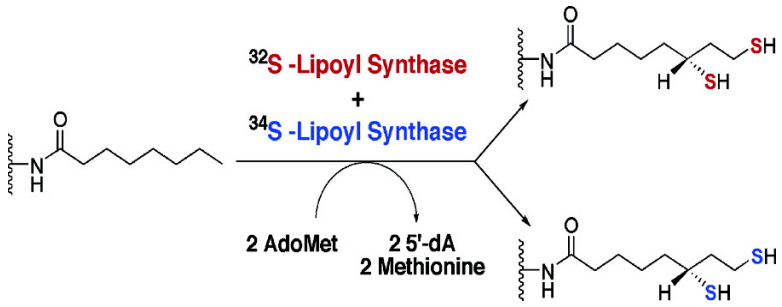
Communication

Mechanistic Investigations of Lipoic Acid Biosynthesis in *Escherichia coli*: Both Sulfur Atoms in Lipoic Acid are Contributed by the Same Lipoyl Synthase Polypeptide

Robert M. Cicchillo, and Squire J. Booker

J. Am. Chem. Soc., **2005**, 127 (9), 2860-2861• DOI: 10.1021/ja042428u • Publication Date (Web): 11 February 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 9 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 02/11/2005

Mechanistic Investigations of Lipoic Acid Biosynthesis in *Escherichia coli*: Both Sulfur Atoms in Lipoic Acid are Contributed by the Same Lipoyl Synthase Polypeptide

Robert M. Cicchillo and Squire J. Booker*

Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received December 16, 2004; E-mail: sjb14@psu.edu

Lipoyl synthase (LS) catalyzes the final step in the de novo biosynthesis of the lipoyl cofactor, which is the insertion of two sulfur atoms into an 8-carbon-saturated fatty acyl chain at C-6 and C-8 (Figure 1).[1,2] Genetic and recent biochemical studies indicate that the enzyme is a member of a newly characterized superfamily of metalloproteins that utilize a $[4Fe-4S]^{+}$ cluster to reductively fragment *S*-adenosyl-L-methionine (SAM) into L-methionine and a 5′-deoxyadenosyl radical (5′-dA•).[3−6] This high-energy radical then initiates catalysis by abstracting a key hydrogen atom from the relevant substrate, or it abstracts a hydrogen atom from the α-carbon of a glycine residue on a cognate protein, creating a stable glycyl radical cofactor.[7] In the LS reaction, the octanoyl acyl chain substrate is attached covalently in an amide linkage to a conserved lysine residue on the protein that is to bear the lipoyl cofactor, indicating that the cofactor is constructed "on site".[2,8] In *Escherichia coli*, three proteins are known to house the lipoyl cofactor: the H protein of the glycine cleavage system, and the E_2 subunits of the pyruvate and α-ketoglutarate dehydrogenase complexes.[9]

Recent studies have provided strong evidence that the active form of LS contains two [4Fe−4S] clusters per polypeptide. One of the clusters is coordinated by cysteines residing in the CX_3CX_2C motif that is common to all radical SAM enzymes, while the other cluster is coordinated by cysteines residing in a CX_4CX_5C motif, which is found only among lipoyl synthases.[10] The presence of two distinct iron−sulfur clusters on LS is reminiscent of biotin synthase (BS), in which the active form of the enzyme is proposed to contain a [2Fe−2S] cluster in addition to the [4Fe−4S] cluster that interacts with SAM.[11] The reaction mechanisms of the two enzymes are presumably similar, given the similarities in reaction type and primary structures (32% sequence similarity);[3] although, BS catalyzes the insertion of only one sulfur atom, between C-6 and C-9 of dethiobiotin, while LS catalyzes the insertion of two sulfur atoms into the octanoyl chain. Although there is not yet conclusive evidence that the additional cluster provides the sulfur in each enzyme system, it is tempting to speculate that the different configurations of the additional cluster somehow relate to the number of sulfur atoms that are mobilized during catalysis.

It has been proposed that during catalysis by LS, the 5′-dA• abstracts hydrogen atoms from C-6 and C-8 of the octanoyl chain, allowing for subsequent sulfur insertion by a mechanism involving carbon-centered radicals.[6,8,12] Consistent with this premise, we have shown recently that synthesis of one lipoyl cofactor requires the expenditure of two molecules of SAM, and that deuterium from [octanoyl-d_{15}]-H protein is transferred to the resulting 5′-dA.[8] In analogy to BS, LS does not catalyze multiple turnovers; 50 μM LS affords only 18 μM of the lipoyl cofactor over 30 min at 37 °C in a kinetic process that displays a pseudo-first-order rate constant of 0.175 min^{-1}.[8] Two hypotheses have been envisaged to account for the meager amount of the lipoyl cofactor generated by LS.[8]

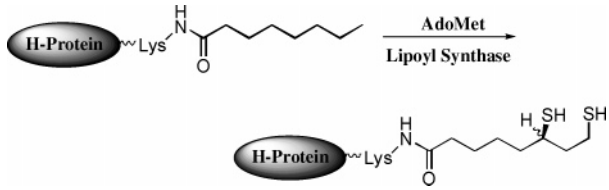


Figure 1. Reaction catalyzed by lipoyl synthase. Two molecules of *S*-adenosyl-L-methionine (SAM) are cleaved to two molecules of 5′-deoxyadenosine (5′-dA) and L-methionine (Met) concomitant with formation of the lipoyl cofactor.

The first hypothesis simply supposes that only 36% of the enzyme is capable of generating the lipoyl cofactor. The second supposes that 72% of the enzyme is in an active state; however, each polypeptide contributes only one sulfur atom to the intact cofactor. This scenario would predict that the maximum concentration of lipoyl cofactor synthesized by LS could be no higher than one-half of the enzyme concentration, or 25 μM.

The above working hypotheses can be differentiated experimentally. The first hypothesis assumes that both sulfur atoms derive from the same polypeptide. The second hypothesis necessitates that after insertion of one sulfur atom into the octanoyl chain, the monothiolated intermediate dissociates from the enzyme and binds another LS polypeptide that is competent for sulfur transfer. Herein, we address this issue directly using two differentially labeled forms of LS: one that is isolated from minimal medium containing $Na_2{}^{34}S$ (98%) as the only sulfur source, and one that is isolated from minimal medium containing natural abundance Na_2S ($^{32}S = 95\%$) as the only sulfur source.[13] Under turnover conditions, in the absence of extraneous sources of sulfur, and using equimolar concentrations of [^{34}S]-LS and [^{32}S]-LS, each synthesized lipoyl cofactor should contain either two atoms of ^{32}S or two atoms of ^{34}S if both sulfurs emanate from the same polypeptide. If each polypeptide contributes only one sulfur atom in the reaction, then the isolated lipoyl cofactor should contain a 1:2:1 ratio of $^{32}S-{}^{32}S$, $^{32}S-{}^{34}S$, and $^{34}S-{}^{34}S$, respectively.

The analysis of oxidized lipoic acid methyl ester (LAME) by GC−MS has previously been described.[14] The relative intensities of the parent ion ($m/z = 220$), which is approximately 40% of the base peak ($m/z = 85$), were used to analyze LS-dependent incorporation of ^{32}S or ^{34}S sulfur into LA by single ion monitoring (SIM) analysis after derivatization with (trimethylsilyl)diazomethane.[15] In Figure 2A, the mass spectrum of LAME synthesized from LS containing natural abundance sulfur is displayed from *m/z* values of 219−228. It is virtually identical to that of commercially available LS derivatized in the same manner (data not shown). As expected, the base peak corresponds to $m/z = 220$, and the peaks at *m/z* values of 221 and 222 (12.2 and 9.01% of the base peak, respectively) represent the contribution of ^{13}C and ^{33}S to the M +

10.1021/ja042428u CCC: $30.25

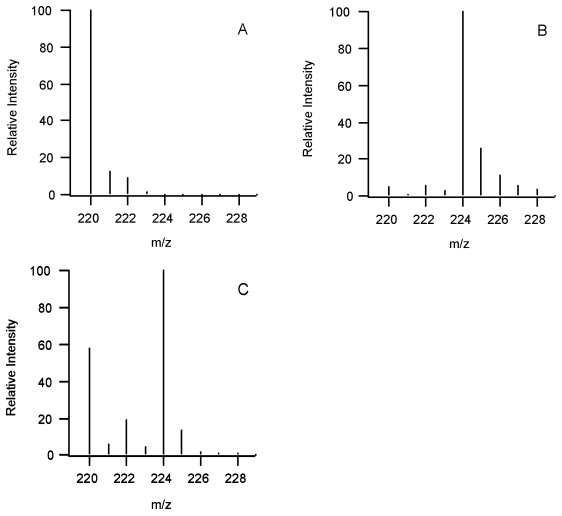


Figure 2. Analysis of the lipoyl synthase reaction by GC−MS. Each reaction contained in addition to 0.1 μmol of octanoyl-H protein, 0.53 μmol of ^{32}S-labeled LS (panel A), 0.53 μmol of ^{34}S-labeled LS (panel B), or a mixture of ^{32}S- (0.16 μmol) and ^{34}S-labeled (0.16 μmol) lipoyl synthases (panel C). The reaction conditions and subsequent workup are described in ref 15.

1 peak, and ^{34}S to the M + 2 peak of LAME. In Figure 2B, the mass spectrum of LAME synthesized from [^{34}S]-containing LS is displayed. The base peak corresponds to $m/z = 224$, indicating insertion of two ^{34}S atoms and clearly supporting the premise that the sulfur atoms in LA derive directly from LS itself. The peak intensities at m/z values of 220, 222, 225, and 226 are higher than what would be expected as a result of contributions by other relevant isotopes of sulfur (^{32}S and ^{33}S) and carbon (^{13}C). In particular, the peak at $m/z = 225$ is approximately twice the intensity predicted based on other isotopic contributions. We attribute this to adventitious contamination, which appears to be significant because of the low concentrations of metabolites analyzed. In Figure 2C, the mass spectrum of LAME synthesized from equimolar concentrations of [^{32}S]-LS and [^{34}S]-LS is displayed. The two most prominent peaks exhibit m/z values of 220 and 224, indicating that most of the LAME synthesized by LS contains two sulfur atoms of identical isotope. Again, the peak at $m/z = 222$ is more intense than what would be expected from analysis of the plots in Figure 2A,B (observed 19.4% of base peak; calculated 10.9% of base peak). This may reflect a small fraction of liberated monothiolated species that are converted to the intact cofactor at another active site, or it may arise from adventitious contamination. The peaks at $m/z = 225$ and 226 correspond to the predicted intensities after correcting for the contribution by ^{13}C to the product. Ideally, the peaks at $m/z = 220$ and 224 should be of equal intensity. The deviation might reflect a slight difference in activity of the two proteins or a slight contamination at $m/z = 224$. Clearly, however, the ratio of the peaks at m/z values of 220, 222, and 224 is far from the 1:2:1, respectively, that is expected for a mechanism in which the monothiolated species are obligatorily released and is consistent with a mechanism in which both sulfurs must derive from the same polypeptide. A similar result might also arise from a mechanism in which sulfurs derive from different LS polypeptides that form kinetically stable dimers. However, molecular sieve chromatographic studies conducted under anaerobic conditions indicate that LS is primarily monomeric (data not shown).

Our studies herein strongly suggest that both sulfurs in the lipoyl cofactor, synthesized by action of lipoyl synthase, emanate from the same polypeptide, again eliciting speculation that the difference in cluster configuration between LS and BS might relate to the number of sulfurs that are mobilized for insertion by each enzyme. If indeed the sulfurs mobilized by LS derive from one of the [4Fe−4S] clusters on the protein, then the mechanism originally proposed by Miller et al.[6] might serve as a good initial working model.

Acknowledgment. This work was supported by a grant (GM-63847) from the National Institutes of Health to S.J.B. We thank Drs. Joseph T. Jarrett and Mike Dotson for advice in preparing Na_2S from elemental sulfur.

References

(1) Booker, S. J. *Chem. Biol.* **2004**, *11*, 10−12.

(2) Zhao, S.; Miller, J. R.; Jiang, Y.; Marletta, M. A.; Cronan, J. E., Jr. *Chem. Biol.* **2003**, *10*, 1293−1302.

(3) Hayden, M. A.; Huang, I.; Bussiere, D. E.; Ashley, G. W. *J. Biol. Chem.* **1992**, *267*, 9512−9515.

(4) Reed, K. E.; Cronan, J. E., Jr. *J. Bacteriol.* **1993**, *175*, 1325−1336.

(5) Sofia, H. J.; Chen, G.; Hetzler, B. G.; Reyes-Spindola, J. F.; Miller, N. E. *Nucleic Acids Res.* **2001**, *29*, 1097−1106.

(6) Miller, J. R.; Busby, R. W.; Jordan, S. W.; Cheek, J.; Henshaw, T. F.; Ashley, G. W.; Broderick, J. B.; Cronan, J. E., Jr.; Marletta, M. A. *Biochemistry* **2000**, *39*, 15166−15178.

(7) Frey, P. A.; Booker, S. J. *Adv. Protein Chem.* **2001**, *58*, 1−45.

(8) Cicchillo, R. M.; Iwig, D. F.; Jones, A. D.; Nesbitt, N. M.; Baleanu-Gogonea, C.; Souder, M. G.; Tu, L.; Booker, S. J. *Biochemistry* **2004**, *43*, 6378−6386.

(9) Vanden Boom, T. J.; Reed, K. E.; Cronan, J. E., Jr. *J. Bacteriol.* **1991**, *173*, 6411−6420.

(10) Cicchillo, R. M.; Gogonea, C.; Lee, K.-H.; Nesbitt, N. M.; Krebs, C.; Booker, S. J. *Biochemistry* **2004**, *43*, 11770−11781.

(11) Ugulava, N. B.; Sacanell, C. J.; Jarrett, J. T. *Biochemistry* **2001**, *40*, 8352−8358.

(12) Busby, R. W.; Schelvis, J. P. M.; Yu, D. S.; Babcock, G. T.; Marletta, M. A. *J. Am. Chem. Soc.* **1999**, *121*, 4706−4707.

(13) $Na_2{}^{34}S$ was prepared from elemental sulfur-34 obtained from Trace Sciences International (Richmond Hill, ON, Canada), using a modification of a previously published procedure.[16] The sodium sulfide produced was analyzed by ICP−MS, which revealed the following isotopic distribution (0.99% ^{32}S, 1.2% ^{33}S, and 98% ^{34}S). The *lipA* gene was expressed in M9 minimal medium as previously described,[8,10] with the exception that $Na_2{}^{34}S$ or Na_2S (1.35 mM final concentration) was substituted for $MgSO_4$, using $MgCl_2$ as the source of magnesium.

(14) Pratt, K. J.; Carles, C.; Carne, T. J.; Danson, M. J. *Biochem. J.* **1989**, *258*, 749−754.

(15) Reactions were carried out as previously described, and protein concentrations were determined using an established correction factor for the Bradford protein assay.[8] Reactions employing either [^{32}S]- or [^{34}S]-LS contained 0.53 μmol of the respective protein and 0.1 μmol of octanoyl-H protein, while the reaction employing both [^{32}S]- and [^{34}S]-LS simultaneously contained 0.16 μmol of each protein in addition to 0.1 μmol of octanoyl-H protein. All reactions were incubated at 37 °C for 30 min, and then acidified by addition of concentrated HCl to a final concentration of 6.4 M. The acidified solutions were autoclaved for 2 h at 121 °C to liberate lipoic acid from the H protein and then extracted four times with equal volumes of benzene. The benzene extracts were concentrated to 0.5 mL, and 1 mL aliquots of (trimethylsilyl)diazomethane (2 M solution in hexanes) and methanol were added to form LAMEs. Derivatizations were carried out at room temperature and were typically complete within 30 min. The solution was evaporated to ~50 μL and injected directly onto a Shimadzu QP5000 GC−MS. Sample introduction was via splitless injection onto an XTI-5 column (30 m × 0.25 μm; Restek Corporation). The injection temperature was 250 °C. The initial column temperature was 50 °C and was held for 1 min after injection before increasing to 180 °C at 15 °C min^{-1}. The temperature was subsequently increased to 275 °C at 5 °C min^{-1}.

(16) Fehér, F. In *Handbook of Preparative Inorganic Chemistry*; Brauer, G., Ed.; Academic: New York, 1963; Vol. 1, pp 358−360.

JA042428U