, this indicated the absence of oxygen. As the cofactor mixture and the enzyme fraction were colored, their anaerobicity could not be checked by the resazurin test, but as they had smaller or equal volumes as the buffer solution which was left for the same time and under the same conditions, it was then assumed that they were also oxygen free.

The Tris-HCl buffer and the enzyme were added to the mixture so that the final concentration of the reagents in the assay (final volume of 700 μL) was 0.1 mM DTB, 0.5 mM AdoMet, 2 mM NADPH, 5 mM DTT, 1 mM L-cysteine, 2 mM FAD, 39 mg/mL F(25−45), and 40 mM Tris-HCl buffer, pH 8. This mixture was then incubated at 37 °C for 4 h. At the end of the reaction, 550 μL was transferred to a Mössbauer cup holder and 130 μL to an EPR tube, and 20 μL was kept for biotin quantification. For the control experiments, the same amount and volume of reagents were used except that the omitted reagents were replaced by water. Nonreacted F(25−45) from the same batch was also transferred to a Mössbauer cup holder and a quartz EPR tube for measurement.

As our glovebox was not equipped to enable freezing of the samples inside the anaerobic chamber, they were transferred outside and immediately frozen and stored in liquid nitrogen. The Mössbauer cups used have been previously checked for their airtightness: cups that were held in the anaerobic chamber for several weeks in advance were filled with anaerobic water, closed, and transferred outside. They were left to stand in air for 30 min, frozen in liquid nitrogen, left to thaw in air for 45 min, refrozen in liquid nitrogen, and introduced back to the glovebox. The water was then tested with the resazurin solution, and as it stayed colorless, this indicated that no oxygen had penetrated inside the cups. To protect the EPR samples from oxygen, the tubes were capped with airtight rubber septums and then wrapped with several layers of Parafilm to enforce their airtightness inside the glovebox.

Formed biotin was assayed microbiologically by the paper disk plate method using *Lactobacillus plantarum* (*29*).

Mössbauer Spectroscopy. Mössbauer spectra were recorded using a spectrometer in the constant acceleration mode. Isomer shifts are given relative to α-Fe at room temperature. The spectra obtained at 20 mT were measured in a He-bath cryostat (Oxford MD 306) equipped with a pair of permanent magnets. High-field measurements (7 T) were performed with a cryostat equipped with a superconducting magnet (Oxford Instruments, Spectromag 4000). Magnetically split spectra were simulated within the spin Hamiltonian formalism (*30*); otherwise, spectra were analyzed by least-squares fits using Lorentzian line shape.

RESULTS

All of the results gathered so far in the literature (see Discussion) concerning the nature and amount of Fe-S centers that biotin synthase can accommodate were principally obtained by treatment of the enzyme with dithionite and/or a large excess of $Fe^{2+/3+}$, S^{2-}, i.e., under conditions which were very far from the physiological ones. In this paper, we examined the fate of the cluster when the $(2Fe\text{-}2S)^{2+}$ enzyme was reduced with NADPH, flavodoxin, and flavodoxin reductase.

To analyze the cluster(s) nature and content, all the reactions and manipulations must be performed under strict anaerobicity, within the glovebox, since the $(4Fe\text{-}4S)^{2+,+}$ center, sensitive to oxygen, can be transformed into $(2Fe\text{-}2S)^{2+}$. We encountered, however, a problem since purified biotin synthase, either the as-isolated $(2Fe\text{-}2S)^{2+}$ form or the enzyme reconstituted anaerobically, containing a mixture of $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$ (*31*) yielded a very weak activity when the assay was performed in the glovebox. With the same sample, the usual activity was observed when the reaction was performed outside, in a septum-closed vial, after degassing with argon (*32*). These observations were very reproducible for any batch of enzyme tested.

The situation was different with a partially purified fraction which we termed F(25−45). This corresponds to a crude extract precipitated between 25% and 45% of ammonium sulfate, followed by aerobic dialysis overnight and then by another overnight stay in the glovebox. With this preparation, the activity in the glovebox was slightly higher than outside (at the protein concentration used in this study, i.e., 39 mg/mL, respectively 68 ± 0.7 μM and 42 ± 1 μM of biotin produced). This confirms what was already very clear from the litterature, that the in vitro system is not yet completely mastered. Therefore, we chose to work with F(25−45) which is active in the glovebox. This fraction contains enough flavodoxin and flavodoxin reductase, since addition of these

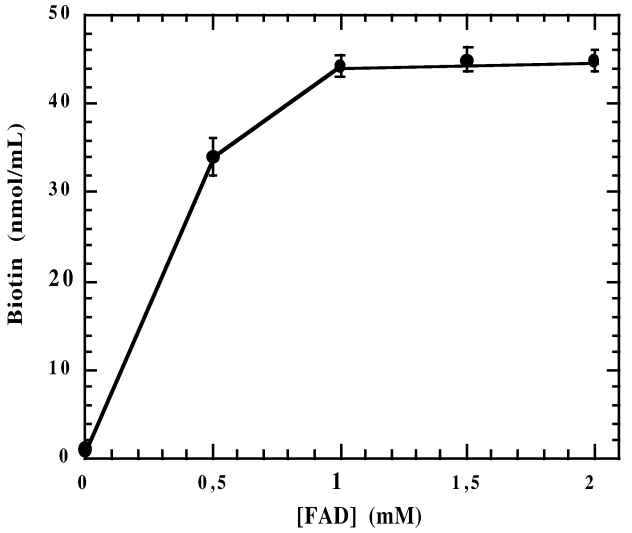


FIGURE 1: Effect of FAD on the activity of F(25−45). The reaction mixture contained 40 mg/mL protein, 0.1 mM DTB, 0.5 mM AdoMet, 2 mM NADPH, 5 mM DTT, 1 mM L-cysteine, and varying amounts of FAD in 40 mM Tris-HCl buffer, pH 8. The reaction mixture was incubated for 4 h at 37 °C. The error bars represent standard deviations and when not shown fall within the symbol.

two proteins had no effect on the activity (results not shown). One has, however, to point out the positive effect of FAD with F(25−45) (Figure 1), also observed with a crude extract (*25*), whereas it does not influence the activity of the pure enzyme (results not shown). FAD might be necessary to saturate the flavoproteins present in F(25−45).

The pure enzyme requires the addition of Fe^{2+} for activity; we verified that addition of Fe^{2+} has no effect on the activity of F(25−45) (data not shown). Indeed, the Mössbauer analysis of this fraction (issued from bacteria grown in the presence of $^{57}FeCl_3$) measured at $T = 77$ K and $T = 4.2$ K (Figure 2) revealed that it contains 41% of the total iron as high-spin Fe^{2+} ($\Delta E_Q = 3.10$ mm/s and $\delta = 1.26$ mm/s) besides 46% as $(2Fe\text{-}2S)^{2+}$ ($\Delta E_Q = 0.51$ mm/s and $\delta = 0.29$ mm/s), 7% as $(4Fe\text{-}4S)^{2+}$ ($\Delta E_Q = 1.11$ mm/s and $\delta = 0.45$ mm/s), and 6% as high-spin Fe^{3+} ($\Delta E_Q = 0.75$ mm/s, $\delta = 0.43$ mm/s, $\Gamma = 0.35$ mm/s). As no EPR signal is detectable,[2] the latter species are believed to represent small superparamagnetic iron clusters. The parameters of the four doublets are similar to those found in whole cells overexpressing biotin synthase (*26*).

As already mentioned, one of our aims was to analyze the state of the cluster(s) by Mössbauer spectroscopy after reaction. The various steps of reaction and the resulting iron composition in F(25−45) are summarized in Table 1. Entry 1 corresponds to untreated F(25−45) (Figure 2). Entry 2 reveals that, by addition of DTT, L-cysteine, and FAD and incubation at 37 °C in the glovebox, $(4Fe\text{-}4S)^{2+}$ clusters are reconstituted, at the expense of "free" Fe^{2+} species and also at the expense of $(2Fe\text{-}2S)^{2+}$ clusters. This result was obtained in the absence of the reductant NADPH; obviously the reconstitution of $(4Fe\text{-}4S)^{2+}$ clusters requires only a source of S^{2-}, probably provided by cysteine desulfurases present in F(25−45). Practically the same result was obtained

[2] $T = 10$ K, microwave power 80 μW, microwave frequency 9.65 GHz, and modulation amplitude 0.5 mT.

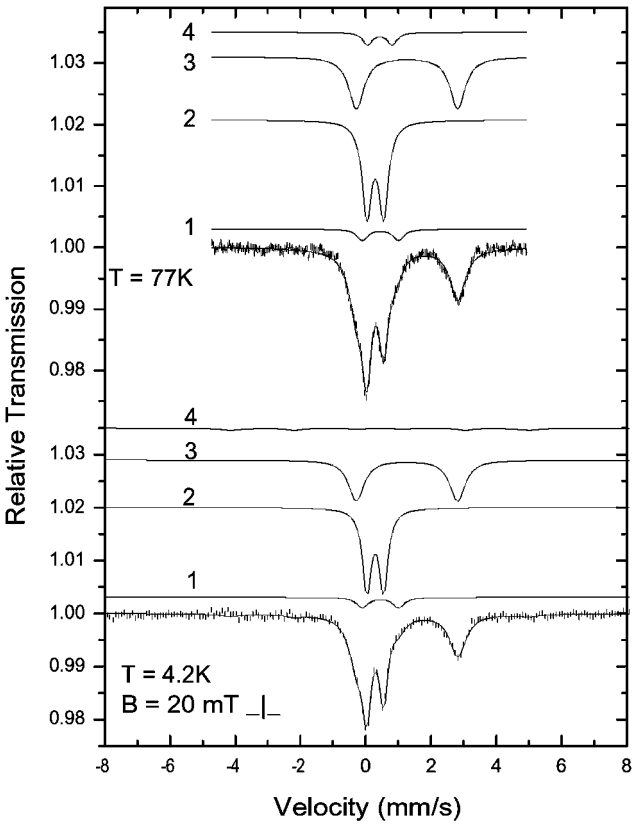


FIGURE 2: Mössbauer spectra of F(25−45). Doublet 1 represents $(4Fe\text{-}4S)^{2+}$ clusters ($\Delta E_Q = 1.11$ mm/s, $\delta = 0.45$ mm/s, $\Gamma = 0.40$ mm/s, relative area = 7%) and doublet 2 $(2Fe\text{-}2S)^{2+}$ clusters ($\Delta E_Q = 0.51$ mm/s, $\delta = 0.29$ mm/s, $\Gamma = 0.34$ mm/s, relative area = 46%). Doublet 3 corresponds to high-spin Fe^{2+} species ($\Delta E_Q = 3.10$ mm/s, $\delta = 1.26$ mm/s, $\Gamma = 0.57$ mm/s, relative area = 41%) with iron not being coordinated to sulfur. Line 4 represents high-spin Fe^{3+} species ($\Delta E_Q = 0.75$ mm/s, $\delta = 0.43$ mm/s, $\Gamma = 0.35$ mm/s, relative area = 6%) which, as no EPR signal is detectable,[2] are believed to represent small superparamagnetic iron clusters.

Table 1: Percentage of the Total Iron Content within the Different Species in F(25−45)[a]

entry	preparation	$(2Fe\text{-}2S)^{2+}$	$(4Fe\text{-}4S)^{2+}$	Fe^{2+}	Fe^{3+}
1[b]	F(25−45)	46 (23)	7 (2)	41	6
		51 (25.5)	10 (2.5)	30	9
2	+DTT + Cys + FAD	28 (14)	62 (15.5)	4	6
3	+DTT + Cys + FAD + DTB	31 (15.5)	57 (14)	4	8
4	+DTT + Cys + FAD + NADPH	24 (12)	62 (15.5)	7	6
5	+DTT + Cys + FAD + AdoMet + NADPH	31 (15.5)	55 (14)	7	7
6	+DTT + Cys + FAD + DTB + NADPH	29 (14.5)	56 (16)	5	10
7[b]	+DTT + Cys + FAD + AdoMet + DTB + NADPH (complete assay)	15 (7.5)	56 (14)	8	21
		18 (9)	54 (13.5)	7	21

[a] Error on the areas is about ±3%. The values in parentheses represent the relative amounts of Fe-S clusters. [b] Results from two different batches. Entries 2−6 correspond to a single experiment.

in the presence of the substrate DTB (entry 3). Addition of NADPH, that is when the complete reducing system is present (entries 4−6), does not influence the composition of the Fe-S centers significantly. This shows for the first time without ambiguity that under the conditions of the assay, in the presence of the physiological reducing system, the

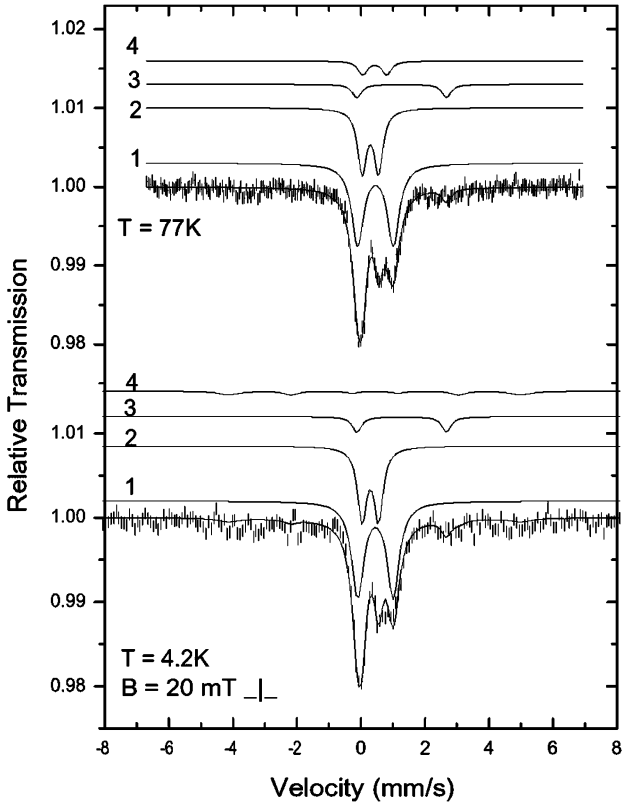


FIGURE 3: Mössbauer spectra of F(25−45) after reaction with the complete assay without DTB (entry 5 in Table 1). Doublet 1 represents $(4Fe\text{-}4S)^{2+}$ clusters ($\Delta E_Q = 1.11$ mm/s, $\delta = 0.45$ mm/s, $\Gamma = 0.45$ mm/s, relative area = 55%), doublet 2 $(2Fe\text{-}2S)^{2+}$ clusters ($\Delta E_Q = 0.51$ mm/s, $\delta = 0.29$ mm/s, $\Gamma = 0.33$ mm/s, relative area = 31%), and doublet 3 high-spin Fe^{2+} species ($\Delta E_Q = 2.80$ mm/s, $\delta = 1.27$ mm/s, $\Gamma = 0.35$ mm/s, relative area = 7%). Line 4 represents high-spin Fe^{3+} species ($\Delta E_Q = 0.75$ mm/s, $\delta = 0.43$ mm/s, $\Gamma = 0.35$ mm/s, relative area = 7%) which, as no EPR signal is detectable,[2] are believed to represent small superparamagnetic iron clusters.

enzyme is in the mixed-cluster state [containing both $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$]. Reduction of the $(4Fe\text{-}4S)^{2+}$ clusters to $(4Fe\text{-}4S)^{1+}$ was never observed. As all Mössbauer spectra recorded for the blank experiments (entries 2−6) were similar, we show only one representative spectrum, i.e., the one corresponding to entry 5 (Figure 3).

After reaction in the complete assay (entry 7), the amount of $(4Fe\text{-}4S)^{2+}$ clusters has remained compared with the blank experiments, whereas the amount of $(2Fe\text{-}2S)^{2+}$ clusters has decreased significantly and is accompanied by an increase in the amount of Fe^{3+} species. Their corresponding spectra at 77 and 4.2 K are shown in Figure 4. Doublet 1 represents $(4Fe\text{-}4S)^{2+}$ clusters, doublet 2 $(2Fe\text{-}2S)^{2+}$ clusters, and doublet 3 high-spin Fe^{2+}. The low-field Mössbauer data would be consistent also with the presence of reduced $(3Fe\text{-}4S)^0$ clusters ($S = 2$). However, the spectrum obtained at 4.2 K and 7 T displayed in Figure 4c rules out the presence of such species within the detection limit of Mössbauer spectroscopy (∼3%). Instead of a magnetic hyperfine splitting characteristic of reduced $(3Fe\text{-}4S)^0$ clusters, components 1 and 2 show a magnetic hyperfine splitting only due to the high external field which proves the assignment to $(4Fe\text{-}4S)^{2+}$ and $(2Fe\text{-}2S)^{2+}$ clusters. Doublet 4 at 77 K has parameters representative for high-spin Fe^{3+} species. Under applied field conditions, the Fe^{3+} species exhibits magnetic

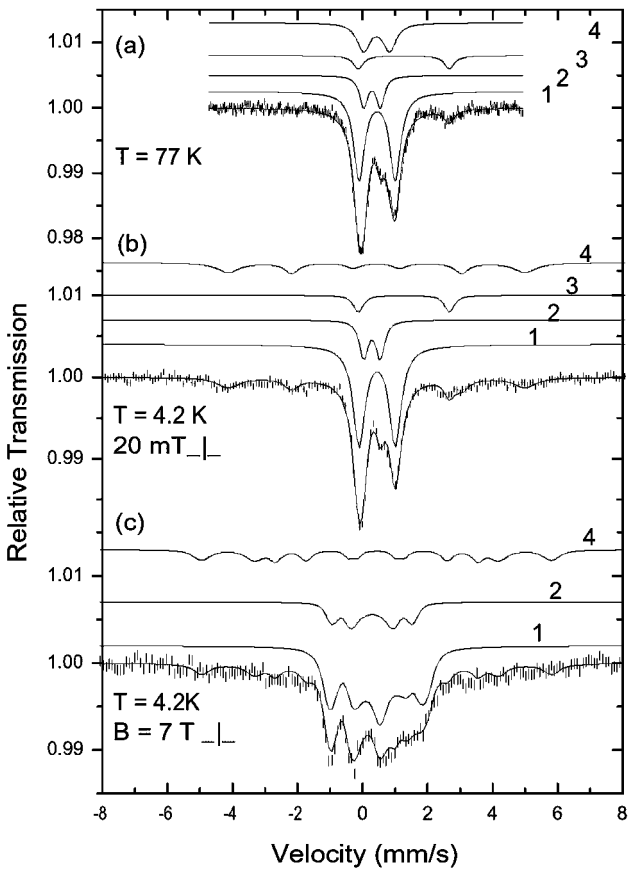


FIGURE 4: Mössbauer spectra of F(25−45) after reaction with the complete assay (entry 7 in Table 1). Doublet 1 represents $(4Fe\text{-}4S)^{2+}$ clusters ($\Delta E_Q = 1.11$ mm/s, $\delta = 0.45$ mm/s, $\Gamma = 0.40$ mm/s, relative area = 56%), doublet 2 represents $(2Fe\text{-}2S)^{2+}$ clusters ($\Delta E_Q = 0.51$ mm/s, $\delta = 0.29$ mm/s, $\Gamma = 0.30$ mm/s, relative area = 15%), and doublet 3 belongs to high-spin Fe^{2+} species ($\Delta E_Q = 2.80$ mm/s, $\delta = 1.27$ mm/s, $\Gamma = 0.37$ mm/s, relative area = 8%). Line 4 represents high-spin Fe^{3+} species ($\Delta E_Q = 0.80$ mm/s, $\delta = 0.43$ mm/s, $\Gamma = 0.48$ mm/s, relative area = 21%). Under strong applied field (7 T perpendicular to the γ beam), the magnetic pattern of lines 1 and 2 exhibits diamagnetic behavior of $(4Fe\text{-}4S)^{2+}$ and $(2Fe\text{-}2S)^{2+}$ clusters. The magnetic hyperfine interaction of the Fe^{3+} species was simulated assuming antiferromagnetically coupled iron clusters (*33*), while that of the Fe^{2+} species was not simulated because of unknown hyperfine coupling constants.

hyperfine splitting (six-line pattern in Figure 4b,c). This observation, together with the fact that no EPR signal was detectable,[2] indicates that the Fe^{3+} species represent small superparamagnetic iron clusters.

DISCUSSION

Before starting the discussion, it is important to point out that the only technique capable of analyzing thoroughly the different iron species is Mössbauer spectroscopy complemented with EPR and that all of the results based only on chemical analysis and UV−vis spectroscopy are certainly less reliable from a quantitative point of view. Before coming to the discussion of our results, a brief summary of what has been published so far on the nature and amount of Fe-S clusters in biotin synthase seems necessary.

In Whole Cells. The in vivo situation has been recently examined. We have analyzed by Mössbauer spectroscopy aerobically grown cells of *E. coli* TK101 overexpressing biotin synthase (grown on a ^{57}Fe-enriched medium) with the

wild-type strain as reference, and we concluded that biotin synthase contains both types of clusters, $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$ in a 3 to 1 ratio (*26*). We cannot exclude that some $(4Fe\text{-}4S)^{2+}$ has been degraded into $(2Fe\text{-}2S)^{2+}$ by oxygen but the $(4Fe\text{-}4S)^{2+}$ is clearly present. As expected, we observed a decrease of the $(4Fe\text{-}4S)^{2+}/(2Fe\text{-}2S)^{2+}$ ratio during purification under semianaerobic conditions using buffers saturated with argon. Until now, no overexpression of biotin synthase has been observed in our attempts to grow the cells anaerobically.

A similar study was simultaneously been carried out by Cosper et al. with *E. coli* C41[DE3] pT7-7. Their conclusion was that biotin synthase contains uniquely $(2Fe\text{-}2S)^{2+}$ centers (*20*). The absence of $(4Fe\text{-}4S)^{2+}$ may be due to a difference of strain and/or overexpression of the protein or of culture conditions. One has to point out that the bacteria were grown under aerobic conditions with strong agitation. The degree of oxygenation of the culture may account for the difference, either because of the oxygen sensitivity of the $(4Fe\text{-}4S)^{2+}$ cluster or for more complex metabolic reasons.

In As-Isolated or Reconstituted Biotin Synthase. On the other hand, there is a general agreement that the pure enzyme isolated under aerobic or semianaerobic conditions contains uniquely the $(2Fe\text{-}2S)^{2+}$ form. Although it is generally postulated that biotin synthase contains one $(2Fe\text{-}2S)^{2+}$ per monomer, the reported experimental values are quite variable from ≈0.2 (*34*−*36*), 0.5 (*19*), 1 (*3*) to 1.2−1.5 (*23*). Our preparations consistently contain 0.75−0.8 cluster per monomer (*16*, *26*, *32*). The deficiency can be due to the use of different strains and cultivation media as well as different culture conditions and induction procedures, resulting for instance in improper folding of the apoprotein leading to vacant sites.

The situation is quite different with the enzyme reconstituted under strict anaerobic conditions in a glovebox (<2 ppm O_2). Ollagnier de Choudens et al. (*21*) have analyzed biotin synthase samples (chemical analysis, Mössbauer, EPR) obtained by reconstitution of the apoenzyme with a 6-fold molar excess of Fe^{2+} or Fe^{3+} and S^{2-} in the presence of DTT. The samples contain 80−90% of the Fe as (4Fe-4S) clusters, reaching one cluster per monomer, with variable amounts of $(4Fe\text{-}4S)^{2+}$ and $(4Fe\text{-}4S)^{+}$ together with some unindentified Fe^{2+} species. Other preparations described in later papers apparently contain also some $(2Fe\text{-}2S)^{2+}$ and a smaller amount of $(4Fe\text{-}4S)^{2+}$, and the $(4Fe\text{-}4S)^{+}$ was barely detectable (*19*, *37*). One can indeed wonder where the "reducing power" is coming from when the $(4Fe\text{-}4S)^{+}$ species is observed, since no strong reductant was added in the experiment.

We have performed the same kind of experiments, starting with the as-isolated enzyme. We never detected the paramagnetic $(4Fe\text{-}4S)^{+}$, and the ratio $(4Fe\text{-}4S)^{2+}$:$(2Fe\text{-}2S)^{2+}$ was always about 1:1 (*31*). Using Mössbauer spectroscopy, Ugulava et al. (*24*), starting from the as-isolated enzyme, found that one $(4Fe\text{-}4S)^{2+}$ could be reconstituted besides the native $(2Fe\text{-}2S)^{2+}$, a result which is quite consistent with ours.

The situation becomes much more complex when biotin synthase is treated with reducing agents.

After Reduction without Addition of Fe^{3+} and S^{2-}. The first detailed investigation of the fate of the $(2Fe\text{-}2S)^{2+}$ cluster during dithionite reduction was carried out by Duin et al. (*18*), using a combination of UV−vis, EPR, resonance Raman, and variable-temperature magnetic circular dichroism spectroscopies. They reported a near-stoichiometric transformation of two $(2Fe\text{-}2S)^{2+}$ clusters into one $(4Fe\text{-}4S)^{2+}$ in a medium containing glycerol and suggested a dimerization of the $(2Fe\text{-}2S)^{2+}$ species at the interface between the two monomers. The $(4Fe\text{-}4S)^{+}$ (0.4 spin/monomer) was also observed in samples reduced with dithionite followed by glycerol treatment.

We confirmed this $(2Fe\text{-}2S)^{2+}$ to $(4Fe\text{-}4S)^{2+}$ transformation by addition of dithionite (2 equiv) in a glycerol-containing medium using Mössbauer spectroscopy (*38*). We could observe the liberation of Fe^{2+} (≈ 25%) which was not reincorporated into clusters (note that this experiment was done in a glovebox but without oxygen control). This experiment also revealed that, by air reoxidation, the $(4Fe\text{-}4S)^{2+}$ cluster was quantitatively transformed into $(2Fe\text{-}2S)^{2+}$ without further liberation of iron. Ugulava et al. (*22*) brought arguments favoring a dissociation−reassociation of iron during this dithionite-promoted $(2Fe\text{-}2S)^{2+}$ to $(4Fe\text{-}4S)^{2+}$ transformation although their experimental data were rather rough. Indeed, the $(2Fe\text{-}2S)^{2+}$ center is destroyed by dithionite since we used this procedure in the presence of EDTA to prepare the apoenzyme whereas EDTA alone has no effect on the cluster. We did not see the liberation−reincorporation process but maybe after 20 min the transformation was already completed.

After Reduction in the Presence of Added Fe^{3+} and S^{2-}. This reconstitution was examined by Ollagnier de Choudens et al. (*21*), who reduced biotin synthase containing the $(2Fe\text{-}2S)^{2+}$ cluster together with unindentified adventitous Fe, which they termed Y species in a 40:60 ratio (areas) by dithionite or photoreduced deazaflavin. They obtained about 80% of $(4Fe\text{-}4S)^{2+,+}$. The Y species was recruited to build the cluster.

Ugulava et al. (*22*) reduced the native $(2Fe\text{-}2S)^{2+}$ enzyme with an excess of dithionite, resulting in the formation of $(4Fe\text{-}4S)^{2+}$ or $(4Fe\text{-}4S)^{+}$ depending on whether the medium contains ethylene glycol. They report that one or two $(4Fe\text{-}4S)^{2+,+}$ per monomer were obtained, depending on the amount of Fe^{3+} and S^{2-} added (*23*).

After Reduction under Physiological Conditions (This Study). One of our goals in this work was to investigate the fate of the Fe-S centers of biotin synthase during reaction. As discussed above, biotin synthase can accommodate both $(4Fe\text{-}4S)^{2+}$ and $(2Fe\text{-}2S)^{2+}$ centers which interconvert in a highly oxygen-sensitive manner. Thus all of the experiments had to be performed under strict anaerobiosis in a glovebox (≤2 ppm oxygen). For this study, we did not use the purified enzyme as its activity inside the glovebox was very low as compared to that obtained outside, i.e., in a septum-closed vial after degassing all the solutions with argon (*32*). We checked using the resazurin test that, under the latter conditions, oxygen was still present. Instead, we employed a partially purified preparation, F(25−45), obtained by precipitation with ammonium sulfate between 25% and 45% of a crude extract overexpressing biotin synthase. This fraction has a slightly higher activity inside the glovebox than outside. The difference in behavior, inside and outside the glovebox, of the two enzyme preparations is under investigation in the laboratory.

The F(25−45) preparation was obtained by overnight dialysis (in air), followed by an overnight stay in the glovebox to remove all traces of oxygen. This fraction apparently contains enough flavodoxin and flavodoxin reductase as well as cysteine desulfurases. Densitometric analysis of an electrophoresis gel stained with Coomassie Brilliant Blue R-250 of F(25−45) revealed that biotin synthase represented about 32% of the total proteins. The total amount of iron determined by chemical analysis for four independent measurements was 3.1 ± 0.04 per monomer. According to the Mössbauer analysis, 46% of the total iron is present as $(2Fe\text{-}2S)^{2+}$, 7% as $(4Fe\text{-}4S)^{2+}$, 41% as Fe^{2+}, and 6% as Fe^{3+} (Figure 2 and entry 1 of Table 1). This corresponds to 0.7 $(2Fe\text{-}2S)^{2+}$ and 0.05 $(4Fe\text{-}4S)^{2+}$ per monomer; the former value is what we consistently obtain in all of our purified preparations. The predominance of $(2Fe\text{-}2S)^{2+}$ is expected since it is well established that, after being allowed to stand in air (during purification), only the $(2Fe\text{-}2S)^{2+}$ center remains. The Mössbauer parameters of the Fe^{2+} and Fe^{3+} species are similar to those found in whole cells (*26*).

When F(25−45) was incubated in the sole presence of DTT, L-Cys, and FAD (Table 1, entry 2), the amount of $(4Fe\text{-}4S)^{2+}$ increased and that of $(2Fe\text{-}2S)^{2+}$ decreased, bringing the $(2Fe\text{-}2S)^{2+}$:$(4Fe\text{-}4S)^{2+}$ ratio to about 0.9:1 with ≈0.4 $(2Fe\text{-}2S)^{2+}$ and ≈0.5 $(4Fe\text{-}4S)^{2+}$ per monomer, i.e., one $(2Fe\text{-}2S)^{2+}$ and one $(4Fe\text{-}4S)^{2+}$ per enzyme dimer. A similar ratio, 1:1.2, was obtained when the purified $(2Fe\text{-}2S)^{2+}$ enzyme was incubated under anaerobiosis with a 4-fold excess of $FeCl_3$ and Na_2S, resulting in ≈0.5 $(2Fe\text{-}2S)^{2+}$ and ≈0.6 $(4Fe\text{-}4S)^{2+}$ per monomer (*31*). A ratio of 0.8:1 for their reconstituted enzyme was reported by Ugulava et al., however with a higher amount of cluster per monomer [0.9 $(2Fe\text{-}2S)^{2+}$ and 0.8 $(4Fe\text{-}4S)^{2+}$ as calculated from their Mössbauer data and chemical Fe analysis]. Using differential labeling ($^{56}Fe/^{57}Fe$), they concluded that the two clusters were assembled at different sites, a statement which can be consistent with mutagenesis data (*24*).

We have followed this hypothesis to interpret our data, since the presence of the two clusters is better rationalized if they occupy two distinct sites. By comparing entries 1 and 2 of Table 1, we observe that the iron gained by the $(4Fe\text{-}4S)^{2+}$ species (55%) corresponds to that loss by the Fe^{2+} pool (37%) and the $(2Fe\text{-}2S)^{2+}$ cluster (18%). If we assume that, in the absence of reducing agent, no dissociation of the $(2Fe\text{-}2S)^{2+}$ cluster occurs, one has to conclude that part of the $(2Fe\text{-}2S)^{2+}$ has served as scaffold for the construction of the $(4Fe\text{-}4S)^{2+}$. This means that the $(2Fe\text{-}2S)^{2+}$ clusters in F(25−45) should occupy both sites, a "permanent" $(2Fe\text{-}2S)^{2+}$ site and a "modulable" one for building the $(4Fe\text{-}4S)^{2+}$ site. This, however, is in contradiction with the recent results in Jarrett's group, who showed that the $(4Fe\text{-}4S)^{2+}$ is built at an empty site (*24*). This point has to be further studied.

Interestingly, we have very recently shown that biotin synthase overexpressed in whole cells also contains a mixture of $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$ (*26*). The fact that in all three situations both clusters are simultaneously present indicates that this mixed state might be essential for the activity of biotin synthase.

The experiment of entry 3 further confirms that if there is sufficient Fe^{2+} and a source of S^{2-}, probably generated from cysteine by a cysteine desulfurase in the presence of DTT, the $(4Fe\text{-}4S)^{2+}$ center can be formed without the requirement of any reducing system. Addition of exogenous Fe^{2+} to our F(25−45) preparation has no effect on its activity whereas the addition of exogenous Fe^{2+} is essential for the activity of the pure enzyme (*16*, *32*). The added Fe^{2+} is certainly used for the formation of the $(4Fe\text{-}4S)^{2+}$ clusters before reaction can take place. It is worth noting that the enzyme obtained in Jarrett's group containing both $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$ does not need any further addition of exogenous Fe^{2+} for activity (*39*).

When NADPH is added (entries 4−6), no significant change in the proportions of the different iron species is observed. Consequently, the role of the reducing system comprising NADPH, flavodoxin, and flavodoxin reductase would be only to reduce the built-up $(4Fe\text{-}4S)^{2+}$ to $(4Fe\text{-}4S)^{+}$. However, no $(4Fe\text{-}4S)^{+}$ could be detected in our Mössbauer analysis and neither with EPR spectroscopy of all the samples analyzed in Table 1. It has already been shown that the $(4Fe\text{-}4S)^{2+}$ can be converted to the $(4Fe\text{-}4S)^{+}$ by strong reducing agents such as dithionite and reduced deazaflavin and that this $(4Fe\text{-}4S)^{+}$ is the actual species which reacts with AdoMet giving rise to a $(4Fe\text{-}4S)^{2+}$ cluster and the cleavage products methionine and $DOA^{\bullet}$ (*37*). In our assays, the greatest probability where we could have observed the $(4Fe\text{-}4S)^{+}$ would have been in entry 4, that is, in the sole presence of the reducing entities, whereas in entry 5 where AdoMet is present, the $(4Fe\text{-}4S)^{+}$ would have already been converted to the $(4Fe\text{-}4S)^{2+}$ form during the long time of incubation (4 h) (*37*). But no $(4Fe\text{-}4S)^{+}$ was detected even in entry 4. This probably means that the physiological reducing system in the absence of AdoMet is incapable of reducing the $(4Fe\text{-}4S)^{2+}$ to the $(4Fe\text{-}4S)^{+}$. Indeed, AdoMet has been shown to bind at a unique Fe site of the $(4Fe\text{-}4S)^{2+}$ cluster, and this is probably the situation before the reducing system can operate (*40*).

After Reaction under Physiological Conditions. When DTB was finally added to the assay (entry 7) so that the reaction could proceed, there was no change in the amount of $(4Fe\text{-}4S)^{2+}$ whereas the amount of $(2Fe\text{-}2S)^{2+}$ decreased significantly and was accompanied by the formation of additional Fe^{3+}. These results support the findings of Jarrett and co-workers, who, on the sole basis of UV−vis and quantitative iron and sulfide analysis, stated that biotin synthesis was accompanied by the destruction of the $(2Fe\text{-}2S)^{2+}$ cluster whereas the $(4Fe\text{-}4S)^{2+}$ was preserved (*39*). They suggested that the $(4Fe\text{-}4S)^{2+}$ would mediate the reductive cleavage of AdoMet and the $(2Fe\text{-}2S)^{2+}$ would be the sulfur donor. Our Mössbauer results bring strong arguments in favor of this hypothesis.

The degradation of the $(2Fe\text{-}2S)^{2+}$ cluster during reaction is probably responsible for the absence of turnover of the enzyme. On the basis of gel densitometric analysis (≈32% of biotin synthase) and activity measurements, an estimation of the turnover of biotin synthase in F(25−45) leads to ≈0.5 mol of biotin/mol of monomer (molecular mass 38.6 kDa), the same as that obtained for the pure enzyme (*32*). This amount of biotin seems to correlate with the amount of sulfur lost from the $(2Fe\text{-}2S)^{2+}$ cluster. By use of a combination of Mössbauer spectroscopy (data of Table 1), gel densitometric analysis, and chemical determination of Fe (3 per monomer), we could calculate the amount of clusters in reduced biotin synthase in the absence of DTB (entry 5) as

well as in the reacted enzyme (entry 7). It can thus be deduced that around 0.25 mol of $(2Fe\text{-}2S)^{2+}$ is destroyed during reaction. We feel nevertheless that it is too early to attempt a strict quantitative analysis. The decrease in the amount of $(2Fe\text{-}2S)^{2+}$ cluster is correlated to an increase of "Fe^{3+}". The nature of this species is not clear. It could prevent the reconstitution of the $(2Fe\text{-}2S)^{2+}$ species, thus explaining the absence of turnover.

Ollagnier et al. recently found activity with a biotin synthase sample bearing a $(4Fe\text{-}4S)^{2+}$ and lacking $(2Fe\text{-}2S)^{2+}$. They formulated another mechanistic proposal in which the $(2Fe\text{-}2S)^{2+}$ center would not be the sulfur donor (*19*). The origin of the discrepancies between the two sets of results remains to be explained.

CONCLUSION

The conversion of $(2Fe\text{-}2S)^{2+}$ into $(4Fe\text{-}4S)^{2+,+}$ clusters in biotin synthase is now well documented. It has often been observed under reducing conditions with dithionite or photoreduced deazaflavin.

In this work, we have analyzed by Mössbauer spectroscopy the fate of the cluster(s) under the assay conditions in a partially purified preparation of biotin synthase. This preparation, which displayed the usually encountered activity, contained the $(2Fe\text{-}2S)^{2+}$ form together with a small amount of $(4Fe\text{-}4S)^{2+}$ as well as free iron, the amount of which was sufficient to observe optimal activity.

We have observed, under strict anaerobic conditions, that in the presence of a S^{2-} source, the system evolved into a 1:1 mixture of $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$, which is believed to be the active cluster state. The latter species was formed at the expense of free Fe^{2+} and also of the $(2Fe\text{-}2S)^{2+}$ center. No variation of this ratio was observed in the presence of the physiological reducing system (NADPH, flavodoxin, flavodoxin reductase), and no $(4Fe\text{-}4S)^{+}$ was detected by EPR.

When the substrate was added, we observed after reaction that the amount of $(2Fe\text{-}2S)^{2+}$ had decreased whereas the $(4Fe\text{-}4S)^{2+}$ has remained. This result is consistent with the hypothesis formulated by Jarrett that the active form of biotin synthase contains both clusters, the $(4Fe\text{-}4S)^{2+}$ mediating the electron transfer and the $(2Fe\text{-}2S)^{2+}$ being the sulfur source for biotin.

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BI034426C