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Mutants of *Escherichia coli* K-12 that are resistant to a selenium analog of lipoic acid identify unknown genes in lipoate metabolism

(lipoate–protein ligases/lipoate biosynthesis/diselenides)

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ABSTRACT **Lipoic acid is a disulfide-containing cofactor required for the reactions catalyzed by α-ketoacid dehydrogenase enzyme complexes. We report the chemical synthesis and biological properties of lipoic acid analogs in which one or both sulfur atoms were replaced by selenium. Replacement of either the C-6 or the C-8 sulfur atom with selenium results in lipoic acid derivatives with apparently unaltered biological properties. However, simultaneous replacement of both sulfur atoms gave an analog (selenolipoic acid) that inhibited growth of wild-type *Escherichia coli* when present in minimal glucose medium at 50 ng/ml. This growth inhibition was reversed by the addition of either excess lipoic acid or acetate plus succinate. Labeling experiments with [^{75}Se]selenolipoic acid showed that this compound was efficiently incorporated into the α-ketoacid dehydrogenase complexes of growing cells. Spontaneously arising selenolipoic acid-resistant (*slr*) mutants were isolated. Two of these isolates resistant to high levels of selenolipoic acid were studied in detail. The *slr-1* mutation, which was mapped to min 99.6 of the *E. coli* chromosome, increased the lipoate requirement of *lipA* strains by 4-fold and appeared to define a gene encoding a lipoate–protein ligase. The *slr-7* mutation, which was mapped to min 15.25 of the chromosome, completely suppressed the lipoate requirement of *lipA* strains and defined a gene of unknown function in the synthesis of lipoic acid.**

(*R*)-(+)-Lipoic acid (6,8-thioctic acid, or 1,2-dithiolane-3-pentanoic acid) is a sulfur-containing coenzyme found in a wide variety of organisms. In *Escherichia coli*, lipoic acid is a cofactor in the reactions catalyzed by pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and the glycine cleavage system (1, 2). Lipoic acid is covalently bound to the E2 subunits within these large multicomponent dehydrogenase complexes via an amide linkage between the carboxyl group of lipoic acid and the ε-amino group of a specific lysine residue (3). During the catalytic cycle of these dehydrogenases the disulfide group of lipoic acid is reductively acylated, thus mediating the transfer of activated substrates among the active sites within each enzyme complex (3).

Although little is known about lipoic acid biosynthesis or the mechanism whereby lipoic acid becomes covalently attached to proteins, labeling experiments indicate that octanoic acid is a direct precursor of lipoic acid (4). At least two genes, *lipA* and *lipB*, are required for the *de novo* synthesis of protein-bound lipoic acid in *E. coli* (5, 6). The *lipA* gene product may be required for the insertion of at least the first sulfur into the octanoic acid backbone, whereas the *lipB* gene product appears to play a role in the covalent attachment of lipoic acid to protein (6). To better characterize the *lipA* and *lipB* genes and perhaps identify other genes in lipoic acid metabolism, we examined the biological effects of lipoic acid analogs in which each (or both) of the sulfur atoms was replaced by selenium atoms (Fig. 1). Sulfur and selenium are similar in their chemical properties and differ only slightly in their electronegativities, covalent radii, and van der Waals radii (7). However, studies with model compounds have shown that analogous sulfur- or selenium-containing compounds have widely different ionization properties. For example, a comparison of the chemical properties of selenocysteine and cysteine showed that the selenohydryl group has a pK_a of 5.2 whereas the sulfhydryl group has a pK_a of 8.3 (8). Although selenolipoic acid (1,2-diselenolane-3-pentanoic acid) was previously synthesized as a model compound to study the steric and electronic effects of diselenides (9, 10), the biological properties of this compound had not been reported.

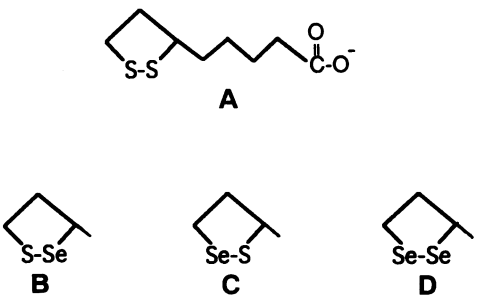

FIG. 1. Structure of lipoic acid (*A*) and the ring structure of 6-seleno-8-thiooctanoic acid, 6-thio-8-selenooctanoic acid, and selenolipoic acid (*B–D*, respectively). The IUPAC names for lipoic acid and selenolipoic acid are 1,2-dithiolane-3-pentanoic acid and 1,2-diselenolane-3-pentanoic acid, respectively.

MATERIALS AND METHODS

Bacterial Strains, Strain Constructions, and Growth Conditions. The bacterial strains used in this study were described previously (5, 6) except as noted. The *lipA* and *lipB* mutations were null mutations (5, 6). Strains KER264 and KER270 were isolated as spontaneous selenolipoic acid-resistant (*slr*) mutants of the prototrophic strain JK1 on minimal E glucose medium. The *lipA* derivatives of strains KER264 and KER270 were obtained by transduction of each *slr* mutant to kanamycin resistance with P1*vir* grown on strain KER176 (5). A P1*vir* stock grown on strain CAG18442 (11) was used to transduce strain KER264 to tetracycline resistance. One candidate which retained selenolipoic acid resistance was designated strain KER282. The Tn*10* element of strain KER282 was 79% cotransduced with *slr-1*. Strain KER305 was obtained by transduction of strain KER296 to tetracycline resistance with P1*vir* grown on strain KER282.

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Strains KER326 and KER327 were obtained by transduction of strain KER270 to kanamycin resistance with P1*vir* stocks grown on strains KER184 and KER310, respectively. Since *slr-7* was found to be linked to the *lipAB* loci, P1*vir* stocks grown on several candidates from each transduction were tested for their ability to transduce strain KER176 to lipoic acid independence. A P1*vir* stock grown on strain KER279 was used to transduce strain GS395 (12) to kanamycin resistance. One candidate which grew on minimal E glucose medium was retained as strain KER335. All cultures were grown at 37°C on either the minimal or the broth medium described previously (6).

Synthesis of Selenium and Sulfur Compounds. All compounds were synthesized from 6,8-dihydroxyoctanoic acid synthesized by an inverse Reformatsky reaction between the methyl ester of adipic hemialdehyde and the *t*-butyl ester of bromoacetic acid (13). Adipic hemialdehyde was synthesized by oxidation with pyridium chlorochromate on alumina (14) of the methyl ester of 6-hydroxyhexanoic acid [obtained by reduction of the monomethyl ester of adipic acid with borane in tetrahydrofuran (15)]. Following the Reformatsky reaction and selective cleavage of the *t*-butyl ester (13), the resulting carboxyl group was reduced with borane in tetrahydrofuran to give methyl 6,8-dihydroxyoctanoate. This compound was then converted to the dimesylate (16) and reacted with the dianion (16, 17) of selenium or sulfur (formed by sodium borohydride reduction of the element) to give methyl selenolipoate or methyl lipoate, respectively. This ester was then cleaved with base (16) to give selenolipoic or lipoic acid. Each of the steps in the synthesis of methyl 6,8-dihydroxyoctanoate proceeded in good yield (generally 80–90%). The product of each step was purified by flash chromatography and characterized by thin-layer and/or gas chromatography. The synthesis of [^{75}Se]selenolipoic acid and of [^{35}S]lipoic acid proceeded as above except that ^{75}Se or ^{35}S was added and the scale was greatly decreased. The mixed sulfur/selenium compounds were synthesized by reaction of the mesylate of the acid resulting from cleavage of the *t*-butyl ester with the dianion of either sulfur or selenium to give the 6-thio or 6-seleno compound, followed by reduction of the carboxyl group with borane in tetrahydrofuran to give the 8-hydroxy-6-thio (or 8-hydroxy-6-seleno) derivative, which was then converted to the mesylate and reacted with the dianion needed to give the mixed sulfur/selenium compound. All of these compounds comigrated with commercial lipoic acid on thin-layer chromatography, and the ultraviolet spectra of the reduced and oxidized forms of selenolipoic acid were identical to those previously reported (18, 19). All compounds were racemic. All reagents were purchased from Aldrich.

Radiolabeling of Cultures with [^{35}S]Lipoic Acid, [^{75}Se]Selenolipoic Acid, or [1-^{14}C]Octanoic Acid. Each strain was cultured at 37°C to 1–2 × 10^9 cells per ml in minimal E glucose medium containing 5 mM acetate, 5 mM succinate, and 3–5 mCi of [1-^{14}C]octanoic acid (55 mCi/mmol; American Radiolabeled Chemicals, St. Louis; 1 mCi = 37 MBq), 0.15 mCi of [^{35}S]lipoic acid (0.44 Ci/mmol), or [^{75}Se]selenolipoic acid at 3–5 × 10^5 cpm/ml (specific activity unknown). Cell pellets were treated with 10% (wt/vol) trichloroacetic acid and the resulting precipitate was washed with acetone, boiled in denaturing SDS buffer, and fractionated in SDS/10% polyacrylamide gels. The radiolabeled lipoyl proteins, selenolipoyl proteins, or octanoyl proteins were visualized by fluorography.

Inhibition Assay. The inhibition assays were performed in minimal E glucose medium with fixed concentrations of racemic lipoic acid and various concentrations of selenolipoic acid. Two milliliters of each medium was inoculated with ≈2 × 10^4 cells of strain KER176 and grown at 37°C. After 40 hr, the turbidity (OD_{600}) of each culture was measured.

Assay of Lipoic Acid Uptake. Cultures were grown overnight in minimal E glucose medium containing 0.1% vitamin-free casein hydrolysate, subcultured into the same medium, and grown to a density of ≈5 × 10^8 cells per ml. The cells were harvested at 10,000 × *g* at 4°C for 10 min, washed twice with ice-cold minimal E medium, resuspended at 2 × 10^9 cells per ml in minimal E glucose medium containing chloramphenicol (50 μg/ml), and starved for lipoic acid for 15 min at room temperature. The starved cells were diluted 2-fold in minimal E medium containing chloramphenicol (50 μg/ml) and [^{35}S]lipoic acid (0.44 Ci/mmol). At various times after the addition of label, 4-ml samples were removed and pipetted onto Gelman GN-6 filters covered with 2 ml of wash buffer [minimal E medium containing 1% (wt/vol) Brij-58]. The filters were washed three times with 2 ml of wash buffer. Filters were air dried before measurement of radioactivity in BioSafe II (Research Products International) liquid scintillation fluid. Alternatively, 4-ml samples were removed and pipetted into cold 10% trichloroacetic acid. The resulting protein pellets were washed twice with 3 ml of ethanol/ether, 1:1 (vol/vol), to remove free lipoic acid and suspended in 0.2 ml of 10% SDS. The detergent-solubilized pellets were boiled and samples were taken for liquid scintillation counting as above.

RESULTS

Effects of Selenium Compounds on Growth of *E. coli*. The four synthetic compounds (Fig. 1) were tested for their ability to stimulate or inhibit growth of *E. coli* on minimal glucose medium. As expected, lipoic acid (Fig. 1*A*) stimulated growth of *lipA* and *lipB* strains and had no effect on growth of wild-type strains. We found that both of the mixed sulfur/selenium compounds (Fig. 1 *B* and *C*) displayed biological properties indistinguishable from those of authentic lipoic acid. That is, both 6-thio-8-selenooctanoic acid and 6-seleno-8-thiooctanoic acid satisfied the lipoic acid requirements of strains KER176 (*lipA*) and KER184 (*lipB*) and failed to inhibit growth of the isogenic wild-type strain JK1. The possibility of *in vivo* conversion of the mixed sulfur/selenium analogs to lipoate was not investigated. In contrast, addition of selenolipoic acid (Fig. 1*D*) at 30–50 ng/ml completely inhibited growth of strain JK1 on minimal glucose medium. This growth inhibition was reversed by the addition of either lipoic acid or acetate plus succinate (supplementation with acetate plus succinate provided metabolic bypasses of the α-ketoacid dehydrogenases, thus eliminating any requirement for lipoic acid-dependent enzymes). We also found that selenolipoic acid could not be utilized as a lipoic acid source by either KER176 (*lipA*) or KER184 (*lipB*). Moreover, this analog inhibited growth of KER176 (*lipA*) even in the presence of lipoic acid and was a more potent inhibitor when lipoic acid was present at low (nearly growth-limiting) concentrations (Fig. 2).

Incorporation of [^{75}Se]Selenolipoic Acid into the α-Ketoacid Dehydrogenases. To test whether selenolipoic acid inhibited growth through formation of selenolipoylated enzyme complexes, we grew strains in the presence of radiolabeled selenolipoic acid or lipoic acid and then assayed for specific attachment to the E2 subunits of the α-ketoacid dehydrogenases by SDS/PAGE (Fig. 3). We found that strains JK1 (wild type), KER176 (*lipA*), KER184 (*lipB*), and KER310 (*lipA lipB*) all efficiently attached [^{35}S]lipoic acid to the pyruvate (E2p) and α-ketoglutarate (E2o) dehydrogenase subunits. [^{75}Se]Selenolipoic acid was also incorporated by all four strains, although with very different efficiencies. Both *lipA* strains accumulated high levels of selenolipoylated proteins, whereas the level accumulated by the wild-type strain was markedly lower and the *lipB* strain accumulated an intermediate level (Fig. 3). Since it has been shown that the

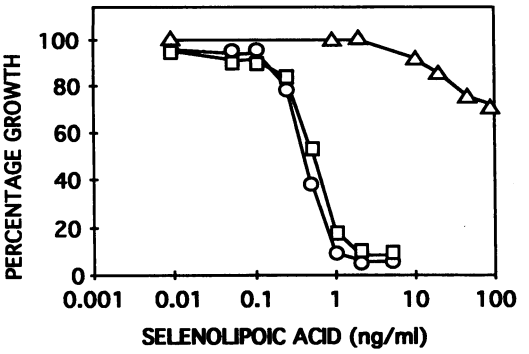

FIG. 2. Growth inhibition of strain KER176 (*lipA*) by selenolipoic acid. Growth tests were performed with fixed concentrations of lipoic acid and various concentrations of selenolipoic acid in minimal glucose medium. Growth is reported as a percentage of that observed in the absence of selenolipoic acid. The concentrations of lipoic acid tested were 0.5 ng/ml (○), 1.0 ng/ml (□), and 5 ng/ml (△).

lipoyl protein content of JK1 > KER184 > KER176 (or KER310) when grown on minimal glucose medium containing acetate plus succinate (6), we conclude that selenolipoic acid incorporation is inversely proportional to the amount of lipoic acid modified protein in each strain.

Characterization of Spontaneous Selenolipoic Acid-Resistant (*slr*) Mutants. When colonies of strain JK1 were streaked for isolation on minimal glucose medium containing selenolipoic acid at 50, 100, or 500 ng/ml, a few selenolipoic acid-resistant colonies appeared after 48 hr at 37°C. Two of these isolates, KER264 (*slr-1*) and KER270 (*slr-7*), were studied in detail. Although both mutants grew well on medium containing selenolipoic acid at 5000 ng/ml (and are thus ≈100-fold more resistant than the parental strain), only the *slr-1* isolate crossfed JK1 in the presence of selenolipoic acid at 200 ng/ml, suggesting that this strain produced a compound that overcame selenolipoic acid inhibition. We were unable, however, to detect the presence of lipoic acid in the spent media of *slr-1* cultures. Also, transduction of a *lipA* null mutation into each of the *slr* mutants gave two strains KER271 (*slr-1 lipA*) and KER279 (*slr-7 lipA*) which had differing growth requirements. Previous work had shown that the parental *lipA* strain required lipoic acid at 0.25 ng/ml to reach half-maximal growth on minimal glucose medium (6). The lipoic acid concentration required for half-maximal growth of the

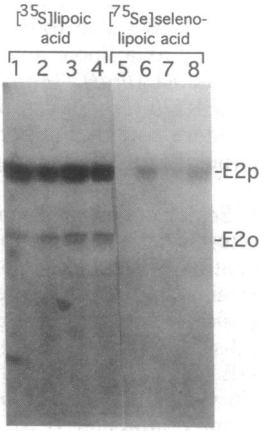

FIG. 3. Radiolabeled E2 proteins of *E. coli* extracts. The strains were labeled with [^{35}S]lipoic acid (lanes 1–4) or [^{75}Se]selenolipoic acid (lanes 5–8) as described in *Materials and Methods*. Proteins were electrophoresed in an SDS/10% polyacrylamide gel and exposed to preflashed film with an intensifying screen. Lanes 1 and 5, JK1 (wild type); lanes 2 and 6, KER176 (*lipA*); lanes 3 and 7, KER184 (*lipB*); lanes 4 and 8, KER310 (*lipA lipB*).

slr-1 lipA double mutant (KER271) was 1 ng/ml, a concentration 4-fold greater than that required by the parental *lipA* strain (KER176). Surprisingly, however, the *slr-7 lipA* double mutant (KER271) grew well without lipoic acid supplementation and also retained selenolipoic acid resistance.

Lipoic Acid Utilization by the *slr-1* Mutant. The increased lipoic acid requirement of strain KER271 (*slr-1 lipA*) led us to assay the kinetics of lipoic acid accumulation by this strain and strain KER176 (*lipA*) (Fig. 4). We also assayed the two strains for the synthesis of [^{35}S]lipoyl protein during the lipoic acid uptake assay. During the first 10 min of the assay, nearly all of the lipoic acid taken up by KER176 (*lipA*) was converted to a trichloroacetic acid-precipitable form (Fig. 4), indicating that following uptake, free lipoic acid was rapidly converted to lipoyl protein. In this same time interval only 60% of the lipoic acid taken up by strain KER271 (*slr-1 lipA*) was converted to a trichloroacetic acid-precipitable form (Fig. 4). These results suggested that the *slr-1 lipA* mutant had an altered ability to convert free lipoic acid to the protein-bound form.

The altered ability of strain KER271 to attach lipoic acid to protein suggested that the mutational defect might also result in altered ability to attach lipoic acid analogs to protein. Thus, we labeled strains KER296 (*lipA fadE*) and KER305 (*lipA fadE slr-1*) with [^{35}S]lipoic acid, [1-^{14}C]octanoic acid, or [^{75}Se]selenolipoic acid and analyzed for radiolabeled E2 proteins (Fig. 5). We found that the *slr-1* strain was partially defective in attachment of lipoic acid and octanoic acid, but severely deficient in attachment of selenolipoic acid (Fig. 5). Thus, it was clear that strains harboring the *slr-1* allele were able to discriminate against selenolipoic acid *in vivo*.

***slr-7* Is a Suppressor of *lipA* Null Mutations.** The surprising result (described above) that the *slr-7* mutation suppressed the lipoic acid requirement of a *lipA150*::Tn*1000*dKn strain when grown on minimal glucose medium was further investigated. A genetic backcross (data not shown) confirmed that KER279 (*slr-7 lipA*) still retained the *lipA* null allele, indicating that the *slr-7* mutation masked the *lipA* defect via true extragenic suppresion. We also found that KER279 grew on minimal succinate medium, but not on minimal acetate media, and thus, suppression was dependent on the carbon source. This suppression could be due to simultaneous metabolic bypasses of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase enzymes or to a restored ability to synthesize lipoic acid. We addressed this question by testing *slr-7* strains for *in vivo* activity of a third lipoic acid-dependent enzyme, the glycine cleavage system (5). We previously reported that *serA* mutants required a specific lipoyl protein subunit to use glycine as a serine source (5). Thus, strains KER332 (*serA lipA*) and KER335 (*serA lipA slr-7*) were constructed and tested for the ability to use

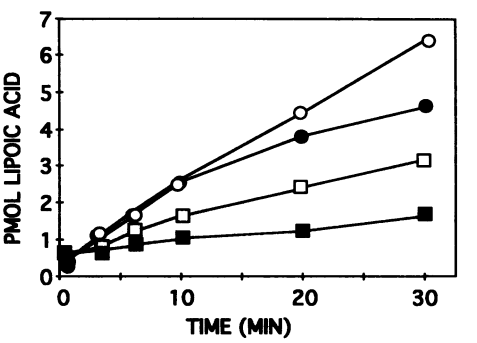

FIG. 4. Comparison of [^{35}S]lipoic acid accumulation by strains KER176 (*lipA*) and KER271 (*lipA slr-1*). Total lipoic acid accumulation: KER176, ○; and KER271, □. Accumulation of protein-bound lipoic acid by KER176, ●; and KER271, ■.

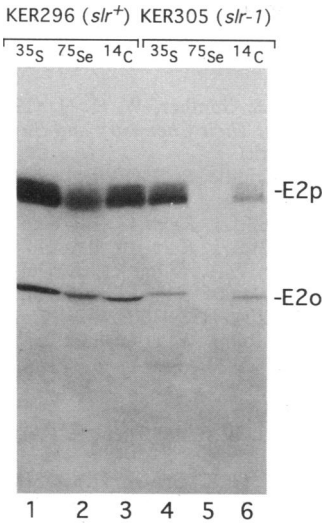

FIG. 5. Radiolabeled E2 proteins in extracts of wild-type and *slr-1* strains. Cultures were labeled with [^{35}S]lipoic acid (lanes 1 and 4), [^{75}Se]selenolipoic acid (lanes 2 and 5), or [1-^{14}C]octanoic acid (lanes 3 and 6). Lanes 1–3, KER296 (*lipA fadE*); lanes 4–6, KER305 (*lipA fadE slr-1*). A *fadE* mutation was included to prevent degradation of octanoic acid by the β-oxidation pathway, allowing the specific incorporation of [1- ^{14}C]octanoic acid into the E2p and E2o dehydrogenase subunits (6).

glycine as a serine source on minimal glucose medium. As expected, strain KER332 was able to use glycine as a serine source only when exogenous lipoic acid was supplied. The *slr-7* strain KER335, however, used glycine as a serine source even in the absence of exogenous lipoic acid. Furthermore, an acid-hydrolyzed protein extract of KER279 (*lipA slr-7*) contained lipoic acid, since the extract allowed growth of KER176 (*lipA*) on minimal succinate medium (20). These results demonstrated that *slr-7* suppression of the *lipA* defect was due to restored synthesis of lipoic acid.

We also used the glycine cleavage assay to determine whether the *slr-7* mutation suppressed the *lipA* defect under anaerobic growth conditions. Recently, we showed that *serA lipA* strains required exogenously supplied lipoic acid in order to utilize glycine as a serine source during anaerobic growth (6). We found that although KER335 (*serA lipA slr-7*) utilized glycine as an anaerobic serine source in the absence of an exogenous lipoic acid supplement, the growth of strain KER335 was much slower than that of strain GS395 (*serA*), indicating that suppression by *slr-7* was partially inhibited by anaerobiosis.

Finally, the *slr-7* mutation was tested for suppression of the *lipB* defect by transduction of the *slr-7* allele into strains KER184 (*lipB*) and KER310 (*lipA lipB*). We found that the *slr-7* mutation could not suppress the *lipB* mutation and also failed to suppress the *lipA* defect when the strain also carried a *lipB* mutation. Thus, *slr-7*-mediated suppression of the *lipA* lesion required a functional *lipB* gene.

Mapping of *slr-1* and *slr-7*. Both *slr* alleles were localized on the *E. coli* genetic map by using the mapping-strain collection of Singer *et al.* (11). Mating experiments with Hfr strains (11) indicated that *slr-1* mapped between min 89 and min 6. Transductional crosses with strain CAG18442 (11) as donor showed that the *slr-1* mutation was 79% (31/39 colonies scored) linked to *thr34*::Tn*10*. Additional P1 crosses with strains CAG18430 and CAG12093 as donors showed that *slr-1* was 38% (37/98) linked to *zjj-202*::Tn*10* and 4% (2/49) linked to *car-96*::Tn*10*. Thus, the *slr-1* mutation mapped to min 99.6. P1 transduction experiments with KER176 and CAG12077 as donors showed that the *slr-7* mutation was 23% (6/27) linked to *lipA150*::Tn*1000*dKn and 96% (48/50) linked to *zbe-280*::Tn*10*, indicating that the *slr-7* mutation was located very close to min 15.25.

Reduction of the Diselenide Bond of Selenolipoic Acid. Our findings that selenolipoic acid was attached to the E2 proteins and that this attachment inhibited growth led us to examine the chemistry of the diselenide moiety present in such selenolipoylated proteins. It seemed likely that the diselenide bond (Fig. 1*D*) could be highly resistant to the reductive acylation of the disulfide bond of lipoic acid which normally occurs during the α-ketoacid dehydrogenase catalytic cycle. Therefore, we examined the reduction of selenolipoic acid *in vitro* by monitoring the loss of absorbance at 442 nm (18, 19). We observed that selenolipoic acid was readily reduced by sodium borohydride (19) but was not reduced by dithiothreitol. Upon reduction with sodium borohydride immobilized on alumina and removal of the reducing agent, the presence of 1 M dithiothreitol had no effect on the rapid and quantitative return of the oxidized spectrum. These experiments used the buffer conditions of Houk and Whitesides (21), and thus it can be calculated from their data that a redox potential (E_0) more negative than −478 mV would be required to reduce selenolipoic acid. Since this value greatly exceeds the reducing potential of most biological reductants, it seems likely that selenolipoylated proteins remain in the oxidized state *in vivo*.

DISCUSSION

Our observations indicate that selenolipoic acid inhibits growth through formation of selenolipoylated α-ketoacid dehydrogenase complexes that are inactive. Labeling experiments clearly demonstrated that [^{75}Se]selenolipoic acid was specifically and efficiently incorporated into the appropriate dehydrogenase complexes. Cell growth was readily restored by the addition either of lipoic acid or of acetate plus succinate, and thus it seems likely that selenolipoylated enzymes are nonfunctional rather than toxic. We believe that the protein-bound selenolipoate moiety is irreversibly oxidized *in vivo* and that the diselenide bond is inert as a substrate for the reductive acylation that is an essential step of the α-ketoacid dehydrogenase catalytic cycle. This hypothesis is also consistent with the finding that selenolipoic acid is a less effective inhibitor when sufficient lipoic acid is also present (Fig. 2).

Several results indicate that *slr-1* is an allele of a gene encoding a lipoate–protein ligase. First, insertion of the *slr-1* allele into a *lipA* background resulted in an increased requirement for lipoic acid. Moreover, the *lipA slr-1* strain was markedly deficient in the synthesis of lipoyl proteins when compared with an isogenic *lipA* strain. The finding that the *lipA slr-1* mutant incorporated [^{35}S]lipoic acid but not [^{75}Se]-selenolipoic acid into the E2 subunits of the α-ketoacid dehydrogenases suggests that the *slr-1* mutant is a substrate specificity mutant that results in the ability to discriminate selenium from sulfur. Indeed, genetic complementation studies indicate that the *slr-1* mutation lies within a gene called *lplA*. The *lplA* gene is defined by null mutants (isolated by a procedure independent of selenolipoic acid) and encodes the lipoate–protein ligase activity detectable in cell extracts (unpublished data). Analysis of independent *lplA* mutants (including the *slr-1* allele) has revealed an unexpected redundancy in the functions of the *lplA* and *lipB* genes; the *lipB* gene appears to act only in the utilization of lipoyl groups generated by the endogenous (*lipA*-dependent) biosynthetic pathway, whereas the *lplA* gene product functions in a separate and independent pathway for the incorporation of exogenously supplied lipoic acid or lipoic acid analogs into E2 proteins (unpublished data).

The function of the gene identified by the *slr-7* mutation is less clear. Here we have shown that the *slr-7* mutation restores lipoate synthesis in *lipA* null strains, presumably by

substituting for the sulfur-insertion activity that *lipA* is believed to encode. Based solely on thiooctanoate feeding experiments with two *lipA* point mutants, Hayden *et al.* (22) have recently concluded that the *lipA* gene product is the only protein required for the sulfur-insertion step(s) of lipoate synthesis. In contrast, we previously found that *lipA* null mutants utilize both 8-thio- and 6-thiooctanoate as alternative lipoate sources (6), thus suggesting the existence of another activity which converts these monothiols to lipoic acid. Perhaps the wild-type product of the gene defined by *slr-7* encodes such a sulfur-inserting activity, and it is an alteration of this activity in the *slr-7* mutant which restores lipoate synthesis to *lipA* null strains. In this regard, it is intriguing that *slr-7*-mediated lipoic acid synthesis seemed to be decreased during anaerobic growth. It may be that the activity encoded by *slr-7* is sensitive to anaerobiosis or simply that the *slr-7* gene is oxygen regulated. Unfortunately, null mutants of the *slr-7* locus are not available and so we cannot yet evaluate the role of this gene in the wild-type sulfur-insertion step(s) of lipoate biosynthesis.

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