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Destruction and Reformation of an Iron-Sulfur Cluster during Catalysis by Lipoyl Synthase

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Abstract

Lipoyl synthase (LipA) catalyzes the last step in the biosynthesis of the lipoyl cofactor, which is the attachment of two sulfhydryl groups to C6 and C8 of a pendant octanoyl chain. The appended sulfur atoms derive from an auxiliary [4Fe–4S] cluster on the protein that is degraded during turnover, limiting LipA to one turnover *in vitro*. We report that the iron-sulfur (Fe-S) cluster carrier protein NfuA, the bacterial ortholog of NFU1, efficiently reconstitutes the auxiliary cluster during LipA catalysis in a step that is not rate-limiting. These results show that enzymes that degrade their Fe-S clusters as a sulfur source can nevertheless act catalytically. Our results also explain why patients containing *NFU1* gene deletions exhibit phenotypes that are indicative of lipoyl cofactor deficiencies.

Lipoic acid is an eight-carbon, straight-chain fatty acid containing sulfhydryl groups at C6 and C8, which undergo reversible disulfide-bond formation to generate a dithiolane ring (1, 2). It is used as a redox-active cofactor in several multienzyme complexes that are involved in the oxidative decarboxylation of various α-keto acids and glycine, as well as in the oxidative degradation of acetoin (3–5). In these complexes, it is attached through an amide linkage to a conserved lysyl residue of a lipoyl carrier protein (LCP), producing a 14 Å "swinging arm" that allows its dithiolane ring to access multiple active sites. A well known role is in the pyruvate dehydrogenase complex (PDC), where it plays a central function in the conversion of pyruvate to acetyl-coenzyme A (6, 7). Other complexes in which it functions in a similar capacity are the α-ketoglutarate dehydrogenase complex (KDC), the branched chain α-ketoacid dehydrogenase complex, the acetoin dehydrogenase complex, and the glycine cleavage system (GCS)(3–5, 8). Deficiencies in the biosynthesis of the lipoyl cofactor or in any of the multienzyme complexes that require it result in a number of diseases, one of which is multiple mitochondrial dysfunctions syndrome, which leads to

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SUPPLEMENTARY MATERIALS:

Materials and Methods

Figs. S1 to S4

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severe developmental delays, seizures, and death (9). In mammals, the complete inability to synthesize lipoic acid is embryonic lethal (10).

Although the lipoyl cofactor is structurally simple, its biosynthesis has been enigmatic. Seminal studies from the Cronan laboratory identified the pathway by which the lipoyl cofactor is biosynthesized *de novo* in *Escherichia coli (E. coli)* and highlighted the roles of two dedicated proteins: octanoyltransferase (LipB), which catalyzes the transfer of an *n*-octanoyl moiety from octanoyl-acyl carrier protein (ACP) to a conserved lysyl residue on an LCP; and lipoyl synthase (LipA, LIAS in humans and Lip5 in yeast), which catalyzes the subsequent attachment of two sulfur atoms at C6 and C8 of the aliphatic chain (11–14). This pathway is conserved in all organisms that synthesize the lipoyl cofactor *de novo*, although slight variations have been noted (11, 15–17).

LipA is a member of the radical *S*-adenosylmethionine (SAM) superfamily of enzymes, and as such, uses a 5′-deoxyadenosyl 5′-radical (5′-dA•) that results from the reductive cleavage of SAM to abstract hydrogen atoms (H•)—first from C6 and then from C8—to activate the aliphatic chain for sulfur attachment (18–21). All radical SAM (RS) proteins contain one [4Fe–4S] cluster that supplies the electron during the reductive cleavage of SAM (22, 23). However, lipoyl synthases contain a second [4Fe–4S] cluster that has been hypothesized to be the source of the attached sulfur atoms (20, 24, 25). Consistent with this hypothesis, LipA typically catalyzes no more than one turnover in *in vitro* reactions due to the obligate destruction of the iron–sulfur (Fe–S) cluster (19, 21, 26). The current working hypothesis for turnover by LipA is shown in Fig. 1 (19, 20, 27). SAM binds in contact with the RS [4Fe–4S] cluster— termed $[4Fe–4S]_{RS}$—and undergoes reductive cleavage to generate a 5′-dA•, which abstracts an H• from C6 of the octanoyllysyl group of an LCP. The resulting C6 radical attacks a bridging μ-sulfido ion of the auxiliary cluster with concomitant reduction of one of the Fe^{3+} ions of the cluster to Fe^{2+} and loss of an Fe^{2+} ion to afford a [3Fe–3S–1(6*S*)- thio-octanoyl-LCP] cluster (19, 20). A second 5′-dA•, generated from the reductive cleavage of a second SAM molecule, abstracts a C8 H•, with subsequent attack of the resulting C8 radical onto a second bridging μ-sulfido ion of the auxiliary cluster. This step results in attachment of the second sulfur atom and concomitant full or partial destruction of the cluster upon protonation of the two sulfur atoms in the nascent lipoyl group.

If the current proposed mechanism for LipA catalysis reflects the *in vivo* mechanism, LipA would be a substrate rather than an enzyme, given that it is irreversibly consumed in the reaction. Hence, it is likely that there is a system responsible for either the repair of a partially degraded cluster or the insertion of a newly assembled [4Fe–4S] cluster into the LipA active site. Although Fe–S cluster assembly can occur spontaneously *in vitro*, it has been established that the *in vivo* process involves a complex network of proteins that is highly regulated (28–33). Herein, we provide evidence that *E. coli* NfuA, an Fe–S cluster-containing protein suggested to serve as an intermediate in Fe–S cluster delivery, confers catalytic properties to *E. coli* LipA.

A number of studies have provided strong evidence that NFU1 and BOLA3 are involved in lipoic acid production in mammalian and yeast cells. For example, mutations in either *NFU1*

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or BOLA3, previously considered to encode alternative scaffold proteins in Fe–S cluster biosynthesis, were found to cause a fatal deficiency of multiple respiratory chain and 2-oxoacid dehydrogenase enzymes. These deficiencies were thought to be due to a lack of the lipoyl group on the LCPs of the PDC and KDC, as well as defects in complexes I and II of the mitochondrial respiratory chain (34, 35). More recent studies directly linked NFU1 and BOLA3 to lipoic acid biosynthesis in mammalian cells and yeast, and it was determined that the proteins participated at some late stage in the process (36).

To assess the effect of NfuA—a bacterial homolog of NFU1—on the LipA reaction, molecular-sieve chromatography (MSC) was carried out to establish first whether NfuA associates with LipA. *E. coli* NfuA was overproduced with an N-terminal hexahistidine (His_6) tag to allow purification by immobilized metal affinity chromatography (fig. S1). The purified protein contains 1.8 ± 0.2 sulfide and 2.3 ± 0.2 iron ions per polypeptide; however, its UV-visible spectrum suggests the presence of [4Fe–4S] clusters (Fig. 2A). Moreover, reconstitution of as-isolated NfuA with additional iron and sulfide does not lead to more cluster incorporation. This stoichiometry is consistent with the presence of one [4Fe–4S] cluster per dimer of polypeptides. LipA alone (dotted trace) elutes at 59.6 mL by MSC, exhibiting an experimentally calculated mass of 44.1 kDa (theoretical mass, 38.2 kDa) based on the elution profiles of a suite of standards (Fig. 2B and fig. S2). NfuA alone (dashed trace) elutes at 64.6 mL, exhibiting an experimentally calculated mass of 28.9 kDa (theoretical mass, 25.6 kDa). The sample containing both LipA and NfuA (solid trace) shows an elution at 54.6 mL, corresponding to an experimentally calculated mass of 67.2 kDa, suggesting a 1:1 heterodimer of LipA and NfuA (theoretical mass, 63.8 kDa). To confirm the results obtained by MSC, fractions from the two peaks observed in the LipA + NfuA trace were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). As shown in Fig. 2C, the major peak (lane 2) contains both NfuA and LipA. In these experiments, NfuA migrates as a monomer and can interact with LipA as a monomer. In previous similar characterizations of NfuA, it was stated to migrate as a homodimer by MSC; however, the experimental methods and data were not provided in detail (37, 38). The UV-vis spectrum of NfuA alone upon elution from the column reveals that it has lost its cluster (fig. S3). By contrast, LipA elutes from the column with both of its clusters intact. However, when NfuA is chromatographed at higher concentrations, as during its purification, it elutes with its [4Fe–4S] cluster intact. These observations are consistent with a comparatively less stable cluster on NfuA that is required for dimerization, as has been suggested previously (37–39).

Shown in Fig. 2D is a reaction depicting LipA turnover using an 8-amino acid peptide substrate analog containing the octanoyllysyl residue (Glu-Ser-Val-(N^6-octanoyl)Lys-Ala-Ala-Ser-Asp) undergoing modification (19). The closed triangles show formation and decay of the monothiol-containing intermediate, while the black squares show formation of the lipoylated peptide product. As is typical, formation of the product levels off around the concentration of the enzyme (50 μM). When NfuA (200 μM polypeptide; 100 μM [4Fe–4S] cluster) is injected into the reaction after a single turnover by LipA (150 min), rapid formation of additional lipoyl-containing product and monothiol-containing intermediate is observed. A similar result is shown in Fig. 2E, wherein NfuA is added at the beginning of the reaction. In this instance, more than two additional turnovers (closed triangles) take place

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above that observed when NfuA is omitted (closed squares). Moreover, when the concentration of LipA in the reaction is lowered (7 μM) as the concentration of NfuA is raised (400 μM), the reaction becomes catalytic (Fig. 2F). The absence of an initial burst of 1 equivalent of product that is followed by a slow phase indicates that cluster transfer from NfuA to LipA is not rate-limiting during catalysis.

The model for LipA catalysis shown in Fig. 1 predicts that upon one full turnover, two sulfide ions from the auxiliary [4Fe–4S] cluster are incorporated into the lipoyl product, while the remaining two sulfide ions are released into solution along with four ferrous ions. To assess this stoichiometry more rigorously, reactions were conducted with NfuA that was overproduced in the absence of its Fe–S cluster and then reconstituted with iron and ^{34}S-labeled sulfide. Shown in Fig. 3A is a reaction with 20 μM LipA containing Fe–S clusters composed of sulfide at natural abundance (~95% ^{32}S) and 100 μM NfuA containing Fe–S clusters composed of ^{34}S-labeled (~99%) sulfide. An initial burst of lipoic acid containing two ^{32}S sulfurs is observed (closed circles), which is consistent with previous results that indicate that the auxiliary cluster of LipA is used as the source of inserted sulfur atoms (19, 20, 40). Surprisingly, however, the amount of lipoic acid containing two ^{32}S atoms is more than 50% greater than that expected (33 μM vs. 20 μM), suggesting that the enzyme can potentially direct all four sulfide ions from the cluster into the lipoyl product rather than release two of them into solution during each turnover. Lipoyl product containing two ^{34}S atoms is also observed (closed triangles), confirming that the [4Fe–4S] cluster on NfuA can be used to reconstitute the auxiliary cluster on LipA. Formation of the lipoyl product containing two ^{34}S atoms takes place with a pronounced lag, which is expected for a reaction in which the Fe–S cluster on LipA is consumed before NfuA transfers a labeled cluster to LipA. In this experiment, the amount of ^{34}S-labeled lipoyl product is limited by the concentration of holo NfuA in the reaction mixture. When the experiment is conducted with excess NfuA (400 μM NfuA and ~7 μM active LipA), production of ~1.5 equivalents of the ^{32}S-labeled lipoyl product (Fig. 3B, closed circles) is followed by production of multiple equivalents of the ^{34}S-labeled lipoyl product (Fig. 3B, closed triangles). Also observed is slow formation of a lipoyl group containing one ^{32}S atom and one ^{34}S atom (Fig. 3A, closed squares), which we believe derives from aberrantly released sulfide that is subsequently used to reconstitute another auxiliary cluster during the reaction. Consistent with this explanation, when reactions are conducted in the presence of 0.8 mM sodium sulfide but in the absence of NfuA, additional formation of product is observed that is beyond that supported in the absence of sodium sulfide (Fig. 3C, open squares vs. closed squares). However, this additional formation of product is not as fast or as extensive as when NfuA is present (Fig. 3C, closed triangles). Similarly, when IscS, cysteine, and DTT are included in reaction mixtures a slight increase in product is observed (Fig. 3D, open triangles).

To show that the effect of NfuA on the LipA reaction involves a direct transfer of the cluster from NfuA to LipA rather than the release of iron and sulfide into solution followed by reconstitution of the auxiliary cluster, two additional experiments were conducted. In one experiment, the effect of NfuA (1 mM) on LipA (100 μM) catalysis was conducted in the presence of 5 mM citrate, which can chelate released iron from NfuA and prevent it from being used in the reconstitution of LipA during turnover (41). As shown in Fig. 3E, the presence of citrate has no effect on NfuA's ability to enhance LipA turnover (open circles

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versus closed circles). In a second experiment, the effect of NfuA (400 μM) on LipA (25 μM) catalysis was conducted in the presence of 1 mM $Na_2{}^{34}S$. If the enhanced effect of NfuA is mediated through release of its iron and sulfide into solution, significant incorporation of ^{34}S in the lipoyl product after the initial turnover would be expected (42). As shown in Fig. 4F, when the reaction is conducted in the presence of $Na_2{}^{34}S$ (black triangles), the formation of lipoyl product containing two ^{32}S atoms is similar to that when the reaction is conducted in the absence of $Na_2{}^{34}S$ (black squares). A very small amount of lipoyl product containing one ^{34}S and ^{32}S atom is observed (closed circles), while virtually no product containing two ^{34}S atoms is observed (open circles). These results indicate that release of iron and sulfide from NfuA into solution followed by its use in reconstituting the auxiliary cluster of LipA is unlikely.

The effect of NfuA on the growth and viability of *E. coli* was assessed in two previous studies (37, 43). In one study, Δ*nfuA* strains grew as well as wild-type strains in rich media, except under conditions of oxidative stress (paraquat administration) or iron starvation (2,2′-dipyridyl administration) (37). In a second study, a Δ*nfuA* strain displayed a growth curve in Luria–Bertani (LB) medium that was similar to that of the wild-type control. However, Δ*nfuA* strains that also carried *isc* operon gene deletions displayed an impaired growth rate (38). Because *E. coli* can incorporate exogenous lipoic acid present in rich or LB media into LCPs via a pathway that is independent of LipA, we investigated whether an *E. coli* Δ*nfuA* strain could grow in M9 minimal medium lacking lipoic acid and using glucose as a carbon source. As shown in Fig. 4A, the Δ*nfuA* strain does not exhibit pronounced growth defects (closed circles) compared to the control (solid squares). Moreover, neither the addition of exogenous lipoic acid nor succinate plus acetate (which bypasses the requirement for lipoic acid) has a significant effect on the overall growth rate. By contrast, *E. coli lipA* mutants cannot grow under these conditions. These results differ from the extreme phenotypes observed in humans or yeast for *NFU1* or *BOLA3* deletion strains (34–36).

The observation that *E. coli* Δ*nfuA* strains do not exhibit severe growth defects in the absence of lipoic acid suggests that a second pathway exists for the regeneration of LipA's auxiliary cluster in *E. coli*. Given that an exacerbated effect was previously observed in an Δ*nfuA*:Δ*iscU* strain compared to strains harboring single deletions of the two genes, we assessed whether *E. coli* IscU could render LipA catalytic in the absence of NfuA (48). *E. coli* IscU was overproduced and isolated in its apo form and then its [4Fe–4S] cluster was reconstituted using previously established methods (44). Indeed, when *E. coli* IscU containing a [4Fe–4S] cluster was included in excess of LipA, additional turnover was observed (Fig. 4B, closed triangles). It has been shown that the co-chaperones HscA and HscB, encoded within the *isc* gene operon, function in facilitating cluster transfer from *E. coli* IscU to its recipient proteins, including NfuA (37, 45). However, no significant increase in rate or product formation was observed when HscA, HscB, $MgCl_2$, and ATP were included in the reaction with holo IscU or holo NfuA (Figs. 4C and 4D, closed triangles). In fact, the inclusion of HscA and HscB was slightly inhibitory (Figs. 4C and 4D, closed triangles).

A similar scrambling study of the effect of IscU on LipA catalysis was also carried out (Fig. 4E). When the reaction was conducted with unlabeled IscU and unlabeled LipA in the

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presence of 1 mM $Na_2{}^{34}S$, almost all of the resulting lipoyl product contained two ^{32}S atoms (closed squares). Very little contained the mixed $^{32}S/^{34}S$ (closed triangles) or the $^{34}S/^{34}S$ (open triangles) product. The effect of citrate on the enhancement of the LipA reaction by IscU could not be conducted; however, because IscU's cluster was unstable under those conditions (fig. S4).

In summary, the resistance to the idea that an Fe-S cluster can act as a sulfur source during the radical-mediated sulfhydrylation of unactivated carbon centers is largely due to the consequence of the enzyme inactivating itself after only one turnover. Our finding that *E. coli* NfuA or IscU can reinstall the Fe-S cluster in *E. coli* LipA after each turnover in a process that is not rate-limiting, suggests that this concern is no longer warranted. Our studies also most likely explain why patients with defects in NFU1, the mammalian ortholog of NfuA, display phenotypes that are consistent with lipoic acid deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One sentence summary

NfuA reconstitutes the auxiliary [4Fe–4S] cluster of lipoyl synthase after each turnover

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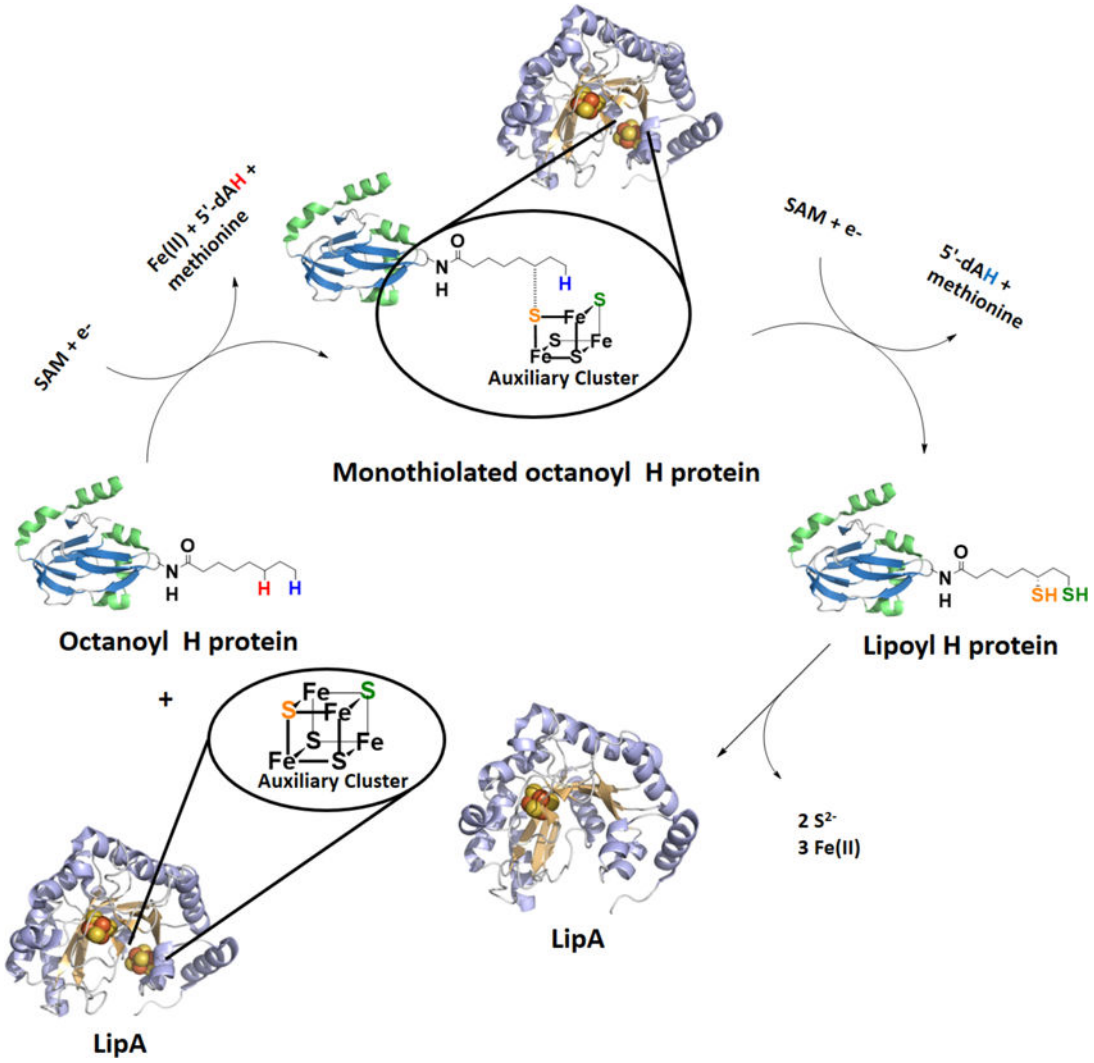

Fig. 1.

Catalysis by LipA. LipA contains two [4Fe–4S] clusters, one of which (the auxiliary cluster shown) is sacrificed during turnover. Catalysis proceeds by reductive cleavage of SAM to render a 5′-deoxyadenosyl 5′-radical (5′-dA•), which abstracts the C6 *pro-R* hydrogen atom of a pendant *n*-octanoamide chain residing on a lipoyl carrier protein (H protein of the glycine cleavage system shown). The resulting C6 substrate radical attacks one of the sulfide ions of the auxiliary cluster, which is followed by loss of an Fe^{2+} ion to afford a [3Fe–3S–1(6*S*)-thio-octanoamide]H protein intermediate (labeled as monothiolated octanoyl H protein). A second reductive cleavage of SAM generates a second 5′-dA•, which abstracts an H• from C8 of the thio-octanoamide H protein intermediate. The resulting C8 substrate radical attacks a second sulfide ion of the auxiliary cluster, which is followed by the addition of two protons and the loss of three Fe^{2+} ions and two S^{-} ions to generate the lipoyl group in its reduced form.

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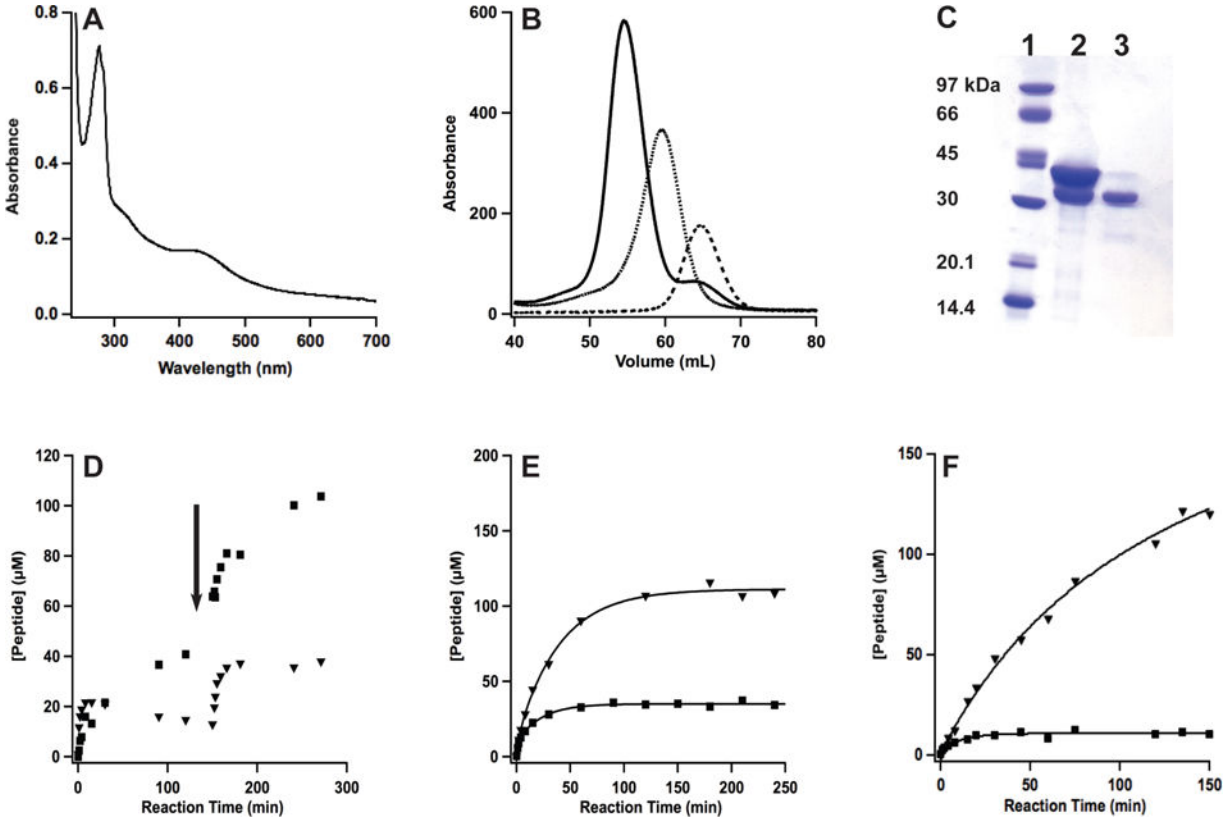

Fig. 2.

(A) UV-visible spectrum of 15 μM as-isolated *E. coli* NfuA. **(B)** Interaction between LipA and NfuA monitored by molecular sievechromatography. Dashed line, 100 μM NfuA alone. NfuA elutes (64.6 mL) with an experimentally calculated molecular mass of 28.9 kDa (theoretical mass, 25.6 kDa). Dotted line, 100 μM LipA alone. LipA elutes (59.6 mL) with an experimentally calculated molecular mass of 44.1 kDa (theoretical mass, 38.2 kDa). Solid line, 100 μM LipA + 100 μM NfuA. A complex between LipA and NfuA elutes (54.6 mL) with an experimentally calculated molecular mass of 67.2 kDa (theoretical mass, 63.8 kDa). **(C)** SDS–PAGE gel of fraction represented by solid line in panel B (lane 2). NfuA standard (lane 3). Molecular mass markers (lane 1). (**D**) Addition of NfuA into a LipA reaction gives rise to additional turnovers. 6(*S*)-thio-octanoyllysyl intermediate (closed triangles). Lipoyllysyl product (closed squares). The reaction contained 50 μM LipA, 600 μM peptide substrate analog (Glu-Ser-Val-[N^6-octanoyl]Lys-Ala-Ala-Ser-Asp), 0.5 μM SAH nucleosidase, 2 mM dithionite and 1 mM SAM. Reactions were conducted at room temperature, and at the designated time indicated by the arrow, 200 μM NfuA was added to the reaction **(E)** Inclusion of NfuA (200 μM) in LipA reaction mixtures gives rise to multiple turnovers. Lipoyllysyl product in the absence of NfuA (closed squares). Lipoyllysyl product in the presence of NfuA (closed triangles). The reaction conditions were as described for panel **D**. **(F)** The LipA reaction under catalytic conditions. The reaction conditions were as described in panel A, except that the concentration of LipA was 10 μM (7 μM active) and the concentration of NfuA was 400 μM.

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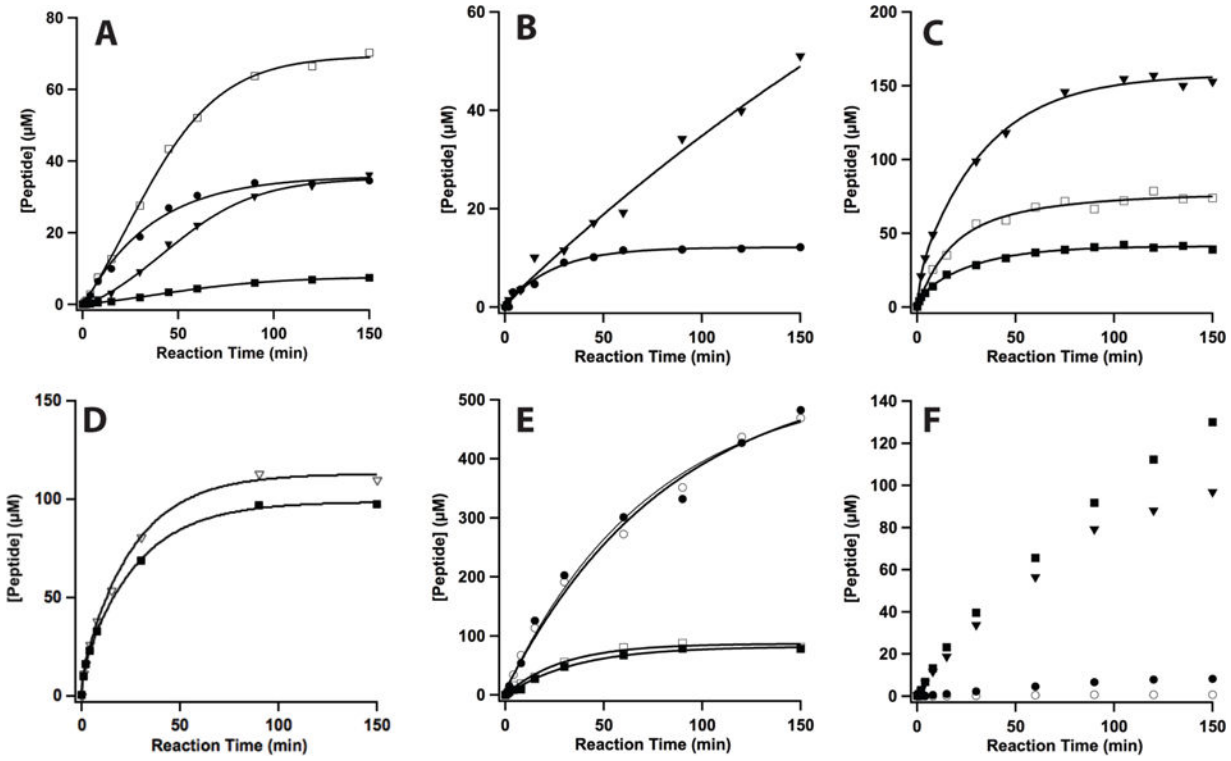

Fig. 3.

(A) Formation of lipoyllysyl product in the presence of NfuA reconstituted with ^{34}S-labeled sulfide. (^{32}S,^{32}S)-containing lipoyllysyl product (closed circles); (^{34}S,^{34}S)-containing lipoyllysyl product (closed triangles); (^{32}S,^{34}S)-containing lipoyllysyl product (closed squares); total lipoyllysyl product (open squares). The reaction contained 20 μM LipA, 100 μM ^{34}S-labeled NfuA, 600 μM peptide substrate analog, 0.5 μM SAH nucleosidase, 2 mM dithionite and 1 mM SAM. **(B)** Formation of lipoyllysyl product in the presence of NfuA reconstituted with ^{34}S-labeled sulfide and under catalytic conditions. The reaction conditions were as described above, except that the concentration of LipA was 10 μM and the concentration of ^{34}S-labeled NfuA was 400 μM. (^{32}S,^{32}S)-containing lipoyllysyl product (closed circles); (^{34}S,^{34}S)-containing lipoyllysyl product (closed triangles). **(C)** Effect of extraneous iron and sulfide on the activity of LipA. LipA reaction (50 μM) in the absence of NfuA or iron and sulfide (closed squares). LipA reaction in the presence of 0.8 mM $FeCl_3$ and 0.8 mM Na_2S (open squares). LipA reaction in the presence of 200 μM NfuA (closed triangles). Other reaction components were as described above in panel A. **(D)** Effect of IscS on the LipA reaction. LipA reaction in the presence of IscS (open triangles). LipA reaction in the absence of IscS (closed squares). Reactions were described as above (panel A), except they contained 100 μM LipA, 200 μM IscS, 5 mM cysteine, 5 mM DTT, and no NfuA. (**E**) Effect of citrate on NfuA's enhancement of LipA catalysis. Reactions included 100 μM LipA (open squares); 100 μM LipA + 5 mM citrate (closed squares); 100 μM LipA + 1 mM NfuA (open circles); 100 μM LipA + 1 mM NfuA + 5 mM citrate (closed circles). (**F**) Reaction of 25 μM LipA + 400 μM NfuA (closed squares); 25 μM LipA, and 400 μM NfuA in the presence of 1 mM $Na_2{}^{34}S$ (closed triangles) in which the lipoic acid product containing two ^{32}S atoms is monitored. Reaction of 25 μM LipA + 400 μM NfuA in the

presence of 1 mM $Na_2{}^{34}S$ monitoring the $^{32}S/^{34}S$ mixed-labeled product (closed circles) and the $^{34}S/^{34}S$-labeled product (open circles).

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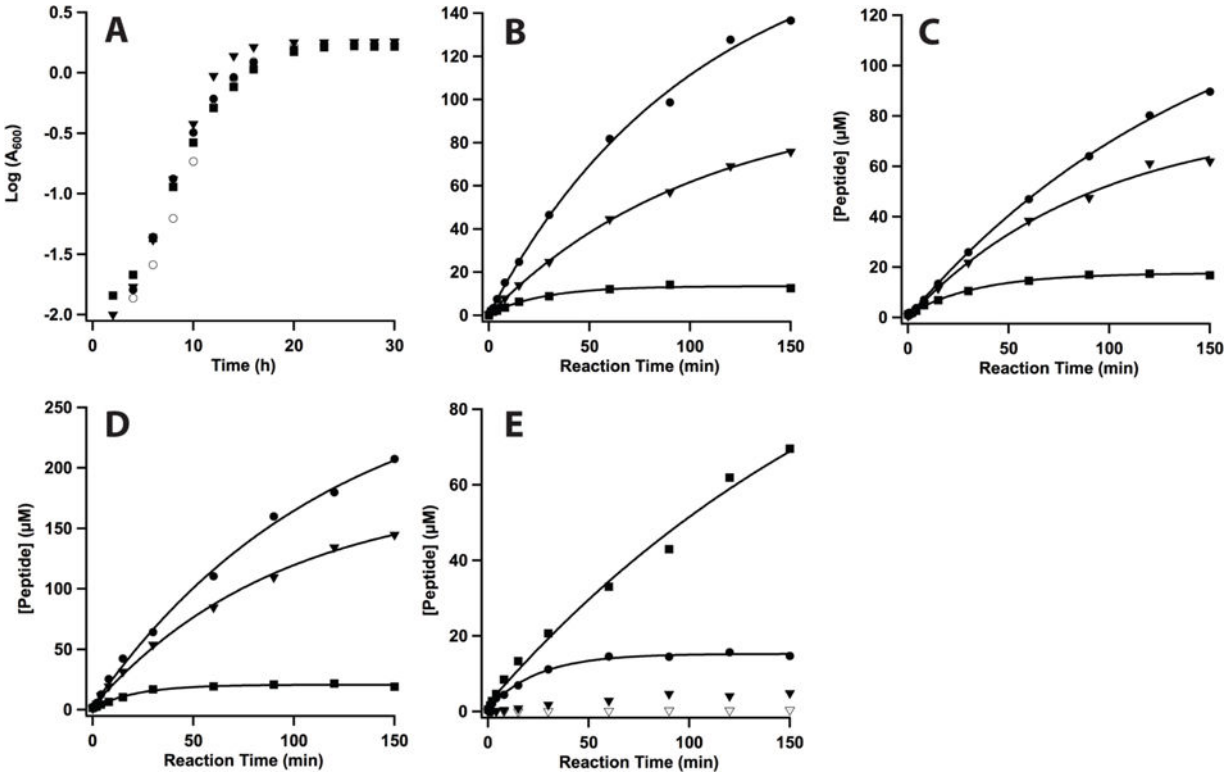

Fig. 4.

(A) Effect of NfuA on growth of *E. coli* in M9 minimal medium using glucose as a carbon source. Wild-type *BW25113* (closed circles), *BW25113*:Δ*nfuA* (solid squares), *BW25113*:Δ*nfuA* + 25 μM lipoic acid (open circles), and *BW25113*:Δ*nfuA* + 5 mM succinate/5 mM acetate (black triangles). **(B)** Effect of IscU on LipA catalysis. LipA only (solid squares). LipA + IscU (solid triangles). LipA + NfuA (solid circles). **(C)** Effect of IscU, HscA, and HscB on LipA catalysis. LipA only (solid squares). LipA + IscU (solid circles). LipA + IscU + HscA and HscB (solid triangles). **(D)** Effect of NfuA, HscA, and HscB on LipA catalysis. LipA only (solid squares). LipA + NfuA (solid circles). LipA + NfuA + HscA and HscB (solid triangles). Reactions contained 20 μM LipA, 400 μM octanoyllysyl-containing peptide substrate, 2 mM SAM, 2 mM dithionite, 2 mM ATP, 100 mM $MgCl_2$, and 0.5 μM SAH nucleosidase. When appropriate, IscU, HscA, and HscB were each added to a final concentration of 200 μM. (**E**) Reaction of 25 μM LipA + 400 μM IscU (closed squares); 25 μM LipA, and 400 μM IscUin the presence of 1 mM $Na_2{}^{34}S$ (closedsquares) in which the lipoic acid product containing two ^{32}S atoms is monitored. Reaction of 25 μM LipA + 400 μM IscU in the presence of 1 mM $Na_2{}^{34}S$ monitoring the $^{32}S/^{34}S$ mixed-labeled product (closed triangles) and the $^{34}S/^{34}S$ doubled-labeled product (open triangles).