Further Investigation on the Turnover of *Escherichia coli* Biotin Synthase with Dethiobiotin and 9-Mercaptodethiobiotin as Substrates†

Bernadette Tse Sum Bui, Manuela Lotierzo, Franck Escalettes, Dominique Florentin, and Andrée Marquet*

Synthèse, Structure et Fonction de Molécules Bioactives, UMR CNRS 7613, Université Paris VI, 4 place Jussieu, 75252 Paris Cedex 05, France

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ABSTRACT: Biotin synthase, a member of the "radical-SAM" family, produces biotin by inserting a sulfur atom between C-6 and C-9 of dethiobiotin. Each of the two saturated carbon atoms is activated through homolytic cleavage of a C−H bond by a deoxyadenosyl radical, issued from the monoelectronic reduction of *S*-adenosylmethionine (SAM or AdoMet). An important unexplained observation is that the enzyme produces only 1 mol of biotin per enzyme monomer. Some possible reasons for this absence of multiple turnovers are considered here, in connection with the postulated mechanisms. There is a general agreement among several groups that the active form of biotin synthase contains one $(4Fe\text{-}4S)^{2+,1+}$ center, which mediates the electron transfer to AdoMet, and one $(2Fe\text{-}2S)^{2+}$ center, which is considered the sulfur source [Ugulava, N. B., Sacanell, C. J., and Jarrett, J. T. (2001) *Biochemistry 40*, 8352−8358; Tse Sum Bui, B., Benda, R., Schünemann, V., Florentin, D., Trautwein, A. X., and Marquet, A. (2003) *Biochemistry 42*, 8791−8798; Jameson, G. N. L., Cosper, M. M., Hernandez, H. L., Johnson, M. K., and Huynh, B. H. (2004) *Biochemistry 43*, 2022−2031]. An alternative hypothesis considers that biotin synthase has a pyridoxal phosphate (PLP)-dependent cysteine desulfurase activity, producing a persulfide which could be the sulfur donor. The absence of turnover was explained by the inhibition due to deoxyadenosine, an end product of the reaction [Ollagnier-de Choudens, S., Mulliez, E., and Fontecave, M. (2002) *FEBS Lett. 535*, 465−468]. In this work, we show that our purified enzyme has no cysteine desulfurase activity and the required sulfide has to be added as Na_2S. It cannot be replaced by cysteine, and consistently, PLP has no effect. We observed that deoxyadenosine does not inhibit the reaction either. On the other hand, if the $(2Fe\text{-}2S)^{2+}$ center is the sulfur source, its depletion after reaction could explain the absence of turnover. We found that after addition of fresh cofactors, including Fe^{2+} and S^{2-}, either to the assay when one turn is completed or after purification of the reacted enzyme by different techniques, only a small amount of biotin (0.3−0.4 equiv/monomer) is further produced. This proves that an active enzyme cannot be fully reconstituted after one turn. When 9-mercaptodethiobiotin, which already contains the sulfur atom of biotin, is used as the substrate, the same turnover of one is observed, with similar reaction rates. We postulate that the same intermediate involving the (2Fe-2S) cluster is formed from both substrates, with a rate-determining step following the formation of this intermediate.

Biotin synthase (BS),[1] an iron−sulfur protein which catalyzes the final step of biotin biosynthesis, namely, the introduction of sulfur onto the two nonactivated carbon atoms, C-6 and C-9 of dethiobiotin (DTB) (*1*, *2*), belongs to the "radical-SAM" enzyme family (*3*).

The nature of the cofactors it contains and of the components required for activity and, more generally, its mechanism of action have been the subject of highly controversial discussions. A consistent picture is now emerging, in accord with the mechanism previously proposed by us (*4*) and by others (*5*). However, several points remain to be definitely established, especially (i) the nature of the iron−sulfur clusters present in the active form of biotin synthase *in vitro* and *in vivo*, (ii) the sulfur source for biotin, and (iii) the reasons for the absence of turnover.

We have reviewed the situation concerning the (Fe−S) centers in a recent paper. The data contained in that paper (*6*) together with those published by Jarrett's (*7*, *8*) and more recently by Johnson's groups (*9*) are becoming more or less consistent. As summarized by Johnson (*9*, *10*), there is now a general agreement between the three groups that the active form of biotin synthase contains one $(2Fe\text{-}2S)^{2+}$ and one $(4Fe\text{-}4S)^{2+}$ cluster, located at two different sites of the protein. This was confirmed by the recently obtained X-ray structure (*11*).

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* To whom correspondence should be addressed. Telephone: +33 1 44 27 55 64. Fax: +33 1 44 27 71 50. E-mail: marquet@ccr.jussieu.fr.

[1] Abbreviations: AdoMet or SAM, *S*-adenosylmethionine; BS, biotin synthase; 5-DAF, 10-methyl-5-deazaisoalloxazine; DEAE, diethylaminoethyl; $AdoCH_2^{\bullet}$, 5′-deoxyadenosyl radical; $AdoCH_3$, 5′-deoxyadenosine; DTB, dethiobiotin; 9-DTBSH, 9-mercaptodethiobiotin; $(9\text{-DTBS})_2$, 9-mercaptodethiobiotin, disulfide form; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; (Fe-S), iron−sulfur cluster; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PLP, pyridoxal 5′-phosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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It should be pointed out that among the different Mössbauer studies carried out on biotin synthase (*6*), ours is the only one using an enzyme which initially has not been "artificially" reconstituted. Hence, this experiment showed what happens to the enzyme when it is placed under assay conditions. Starting from a partially purified preparation of biotin synthase containing besides the $(2Fe\text{-}2S)^{2+}$ form of the enzyme, Fe^{2+}, dithiothreitol (DTT), cysteine, and cysteine desulfurases as a source of sulfide, we observed that just by leaving the sample under strict anaerobic conditions, a 1:1 mixture of $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$ clusters was formed, at the expense of the free iron and also of the $(2Fe\text{-}2S)^{2+}$ center. This 1:1 mixture remains intact in the complete assay mixture, as long as the substrate is not present. Our Mössbauer study of whole cells overexpressing the enzyme and of the corresponding crude extracts revealed that this mixture of clusters was also present, although in a different ratio (*12*).

It is now clear that the $(4Fe\text{-}4S)^{2+}$ cluster, liganded by the three cysteines of the CX3CX2C motif, conserved in all members of the radical-SAM family, is mediating the one electron transfer to SAM (or AdoMet) to generate the deoxyadenosyl radical ($AdoCH_2^{\bullet}$) (*13*). The X-ray structures of biotin synthase, as well as those of two other enzymes of the family, HemN (*14*) and MoaA (*15*) reveal that AdoMet is suitably positioned for electron transfer and is bound to the unique iron of the cluster, the one which is not coordinated by cysteine.

Evidence is available for the participation of the $(2Fe\text{-}2S)^{2+}$ center as the sulfur source. Our early experiments indicated that the sulfur introduced into DTB was present in biotin synthase itself (*16*). We postulated that it could be either the (Fe-S) center or a sulfur atom covalently bound to the protein as persulfide for instance. We have then shown that biotin synthase containing a labeled $(2Fe\text{-}2[^{34}S])^{2+}$, cluster which was reconstituted from the apoprotein, produced [^{34}S]biotin with a high level (≈60%) of isotope incorporation (*17*). The possibility of formation of a persulfide during these reconstitution experiments, realized by incubating the apoenzyme with Na_2S and $FeCl_3$ under strongly reducing conditions (5 mM DTT), was considered as very unlikely, and we concluded that the reconstituted (Fe-S) center was probably the sulfur source (at that time, a precise view of the nature of the clusters was lacking).

Later, by following changes in the Fe and S content and the UV−visible spectrum during the time course of the assay, Jarrett and co-workers reported the formation of a mixed $(2Fe\text{-}2S)^{2+}$:$(4Fe\text{-}4S)^{2+}$ cluster in a 1:1 ratio. Since during reaction, the $(4Fe\text{-}4S)^{2+}$ cluster was preserved and the $(2Fe\text{-}2S)^{2+}$ was destroyed, they concluded that the latter was the sulfur donor (*18*). Our Mössbauer studies (*6*), followed by those of Jameson *et al*. (*10*), confirmed that part of the $(2Fe\text{-}2S)^{2+}$ center disappeared during the reaction, although it is at present difficult to establish a quantitative correlation between its consumption and the formation of biotin.

A different conclusion has been reached by the group of Fontecave who propose that biotin synthase has a pyridoxal phosphate (PLP)-dependent cysteine desulfurase activity (*19*). A persulfide formed on one of the cysteines of the enzyme would be the immediate sulfur donor. We (*20*) and others (*21*−*23*) have also reported that addition of cysteine improved the activity in an *in vitro* assay conducted with an ≈90% pure enzyme. In this paper, we describe experiments performed with another enzyme, namely, the His-tagged (HT) protein cloned in Jarrett's group (*24*), and we show that cysteine has no effect on the activity. Consistently, no effect of PLP was observed. Addition of sodium sulfide is essential to observe an activity. Thus, this biotin synthase has no desulfurase activity, and we conclude that our previous enzyme preparations were very likely contaminated by desulfurases. The HT enzyme, in our *in vitro* assay, has a maximum turnover of one. Likewise, in the different groups working with a well-defined assay, the amount of biotin formed per enzyme monomer never exceeds one.

The objective of this work is to investigate the factors that might be responsible for the absence of multiple turnovers. We show that this is not due to the inhibition by deoxyadenosine ($AdoCH_3$), one of the reaction products, as proposed by the group of Fontecave (*25*). We also examined whether one of the cofactors was limiting: addition of all cofactors made the enzyme react again but not as efficiently as in the first run. Another approach made use of 9-mercaptodethiobiotin (9-DTBSH) which was previously shown to be converted into biotin, either *in vivo* (*26*) or *in vitro* with a crude cell-free extract (*27*). The properties of this compound had never been studied before with the pure enzyme and the well-defined assay system. The results show that 9-DTBSH is a substrate of the enzyme. Since it already contains the sulfur atom, one could have expected a turnover higher than one, but this is not the case.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from either Sigma-Aldrich Chemical Co., Merck eurolab, or Acros Organics unless otherwise stated. 5′-Deoxyadenosine and 10-methyl-5-deazaisoalloxazine (5-DAF) were synthesized according to the procedures of Wang *et al*. (*28*) and Janda and Hemmerich (*29*), respectively. 5-DAF was solubilized in dimethyl sulfoxide to yield a 2.4 mM stock solution.

Synthesis and Purification of 9-Mercaptodethiobiotin (9-DTBSH). 9-DTBSH was synthesized as described by Even *et al*. (*30*). 9-DTBSH methyl ester (20 mg, 77 μmol) in 1 mL of methanol was saponified in the presence of 0.1 N NaOH (4 equiv) with overnight stirring. The acid was then converted to the disulfide $(9\text{-}DTBS)_2$, under stirring in air for 24 h, after adjusting the pH to 9−10. The reactions were monitored on TLC plates (95:5:5 CH_2Cl_2/EtOH/AcOH). The products were detected with 4-(dimethylamino)cinnamaldeyde. The R_f values of 9-DTBSH methyl ester, 9-DTBSH, and $(9\text{-}DTBS)_2$ were 0.6, 0.4, and 0.2, respectively.

The purification of $(9\text{-}DTBS)_2$ (15 mg) was carried out on an HPLC reversed phase C8 7 μm SymmetryPrep column (7.8 mm × 300 mm, Waters). The solvent system consisted of aqueous 0.02% TFA and acetonitrile (85:15, v/v) (eluent A) and 0.02% TFA and acetonitrile (50:50, v/v) (eluent B). Solvents were filtered through a 0.45 μm nylon membrane filter (Whatman, Maidstone, England) under vacuum prior to use. Separations were carried out at a flow rate of 2.5 mL/min using a 35 min linear gradient beginning with 100% eluent A and ending with a final eluent B:eluent A ratio of 70:30 (v/v). The optical density was monitored at 210 nm. The retention time for the $(9\text{-}DTBS)_2$ was approximately 19 min. The purified product (7.4 mg) was solubilized in

alkaline water (pH 9.0) as a stock solution of 11 mM $(9\text{-DTBS})_2$ which was used for the enzymatic activity experiments.

Bacterial Strains and Enzyme Purification. Escherichia coli TK101pI$_3$BLS$_2$ overexpressing biotin synthase (BS-WT) (*31*) was a generous gift from Y. Izumi (Tottori University, Tottori, Japan). Purification of BS-WT, flavodoxin, and flavodoxin reductase has been described previously (*21*, *32*). *E. coli* strain BL21(DE3)pLysSpJJ15-4A overexpressing biotin synthase bearing a His_6 tag at its N-terminus (BS-HT) (*24*) was a generous gift from J. Jarrett (University of Pennsylvania, Philadelphia, PA). Cells were cultivated as described previously (*24*), suspended in 50 mM Tris-HCl (pH 8.0) (buffer A), and sonicated. After centrifugation to remove cell debris, the supernatant was diluted with 3 volumes of the same buffer and centrifuged again to remove any precipitated proteins. The diluted cell-free extract was loaded onto a Ni^{2+}-chelating Sepharose (Pharmacia-Amersham) column (2.5 cm × 5 cm), previously equilibrated with buffer A containing 0.5 M NaCl and 100 mM imidazole. Under the injection conditions that were used, most of the contaminating proteins did not bind and were eliminated in the nonretained fraction. Bound proteins were eluted with the same buffer containing 200 mM imidazole. After concentration on Centriprep 30 concentrators (Millipore), the enzyme was loaded on a Sephadex G-25 column equilibrated with buffer A to remove imidazole. The protein was concentrated with a Centriprep 30 concentrator and frozen as small aliquots in liquid nitrogen before being stored at −80 °C.

Preparation of the Apoenzyme. Solutions of 115 μM BS-HT with 10 mM EDTA in 100 mM Tris-HCl (pH 8.0) (final concentrations) and 5-DAF (2.4 mM in DMSO) were degassed separately for 30 min at room temperature under a stream of wet argon. With a gastight syringe, 5-DAF (final concentration of 24 μM) was added to give a final volume of 300 μL of the mixture. Photoreduction was started by irradiation with a white fluorescent tube (18 W) placed 10 cm away. After 1 h, the mixture was desalted on a PD-10 column (Pharmacia-Amersham) equilibrated with buffer A and the enzyme concentrated on a Centricon 30 centrifugal filter device (Millipore). The absence of (Fe-S) clusters was verified by UV−vis spectroscopy.

Enzymatic Assay with DTB. The standard reaction mixture in a final volume of 100 μL containing 9 μM BS-HT, 100 μM DTB, 100 μM AdoMet, 2 mM NADPH, 2 μM flavodoxin, 0.05 μM flavodoxin reductase, 2 mM DTT, and 200 μM $Fe(NH_4)_2(SO_4)_2$ in 40 mM Tris buffer (pH 8.0) was placed in a 1.5 mL septum-capped Eppendorf tube and was degassed for 10 min at room temperature by a continuous stream of wet argon until the red color, which appeared upon addition of Fe^{2+}, turned colorless. Then Na_2S (prepared in argon-saturated buffer A) was added with a gastight syringe to give a final concentration of 100 μM, and the mixture was left under argon for a further 15 min. The reaction mixture was incubated at 37 °C for 4 h and the reaction stopped in ice. After centrifugation of the sample at 13000*g* for 5 min at 4 °C, the amount of biotin that formed was determined on the supernatant by a microbiological assay using *Lactobacillus plantarum* (*33*).

Effect of L-Cysteine, Na_2S, PLP, and DOAH on the Activity of BS-HT. A 5 mM stock solution of DOAH was prepared by dissolving it in hot water. A 10 mM stock solution of PLP in water was prepared by adding 0.1 M NaOH for solubilization. The concentrations of Cys, Na_2S, PLP, and DOAH present in the assay varied from 0.2 to 1 mM, 0.05 to 0.8 mM, 2 to 40 μM, and 2.5 to 30 μM, respectively.

Experiments for Determining Whether BS-HT Can Perform Successive Enzymatic Cycles. Two types of experiments were run. (i) The enzymatic assay was performed as described above, and after reaction for 4 h, the Eppendorf tubes were removed from the incubation bath. Then either water (control), all components (DTB, AdoMet, NADPH, flavodoxin, flavodoxin reductase, DTT, Fe^{2+}, and Na_2S), a mixture of DTT, Fe^{2+}, and Na_2S, or a mixture of all components except DTT, Fe^{2+}, and Na_2S was added to separate Eppendorf tubes with a gastight syringe. The volumes were adjusted with water so that the final reaction volumes were similar. After the mixtures had been degassed for 30 min under argon, the Eppendorf tubes were incubated for a further 4 h at 37 °C before biotin quantification. (ii) For enzyme recovery, the assays were performed as described above except that the final volume was scaled up to 500 μL. Depending on the method of recovery (outlined below), a total varying from 10 to 40 Eppendorf tubes was used. After reaction, the contents from all the Eppendorf tubes were assembled, and 20 μL was saved for biotin quantification. The rest were desalted either by three rounds of dilution and concentration in a Centriprep 30 concentrator or by being passed through Sephadex G-25 (the volume of the gel was 10 times that of the reaction mixture) or a Q-Sepharose high-performance anion exchanger, which according to Johnson and co-workers (*9*) was more efficient than gel filtration for removing iron and sulfide containing impurities. The enzyme was then brought to the appropriate concentration for the next cycle with either a Centriprep, Centricon, or a Microcon YM-30. In parallel, control experiments including all the assay components except DTB were carried out at the same time and the reaction mixtures treated similarly. Assay and control experiments for the second cycle were carried out as for the first one in 500 μL reaction mixtures. The enzymes were recovered after the second cycle using the same purification procedures that were used after the first cycle to be utilized in the third one.

Comparison of DTB and 9-DTBSH as Substrates in the Enzymatic Assay. Enzymatic assays, consisting of varying concentrations of DTB (2.5−400 μM) and $(9\text{-DTBS})_2$ (10−350 μM), were run in parallel at 37 °C for 6 h. Since DTBSH is easily oxidized to $(9\text{-DTBS})_2$, it was added to the assay in the oxidized form. The presence of 2 mM DTT in the reaction medium was sufficient for its *in situ* reduction to 9-DTBSH. This was checked by separately incubating a sample containing 300 μM $(9\text{-DTBS})_2$ and 2 mM DTT in 50 mM Tris-HCl buffer (pH 8.0) under the usual assay conditions. TLC analysis (95:5:5 CH_2Cl_2/EtOH/AcOH) of the sample showed the conversion of the disulfide to the −SH form after approximately 30 min and the stability of the reduced form during the 6 h incubation time.

Time Course for the Formation of Biotin with either DTB or 9-DTBSH as the Substrate. Enzymatic assays consisting of 100 μM DTB and 300 μM $(9\text{-DTBS})_2$ were run in parallel at 37 °C. At various time intervals, the Eppendorf tubes were removed from the water bath and the reactions stopped in ice before quantification of biotin.

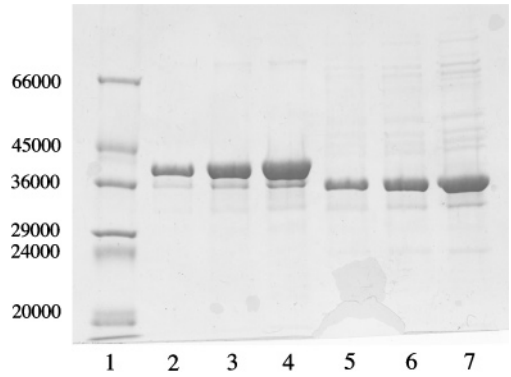


FIGURE 1: SDS−PAGE of purified biotin synthase: lane 1, mixture of molecular mass markers; lanes 2−4, 3, 6, and 10 μg of BS-HT, respectively; and lanes 5−7, 3, 6, and 10 μg of BS-WT, respectively.

Analytical Methods. Protein concentrations were measured by the method of Bradford (*34*) using bovine serum albumin as a standard. Iron was assayed by the method of Fish (*35*), and inorganic sulfide was quantified as described by Beinert (*36*). SDS−PAGE was performed in a 10% gel as described by Laemmli (*37*). Proteins were stained with Coomassie Brilliant Blue. UV−visible absorption spectra were recorded on a Uvikon 930 spectrophotometer.

RESULTS

Biotin Synthase Has No Cysteine Desulfurase Activity. During the past years, we have been working with a preparation of biotin synthase isolated from a recombinant strain of *E. coli* TK101pI$_3$BLS$_2$ (BS-WT). The purification procedure, which involved three chromatographic steps comprising DEAE, Phenyl, and Mono-Q Sepharose, yielded an enzyme preparation with a purity of ≈90%, as judged from SDS−PAGE (Figure 1), with consistent values of 0.8 (2Fe-2S)$^{2+}$ cluster per monomer. The assay was adapted from the one described by Sanyal *et al.*, who were the first to obtain an activity with the purified *E. coli* enzyme (*21*). After optimization of the concentrations of the different cofactors and enzymes, the maximum amount of biotin formed was, in our hands, 0.5 equiv per monomer (*20*).

We are now working with a His-tagged enzyme (BS-HT), cloned in Jarrett's group. The enzyme was purified in a single step on a Ni^{2+}-chelating Sepharose column (Figure 1). Chemical analyses of the iron and sulfide content gave 1.5 Fe and 1.5 S^{2-} per monomer, lower than that reported by Ugulava *et al*. which was 2.5−3 (*7*). We have no explanation for this difference since we found a similar extinction coefficient value at 452 nm of 7.7 ± 0.2 mM^{-1} cm^{-1} as compared to a value of 8.4 ± 0.8 mM^{-1} cm^{-1} (*7*).

When our optimized assay system for BS-WT was applied to BS-HT, only negligible activity was detected. However, if we use the assay system described by Ugulava *et al.* (*7*) whose only difference resides in the use of Na_2S instead of cysteine, a turnover of one was obtained after reoptimization of the concentrations. Figure 2 shows that Na_2S always has a positive effect, with an optimal concentration of 100 μM, whereas cysteine has no effect. This result ruled out the proposal advanced by Fontecave and co-workers that biotin synthase has a cysteine desulfurase activity (*19*). Despite that, we verified whether PLP had any influence on our assay.

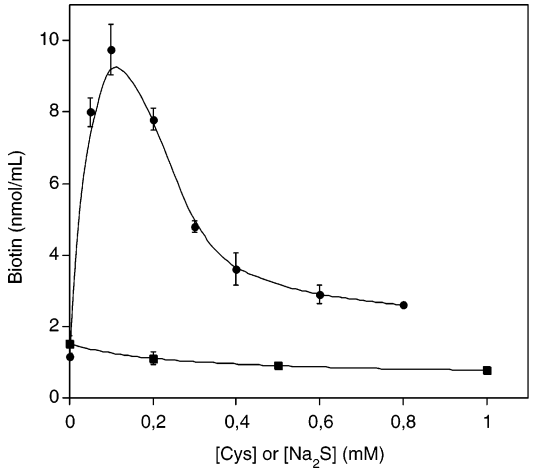


FIGURE 2: Effect of Na_2S (●) and L-cysteine (■) on the activity of BS-HT. The reaction mixture contained 9 μM BS-HT, 100 μM DTB, 100 μM AdoMet, 2 mM NADPH, 2 μM flavodoxin, 0.05 μM flavodoxin reductase, 2 mM DTT, 0.2 mM $Fe(NH_4)_2(SO_4)_2$, and either L-Cys or Na_2S at various concentrations in 40 mM Tris-HCl buffer (pH 8.0). The reaction mixture was incubated for 4 h at 37 °C. Data are means from three independent experiments with each point determined in duplicate or triplicate. The error bars represent standard deviations and when not shown fall within the symbol.

Varying concentrations of PLP ranging from 0.2 to 4 equiv with respect to BS were added together with 0.5 mM cysteine. We did not observe any effect of PLP on the activity, with the His-tagged and WT enzymes (graphs not shown). Thus, these results indicate that BS-HT has no PLP cysteine desulfurase activity and imply that our WT preparations were very likely contaminated with desulfurases containing sufficient amounts of endogeneous PLP.

DOAH Is Not an Inhibitor. According to the mechanism proposed by Fontecave and co-workers which postulates that the added cysteine generates a persulfide on the enzyme, the sulfur source should be constantly regenerated and a catalytic system should be obtained. However, in their assay, the turnover did not exceed one either. They attributed this lack of turnover to the inhibition caused by DOAH, an end product of the reaction (*25*). To verify this point, we added DOAH (0.25−3 equiv) to the assays of the HT and WT enzymes, but no effect was observed (graphs not shown).

Effect of the Addition of Fresh Cofactors after One Cycle. Another possibility that could account for the single turnover would be that one or several cofactors were limiting, consumed during reaction. Thus, all the components of the assay were added to the reaction medium after the enzyme has turned once to determine if the reaction could start again (Figure 3). After one turn, the amount of biotin formed per enzyme monomer was, as usual, equal to one. When all components (DTB, AdoMet, NADPH, flavodoxin, flavodoxin reductase, DTT, Fe^{2+}, and S^{2-}) were added, a further amount of ≈0.4 mol of biotin per mole of monomer was produced (column 2), indicating that the enzyme could perform a second cycle but not as efficiently as the first. In the control experiment (addition of water, column 1), no more biotin was produced. Column 3 shows that addition of a mixture of DTB, AdoMet, NADPH, flavodoxin, and flavodoxin reductase has no effect, indicating that none of these components was limiting. On the other hand, upon addition of DTT, Fe^{2+}, and S^{2-} (column 4), additional biotin was

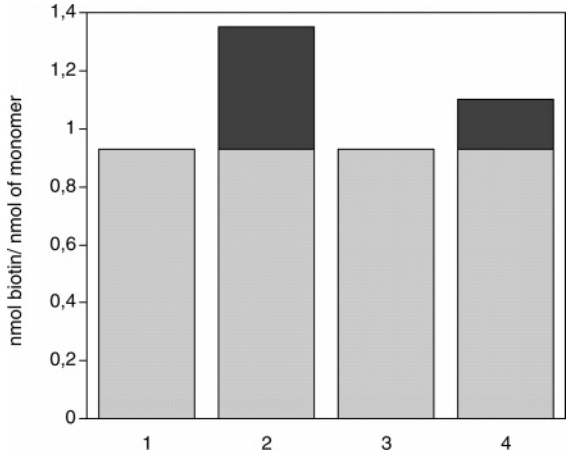


FIGURE 3: Effect of adding fresh components at the concentrations used in the assay to the reaction mixture after one cycle (represented as light gray). (1) addition of water (control), (2) all components (DTB, AdoMet, NADPH, flavodoxin, flavodoxin reductase, DTT, Fe^{2+}, and S^{2-}), (3) all except DTT, Fe^{2+}, and S^{2-}, and (4) DTT, Fe^{2+}, and S^{2-}. After addition, the assay was incubated for 4 h at 37 °C for the next cycle. Data are means from four independent experiments with each point determined in triplicate or quadruplicate. Errors in the values of columns 1−4 are ±0.06, ±0.05, ±0.04, and ±0.07, respectively.

formed. Although reproducible, it is not clear why the value of column 2 was not reached.

Attempts To Free the (2Fe-2S)$^{2+}$ Site. Our previous Mössbauer study (*6*) showed that there was a decrease in the (2Fe-2S)$^{2+}$ cluster content after reaction. The depletion of sulfur from the cluster for incorporation into biotin would explain the absence of turnover of the enzyme. In that case, to render the enzyme catalytic, the cluster has to be regenerated. In the assay, 20- and 10-fold excesses of Fe^{2+} and S^{2-} over the enzyme monomer, respectively, are present. When we use the as-isolated enzyme containing one (2Fe-2S)$^{2+}$ cluster per monomer, some of the added Fe^{2+} and S^{2-} is consumed for building the (4Fe-4S)$^{2+}$ cluster, but the rest does not seem to be capable of reconstructing the (2Fe-2S)$^{2+}$ cluster. The Mössbauer study also revealed the appearance of a new iron signal after reaction. The structure of this species could not be determined, but from high-field measurements, they could correspond to small superparamagnetic "Fe^{3+}" clusters. We postulated that this Fe^{3+} species could block the site and prevent any further reconstitution. Therefore, removal of this species would lead to an enzyme capable of performing a second turnover. Three methods were attempted. The reaction mixture was (i) introduced into Centriprep-30 concentrators and subjected to three successive dilution−concentration cycles, (ii) filtered on a Sephadex G-25 column, and (iii) passed through a high-performance Q-Sepharose column.

The recovered enzymes, which were completely free of any biotin as determined by the *L. plantarum* test, were made to react in a second run. Whichever purification procedure was employed, the amount of biotin produced was always 0.3−0.4 mol/monomer. On the other hand, the enzyme recovered from control experiments consisting of all assay components except DTB produced a turnover of one in the second cycle. When the enzyme recovered from the second run was made to react for a third cycle, negligible activity, i.e., ≈0.1 mol of biotin per mole of monomer, was measured as compared to a value of 0.6−0.7 found for the control.

Table 1: Activity (nanomoles of biotin formed per nanomole of monomer) of BS-HT Recovered after Successive Enzymatic Cycles

cycle number	method of purification	enzyme recovered from complete[a] assay	enzyme recovered from control[b] assay
1		0.98 ± 0.02	0[c]
2	Centriprep 30	0.35 ± 0.04	0.98 ± 0.02
	Sephadex G-25	0.36 ± 0.03	1.0 ± 0.04
	Q-Sepharose	0.36 ± 0.03	0.95 ± 0.05
3	Centriprep 30	0.09 ± 0.02	0.65 ± 0.05
	Sephadex G-25	ND[d]	ND[d]
	Q-Sepharose	0.13 ± 0.01	0.63 ± 0.03

[a] In the presence of all components of the assay. [b] In the presence of all components of the assay except DTB. [c] No recovery, activity of the as-isolated enzyme. [d] Not determined.

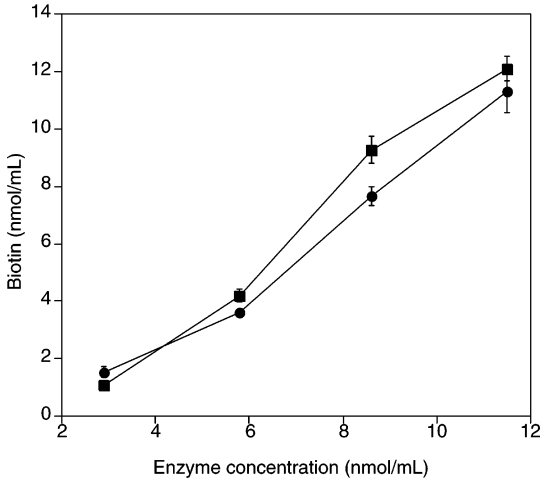


FIGURE 4: Comparison of the activities of apo (●) and as-isolated (■) BS-HT. The reaction mixture contained varying concentrations of either the apo or the as-isolated enzyme, 100 μM DTB, 100 μM AdoMet, 2 mM NADPH, 2 μM flavodoxin, 0.05 μM flavodoxin reductase, 2 mM DTT, 0.2 mM $Fe(NH_4)_2(SO_4)_2$, and 0.1 mM Na_2S in 40 mM Tris-HCl buffer (pH 8.0). The reaction mixture was incubated for 4 h at 37 °C. Data are means from three independent experiments with each point determined in duplicate. The error bars represent standard deviations and when not shown fall within the symbol.

The activities of the recovered enzymes after successive cycles are summarized in Table 1.

Hence, we see that in all these experiments, the additional amount of biotin produced per enzyme monomer is always ≈0.4, whatever the treatment of the reacted medium: addition of fresh cofactors directly to the reacted enzyme (Figure 3) or to the reacted enzyme after attempts at its purification (Table 1).

On the other hand, the apoenzyme, obtained by removing the (2Fe-2S)$^{2+}$ cluster from the as-isolated enzyme, produces the same activity as the latter under the standard assay conditions (Figure 4). The kinetics of biotin production is also similar for these two forms (data not shown). These findings indicate that the active cluster species [supposed to be the 1:1 mixture of (2Fe-2S)$^{2+}$ and (4Fe-4S)$^{2+}$ clusters] is also assembled in the apoenzyme, whose (2Fe-2S)$^{2+}$ and (4Fe-4S)$^{2+}$ sites were originally free.

Consequently, the fact that with the reacted enzyme this reconstitution does not take place is significant.

Experiments with 9-Mercaptodethiobiotin (9-DTBSH). In preceding papers, we showed that 9-[^{34}S]DTBSH was incorporated into biotin in whole cells (*26*) and crude extracts

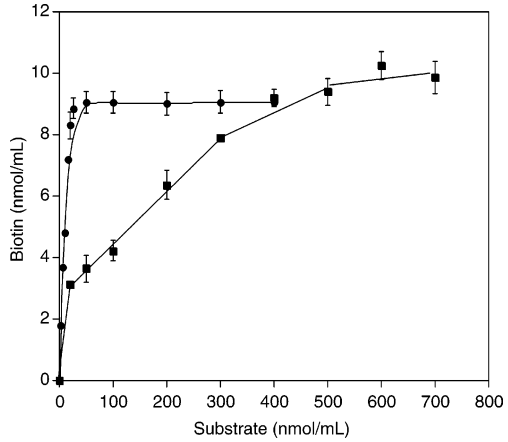


FIGURE 5: Comparison of 9-DTBSH (■) and DTB (●) as substrates of BS-HT. The reaction mixture contained 9 μM enzyme, varying concentrations of either DTB or $(9\text{-DTBS})_2$, 100 μM AdoMet, 2 mM NADPH, 2 μM flavodoxin, 0.05 μM flavodoxin reductase, 2 mM DTT, 0.2 mM $Fe(NH_4)_2(SO_4)_2$, and 100 μM Na_2S in 40 mM Tris-HCl buffer (pH 8.0). The reaction mixture was incubated for 6 h at 37 °C. Data are means from three independent experiments with each point determined in duplicate or triplicate. The error bars represent standard deviations and when not shown fall within the symbol.

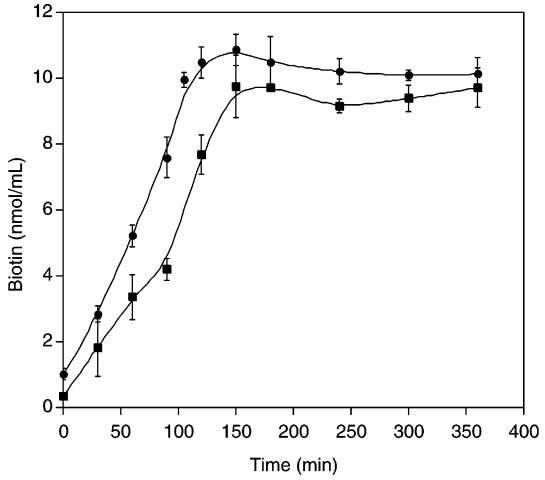


FIGURE 6: Time course for the formation of biotin. BS-HT (9 μM) was incubated with 300 μM $(9\text{-DTBS})_2$ (■) or 100 μM DTB (●) under conditions described in the legend of Figure 5. At the indicated times, the Eppendorf tubes were removed from the water bath at 37 °C and stored in ice. Data are means from three independent experiments with each point determined in duplicate or triplicate. The error bars represent standard deviations and when not shown fall within the symbol.

(*27*) of *Bacillus sphaericus*, with conservation of 75% of the label. Although it could never be isolated, we considered that 9-DTBSH was a likely intermediate of the reaction, probably not in its free form, but remaining attached to the protein. This compound had till now never been tested with a well-defined *in vitro* system, and the first results are presented here. The main question concerned the turnover: starting from a substrate already containing the sulfur atom present in biotin, would we observe a catalytic activity?

9-DTBSH was synthesized as previously described (*30*) and purified by HPLC as the oxidized disulfide form, since the free thiol was quickly oxidized in air. We checked by TLC that the conversion of the disulfide into the free thiol was rapid under the assay conditions (2 mM DTT). Thus, all the experiments were carried out with the disulfide, assuming that $2[(9\text{-DTBS})_2] = [9\text{-DTBSH}]$. Figure 5 shows the behavior of 9-DTBSH compared to that of DTB. It should be noted that the presence of AdoMet is essential for observation of an activity with the thiol (data not shown). Although the latter proved to be a good substrate of biotin synthase, its apparent K_m was much higher than that of DTB (~20 times). Noteworthy with this substrate is the fact that the same turnover of one is observed.

We also compared the rate of biotin formation from DTB and 9-DTBSH. Figure 6 shows that the rate of biotin formation from the thiol is slightly slower at the beginning of the reaction, the slopes becoming parallel after 90 min and the reaction being complete after 3 h.

DISCUSSION

We addressed in this paper some of the problems which are still open in the mechanism of action of biotin synthase. Whereas there is a general agreement about the first step of the reaction, namely, the homolytic cleavage of a C−H bond at C-9 of DTB by a deoxyadenosyl radical, the sulfur source is still up for debate and two apparently exclusive mechanisms have been proposed for the formation of the C−S bonds.

Our early experiments with a well-defined *in vitro* system led to the conclusion that the sulfur source was biotin synthase itself, either its (Fe-S) center or sulfur species covalently bound to the protein (*16*). Gibson *et al.* performed independent experiments which confirmed that biotin synthase was the sulfur donor, and suggested that it could be the (Fe-S) center (*38*), but their results also did not allow one to choose between the two hypotheses. Our later finding that biotin synthase containing a ^{34}S or ^{35}S-labeled $(2Fe\text{-}2S)^{2+}$ center, reconstituted from the apoprotein, produced labeled biotin led us to favor the hypothesis of the (Fe-S) center being the sulfur source (*17*, *20*). At that time, the exact nature of the (Fe-S) clusters in the active enzyme was unknown. It is now generally accepted that several forms of biotin synthase can be obtained: the as-isolated enzyme, after aerobic or semianaerobic purification, contains one $(2Fe\text{-}2S)^{2+}$ cluster per polypeptide. Reconstitution of the apoenzyme with Fe^{2+} and S^{2-} under strictly anaerobic conditions leads to a form containing one $(4Fe\text{-}4S)^{2+}$ center (*9*, *19*), whereas reconstitution of the as-isolated enzyme under the same conditions generates an enzyme containing two iron−sulfur clusters per monomer, one $(2Fe\text{-}2S)^{2+}$ and one $(4Fe\text{-}4S)^{2+}$, located at different sites (*7*−*9*). According to Jarrett's group, this form is active without addition of iron and sulfide, and it is considered the active form *in vivo*. This was recently confirmed by Johnson and co-workers (*10*).

For our part, we followed a slightly different approach, examining the fate of the $(2[^{57}Fe]\text{-}2S)^{2+}$ enzyme under the assay conditions by Mössbauer spectroscopy (*6*). For that, we used a partially purified preparation also containing endogeneous $^{57}Fe^{2+}$. After the mixture had stood under strictly anaerobic conditions, a 1:1 mixture of $(4Fe\text{-}4S)^{2+}$ and $(2Fe\text{-}2S)^{2+}$ was observed. The $(4Fe\text{-}4S)^{2+}$ center was formed at the expense of the "free" Fe^{2+} and some of the initial $(2Fe\text{-}2S)^{2+}$ cluster. Investigation of the nature of the cluster(s) of biotin synthase in aerobically grown cells also revealed the presence of a mixture of $(2Fe\text{-}2S)^{2+}$ and $(4Fe\text{-}4S)^{2+}$ centers in a 3:1 ratio (*12*). We attributed the lower

proportion of the $(4Fe\text{-}4S)^{2+}$ species to its high oxygen sensitivity, but from the Mössbauer spectrum, there was no doubt that it was present. On the other hand, in a similar study, Cosper *et al*. observed only the $(2Fe\text{-}2S)^{2+}$ center, even in anaerobically grown cells (*9*). The origin of the divergence is not understood at the moment.

In summary, we believe that the mixed-cluster state is the active one and that it is present *in vivo*. We have previously described that the as-isolated $(2Fe\text{-}2S)^{2+}$ enzyme is active without addition of sulfide or cysteine (*16*), although to a lesser extent than in their presence. This means that the clusters can rearrange into their active configuration during the assay. In this work, we have also shown that the apoenzyme is as efficient as the as-isolated enzyme under the assay conditions (in the presence of Fe^{2+} and S^{2-}), with the same turnover (Figure 4) and kinetics, indicating that both iron−sulfur centers are easily reconstituted in the apo form.

In the crystal structure of biotin synthase which has recently been determined, with DTB and AdoMet included, the two clusters are clearly present (*11*). As expected from previous studies (*23*), the $(4Fe\text{-}4S)^{2+}$ center is liganded by the cysteines of the CX3CX2C motif common to all radical-SAM enzymes. The fourth ligand is provided by AdoMet, and this configuration, also visible on the X-ray structures of two other AdoMet-dependent enzymes, HemN (*14*) and MoaA (*15*), may be common to the whole family. This supports the well-established conclusion that the role of the $(4Fe\text{-}4S)^{2+}$ center is to transfer electrons for the reductive cleavage of AdoMet to $AdoCH_2^{\bullet}$. The $(2Fe\text{-}2S)^{2+}$ center of biotin synthase is liganded by three other conserved cysteines and, less expectedly, by a conserved arginine (*11*).

There are many arguments to postulate that this $(2Fe\text{-}2S)^{2+}$ center is the sulfur-donating species. We have shown that the sulfur of biotin should arise from an (Fe-S) center. It is obvious now that it is not from the $(4Fe\text{-}4S)^{2+}$ cluster, which mediates electron transfer. Thus, it has to be the $(2Fe\text{-}2S)^{2+}$ cluster. This was supported by experiments from Jarrett and co-workers who have shown, based on UV−visible absorption changes and Fe and S analyses, that after turnover, the $(4Fe\text{-}4S)^{2+}$ cluster remained intact, whereas the $(2Fe\text{-}2S)^{2+}$ cluster was destroyed (*18*). We have confirmed this result in the Mössbauer study mentioned above (*6*). Starting from the 1:1 mixture of the two clusters, we observed that after reaction with DTB, the amount of the $(4Fe\text{-}4S)^{2+}$ cluster was unchanged whereas that of the $(2Fe\text{-}2S)^{2+}$ cluster had decreased, although not completely. The same uncomplete consumption of the $(2Fe\text{-}2S)^{2+}$ center was reported by Jameson *et al*. (*10*). It should be pointed out that in the crystal structure of the BS−DTB−AdoMet complex, the methyl group of DTB, which is assumed to be the first site of functionalization (*26*, *27*), is properly oriented toward one of the sulfur atoms of the $(2Fe\text{-}2S)^{2+}$ center.

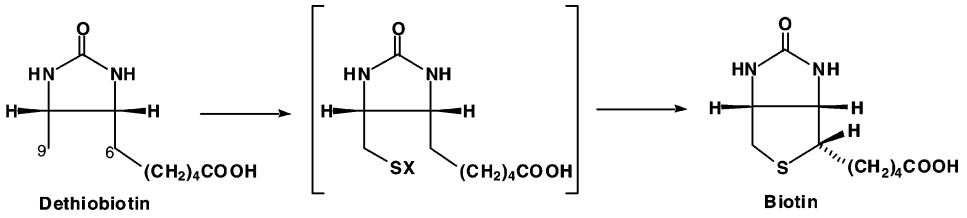
In contrast, Fontecave and co-workers (*19*), starting from a reconstituted enzyme containing mostly a $(4Fe\text{-}4S)^{2+}$ center, found that it possessed a PLP-dependent cysteine desulfurase activity. They proposed that a protein-bound persulfide was generated, either on Cys 97 or Cys 128, which would trap the C-9 radical. Although there is in the sequence of BS no obvious PLP binding site, the conserved Lys 49 was considered as a good candidate for formation of the aldimine with PLP. However, the X-ray structure (*11*) reveals that Lys 49 and the other lysines are situated on the surface of the protein, far from the active site. Cys 97 and Cys 128 were found to be essential by site-directed mutagenesis, but the X-ray structure shows that they are ligands of the $(2Fe\text{-}2S)^{2+}$ cluster. Of course, one can argue that if the $(2Fe\text{-}2S)^{2+}$ site is empty, then the free cysteines can play another role. However, a thorough investigation carried out in the group of Johnson showed no evidence for any PLP binding or PLP-induced Cys desulfurase activity, even with the enzyme containing only the $(4Fe\text{-}4S)^{2+}$ cluster (*9*).

In our hands, working with the His-tagged biotin synthase cloned in Jarrett's group, we found no effect of cysteine on the activity of the as-isolated enzyme. To observe an activity, it is necessary to add sulfide together with iron to generate the $(4Fe\text{-}4S)^{2+}$ center. In previous experiments performed with an enzyme from another source, we observed a positive effect of cysteine, but this was probably due to a contaminating Cys desulfurase activity. In accord with the absence of a cysteine effect on the HT enzyme, we observed no influence of PLP. With the persulfide hypothesis (*19*), it is difficult to rationalize the absence of turnover, which was explained by the authors, as being due to a strong inhibition of $AdoCH_3$, one of the reaction products (*25*). When $AdoCH_3$ was added to our assay, no inhibition was observed. Thus, we conclude that the mechanism proposed by Fontecave is not valid with our material, and we will further consider the $(2Fe\text{-}2S)^{2+}$ hypothesis.

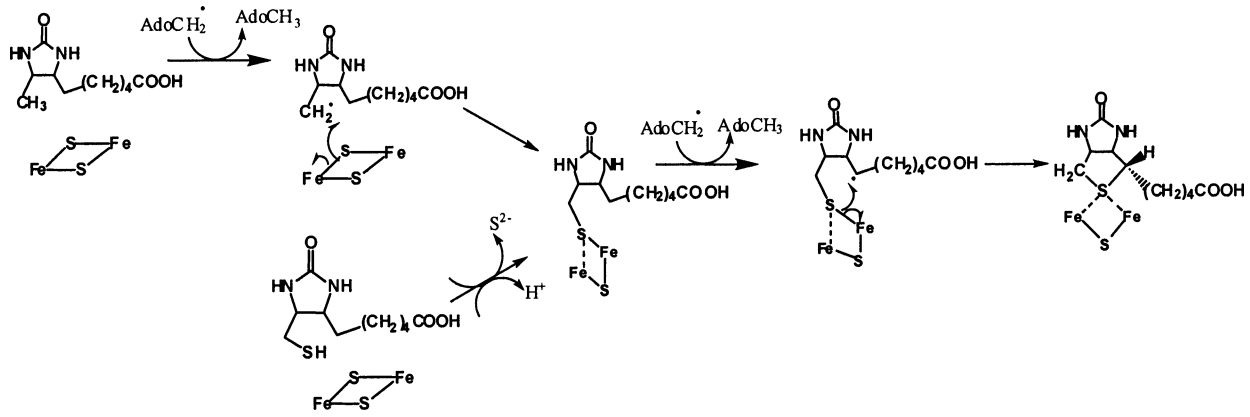
Ugulava *et al*. found a correlation between the absorbance decrease at 460 nm [as a measure of the $(2Fe\text{-}2S)^{2+}$ content] and the production of biotin (*7*). In our Mössbauer study, carried out with a partially purified enzyme, such a quantitative correlation was difficult to establish, but clearly, some $(2Fe\text{-}2S)^{2+}$ was still present at the end of the reaction. The same observation was reported by Jameson *et al*. (*10*). However, the accuracy of the measurements does not allow a safe discussion about this. The kinetic aspects were also examined. Whereas Ugulava *et al*. found that the production of biotin followed the rate of degradation of the $(2Fe\text{-}2S)^{2+}$ center, Jameson *et al*. observed that the initial rate of degradation was much faster than the rate of biotin formation. As they mentioned, one of the possible explanations could be that a subsequent step is rate-limiting, for instance, the formation of the second C−S bond. The results presented in this paper with 9-DTBSH as a substrate seem to confirm this hypothesis (see below).

Contrary to what is cited by Johnson and co-workers (*10*), we have shown that this compound labeled with ^{34}S or ^{35}S was transformed into labeled biotin by resting cells of *Bacillus sphearicus*, whereas both isomers of 6-DTBSH were not (*26*). The proposal that the first step in the activation of DTB is the formation of a radical at C-9 relies on this observation. In our preliminary *in vitro* studies, with a cell-free extract of *B. sphaericus*, we also observed the conversion of [^{34}S]-9-DTBSH to [^{34}S]biotin (*27*). Attempts to isolate this intermediate (or a derivative) failed in our hands (*39*). There are two reports in the literature of its detection by HPLC (*5*, *40*), but these results should be confirmed, since the structure could not be unambiguously established due to the extremely small amounts of product detected. One of these reports (*40*) concerns a study performed with lavender cells, but more recently, the same group found that with *Arabidopsis thaliana*, this intermediate did not accumulate

Scheme 1: Reaction Catalyzed by Biotin Synthase



Scheme 2: Proposed Mechanism for the Formation of Biotin from DTB and 9-DTBSH



in vitro (*41*). We rather think that in *E. coli*, the free thiol is never produced. One can imagine that if the radical at C-9 of DTB is trapped by the cluster, the two moieties remain attached until cyclization takes place. As we assume that cyclization involves the C-6 radical, the free thiol is not a good candidate for an intermediate, since transfer of its hydrogen to C-6 would be the preferred pathway (*42*).

In this work, we have compared DTB and 9-DTBSH using the pure His-tagged enzyme. First, we confirmed that cyclization of 9-DTBSH requires the presence of AdoMet in the assay. This is consistent with our previous findings (*43*) that 2 equiv of AdoMet is necessary to activate the two carbon atoms. The results reported in Figure 5 show that, with 9-DTBSH, i.e., when the sulfur atom is already present in the substrate, the same turnover of one is reached. The reaction stops at this level, and the limitation cannot be overcome. Thus, we assume that the reaction must proceed through an intermediate also present in the pathway starting from DTB. The apparent K_m value which can be estimated from Figure 5 is much higher, roughly 20 times, than that of DTB. When the kinetics at saturating concentrations for both substrates were measured (Figure 6), comparable rates were observed.

Thus, we can propose the following scenario (Scheme 2): the radical generated at C-9 of DTB is trapped by a sulfide bridge of the $(2Fe\text{-}2S)^{2+}$ cluster, and in most systems, the two moieties remain bound until cyclization occurs (*18*). This would explain why free 9-DTBSH cannot be isolated. If 9-DTBSH is used as substrate, one can postulate for instance that the −SH group of DTBSH exchanges with a sulfide bridge of the cluster, leading to a species similar to that produced from DTB. With this hypothesis, the different apparent K_m of 9-DTBSH is not unexpected. If the further cyclization step is rate-determining in both cases, the parallel kinetic behavior of the two substrates can be rationalized. This could explain the same turnover of one, since in this hypothesis, the $(2Fe\text{-}2S)^{2+}$ cluster is also destroyed, even if the sulfur is already present in the substrate. However, this absence of turnover, now found with two substrates, remains an open problem.

To verify whether this single turnover was a consequence of the consumption or degradation of any of the cofactors necessary for activity, fresh ones were added to the assay after one cycle. A further formation of ≈0.4 mol of biotin/mol of monomer was obtained (Figure 3, column 2). If DTT, Fe^{2+}, and S^{2-} were omitted, no additional biotin was produced (column 3). When they were added without the other cofactors (column 4), some biotin was produced, but slightly less than that shown in column 2. The origin of this difference, which is larger than the estimated errors, is not clear. It may depend on the availability of Fe^{2+} and S^{2-}, probably associated with some constituents of the assay mixture.

We then considered a second hypothesis, namely, that the active site was blocked by the destroyed $(2Fe\text{-}2S)^{2+}$ cluster. We have observed by Mössbauer spectroscopy that, after reaction, together with the disappearance of the $(2Fe\text{-}2S)^{2+}$ center, a new species was formed, with parameters representative for high-spin Fe^{3+} species, exhibiting magnetic hyperfine splitting under applied field conditions, but with no detectable EPR signal. It was postulated on this basis that it could represent small superparamagnetic iron clusters (*6*). Johnson and co-workers (*10*) also observed the formation of a new species after turnover, however with different Mössbauer parameters, characterized as $FeIIS_4/N/O$. The binding site of these species has not been established. If we postulate that it remains at the $(2Fe\text{-}2S)^{2+}$ site, it could block it and prevent any cluster reconstitution, even in the presence of an excess of iron and sulfide. If this hypothesis is correct, removal of the Fe^{3+} species should allow the enzyme to perform a new turnover. We tried three different methods for removing this species. The treated enzymes were then made to react for a second cycle, in the presence of all the components of the assay. In all three cases, the recovered

enzymes produced ≈0.4 mol of biotin/mol of monomer. When the whole operation was repeated, negligible activity was measured. This value of 0.4 is difficult to explain: a value of one should have been observed if we had reconstituted the 1:1 $(2Fe\text{-}2S)^{2+}/(4Fe\text{-}4S)^{2+}$ mixture. On the other hand, we have found that in the first run, 1 equiv of biotin per monomer was produced. If we assume that it has consumed one $(2Fe\text{-}2S)^{2+}$ cluster, which cannot be reconstituted, no biotin should be produced in the second run. The production of 0.4 mol of biotin may be due to the remaining $(2Fe\text{-}2S)^{2+}$ center that was observed after the first run in the Mössbauer studies (*6*, *10*). The reasons for its uncomplete consumption are not clear, and as pointed out above, the precision of these experiments is not sufficient to draw definite quantitative conclusions. An only partial reconstitution can also be considered if the protein suffered from some covalent or conformational modification.

A point which still remains under debate is whether the ultimate sulfur donor is the $(2Fe\text{-}2S)^{2+}$ cluster, as postulated by us and by Jarrett, or a protein-bound sulfur, resulting from the degradation of the cluster, as suggested by Johnson. His assumption relies on the observation that protein-bound poly- and persulfides can be generated in the apoprotein following (Fe-S) center degradation as in the case of aconitase (*44*). However, in the cited work, the apoprotein was obtained by oxidation with a large excess of ferricyanide in the presence of EDTA, whereas we are using strong reducing conditions, under which the presence of disulfide bonds or S^0 species is rather unlikely. Thus, we still favor the hypothesis of the $(2Fe\text{-}2S)^{2+}$ center as the ultimate sulfur donor.

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