Lipoic Acid Biosynthesis: LipA Is an Iron−Sulfur Protein

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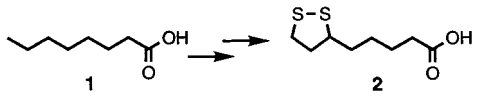
Lipoic acid (**2**) is a crucial cofactor in the pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH) multienzyme complexes responsible for the production of acetyl-CoA in metabolic pathways. Despite the wealth of information available about these dehydrogenases, there is little known about the enzymes involved in the biosynthesis of **2**. The eight-carbon backbone of **2** derives from octanoic acid (**1**) as it had previously been shown that sodium [1-^{14}C]octanoic acid was specifically incorporated into lipoic acid in *E. coli* at a level of 0.17%.[1−3] Perhaps the most intriguing component of lipoic acid biosynthesis is the formation of the two carbon−sulfur bonds that must occur at unactivated carbon atoms to afford **2** (see Scheme 1). Three genes have been linked to the lipoic acid biosynthetic pathway: *lipA*, *lipB*, and *lplA*. The latter two have been identified as lipoyl ligases that transfer lipoyl groups to the E2 subunits of PDH and KGDH.[4,5] Genetic studies on *E. coli* showed that cells with mutations in *lipA* did not produce lipoic acid,[6−8] and that of the three genes mentioned, only *lipA* is directly involved in the biosynthesis of lipoate.[4,7−9] The *lipA* gene product, lipA, is thought to catalyze one[4] or both[8,9] of the carbon−sulfur bond formations in vivo. Neither the reaction mechanism nor the identity of the sulfur donor is known; in fact, there is even debate over the actual biological form of lipoate produced in microorganisms (and thus possible substrates for lipA).[10]

LipA contains some amino acid sequence homology to biotin synthase (bioB), a carbon−sulfur bond-forming enzyme that catalyzes the conversion of dethiobiotin to biotin.[11−13] The similarity in chemistry between the biosynthesis of the dithiolane ring of lipoate and the thiophane ring of biotin suggests there might be functional parallels between the enzymes that produce these compounds. BioB contains an Fe−S cluster,[11] and there has been speculation that lipA does as well,[4,9] as they have in common the iron−sulfur cluster binding motif, CXXXCXXC. Herein we report that lipA is, in fact, an iron−sulfur protein containing a cluster similar to that of bioB, strongly suggesting that it belongs to a family of proteins that use Fe−S clusters to activate C−H bonds.

Scheme 1



LipA was obtained from an *E. coli* overproducer[14] and purified to homogeneity as judged by SDS-PAGE.[15] The purified protein has a dark, reddish-brown color, and initial metal analysis using inductively coupled plasma atomic emission spectroscopy (ICP) confirmed the presence of Fe and showed no evidence of other metals. Determination of the Fe stoichiometry using ferrozine[16] and the bicinchoninic acid (BCA) protein assay[17] coupled with a correction factor of 0.66 from quantitative amino acid analysis (University of Michigan Core Facility) revealed 1.8 ± 0.2 mol of Fe/mol of lipA. Acid-labile sulfide analysis[19] revealed the presence of sulfide at a level of 2.2 ± 0.4 S^{2-}/mol of lipA. These values suggest there are 2Fe and 2S per lipA monomer.

The electronic absorption spectrum of lipA is shown in Figure 1a. In addition to the protein absorbance at 278 nm, the spectrum shows absorbance maxima at 330 and 420 nm, consistent with a $[4Fe\text{-}4S]^{2+}$ cluster.[20,21] X-band EPR measurements on the reduced protein show no signal, consistent with a diamagnetic form of the protein (data not shown). To characterize the cluster further, resonance Raman (RR) spectroscopy was used to distinguish between the possible diamagnetic ground states. RR spectra were collected in the Fe−S stretching region (200−450 cm^{-1}) by using 426 nm light from a dye (Stilbene 420) laser pumped with the UV output from an Ar^+ laser (Figure 2a). The RR spectrum is characteristic of a $[4Fe\text{-}4S]^{2+}$ cluster, in agreement with the UV−visible optical properties. The vibrational modes can be tentatively assigned by assuming idealized T_d symmetry in analogy to synthetic analogues.[23] The RR spectrum is dominated by a vibration at 337 cm^{-1}, which we attribute to the breathing mode of the Fe_4S_4 cubane ($A_1{}^b$).[24] In addition, the fact that it is in the range of 333−338 cm^{-1} is indicative of exclusive cysteinyl ligation of the Fe−S center.[23,25,26] The overall shape of the RR spectrum is very similar to those of other proteins that are known to contain $[4Fe\text{-}4S]^{2+}$ clusters.[23,26−28] The bands at 358 and 381

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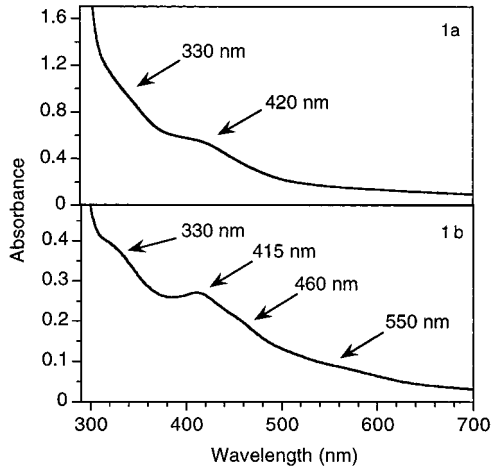

Figure 1. UV−visible absorption spectra for 0.3 mM dimeric lipA (a) and 0.5 mM lipA enriched in monomer with some contaminating dimer (b). Spectra were recorded on a Cary 3E spectrophotometer at 10 °C.

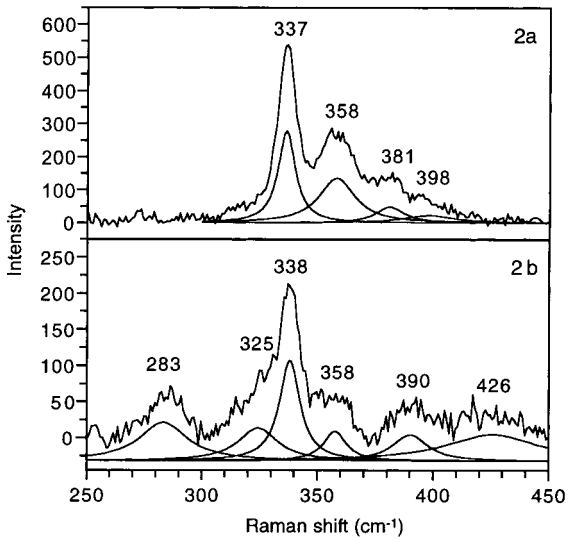

Figure 2. Resonance Raman spectra of dimeric lipA (a) and a sample enriched in monomeric lipA with some contaminating dimer (b). The data were collected using the experimental setup described elsewhere.[22] The samples (110 μL, 0.5 mM) were contained in a spinning cell under Ar atmosphere at 4 °C. The laser power was 35 mW at the sample. The spectral slit width was 3 cm^{-1}. Accumulation time was 2 (a) and 5 h (b). Spectra were corrected for contributions from the buffer and baseline corrected, but not smoothed. The spectra were deconvoluted with Lorentzian line shapes using a least-squares fitting routine.

cm^{-1} are likely the T_2 terminal and bridging modes, respectively, while the weaker vibration at 398 cm^{-1} is most likely the A_1 terminal sulfur breathing mode.

LipA is normally purified as a dimer, based on a standard curve of gel filtration elution times of various molecular weight protein standards; however, in some lipA preparations, a small amount of protein corresponding to monomeric lipA was also isolated. The electronic absorption of monomeric lipA with some dimeric lipA is shown in Figure 1b. A shoulder evident at 460 nm and a broad absorbance at 550 nm are indicative of the presence of a $[2Fe\text{-}2S]^{2+}$ cluster.[30] This sample was also analyzed using RR spectroscopy to probe the structure of the Fe−S cluster, and its spectrum is shown in Figure 2b. The spectrum contains the Fe−S terminal vibration (B_{3u}^t) at 283 cm^{-1} that is characteristic of a $[2Fe\text{-}2S]^{2+}$ cluster.[24,31−33] In addition, it occurs within the range (281−291 cm^{-1}) indicative of complete cysteinyl coordination.[29] The vibrations at 325 and 426 cm^{-1} are also attributed to the 2Fe-2S cluster.[26,31,33] In 2Fe-2S clusters, excitation with 400−460 nm light results in enhancement of the B_{3u}^t vibration over the 337 cm^{-1} vibration.[31] Since the latter vibration is stronger in our spectrum than the 283 cm^{-1} vibration with 426 nm excitation, we conclude that $[4Fe\text{-}4S]^{2+}$ clusters are also present in our sample. Therefore, the vibrations at 337, 358, and 390 cm^{-1} cannot be solely assigned to the $[2Fe\text{-}2S]^{2+}$ cluster, and some contribution may come from a $[4Fe\text{-}4S]^{2+}$ cluster. The presence of $[2Fe\text{-}2S]^{2+}$ clusters in lipA monomeric subunits is not unexpected; however, the presence of some population of $[2Fe\text{-}2S]^{2+}$ clusters in dimeric lipA cannot be ruled out. Dithionite alone does not induce dimerization of monomeric lipA.

The combination of resonance Raman and electronic absorption spectroscopies provides a sensitive structural probe of Fe−S cluster type. This qualitative analysis coupled with the analytical data indicate that lipA is an iron−sulfur protein. Furthermore, the main form of lipA appears to be a homodimer containing a $[4Fe\text{-}4S]^{2+}$ cluster. The presence of a small population of monomeric $[2Fe\text{-}2S]^{2+}$ cluster indicates that lipA may be analogous to proteins that apparently form $[4Fe\text{-}4S]^{2+}$ clusters at their dimeric interface. One example of such an Fe−S cluster is found in bioB. BioB[26] along with the pyruvate formate-lyase-activating enzyme (PFL-AE),[19,27] anaerobic ribonucleotide reductase (ARR),[33] and lysine 2,3-aminomutase[34] all contain $[4Fe\text{-}4S]^{2+}$ clusters in their active forms. In addition, all of these enzymes are (*S*)-adenosyl methionine-dependent and use their Fe−S clusters to homolytically abstract hydrogen atoms at unactivated carbons in a variety of radical-dependent reactions. The chemistry required for sulfur insertion into the 6 and 8 positions of octanoate in the biosynthesis of lipoic acid almost certainly involves a radical-based mechanism. This is supported by bioorganic studies in which lipoic acid was isolated from *E. coli* that had been administered uniformly deuterated octanoic acid. These studies showed that sulfur atom insertion into octanoic acid resulted in the removal of only the hydrogens at the replaced positions,[3] and not any adjacent hydrogens.[1]

Fe−S clusters found in nature have shown great functional diversity. Whether the Fe−S cluster found in lipA is involved in catalysis, purely structural, or in some way regulatory has yet to be elucidated. If the Fe−S cluster is catalytic, there are two reasonable possibilities: (*i*) lipA could directly catalyze radical formation on a substrate followed by sulfur insertion (e.g., bioB) or (*ii*) function like an activase by initiating radical formation on another (as yet undiscovered) protein (e.g., PFL-AE and ARR). Either way, lipA would emerge as the fifth member of this newly recognized family of proteins.

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