LETTER

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Small-Molecule Diselenides Catalyze Oxidative Protein Folding *in Vivo*

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ABSTRACT Prokaryotic cells normally rely on periplasmic oxidoreductases to promote oxidative protein folding. Here we show that simple diselenides can also facilitate the conversion of dithiols to disulfides *in vivo*, functionally replacing one such oxidoreductase, DsbA, in the oxidative folding of diverse proteins. Structurally analogous disulfides provide no detectable effect when used at concentrations that gave optimal activity with diselenides, and even at 100- to 1000-fold higher levels they show only partial activity. The low concentrations of diselenides needed to fully negate typical DsbA knockout phenotypes suggest catalysis *in vivo*, a property that sets these additives apart from other small molecules used in chemical biology. Supplementing growth media with cell-permeable organocatalysts provides a potentially general and operationally simple means of fine-tuning the cellular redox environment.

Disulfide bonds are essential for the stability and function of many secreted proteins. Because the spontaneous oxidation of cysteine thiols is slow and nonspecific, formation of disulfide cross-links in the cell is enzyme-catalyzed. The CXXC family of oxidoreductases promote oxidative folding *via* thiol-disulfide exchange reactions between a Cys-Xaa-Xaa-Cys active site motif and substrate (*1*). For instance, the promiscuous enzyme DsbA effects thiol oxidation in the periplasmic space of *Escherichia coli via* direct transfer of an active-site disulfide bond to a reduced protein. Reoxidation of DsbA is accomplished by a second oxidoreductase, DsbB, which shuttles electrons to an acceptor quinone. Thus, DsbA and DsbB act as a redox relay between the thiols of newly secreted proteins and the respiratory chain of *E. coli*. Upon oxidation, disulfide bond isomerization may be necessary to attain the native state, and these rearrangements are catalyzed by the CXXC oxidoreductase DsbC.

High concentrations of exogenously added disulfides can influence protein folding in the periplasm of *E. coli*. Indeed, it was shown that either 0.4 mM cystamine or 0.1 mM cystine complements a DsbB knockout strain, restoring a typical *E. coli* phenotype (*2*). Cystine (0.1 mM) can also partially complement a DsbA deficiency in *E. coli* upon heterologous expression of the weakly active DsbA from *Staphylococcus aureus* (*3*). This ability of disulfide additives to work together with CXXC foldases has been utilized for biotechnological production of difficult-to-fold proteins. For example, the heterologous coproduction of pectate lyase C (PelC) and protein disulfide isomerase (PDI) in a DsbA-deficient *E. coli* strain showed a 2-fold improvement in the yield of PelC upon the addition of oxidized glutathione (1.0–5.0 mM) (*4*). Thiols can also modulate oxidative protein folding in the periplasmic space of *E. coli*, presumably by increasing the rate of disulfide bond isomerization steps. For example, the dithiol BMC ((±)-*trans*-1,2-bis(2-mercaptoacetamido)-cyclohexane) has been developed as a CXXC-motif mimic (*5*). When added to the growth medium of *E. coli*, BMC (2.0–50 μM) boosts production of proinsulin by 60% (*6*). Similarly, reduced glutathione (5.0 mM) improves the yield of two disulfide-bond-rich protease inhibitors, the Ragi bifunctional inhibitor and bovine pancreatic trypsin inhibitor, by 14-fold and 3-fold, respectively (*7, 8*). However, the potential benefit of thiol additives can be highly case-dependent, as the yields of active alkaline phosphatase and β-lactamase decrease with increasing glutathione concentration (1.0–100 mM) (*9*). From these examples it is clear that small-molecule thiols and disulfides are capable of influencing oxidative protein folding *in vivo*, but these effects mostly require near-millimolar concentrations to achieve modest gains.

Diselenides possess significant advantages over disulfides for protein folding (*10*). As a result of the low pK_a value of the

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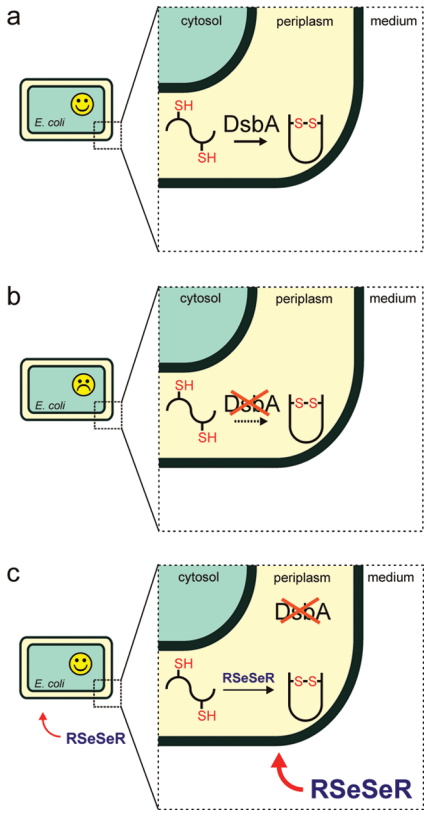

Figure 1. Oxidative protein folding in *E. coli*. a) The periplasmic protein DsbA accelerates the conversion of thiols to disulfides in substrate proteins. b) DsbA-deficient strains exhibit greatly decreased levels of oxidative folding. c) Diselenides such as selenocystamine, which catalyze the oxidation of thiols to disulfides, can cross the outer bacterial membrane and complement the DsbA deficiency. Analogous disulfides are much less effective as alternative oxidants.

selenol leaving group, diselenide bonds are kinetically superior to disulfides for the oxidation of thiols. Moreover, selenols are rapidly (re)oxidized to diselenides by atmospheric oxygen, rendering oxidative protein folding reactions catalytic in diselenide (*11*). As a result, redox buffers based on selenoglutathione, a diselenide-bond-containing variant of oxidized glutathione, afford the same folding rates and yields as a standard glutathione redox buffer but at substantially lower concentrations (*11*). These favorable properties would also be expected to benefit oxidative protein folding *in vivo*. As with the sulfur-containing additives mentioned above, low molecular weight diselenides should be able to cross the outer membrane of *E. coli* and function as periplasmic oxidants (Figure 1). We hypothesized that small-molecule diselenide catalysts might therefore be able to replace natural enzymes like DsbA.

Though not lethal to *E. coli*, deletion of the gene encoding DsbA significantly retards disulfide bond formation in periplasmic proteins (*12*). The magnitude of the impairment can be conveniently assessed using a redox sensor based on an engineered MalF-β-galactosidase fusion protein (*13*). In DsbA-deficient cells, the sensor remains reduced and the β-galactosidase component remains active with chromogenic substrates (Figure 2, panel a). Oxidation of the sensor prevents proper folding of the β-galactosidase domain and no enzyme activity is observed in DsbA knockout strains (THZ2 and JCB817) harboring a plasmid encoding wild-type DsbA (pDsbA3). This observation is consistent with previous findings that, upon transformation with pDsbA3, these strains produce 65 times more DsbA than wild-type *E. coli* and show the same behavior in a motility assay as the *dsba*$^+$ strain JCB816 (*14*). We have employed this MalF-β-galactosidase redox sensor to screen a range of diselenides and structurally analogous disulfides as alternative, exogenously added sources of periplasmic oxidative activity.

Selenocystamine was the most effective oxidant tested, decreasing β-galactosidase activity of DsbA-deficient cells more than 100-fold when present at 10 μM concentration in the medium. This effect is similar to that observed with cells overexpressing DsbA (Supporting Figure S1). Lower concentrations are also effective. For example, 1.0 μM caused a 4-fold decrease in β-galactosidase activity (Figure 2, panel a). Comparable amounts of cystamine had no effect. With one exception, all other tested diselenides also cause substantial decreases in β-galactosidase activity relative to both the negative control and disulfide reagents (Figure 2, panel a). While the efficacies of these other diselenides are all somewhat lower than that of selenocystamine, the observed activities demonstrate that diselenides are generally much more active in this assay than disulfides. Selenocystine provides the lone exception among the diselenides we tested because this amino acid is very toxic to *E. coli* (*15*). The observed toxicity probably originates from mischarging of cysteinyl-tRNA, which would lead to partial, nonspecific replacement of cysteine by selenocysteine in the bacterial proteome.

The primacy of selenocystamine in this assay was not anticipated on the basis of its folding activity *in vitro*. For example, selenoglutathione is more effective than selenocystamine for the oxidative folding of purified ribonuclease A (RNase A) (*11*). The advantage of using selenocystamine for folding *in vivo* may stem from its net positive charge, which presumably leads to its preferential accumulation inside the periplasm at the negatively charged phospholipid bilayer compared to the other selenium compounds.

The *in vivo* benefit provided by exogenously added selenocystamine appears to be general. The enzyme alkaline phosphatase requires two structural disulfide bonds to attain an active conformation, and DsbA-deficient cells display strongly decreased alkaline phosphatase activity compared to cells producing active DsbA (*16*). As shown in Figure 2, panel b, the addition of 1.0 μM selenocystamine to a DsbA knockout strain provides the same level of alkaline phosphatase activity as does plasmid-encoded DsbA. In contrast, 1.0 μM cystamine is ineffective. At a 1000-fold higher concentration (1.0 mM), the disulfide partially complements the DsbA deficiency, but even then it is still ca. 6-fold less

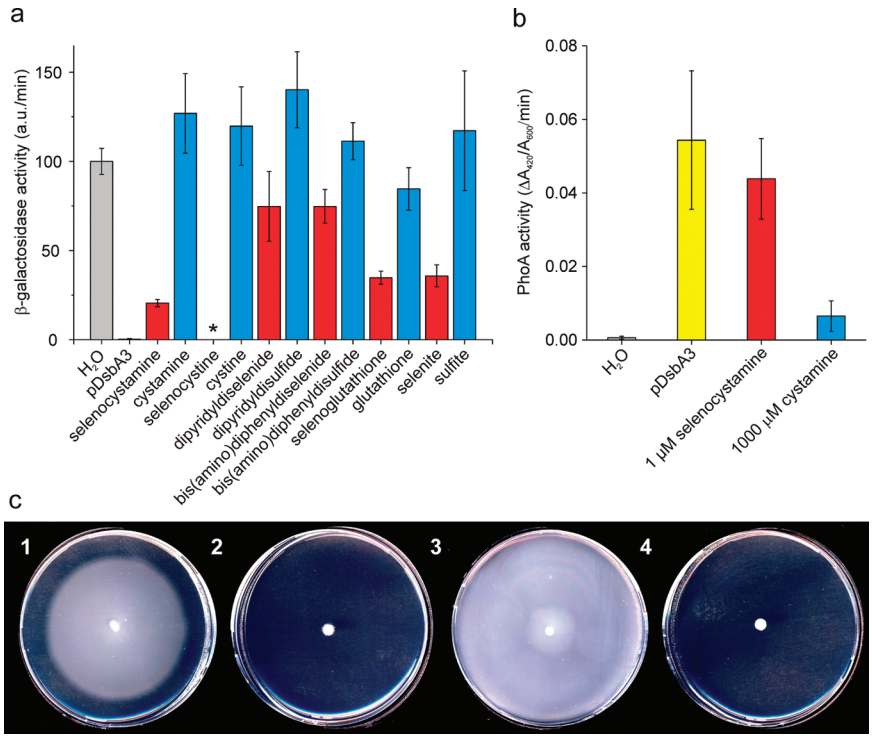

Figure 2. Catalysis of disulfide bond formation *in vivo* and *in vitro*. a) β-Galactosidase activity of a DsbA-deficient *E. coli* strain (THZ2) grown at 37 °C for 16 h in maltose-containing M63 medium in the presence of various disulfides and diselenides at 1.0 μM concentration. Activity was monitored fluorometrically using 4-methylumbelliferyl galactopyranoside as a substrate. A MalF-β-galactosidase fusion protein is inactive when wildtype DsbA is expressed from plasmid pDsbA3. The observed values are the average of at least three independent measurements. An asterisk indicates that activity could not be measured because the compound in question kills the host. β-Galactosidase activity data for supplementation at various concentrations of disulfides and diselenides can be found in Supporting Figures S1 and S2. b) Alkaline phosphatase (PhoA) activity of THZ2 cells grown in M63 medium in the presence of cystamine or selenocystamine at 37 °C for 24 h. Activity was monitored spectrophotometrically using *p*-nitrophenylphosphate as a substrate. The observed values are the average of at least three independent experiments. Supporting Figure S3 shows the dependence of alkaline phosphatase activity on disulfide and diselenide concentration. c) Restoration of motility to a DsbA-deficient *E. coli* strain (THZ2). (1) THZ2 cells transformed with pDsbA3 were spotted on M63 0.4% (w/v) agar plates. For all other plates, untransformed THZ2 cells were spotted on M63 0.4% (w/v) agar containing (2) no additives, (3) 1.0 μM selenocystamine, or (4) 1.0 μM cystamine. Plates were incubated for 24 h at 37 °C. Motility data at various concentrations of disulfides and diselenides are summarized in Supporting Tables S1 and S2.

effective than 1.0 μM selenocystamine (Figure 2, panel b).

The potency of selenocystamine is most strikingly demonstrated by the restoration of motility to DsbA-deficient cells (Figure 2, panel c). *E. coli* propels itself by rotary engine-driven propellers called flagella (*17*). Successful assembly of these supramolecular structures requires DsbA-catalyzed formation of a stabilizing structural disulfide in the flagellar FlgI protein (*18*), which constitutes the P-ring of the motor in the outer membrane. When plated overnight on soft agar, DsbA-producing bacteria swim to the edges of the Petri dish. DsbA-knockout cells remain viable but are not chemotactic because they cannot oxidize FlgI. Supplementing the plates with 1.0 μM selenocystamine restores full motility to the DsbA-deficient host (Figure 2, panel c). This compound apparently provides sufficient oxidative power to the bacterial periplasm to enable proper assembly of the flagellar motor. Cystamine does not complement the DsbA deficiency, even at a 1000-fold higher concentration.

The three phenotypic assays described here demonstrate that the utility of low molecular weight diselenides as folding catalysts extends beyond the test tube. The ability of these compounds to functionally replace the natural enzyme DsbA in living cells for the folding of diverse (unrelated) proteins can be ascribed, at least in part, to intrinsically high thiol-oxidase activity. Diselenides are more electrophilic than disulfides and therefore react much faster with protein cysteines. Perhaps more crucially, diselenides are rapidly regenerated by molecular oxygen, which enables the oxidation of multiple cysteine pairs by a single diselenide and presumably lowers the concentration of diselenide required for effective protein oxidation. The catalytic potential of diselenides is tellingly illustrated by the large rate acceleration observed *in vitro* for the aerobic oxidation of dihydrolipoic acid (Figure 3). In contrast, disulfides are not catalysts of this process and only act as stoichiometric oxidants. Thus, the enormous difference in the abilities of diselenides and disulfides to complement a DsbA knockout is rooted in their different modes of action.

In our model for the catalysis of protein oxidation by diselenides, the catalyst is directly regenerated by atmospheric O_2. Thus, the participation of DsbB, which normally reoxidzes DsbA, should not be required for cellular activity. To test this hypothesis, we fed diselenides to *E. coli* cells deficient in both DsbA and DsbB. Upon addition of

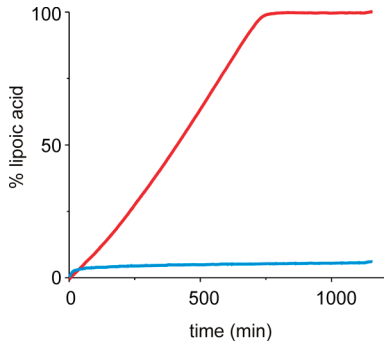

Figure 3. *In vitro* oxidation of 1.0 mM dihydrolipoic acid by molecular oxygen (at pH 7.0, 25 °C), catalyzed by 10 μM selenocystamine (in red) or 10 μM cystamine (in blue) and monitored spectrophotometrically at 330 nm.

1.0 μM selenocystamine, the double knockout and the single DsbA knockout showed the same motility, demonstrating that diselenides bypass the entire pathway for protein oxidation (Supporting Table S2).

In this context, Cu^{2+} provides an interesting comparison, as it is also known to catalyze the air oxidation of thiols (*19*). Indeed, it was recently shown that $CuCl_2$ can complement a DsbA and DsbB double deficiency *in vivo*, restoring motility to near the wild-type level at a concentration of 0.2 mM (*20*). However, at the optimal concentration for selenocystamine (1.0 μM), we observed no complementation of DsbA null strains by either $CuSO_4$ or $CuCl_2$ (Supporting Table S1). Moreover, *in vitro* experiments show that Cu^{2+} is a poor oxidative protein folding catalyst (*20*) and limited in its use due to unspecific oxidation of other amino acids (*21*). The oxidative folding activity of Cu^{2+} thus lags behind that of selenocystamine, but is reminiscent of that displayed by selenite (SeO_3^{2-}), an inorganic form of selenium (Figure 2, panel a, Supporting Figure S1, and Supporting Table S1). Like Cu^{2+}, selenite has long been known to catalyze the oxidation of thiols in the test tube (*22*), and was more recently shown to possess modest catalytic activity for the oxidative folding of RNase A *in vitro* (*23*).

For multiply disulfide-bonded proteins such as alkaline phosphatase, *in vivo* oxidative protein folding may further benefit from the nucleophilic selenols that are transiently liberated upon substrate oxidation, since these species can promote disulfide bond shuffling, not unlike the periplasmic isomerase DsbC (*24*). The intrinsically lower pK_a value of selenols relative to thiols provides an advantage for catalysis of protein disulfide bond isomerization, which compounds the advantage gained from enhanced protein oxidation by diselenides. Extrapolating from the favorable synergy achieved with protein disulfide isomerase *in vitro* (*11*), productive interactions of the diselenide (or selenol) with other periplasmic oxidoreductases may also contribute to folding efficiency. Indeed, the surprisingly low diselenide concentrations needed to replace endogenous DsbA point to a general increase in periplasmic thiol-disulfide exchange rates. Future complementation studies using DsbC knockout strains may help to illuminate this point.

The bacterial periplasm is a convenient reaction chamber whose properties can be manipulated in a variety of ways. For example, the redox environment of the periplasmic space can be adjusted by overexpression or removal of specific oxidoreductases (*7, 8*), which can have dramatic consequences for the folding of heterologously overproduced proteins. The exogenous addition of small-molecule diselenides provides an alternative, operationally simple approach for altering the concentration of folding catalyst. Diselenides (such as selenocystamine) will likely prove to be superior to disulfides as reagents for biotechnological protein production. Their appeal is enhanced by the low concentrations (micromolar range) sufficient for replacing DsbA in bacteria as well as the lack of any obvious toxicity (selenocystine excepted, as noted above). An attractive potential application would be to supplement endogenous levels of DsbA in standard strains of *E. coli* with exogenously provided selenocystamine (which is commercially available) to improve the production efficiency of disulfide-containing proteins. It remains to be seen whether diselenides can act constructively together with DsbA, but the observation that selenocystamine and PDI can act as a dual catalyst system *in vitro* (*11*) is cause for optimism. Diselenide supplementation could also be used in combination with modulation of specific oxidoreductases to increase protein production efficiency. For some proteins (such as the ragi bifunctional inhibitor and tissue plasminogen activator (*7, 25, 26*)), DsbA is deleterious for folding, and higher yields of properly folded protein are obtained in DsbA-deficicient cells. The production of these proteins in a DsbA-knockout strain supplemented with selenocystamine (or some other diselenide) could provide especially interesting test cases. To further optimize activity for a particular application, diselenide reactivity can easily be fine-tuned through substituent or electrostatic effects.

Oxidative protein folding activity extends the fascinating spectrum of known biological phenotypes induced by diselenides (*27*). In the future, it may be possible to harness the rich chemistry of selenium to provoke deliberate shifts in redox balance as a means of probing cellular responses to oxidative stimuli. Because oxidative stress is intimately linked with the anticancer properties of selenium-containing molecules (*28*), the thiol-oxidase activity of diselenides may even have some medical relevance. For these and other applications, diselenides represent an attractive chemical tool for exploring and exploiting biological redox phenomena.

METHODS

General. Strains used in this work are *E. coli* THZ2 (dsba::kan, recA::cam, λmalF-lacZ102) (*29*), *E. coli* JCB817 (araD139, Δ[ara-leu]7679, galU, galK, Δ[lac]174, rpsL, thi-1, phoR dsbA−) (*30*), and *E. coli* JCB818 (MC1000 *phoR* λ102

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dsbA::kan1 dsbB::kan) (*12*). THZ2, JCB817, and JCB818 strains and the pDsbA3 plasmid were kindly provided by R. Glockshuber (ETH Zürich, Switzerland).

Chemicals and Media. All chemicals were obtained as biochemical grade reagents from Fluka, Sigma-Aldrich, ABCR, Acros, and Axon-lab. The concentration of disulfides and diselenides in stock solutions was determined by a colorimetric assay (*31*). *E. coli* M63 minimal medium (*2, 14*) contains per liter 13.6 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.5 mg $FeSO_4 \cdot 7H_2O$ and was adjusted to pH 7.0 with KOH. M63 motile agar plates (*2*) contain 50 mg L^{-1} of all proteinogenic amino acids except methionine and cysteine, and per liter, 10 mL 20% (w/v) glucose, 1.0 mL 1.0 M $MgSO_4 \cdot 7H_2O$, 1.0 mL 5.0 mg mL^{-1} thiamine, 1.0 mL 50 mg mL^{-1} kanamycin, and 0.4% (w/v) agar.

β-Galactosidase Assay. A fluorescence-based 96-well plate liquid culture assay was used to measure β-galactosidase activity according to a previously published procedure (*32*). Briefly, sterile tubes filled with 5.0 mL of M63 minimal medium, supplemented with amino acids, glucose, $MgSO_4$, thiamin, and kanamycin, as described above, as well as 0.4% (w/v) maltose, were inoculated with a single colony and shaken overnight at 37 °C. In the case of THZ2 cells containing the pDsbA3 plasmid, the media additionally contained 0.1 mg mL^{-1} ampicillin. After incubation, 200 μL aliquots of this preculture were added to 2.0 mL of M63 media, supplemented with the nutrients above plus cystamine or selenocystamine (final concentrations of 100, 10, 1.0, or 0.1 μM). These supplemented cultures were shaken overnight at 37 °C and afterward diluted 6-fold into black fluorescence 96-well plates with transparent bottoms containing Z-buffer (100 mM sodium phosphate, 10 mM KCl, 1.0 mM $MgSO_4$, pH 7.0). The OD_{600} values were the same for all samples (except those in which the cells had died). 4-Methylumbelliferyl galactopyranoside in DMSO was added by multichannel pipet to a final concentration of 0.1 mg mL^{-1}, and the change in fluorescence (ex. 360 nm, em. 460 nm) was measured over 15 min with intermittent shaking on a Thermo Varioskan plate-reader.

Alkaline Phosphatase Assay in 96-Well Plates. Two disulfide bonds are essential for the activity of the periplasmic protein alkaline phosphatase, making this enzyme a reporter for oxidative protein folding in *E. coli* (*16, 33*). Alkaline phosphatase assays were carried out in parallel with the β-galactosidase assay. Aliquots of the same preculture were diluted 20-fold into separate transparent 96-well plates containing buffer (1.0 M Tris-HCl, 0.1 mM $ZnCl_2$, pH 8.0) and 0.005% (w/v) SDS. To this mixture was added *p*-nitrophenylphosphate to a final concentration of 1.5 mM, and the change in absorbance was monitored at 420 nm for 1 h at RT, with intermittent shaking on a Thermo Varioskan plate-reader.

Alkaline Phosphatase Assay in Cuvettes. Restoration of alkaline phosphatase activity to THZ2 and JCB817 cells (which lack DsbA) was monitored spectrophotometrically (*16*). Cultures (5.0 mL) in M63 minimal medium, supplemented with different disulfides and diselenides, were grown at 37 °C overnight. A 900 μL aliquot was incubated with 0.1 M iodoacetic acid for 20 min at 0 °C, centrifuged at 16,000 × *g* for 5 min, washed with ice-cold 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgSO_4$, and then resuspended in 1.0 M Tris-HCl buffer (pH 8.0) containing 0.1 mM $ZnCl_2$. After their OD_{600} values were recorded, the cell suspensions were diluted 100-fold and permeabilized with 5% (v/v) chloroform and 0.005% (w/v) SDS for 5 min at RT. *p*-Nitrophenylphosphate was added to a final concentration of 1.5 mM, and the absorbance at 420 nm was continuously monitored for 30 min at 37 °C. The activity per minute was corrected by the measured OD_{600} of the resuspended cells.

Motility Assay. The ability of thiols, disulfides, and diselenides to restore motility to *E. coli* JCB817 and THZ2 cells (which lack DsbA) was tested on 0.4% (w/v) agar M63 minimal plates, supplemented with all proteinogenic amino acids except methionine and cysteine according to a previously published procedure (*2*). The reagents were poured into the plates at the concentrations shown in Supporting Table S1. A 2.0 μL spot of a fresh overnight preculture (in LB medium containing 50 mg L^{-1} kanamycin) was spotted in the middle of the plates prior to incubation at 37 °C. The plates were not stacked. The diameter of the observed *E. coli* swarm was measured after 24 h. Experiments were performed in duplicate.

***In Vitro* Dihydrolipoic Acid Oxidation by Molecular Oxygen.** The aerobic oxidation of dihydrolipoic acid (*34, 35*) was measured spectrophotometrically at 330 nm and 25 °C. Dihydrolipoic acid was added to solutions containing either cystamine (10 μM) or selenocystamine (10 μM) in 100 mM Tris-HCl buffer (pH 7.0) containing 2.0 mM EDTA. Initial velocities were determined over the first 10% of the reaction and corrected for spontaneous background oxidation under the same conditions. The concentrations of dihydrolipoic acid, cystamine, and selenocystamine were determined spectrophotometrically (*31*), and the pH was measured before and after the reaction. The oxidation of dihydrolipoic acid was independently monitored by RP-HPLC. Reaction mixtures were quenched with 1.0 M HCl, and reduced and oxidized lipoic acid were separated on a Waters Polarity column (100 × 4.6 mm, 3 μm, dC_{18}) using a linear gradient from 95% water, containing 0.1% (v/v) trifluoroacetic acid, to 50% acetonitrile, containing 0.05% (v/v) trifluoroacetic acid, over 35 min at a flow rate of 1.2 mL min^{-1}. The effluent was monitored at 220 and 330 nm. A calibration curve was generated from a dilution series of pure standards. The results obtained by monitoring the oxidation of dihydrolipoic acid spectrophotometrically and by RP-HPLC show excellent agreement.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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