Mössbauer studies of *Escherichia coli* biotin synthase: evidence for reversible interconversion between $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters

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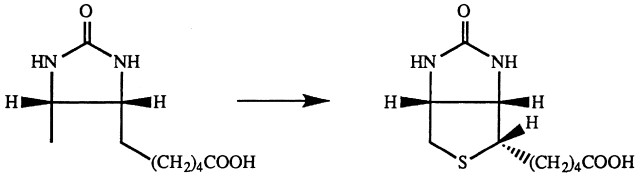
Received 28 July 1999; received in revised form 13 September 1999

Abstract The nature and properties of the iron-sulphur (Fe-S) cluster in as-prepared and reduced biotin synthase of *Escherichia coli* have been investigated by Mössbauer spectroscopy. Our data clearly demonstrate that in the as-prepared sample, the cluster is present as $[2Fe\text{-}2S]^{2+}$ with isomer shift, $\delta = 0.29$ mm/s and quadrupole splitting, $\Delta E_Q = 0.53$ mm/s, indicating incomplete cysteinyl-S coordination. Anaerobic reduction by dithionite in the presence of 55% (v/v) glycerol converts this form to $[4Fe\text{-}4S]^{2+}$ ($\delta = 0.45$ mm/s and $\Delta E_Q = 1.11$ mm/s) and is accompanied by some destruction to Fe^{2+}. This cluster conversion is reversible and when exposed to air, the $[4Fe\text{-}4S]^{2+}$ cluster is quantitatively reconverted to the $[2Fe\text{-}2S]^{2+}$ cluster without any further cluster degradation.

Key words: Biotin synthase; Iron-sulfur cluster; Mössbauer spectroscopy

1. Introduction

The final step of the biosynthesis of biotin which involves the insertion of sulphur into dethiobiotin in catalysed by biotin synthase.



Dethiobiotin **Biotin**

The enzymes from *Escherichia coli* [1] and *Bacillus sphaericus* [2] have been purified and found to be homodimers containing an Fe-S cluster. In vitro activity has an absolute requirement for *S*-adenosylmethionine (AdoMet) [3,4] and an electron source [2,5–7]. It has now been established that biotin synthase belongs to a family of Fe-S enzymes with radical-based mechanisms that include pyruvate formate lyase (PFL) [8,9], anaerobic ribonucleotide reductase (ARNR) [10] and lysine 2,3-aminomutase (LAM) [11]. In all four systems, AdoMet is reduced and cleaved to methionine and a deoxyadenosyl radical [10,12–14]. The Fe-S cluster present in PFL activase [8], ARNR [10] and LAM [15] participates in the one-electron transfer to AdoMet and one can assume that it has the same redox role in biotin synthase [16].

Early spectroscopic studies of aerobically purified *E. coli* biotin synthase revealed the presence of one $[2Fe\text{-}2S]^{2+}$ cluster per monomer. The EPR study of the dithionite reduction product showed a weak signal (0.05–0.1 spin/monomer) attributed to $[2Fe\text{-}2S]^{+}$ [1]. A more detailed investigation of the Fe-S cluster was then carried out by Johnson et al. [17]. They demonstrated using EPR and variable-temperature magnetic circular dichroism (VTMCD) that the cluster signal was due to a $S = 1/2[4Fe\text{-}4S]^{+}$ cluster instead of $S = 1/2[2Fe\text{-}2S]^{+}$.

Furthermore, they deduced from UV-visible absorption and resonance Raman studies that two $[2Fe\text{-}2S]^{2+}$ clusters were converted into one $[4Fe\text{-}4S]^{2+}$ during anaerobic reduction with dithionite in the presence of 60% (v/v) ethylene glycol or glycerol. In this paper, we confirm this result using Mössbauer spectroscopy which allows quantification of the different iron species. We also present convincing evidence of the reversibility of the process upon exposure of the sample to air.

2. Materials and methods

2.1. Materials

The work described here was carried out using *E. coli* TK101 pI3BLS2, which hyperproduces biotin synthase [18]. Purification of the enzyme has been described earlier [1] but after the phenyl-Sepharose step, a chelating Sepharose-Co^{2+} column followed by a FPLC Mono-Q HR 10/10 Pharmacia column, as described for the *B. sphaericus* enzyme [19], was additionally employed to obtain pure protein. All chemicals were purchased either from Sigma, Prolabo, Acros or Aldrich. The sodium dithionite used for the reduction experiment was prepared at a concentration of 70 mM in argonified 50 mM Tris buffer pH 8.0.

2.2. Analytical methods

Protein concentration was measured by the method of Bradford using bovine serum albumin as a standard [20]. Iron was assayed by the method of Fish [21] and inorganic sulphide was quantified as described by Beinert [22].

2.3. Preparation of apo-biotin synthase

Apo-biotin synthase was obtained by reductive degradation of the Fe-S cluster with a 50-fold excess of sodium dithionite and EDTA as already described [16].

2.4. Preparation of $^{57}FeCl_3$

^{57}Fe (18 mg) was converted into its chloride by dissolving it in 1 ml of concentrated (36%) hydrochloric acid at 80°C. A solution of 1 M Trizma base was then added to bring the $^{57}FeCl_3$ to pH 7.5. The mixture was finally diluted with Tris buffer 50 mM pH 7.5 to con-

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Abbreviations: Fe-S, iron-sulphur cluster; AdoMet, *S*-adenosylmethionine; PFL, pyruvate formate lysate; ARNR, anaerobic ribonucleotide reductase; LAM, lysine 2,3-aminomutase; EPR, electron paramagnetic resonance; VTMCD, variable-temperature magnetic circular dichroism; DTT, dithiothreitol; FNR, fumarate nitrate reduction

0014-5793/99/$20.00
PII: S0014-5793(99)01300-9

stitute a 20 mM stock solution. Aliquots were frozen and stored at −20°C until use.

2.5. Chemical reconstitution of ^{57}Fe-S centre into apoenzyme

Aliquots (500 μl) of apo-biotin synthase (213 μM), 200 mM dithiothreitol (DTT), 20 mM $^{57}FeCl_3$ and 20 mM Na_2S were deoxygenated in separate Eppendorf tubes under a stream of wet argon for 30 min. After this time, DTT was added to the apoenzyme to a final concentration of 5 mM and this mixture was incubated for a further 30 min. Four-fold excesses of $^{57}FeCl_3$ and Na_2S with respect to the enzyme were then added to the solution. The mixture was allowed to proceed for 90 min at room temperature after which EDTA (2 mM final concentration) in buffer was added and incubated for 30 min to chelate the free iron. The mixture was desalted on a PD 10 column (Pharmacia) with Tris 50 mM pH 7.5 containing 2 mM DTT and concentrated on a Centricon 30 microconcentrator (Amicon).

2.6. Mössbauer spectroscopy

Mössbauer spectra were recorded using a conventional 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). The spectra obtained in a small applied field (20 mT) were simultaneously fitted with the same set of four subspectra, i.e. for $[2Fe\text{-}2S]^{2+}$, for $[4Fe\text{-}4S]^{2+}$, and for two slightly different Fe^{2+} sites. The high-field spectra (7 T) were fitted only according to their diamagnetic contributions from $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$, while the unspecified paramagnetic background was kept constant, i.e. 24% for Fig. 1C′ as derived from the corresponding measurement in the small applied field (Fig. 1C) and 24% for Fig. 1D′.

3. Results

The 4.2 K Mössbauer spectrum of 0.8 mM ^{57}Fe-reconstituted biotin synthase (1.7 Fe and 1.7 S^{2-}) in the presence of 55% (v/v) glycerol, recorded in a small external field of 20 mT (Fig. 1A) exhibits a doublet with quadrupole splitting $\Delta E_Q = 0.53$ mm/s and isomer shift $\delta = 0.29$ mm/s. These parameters are typical for $[2Fe\text{-}2S]^{2+}$ clusters with incomplete cysteinyl-S coordination. When this enzyme is reduced anaerobically with 2 equivalents of dithionite for 20 min, the $[2Fe\text{-}2S]^{2+}$ clusters are partially converted to $[4Fe\text{-}4S]^{2+}$ clusters ($\delta = 0.45$ mm/s and $\Delta E_Q = 1.11$ mm/s) with the additional formation of Fe^{2+} (Fig. 1B). This spectrum can be represented as a superposition of four components, i.e. contributions from the native $[2Fe\text{-}2S]^{2+}$ clusters, from $[4Fe\text{-}4S]^{2+}$ clusters and from two different Fe^{2+} species with $\Delta E_Q = 3.2$ and 3.3 mm/s and $\delta = 0.89$ and 1.4 mm/s. The latter two account for 26% of total iron and are probably due to cluster destruction (Table 1).

In order to follow the reduction with time, the sample was subsequently thawed in a glove box for a further 30 min, 1 h, 3 h 20 min (Fig. 1C) and 16 h at room temperature. This resulted in an increase of $[4Fe\text{-}4S]^{2+}$ at the expense of $[2Fe\text{-}2S]^{2+}$ clusters without further degradation into Fe^{2+}. Table 1 summarises the iron concentration corresponding to the two cluster species occurring during the time course of reduction. After the third step (equivalent to 1 h 50 min) no significant reduction seemed to occur any more and the proportion of $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ remained practically constant; the $[2Fe\text{-}2S]^{2+}$ cluster then account for 48 (2)%, the $[4Fe\text{-}4S]^{2+}$ cluster for 27 (2)% and the Fe^{2+} species for 26 (2)% of the overall iron content. The incomplete reduction may be due to an insufficient amount of dithionite but we decided not to add more so as not to cause further degradation of the remaining

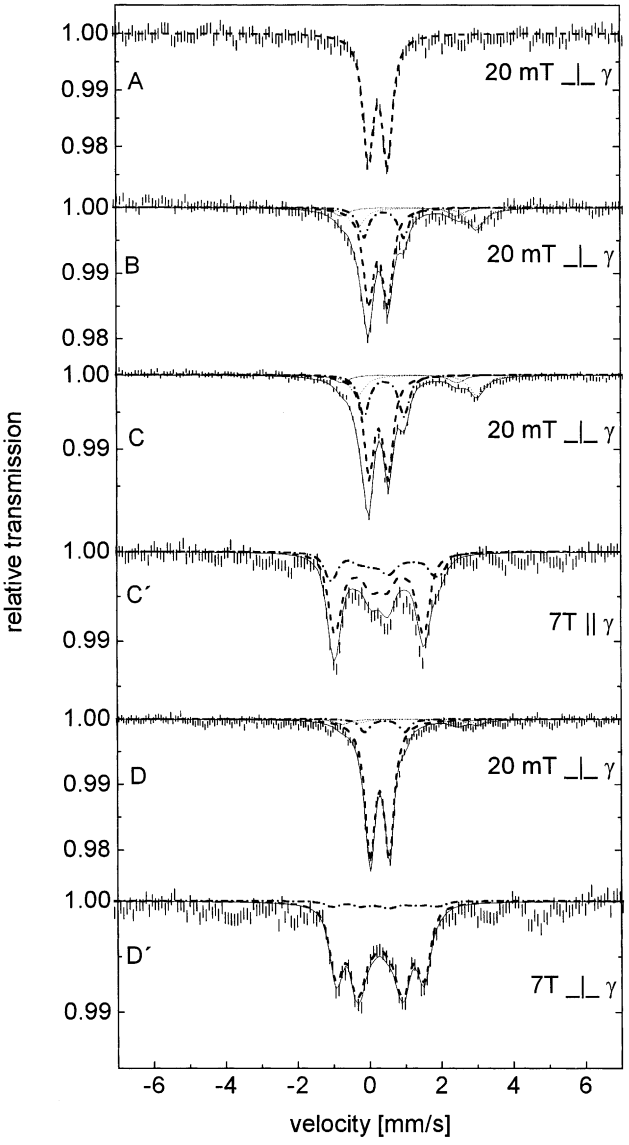

Fig. 1. Mössbauer spectra recorded at 4.2 K of biotin synthase, (A) as isolated; (B) after 20 min anaerobic reduction with 2 equivalent dithionite at room temperature; (C) after additional 4 h 50 min reaction time; (C′) same reaction conditions as in (C), spectrum recorded in a high field as indicated; (D) reduced sample exposed to air for 6 h 30 min; (D′) same reaction conditions as in (D), spectrum recorded in a high field as indicated. The subspectrum of the $[2Fe\text{-}2S]^{2+}$ cluster (dashed lines) was simulated with $\delta = 0.29$ mm/s and $\Delta E_Q = 0.52$ mm/s. The subspectrum of the $[4Fe\text{-}4S]^{2+}$ cluster (dashed dotted lines) was simulated with $\delta = 0.45$ mm/s and $\Delta E_Q = 1.11$ mm/s. The remaining subspectra in B–D belong to Fe^{2+}. The missing parts of total area in C′ belonging to paramagnetic Fe^{2+} species (24%) and in D′ belonging to Fe^{2+} and Fe^{3+} (24%) were not explicitly simulated. The fit of spectrum C′, recorded in high applied field, yielded for the asymmetry parameter η the values $\eta = 1$ for $[2Fe\text{-}2S]^{2+}$ and $\eta = 0.6$ for $[4Fe\text{-}4S]^{2+}$. The same values are used for simulating the two subspectra in D′.

clusters. The assignment of subspectra to $[2Fe\text{-}2S]^{2+}$, $[4Fe\text{-}4S]^{2+}$ and Fe^{2+} is evident from the fitted δ and ΔE_Q parameters [23,24] and was further confirmed by testing the magnetic properties of the various species. The diamagnetism of $[2Fe\text{-}2S]^{2+}$ (53 (2)% of the iron content) and $[4Fe\text{-}4S]^{2+}$ (23 (2)% of the iron content (Fig. 1C′) was established by application of an external field (4.2 K, 7 T∥γ); the paramagnetic

Table 1
Percentage of the observed Fe content, distributed over different species, during the time course of reduction followed by subsequent air reoxidation

Reduction	Fig. 1	$[2Fe\text{-}2S]^{2+}$	$[4Fe\text{-}4S]^{2+}$	Fe^{2+}
As isolated ($t=0$)	A	100	0	0
20 min[a]	B	56	18	26
+30 min[a]	not shown	52	22	26
+1 h[a]	not shown	48	27	26
+3 h 20 min[a,b]	C, C′	53	23	24
+16 h[a]	not shown	49	29	22
Reoxidation	Fig. 1	$[2Fe\text{-}2S]^{2+}$+unspecified Fe^{3+}	$[4Fe\text{-}4S]^{2+}$	Fe^{2+}
30 min[a]	not shown	50	27	23
+1 h[a]	not shown	61	20	19
+2 h[a]	not shown	70	13	17
+3 h[a]	D	82	9	9
Reoxidation	Fig. 1	$[2Fe\text{-}2S]^{2+}$	$[4Fe\text{-}4S]^{2+}$	Fe^{2+}+Fe^{3+}
same as D[a]	D′[c]	70	6	24

[a]The precision of the calculated percentages is of the order of 2%.
[b]Relative amounts additionally established by measurement in applied field (Fig. 1C′).
[c]Measurement in applied field discriminates diamagnetic $[2Fe\text{-}2S]^{2+}$ and paramagnetic unspecified Fe^{3+}, which are lumped together in D.

Fe^{2+} species which were not explicitly simulated in Fig. 1C′ accounted for 24% of the iron content (Fig. 1C).

Previously, Johnson and collaborators described that $[4Fe\text{-}4S]^{2+}$ was partially reconverted to $[2Fe\text{-}2S]^{2+}$ upon air exposure. Their assumption was based on a comparison of the UV-visible absorption and resonance Raman spectra of the air reoxidised sample with that of an as-prepared sample [17,25].

We also checked the behaviour of the sample during reoxidation using Mössbauer spectroscopy. The reduced sample was thawed and exposed to air, with stirring, for 30 min, then for a further 1 h, 2 h and 3 h (Fig. 1D). Table 1 summarises the reconversion of $[4Fe\text{-}4S]^{2+}$ to $[2Fe\text{-}2S]^{2+}$ and the simultaneous oxidation of unspecified Fe^{2+} to Fe^{3+} during the time course of reoxidation. Measurement in applied field makes it possible to discriminate diamagnetic $[2Fe\text{-}2S]^{2+}$ clusters from paramagnetic unspecified Fe^{3+} species (Fig. 1D′). Switching the direction of the applied field in D′ to a perpendicular orientation with respect to the γ-beam, as compared to C′ where the applied field was parallel to the γ-beam, provides an additional means for discriminating $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters due to their different asymmetry parameters η, i.e. $\eta=1$ for $[2Fe\text{-}2S]^{2+}$ and $\eta=0.6$ for $[4Fe\text{-}4S]^{2+}$ (see Fig. 1C′). The overall symmetric line intensity pattern of the four absorption lines (corresponding to $\eta=1$) in Fig. 1D′ in the velocity range from −2 mm/s to +2 mm/s proves that the diamagnetic part of the spectrum mainly represents $[2Fe\text{-}2S]^{2+}$ clusters. Thus, after a total time of 6 h 30 min, there was almost quantitative backconversion of $[4Fe\text{-}4S]^{2+}$ to $[2Fe\text{-}2S]^{2+}$, while the amount of paramagnetic iron (Fe^{2+} and Fe^{3+}) remained constant (Table 1).

We also tested the possibility of $[4Fe\text{-}4S]^{2+}$ cluster degradation to $[3Fe\text{-}4S]^{0}$ or $[3Fe\text{-}4S]^{1+}$ clusters during the course of reoxidation. Using the relevant spin-Hamiltonian parameters for these clusters [26,27], we simulated spectra for 4.2 K and an applied field of 7 T⊥γ (not shown) and compared them with the line intensities of the measured spectrum (Fig. 1D′). Especially the measured line intensity ratios of lines at velocities −4 mm/s, −2 mm/s, +0.3 mm/s and +3 mm/s do not allow an assignment to 3Fe clusters. However, because of the modest resolution on the left and right velocity range of the central four lines we do not rule out a contribution from 3Fe clusters to the magnetic hyperfine pattern in Fig. 1D′ smaller than 5% of the total iron content.

4. Discussion

This study of *E. coli* biotin synthase using Mössbauer spectroscopy brings new results, complementary to those obtained by Johnson and coworkers [17] relying on UV-visible, EPR, resonance Raman and VTMCD techniques.

We confirm that the semi-anaerobically isolated enzyme contains a $[2Fe\text{-}2S]^{2+}$ cluster and that its reduction with dithionite in the presence of 55% (v/v) glycerol leads to its conversion into a $[4Fe\text{-}4S]^{2+}$ centre. Contrary to other techniques, the simulation of Mössbauer spectra which are measured in small and in high applied fields allows the quantification of the different species. It is clearly seen that the $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ conversion is accompanied by cluster destruction leading to about 25% unspecified paramagnetic iron (Fe^{2+}) species (Table 1) which, according to their Mössbauer parameters (δ and ΔE_Q) are penta- and hexa-coordinated and most likely non-sulphur ligated.

Reoxidation with air of the reduced sample induces a reverse conversion of $[4Fe\text{-}4S]^{2+}$ into $[2Fe\text{-}2S]^{2+}$, as already stated by Johnson and collaborators [17,25]. We were able to establish that the conversion is almost quantitative with the amount of unspecified paramagnetic iron (Fe^{2+} and Fe^{3+}) remaining constant. The isomer shift and quadrupole splitting values of the re-formed $[2Fe\text{-}2S]^{2+}$ cluster are exactly the same as those of the native enzyme, indicating the same ligand coordination in the native and reoxidised enzyme. This is contradictory to the results obtained from resonance Raman study in which different spectra were obtained for the as-isolated and reoxidised samples, the authors suggesting that the re-formed clusters could be differently ligated [25].

The observed isomer shift ($\delta=0.29$ mm/s) of the native enzyme indicates an incomplete cysteinyl-S coordination in agreement with resonance Raman spectroscopy [17], but the nature of the other ligand(s), either O or N, cannot be determined. However, this value lies between the isomer shifts of the two Fe^{3+} sites of the oxidised Rieske protein ($\delta=0.24$ mm/s for 4S coordination and $\delta=0.32$ for 2S and 2N coordination) [28]. Concerning the $[4Fe\text{-}4S]^{2+}$ species, the isomer

shift does not indicate deviation from complete cysteinyl coordination. The nature of the ligands is more easily deduced from resonance Raman spectra and on this basis, Johnson et al. [17] propose for $[2Fe\text{-}2S]^{2+}$ three cysteinyl and one oxygenic ligands and for the $[4Fe\text{-}4S]^{2+}$ cluster complete cysteinyl coordination.

The mechanism of the $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ conversion, either dimerisation or decomposition followed by reassembly, is still an open question. We observed some destruction of the cluster during the first 20 min of the reduction but on the other hand, no further degradation was apparently observed during reoxidation. The localisation of $[4Fe\text{-}4S]^{2+}$ within a monomer or bridging subunits [17] has not yet been determined either.

The same $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ conversion also occurred during reduction of PFL activase [9] and ARNR β_2 subunits [24]. For PFL activase, the reversibility has not been demonstrated but the presence of $[2Fe\text{-}2S]^{2+}$ in the as-prepared enzyme was attributed to oxidation. For ARNR, it has very recently been shown that during exposure to air, the 4 Fe clusters are converted back to 2 Fe cluster [26]. This phenomenon was also observed in the *E. coli* transcription factor FNR (fumarate nitrate reduction) [29] where it plays a very important physiological role as oxygen sensor. A already suggested [25], this type of cluster conversion might also have the same function in the AdoMet-dependent enzymes to regulate enzyme activity in response to oxidation, without irreversible cluster degradation. Mössbauer study is lacking in the case of PFL activase but it is interesting to point out that the Mössbauer parameters of $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ are nearly the same for biotin synthase, ARNR β subunit [24] and FNR [29].

The nature of the Fe-S centre of the active biotin synthase also remains to be established, although as already pointed out [17], it is likely that the $[4Fe\text{-}4S]^{2+}$ cluster present in the reduced state is the active form. We have recently shown that the sulphur source in biotin is probably the Fe-S cluster itself [16], the enzyme being at the same time substrate. This unprecedented process remains to be mechanistically understood and we hope that Mössbauer spectroscopy will allow us to detect changes in the coordination and (or) oxidation state of the cluster iron in the presence of the different cofactors of the reaction.

Acknowledgements: A.X.T. and A.M. respectively acknowledge the financial support by the DFG and the CNRS and MENRT.

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