The lipoate synthase from *Escherichia coli* is an iron-sulfur protein

Sandrine Ollagnier-de Choudens, Marc Fontecave*

Laboratoire de Chimie et Biochimie des Centres Rédox Biologiques, DBMS-CB, CEA/CNRS/Université Joseph Fourier, 17 Avenue des Martyrs, 38054 Grenoble, Cedex 09, France

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Abstract Lipoate synthase catalyzes the last step of the biosynthesis of lipoic acid in microorganisms and plants. The protein isolated from an overexpressing *Escherichia coli* strain was purified from inclusion bodies. Spectroscopic (UV-visible and electron paramagnetic resonance) properties of the reconstituted protein demonstrate the presence of a (2Fe-2S) center per protein. As observed in biotin synthase, these clusters are converted to (4Fe-4S) centers during reduction under anaerobic conditions. The possible involvement of the cluster in the insertion of sulfur atoms into the octanoic acid backbone is discussed.

Key words: Lipoate synthase; Iron-sulfur protein; C-S bond formation; Electron paramagnetic resonance

1. Introduction

Lipoic acid, 6,8-thiooctic acid, is a widely distributed protein-bound dithiol-containing cofactor found in most prokaryotic and eukaryotic microorganisms as well as in many plant and animal tissues [1]. In *Escherichia coli*, it is required in the energy metabolism for the activity of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and glycine cleavage enzyme complexes [2–4]. Several isotope experiments have suggested that in *E. coli*, octanoic acid is the direct precursor of lipoic acid and that 8-thiooctanoic and 6-thiooctanoic acids are possible intermediates [5]. Furthermore, molecular genetic studies have demonstrated that the *lipA* gene product, named lipoate synthase (LS), is involved in the insertion of at least one of the sulfur atoms into the octanoate backbone [6]. Whether a second enzyme is required for the insertion of the second sulfur atom is still unclear. A highly homologous enzyme from *Arabidopsis thaliana* has recently been cloned and the corresponding cDNA proved to be able to complement a *lipA* mutant of *E. coli* defective in lipoic acid synthase [7].

While a great deal is known on the genetics of the lipoic acid synthase system, very little is known on the enzyme itself and on the reaction mechanism. The enzyme was not available in a pure form and an in vitro assay system for lipoic acid synthesis has not been established.

Conversion of C-H bonds into C-S bonds is a fascinating chemical problem and lipoic acid synthase provides an interesting example of a biological solution to that challenge. A similar reaction, the insertion of a sulfur atom into dethiobiotin, is catalyzed by the biotin synthase (BS). This enzyme is a (2Fe-2S) protein, in which the iron atoms are supposed to be chelated by cysteines from the CXXXCXXC sequence, in the N-terminal part of the protein [8]. The fourth ligand is still not identified. This sequence has been shown to be present in the anaerobic ribonucleotide reductase (ARR) [9], pyruvate formate lyase (PFL) activating enzyme [10] and in the transcription factor FNR [11], which all are iron-sulfur proteins. As this sequence is also present in the *lipA* gene product [6], a likely working hypothesis is that LS is also an iron-sulfur protein.

Here, we report the purification of the LipA protein and some of its spectroscopic properties which, for the first time, unambiguously show that LS is an iron-sulfur enzyme.

2. Materials and methods

2.1. Materials

A BL21(DE3) *E. coli* strain was obtained from Promega. Plasmid pKR116, carrying the LS gene (*lipA*) [6] was a gift from S. Cronan laboratory (Illinois University). Isopropyl-β-D-thiogalactopyranoside was from Eurogentec, $^{56}Fe(NH_4)_2(SO_4)_2$ and Na_2S were from Aldrich, sodium dithionite was from Fluka and 5-deaza-7,8-demethyl-10-methyl-isoalloxazine (5-DAF) was available in the authors' laboratory.

2.2. Bacterial growth

BL21(DE3)pKR116 cells were grown in LB medium (1.5 l) containing 0.2% glucose and 100 μg/ml ampicillin at 37°C under aeration. When the A_{600} reached 0.4–0.5, the culture was induced with 1 mM isopropyl-β-D-galactoside. After 3 h of growth, the cells (5 g) were centrifuged, washed twice with 50 mM Tris-HCl pH 7.5, resuspended in 25 ml of 50 mM Tris-HCl pH 7.5, 5 mM dithiothreitol (DTT) and sonicated. After centrifugation at 14 000 rpm, 4°C, for 30 min, both the pellet and supernatant were analyzed by SDS-PAGE.

2.3. Purification of the LipA protein

The pellet was dissolved in denaturing buffer (50 mM Tris-HCl pH 7.4, 6 M guanidine-HCl, 100 mM DTT) in order to reach a final protein concentration of 5 mg/ml. The solution was maintained at room temperature for 1 h. Unsoluble material was discarded by centrifugation and the clear supernatant loaded onto a Superdex-75 gel filtration column, 16/60 (Pharmacia Biotech) previously equilibrated with 50 mM Tris-HCl pH 7.4, 6 M guanidine-HCl, 5 mM DTT. Protein fractions were eluted at 0.8 ml/min with the same buffer using the FPLC system. Fractions corresponding to purified denaturated LS, as judged by SDS-PAGE, were pooled and used for subsequent refolding experiments. The typical yield of such a step from 5 g of cells is 40 mg of protein.

2.3.1. Refolding of denaturated LS. Denaturated LS was allowed to refold by dilution of the protein solution to 40 μg/ml in 6 M guanidine-HCl pH 7.4 buffer. Refolding was performed at 4°C under aerobic conditions by dialysis against 50 mM Tris-HCl pH 8.6 buffer during 14-16 hours. After dialysis, the refolded protein was cleared by

*Corresponding author. Fax: (33) (4) 76889124.
E-mail: fontecav@cbcrb.ceng.cea.fr

Abbreviations: LS, lipoate synthase; BS, biotin synthase; ARR, anaerobic ribonucleotide reductase; PFL, pyruvate formate lyase; EPR, electron paramagnetic resonance; DTT, dithiothreitol

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centrifugation and concentrated to 2 ml by ultrafiltration with a Centricon-30 (Amicon) device. Approximately 28 mg of protein were then recovered.

2.3.2. Superdex-75 filtration. Finally, LS was chromatographed using a Superdex-75 column equilibrated with 50 mM Tris-HCl pH 8.6, at 0.8 ml/min. Fractions of 2 ml were collected and those corresponding to pure LS were pooled and frozen in liquid nitrogen. 18 mg of pure protein was recovered under these conditions.

2.4. Reconstitution of LS

Reconstitution was done at 4°C under anaerobic conditions. The protein solution was continuously purged with argon on a manifold and treated in an ice bath under strict anaerobiosis for 3 h with 5 mM DTT and a six molar excess each of Na_2S and $^{56}Fe(NH_4)_2(SO_4)_2$, added in that order. After addition of iron, the solution became brown. Then, 2 mM EDTA was added and the solution was further incubated for 30 min. The capped solution is then transferred to an anaerobic hood and passed through a G25 column (50 ml) equilibrated with 100 mM Tris-HCl pH 8. The colored fractions were concentrated aerobically over a YM10 Diaflo membrane (Amicon), with recovery of 80% of the protein.

2.5. Analysis

The protein concentration was determined by the method of Bradford [12], standardized by amino acid analyses. Protein-bound iron was determined under reducing conditions with bathophenantroline disulfonate after acid denaturation of the protein [13] and labile sulfide by Beinert's method [14].

2.6. Electron paramagnetic resonance (EPR) spectroscopy

The EPR first derivative spectrum was recorded on a Varian E109 (9.5 GHz) EPR spectrometer equipped with an ESR 900 helium flow cryostat (Oxford Instruments). The double integral of the EPR signal was evaluated by using a computer on-line with the spectrometer. The spin concentration in the protein sample was determined by calibrating double integration of the EPR spectrum with a standard sample of a Cu-EDTA (100 μM).

A reduction experiment was performed inside a glove box (Jacomex BS531 NMT) in a N_2 atmosphere containing less than 2 ppm O_2. Stock solutions of dithionite were prepared in 30 mM Tris-HCl pH 8. 5-DAF was dissolved in DMSO, diluted with water to 500 μM and stored inside the box in the dark. LS (70 μM) was prepared in 30 mM Tris-HCl pH 8. Then, either dithionite (5–10 molar excess) or 5-DAF (catalytic amount) were added to the protein solution. After 60 min incubation with dithionite or irradiation with 5-DAF, EPR tubes were frozen in liquid nitrogen inside the box and analyzed.

2.7. UV-visible absorption

UV-visible spectra of the LS, in 30 mM Tris-HCl pH 8, were recorded with a Cary 1 Bio (Varian) spectrophotometer.

3. Results

3.1. Purification and characterization of the product of the lipA gene

pKR116 which carries the *lipA* gene under the control of the T7 promoter was used to transform *E. coli* BL21(DE3) cells. Induction with IPTG resulted in the overexpression of a 36 kDa polypeptide as observed by SDS-PAGE of whole cells. The N-terminal sequence of the overexpressed protein was determined, confirming its identity to the product of the *lipA* gene. Unfortunately, the overproduced LipA protein formed insoluble aggregates which appeared almost exclusively into the pellet of centrifuged bacterial extracts. Even when the cells were induced at lower temperatures or lower IPTG concentrations or in other DE3 strains, the formation of inclusion bodies, containing almost exclusively LipA, could not be decreased. Thus, the protein was purified from the inclusion bodies, after solubilization with 6 M guanidine-HCl pH 7.4, 100 mM DTT.

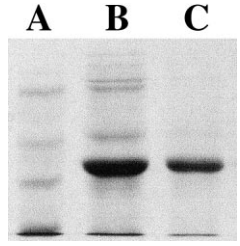

Fig. 1. SDS gel electrophoresis of LS after renaturation of the protein extracted from inclusion bodies (B) and after purification on a Superdex-75 column (C). (A) Size markers (67, 43, 30 and 20 kDa, from the top to the bottom).

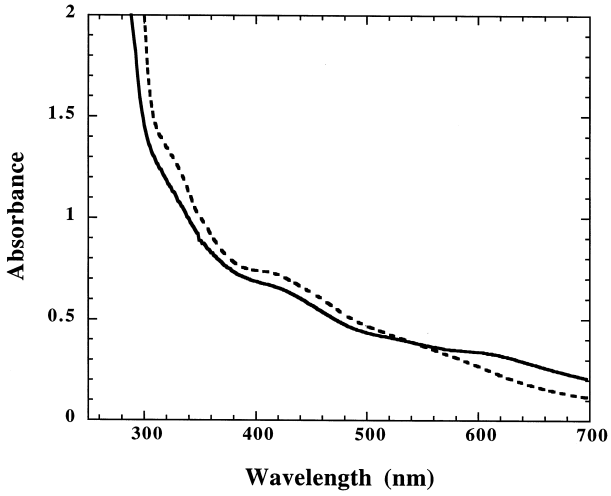

Fig. 2. UV-visible light absorption spectra of the as-isolated protein (300 μM, 0.56 Fe/protein) (dashed line) and the reconstituted (60 μM, 2.3 Fe/protein) (solid line) LS in 100 mM Tris-HCl pH 8.

The solubilized protein was first purified by gel filtration on a Superdex-75 column equilibrated and eluted with 50 mM Tris-HCl pH 7.4, 6 M guanidine-HCl, 5 mM DTT. Renaturation was obtained by dialyzing the protein against 50 mM Tris-HCl pH 8.6. Then, a second Superdex-75 chromatographic step, run with the same buffer, led to a 95% pure soluble protein, as shown by SDS gel electrophoresis (Fig. 1). From 5 g of cells, 18 mg of pure LipA protein could be obtained.

The protein was slightly brownish and was shown to contain comparable amounts of iron and sulfide (0.5–0.6 atoms per polypeptide chain) as shown from specific iron and sulfide quantitation assays [13,14]. This strongly suggested the presence of an iron-sulfur center in the protein as expected from the sequence similarities with the ARR and the BS. In order to reconstitute a full iron-sulfur center, the protein was incubated anaerobically with a small excess of iron and sulfide for 180 min and desalted on a Sephadex-G25 column, under conditions that we previously used to reconstitute the iron-sulfur center of the ARR. As found for the ARR, reconstituted LS could not retain more than 1.8–2.3 Fe and S atoms per polypeptide chain.

3.2. Spectroscopic properties of the iron-sulfur center of LS

3.2.1. Light absorption spectroscopy. Fig. 2 shows the light absorption spectra of both as-isolated (0.56 Fe per protein) and reconstituted (2.3 Fe per protein) LS. Both display sulfide to iron charge transfer bands at 420 nm ($\varepsilon = 4500\ M^{-1}\ cm^{-1}$).

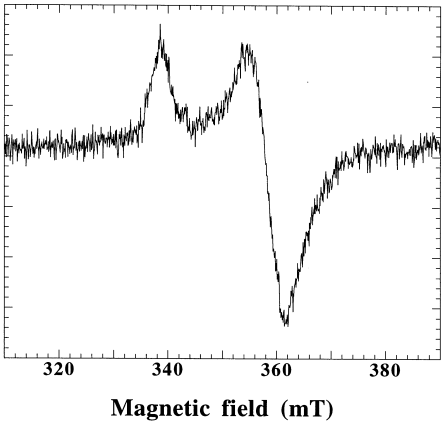

Fig. 3. X-band EPR spectrum of the reduced reconstituted LS (70 μM) in 100 mM Tris-HCl pH 8. Temperature: 10 K, microwave power: 0.1 mW, amplitude modulation, 10 Gauss, receiver gain: 7×10^5.

The reconstituted protein contains additional bands at 540 ($\varepsilon = 2800$ M^{-1} cm^{-1}) and 600 nm ($\varepsilon = 2500$ M^{-1} cm^{-1}), the intensities and energies of which are consistent with the presence of an iron-sulfur center. This spectrum looks very much like that of the oxidized ARR and it is quite similar to other $(2Fe\text{-}2S)^{2+}$ proteins. During anaerobic reduction, a bleaching of the solution is observed with loss of the visible absorption bands.

3.2.2. EPR spectroscopy. Both as-isolated and reconstituted proteins were EPR silent. However, reduction with dithionite or photoreduced 5-DAF under anaerobic conditions generated a $S = 1/2$ species, characterized by an axial EPR signal with g values at $g = 2.03(8)$ and 1.92(5) (Fig. 3). The temperature dependence and the microwave power saturation properties of the signal were in agreement rather with a reduced $(4Fe\text{-}4S)^{+}$ center than with a $(2Fe\text{-}2S)^{+}$ center. The signal integrates for 30% of the total iron contained in the protein, showing that the iron atoms have not been fully reduced.

4. Discussion

LS from *E. coli* has been purified from inclusion bodies generated during growth of an *E. coli* strain overexpressing the *lipA* gene product. Thus, for the first time, a purified soluble form of the enzyme could be obtained, allowing its characterization. It is quite remarkable that after denaturation and refolding, significant amounts of iron and sulfide were still bound to the protein. In fact, anaerobic incubation with iron and sulfide generated an enzyme which could chelate two Fe and two S atoms per polypeptide chain. We take the observation that the protein has retained its capacity to bind iron and sulfide as an evidence that it folded correctly during the renaturing process. In agreement with the presence of iron and sulfide, the protein is dark brown and displays a band at 420 nm in its light absorption spectrum, characteristic of a sulfide to iron charge transfer. Finally, during reduction under anaerobic conditions, a $(4Fe\text{-}4S)^{+}$ center is generated, as shown from the appearance of a characteristic EPR signal.

We thus suggest that LS contains (2Fe-2S) centers which during reduction are converted into (4Fe-4S) centers, at least partially.

It is thus obvious that the iron-sulfur cluster of LS has many similarities with the iron-sulfur centers of BS, PFL activating enzyme and the ARR. All these enzymes have the properties to assemble both (2Fe-2S) and (4Fe-4S) clusters depending on the redox conditions [15–17]. Also in those cases, iron cannot be fully reduced probably due to a very low redox potential of the (4Fe-4S) cluster. If we add the information that a CXXXCXXC motif, probably involved in Fe chelation, is common to all four proteins, it is tempting to suggest that they all belong to the same class of enzymes.

So far, we have not been able to assay the purified protein reported here for lipoic acid synthesis. However, considering the structural similarities discussed above, it is a likely working hypothesis that LS also shares catalytic properties with PFL activating enzyme and ARR and even more with BS since the latter also catalyzes a C-H bond to C-S bond conversion.

PFL activase, ARR and BS use their iron-sulfur center to catalyze the one-electron reduction of *S*-adenosylmethionine as a mechanism for generating a putative 5′-deoxyadenosyl radical [18–20]. The latter is supposed to abstract a hydrogen atom either on a glycine residue of the protein (ARR, PFL) or on the substrate (BS). A similar mechanism might be operative in lipoic acid synthesis with the intermediate formation of an octanoate radical, as the precursor of 6- or 8-thiooctanoic acid.

An intriguing problem remains in the case of BS and LS, the origin of the sulfur atom. Recent studies have made the interesting suggestion that in the case of BS, the Fe-S center might be the actual S donor [21]. The availability of a new enzyme, LS, might be useful for better understanding the biological C-S bond formation reactions.

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