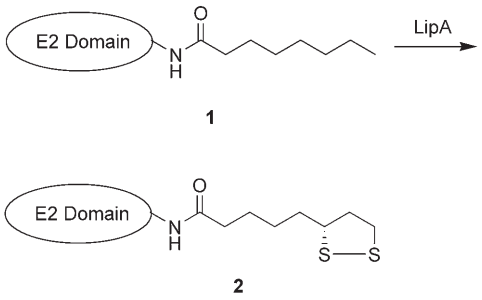

Scheme 1. Insertion of sulfur atoms into octanoyl substrates to form lipoyl groups.

Biosynthesis

DOI: 10.1002/ange.200601910

Lipoyl Synthase Inserts Sulfur Atoms into an Octanoyl Substrate in a Stepwise Manner**

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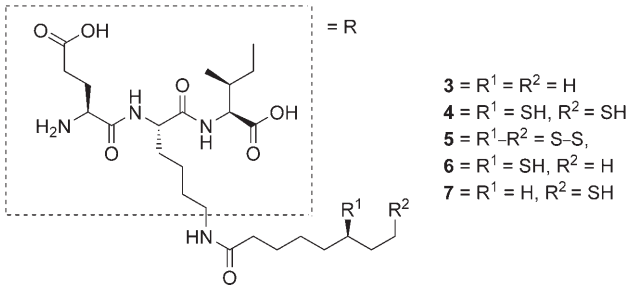
Lipoyl synthase (LipA) is required for the final step in the biosynthesis of lipoyl groups, the insertion of sulfur atoms at C6 and C8 of octanoyl groups (Scheme 1).[1] The octanoyl groups are found attached through an amide linkage to a lysine residue in a sequence motif that is conserved within a small family of protein domains[2] that include the H-protein of the glycine cleavage system[3] and the E2 subunit of oxoacid dehydrogenases.[4] After attachment of the octanoyl groups,[5–7] the substrates undergo sulfur insertion by LipA,[1] a member of the "radical *S*-adenosyl L-methionine" family.[8] Biochemical studies have shown that it contains two essential [4Fe-4S] clusters[9] and that one of these clusters is used to generate 5′-deoxyadenosyl radicals (Ado•) through the reductive cleavage of S-adenosyl L-methionine (AdoMet). The lipoyl forming reaction requires two hydrogen atoms to be removed from the octanoyl group, one from C6 and the other from C8, so that the overall reaction utilizes two equivalents of AdoMet to form each lipoyl group.[10] LipA has been shown to donate both of the inserted sulfur atoms[11] that were proposed to originate from a [4Fe-4S] cluster.[12] Recently, we reported that a short octanoyl peptide, which corresponds in sequence to the lipoylation site of the E2 subunit, could function as a substrate for LipA.[13] Peptide substrates have now been used to investigate the order of sulfur-insertion steps and hence clarify the structure of a key intermediate on the reaction pathway.

The formation of lipoyl groups was monitored in a reaction by using *Sulfolobus solfataricus* P2 LipA with an octanoyl substrate tripeptide (0.5 mole equiv; **3**,), AdoMet,



and dithionite as a reductant. LipA is not catalytic during assays in vitro, producing substoichiometric quantities of lipoyl products with either octanoyl-protein[10] or octanoyl-peptide substrates.[13] To investigate the formation of any intermediate species, the reaction was stopped by acidification before reaching completion (after 20 min), the precipitated protein was pelleted by centrifugation, and the supernatant analyzed by LCMS. Four peptide species were eluted over the time range from 22 to 25 min (Table 1). The peptide at 24.4 min corresponds to the unreacted octanoyl substrate **3**, whereas new species at 22.4 and 23.4 min correspond to the expected protonated product masses of lipoyl **5** and dihydrolipoyl products **4**, respectively. The final species at 23.2 min coelutes with the dihydrolipoyl product and corresponds to a monothiolated species (either **6** or **7**). Analysis of later time points (up to 2 h) showed that the amount of this monothiolated species decreased with time and that there was a

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[**] This research was supported by grants from the Royal Society and Engineering and Physical Sciences Research Council to PLR.

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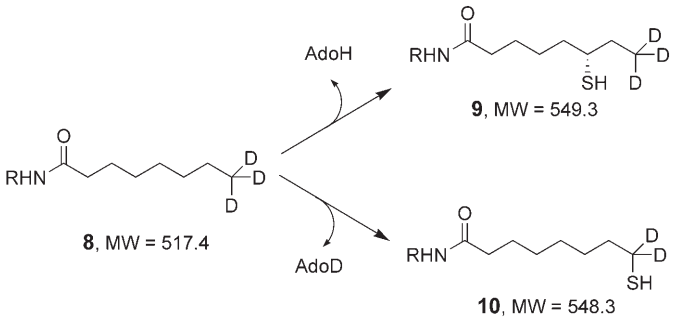
Table 1: LCMS analysis of substrate and product peptides from the LipA reaction.

t_R [min]	Observed mass [m/z]	Calculated mass for MH^+ [Da]	Difference from substrate	Comment
22.4	577.5	577.3	+ 2S−2H	lipoyl product, **5**
23.2	547.3	547.3	+ S	6-thiooctanoyl peptide, **6** or 8-thiooctanoyl peptide, **7**
23.4	579.2	579.3	+ 2S	dihydrolipoyl product, **4**
24.4	515.3	515.3	–	substrate, **3**

concomitant increase in the lipoyl products. This strongly suggests that the monothiolated species represents an intermediate. A similar monothiolation reaction has been observed by mass spectrometry for *E. coli* LipA reacting with an octanoyl protein domain.[10]

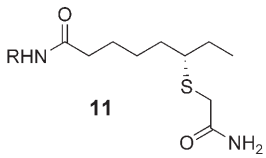
To investigate the nature of the interaction of the monothiolated species with LipA, the assay protocol was modified by using a 5-kDa molecular-weight cut-off filter to remove the LipA. Under these conditions, the proportion of monothiolated species observed by LCMS of the filtrate was greatly reduced (< 10%), indicating that under nondenaturing conditions, the intermediate had remained bound to the native protein and therefore acidic conditions were required for efficient release of the intermediate from LipA. The observation that both of the transferred sulfur atoms originate from the same LipA molecule[11] is also consistent with a tightly bound intermediate that is not in rapid equilibrium with the bulk solution.

If the reaction proceeds by an ordered insertion of sulfur atoms, two potential structures for the monothiolated species can be envisaged: insertion of a sulfur atom at either C6 or C8 of the octanoyl chain. In an effort to identify the order of C–S bond formation, the 8,8,8-trideuterooctanoyl peptide **8** was synthesized and incubated with LipA. With this substrate, two monothiolated peptides that differ in molecular weight could arise from abstraction of a hydrogen atom from C6 or a deuterium atom from C8 (Scheme 2). The results showed the exclusive removal of a hydrogen atom from C6, supporting the formation of a 6-thiooctanoyl intermediate from this substrate (m/z for $MH^+ = 550.3$). Relative to the proteo substrate **3**, the deutero substrate **8** resulted in minute quantities of the lipoyl product. This small amount could not be accurately quantified by using the LCMS data, but the observation is consistent with a large isotope effect on the second sulfur-insertion step.[14]



Scheme 2. Potential outcomes from sulfur insertion into an 8,8,8-trideuterooctanoyl substrate peptide.

The possibility of a mechanism proceeding with a random order of sulfur insertion, biased by the previously observed[14] isotope effect at C8, cannot be excluded by this experiment. This issue was resolved by the spectroscopic determination of the regiochemistry of the initial sulfur insertion. Sufficient material for this analysis was obtained by combining the products from 14 reactions with the proteo-substrate **3**. To ensure the isolation and characterization of the monothiolated peptide without potentially confusing disulfide formation, the crude reaction products were reduced, alkylated with iodoacetamide, and then purified by HPLC. A COSY spectrum facilitated the identification of the signals from protons attached to C6, C7, and C8 of the monothiolated octanoyl group. These spectra indicate unequivocally the exclusive formation of the derivative of the 6-thio intermediate **11** (see Figure 1).



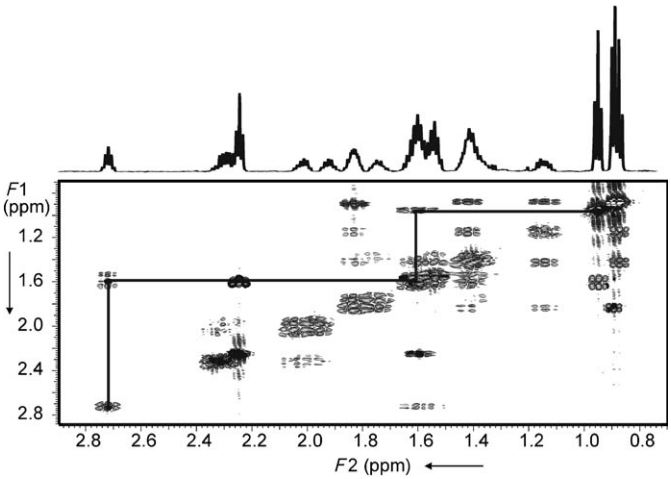
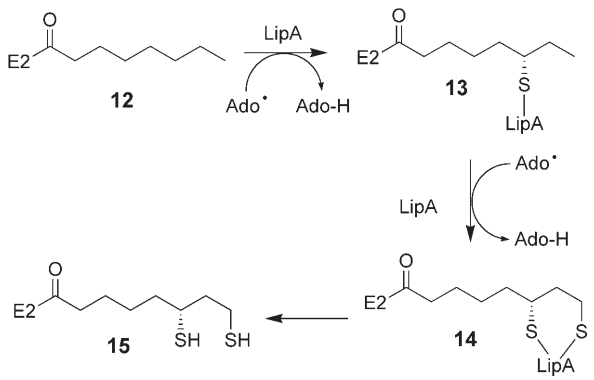


Figure 1. H,H COSY spectrum of the alkylated 6-thio-octanoyl tripeptide **11**. Lines have been added to indicate the key correlations: 0.95 (octanoyl C8 methyl) shows a cross-peak to 1.58 (octanoyl C7 CH_2), which in turn shows a cross-peak to the multiplet at 2.72 (octanoyl C6, CH).

Studies in vivo comparing the efficiency with which deuterium-labeled 6- and 8- thiooctanoic acids were converted into lipoic acid have shown that both can function as precursors, but intriguingly, the 8-thio compound is more efficiently incorporated.[15] Although there may be sufficient flexibility in the active site to accommodate either monothiolated compound, it is clear that when LipA is presented with an octanoyl substrate, the substrate is first converted to

an enzyme bound 6-thiooctanoyl intermediate, which is subsequently converted to the lipoyl product.

The accumulation of the 6-thiooctanoyl intermediate, together with the observed substantial deuterium isotope effect at C8, is consistent with a mechanism (Scheme 3) in



Scheme 3. Proposed mechanism for lipoyl group formation by LipA. The regiochemistry for the sulfur-insertion steps are shown. The 5′-deoxyadenosyl radicals are derived from the reductive cleavage of S-adenosyl methionine.

which sulfur is first inserted at C6 to form an enzyme bound intermediate **13**. In a subsequent rate determining step, the second sulfur atom is inserted at C8, suggesting an energy profile for the reaction that appears to reflect the relative bond strengths of the primary (C8) and secondary (C6) C–H bonds that must be cleaved to form lipoyl groups.

Received: May 15, 2006
Published online: July 11, 2006

Keywords: biosynthesis · cofactors · enzyme models · insertion · lipoic acid

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