Reduction of Methionine Sulfoxide to Methionine by *Escherichia coli*

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L-Methionine-*dl*-sulfoxide can support the growth of an *Escherichia coli* methionine auxotroph, suggesting the presence of an enzyme(s) capable of reducing the sulfoxide to methionine. This was verified by showing that a cell-free extract of *E. coli* catalyzes the conversion of methionine sulfoxide to methionine. This reaction required reduced nicotinamide adenine dinucleotide phosphate and a generating system for this compound. The specific activity of the enzyme increased during logarithmic growth and was maximal when the culture attained a density of about 10^9 cells per ml.

The unique role of methionine as the initiator of protein synthesis in both procaryotes and eucaryotes is well documented (19). In addition, methionine residues are known to play an essential role in maintaining the biological activity of many proteins; for example, chemical oxidation of specific methionine residues in proteins to methionine sulfoxide results in a loss of biological activity (4, 6, 8, 11–14, 16, 18, 20). This reaction can be reversed, since it has been shown that chemical reduction of the methionine sulfoxide residues back to methionine restores the biological activity of adrenocortico-tropic hormone (8) and various other proteins (4, 11, 12, 18). Although methionine sulfoxide in proteins can be easily generated by chemical means, there is evidence that this oxidation takes place in cells. It has recently been reported (22) that methionine sulfoxide residues are present in proteins of cataractous lenses, but not normal lenses. A related finding is that after the administration to rabbits of 3-aminotriazole (a catalase inhibitor and a potent cataractogenic agent) there is a two- to threefold increase in the H_2O_2 concentration of eye tissues (1), and H_2O_2 is known to be an excellent methionine-oxidizing agent. It is reasonable to assume that cells must have mechanisms to both prevent methionine residues in proteins from being oxidized and also to reduce any methionine sulfoxide once formed.

Concerning the latter point, Black et al. (2) purified, from yeast extracts, three proteins which were required in addition to NADPH to reduce methionine sulfoxide to methionine. Two of these proteins were later shown to be identical to thioredoxin and thioredoxin reductase (17). A

previous report from this laboratory has described an inactive form of ribosomal protein L12 (15). Since the methionine residues in L12 are essential for the biological activity of this protein (4, 14), the possibility was considered that the inactive L12 contained methionine sulfoxide residues. Although at the present time this seems unlikely, it was found during the course of these experiments that *Escherichia coli* extracts have a very active system that converts methionine sulfoxide to methionine. The present report describes the enzymatic reduction of methionine sulfoxide to methionine by growing cells of *E. coli* and by cell-free extracts.

MATERIALS AND METHODS

Organisms and preparation of extract. *E. coli* B and a methionine auxotroph of *E. coli* K-12 (strain 2276) were grown at 37°C in a glucose-salts medium (3) on a New Brunswick rotary shaker. The cells were harvested by centrifugation, washed with a buffer containing 10 mM each Tris-chloride (pH 7.4), $MgCl_2$, and NH_4Cl, and suspended in an equal volume (g/ml) of this buffer. The cells were disrupted by sonication (3 × 20 s) in a Bronwill sonifier, and this homogenate was used in the enzyme assays.

Incubation conditions and assay for methionine. The reaction mixture contained in a final volume of 200 μl: 20 μmol of potassium phosphate buffer (pH 6.9), 0.1 μmol of NADPH, 4 μmol of glucose 6-phosphate, 1 μg of glucose 6-phosphate dehydrogenase, and 2.5 μmol of L-methionine-*dl*-sulfoxide. After incubating at 37°C for 60 min (unless stated otherwise), the reaction was stopped by the addition of 2 ml of 10% Cl_3CCOOH and centrifuged. A sample of the supernatant was removed, and the amount of methionine was determined by the procedure of Dubnoff and Borsook (9) as follows. A sample of the Cl_3CCOOH supernatant, 0.2 ml of 5 M NaOH, and 0.1 ml of 1% sodium nitroprusside were combined and mixed. This was followed by the addition of 1 ml of a 9:1 mixture

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of concentrated HCl and 85% phosphoric acid, and the volume was adjusted to 2 ml. After 10 min the samples were read at 540 nm. The concentration of methionine was calculated from a standard curve, which was linear between 50 and 400 μg of methionine.

Chemicals. NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and L-methionine-*dl*-sulfoxide were purchased from Sigma Chemical Corp.

RESULTS AND DISCUSSION

A convenient and rapid method to test whether *E. coli* possesses the enzymatic mechanism to convert methionine sulfoxide to methionine is to determine whether a methionine auxotroph can grow in the presence of methionine sulfoxide. Figure 1A shows that L-methionine-*dl*-sulfoxide is as effective as L-methionine in supporting the growth of such an auxotroph, and the kinetics of growth with each substrate are identical (Fig. 1B). Racemic methionine sulfoxide was used in these experiments, which suggests either that *E. coli* contains a racemase capable of converting L-methionine-*d*-sulfoxide to L-methionine-*l*-sulfoxide or that the reduction is not stereospecific. No growth was observed in the absence of methionine or when methionine sulfone was used as the substrate. Since methionine sulfoxide cannot be used as a substrate to acylate tRNA^{met} (data not shown), it cannot be incorporated into proteins, and therefore these results indicated that *E. coli* contains an enzymatic system that reduces methionine sulfoxide to methionine.
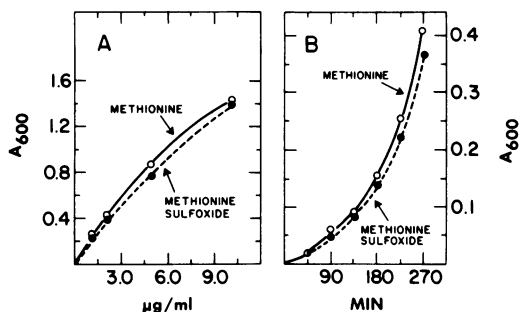

FIG. 1. *(A) Effect of concentration of L-methionine or L-methionine-dl-sulfoxide on the growth of an E. coli methionine auxotroph. E. coli 2276 (met⁻, thiamine⁻) was grown in a glucose salts medium supplemented with either methionine or methionine sulfoxide. The optical density at 600 nm (A$_{600}$) was measured after 16 h of growth at 37°C. (B) Time course of the growth of E. coli 2276 in the presence of either L-methionine or L-methionine-dl-sulfoxide. The organism was grown in the medium described in the text in the presence of 10 μg of either L-methionine or L-methionine-dl-sulfoxide per ml. Samples were removed at the times indicated, and the optical density at 600 nm was measured. (○——○) Methionine; (●——●) methionine sulfoxide.*

This was demonstrated directly by incubating methionine sulfoxide with a broken-cell preparation of *E. coli* B. This preparation was able to catalyze the reduction of methionine sulfoxide to methionine (Table 1). After centrifugation of the extract, little activity was found in either the particulate or supernatant fraction, but the activity could be restored by combining the fractions. These data strongly suggest that a multienzyme system is required for the reduction of methionine sulfoxide to methionine. Table 2 shows that the appearance of methionine in this in vitro system was found to require cell extract, NADPH, and glucose 6-phosphate. The omission of the NADPH-regenerating system resulted in an eightfold decrease in activity. An NADH-generating system is only about 20% as effective as the NADPH system. The rate of the

TABLE 1. *Reduction of methionine sulfoxide by an E. coli broken-cell preparation*[a]

Condition	Activity (%)
Broken cells	100
Particulate fraction	27
Supernatant fraction	19
Particulate fraction and supernatant fraction	92

[a] A broken-cell preparation of *E. coli* B was prepared as described in the text and centrifuged at 15,000 × g for 10 min. The supernatant was removed, and the particulate fraction was washed twice with 2 ml of a buffer containing 10 mM each Tris-chloride (pH 7.4), MgCl$_2$, and NH$_4$Cl. The particulate fraction was suspended in 1 ml of the same buffer. The incubations were carried out for 60 min and assayed as described in the text. One hundred percent activity represents the formation of 1.7 μmol of methionine. The amount of protein present in each of the fractions was broken cells, 1.9 mg; particulate, 0.9 mg; supernatant, 0.8 mg.

TABLE 2. *Requirements for the conversion of methionine sulfoxide to methionine*[a]

Expt	System	Methionine (μmol)
1	Complete	0.40
2	Minus cell extract	0
3	Minus NADPH	0.16
4	Minus glucose 6-phosphate	0.11
5	Minus NADPH-generating system	0.05
6	Plus NADH-generating system	0.08

[a] The incubation conditions and assay are described in the text. Each incubation contained 1 mg of *E. coli* broken-cell suspension and was carried out for 60 min at 37°C. In experiment 5 NADPH, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were omitted. In experiment 6 the NADPH-generating system was replaced with an NADH-generating system which included NADH (0.5 μmol), lactate (4 μmol), and lactic dehydrogenase (10 μg).

reaction was linear up to at least 2 mg of protein and 1 h of incubation. The broken-cell extract was about ⅓ as active as a comparable unbroken-cell suspension.

Figure 2 shows that in this in vitro system methionine sulfone is not converted to methionine, and that the same amount of methionine is formed when either L-methionine-*dl*-sulfoxide or L-methionine-*l*-sulfoxide is used as substrate.

In an effort to investigate whether the amount of this activity varies with the growth of the organisms, cells were harvested at varying times and the activity of the enzyme was measured. Figure 3 shows that there is about a fourfold increase in the specific activity of the enzyme as the organism grows from 0.2 to 1.0 optical density units measured at 600 nm. This increased activity slowly returns to its initial value during the late log and stationary phases of growth.
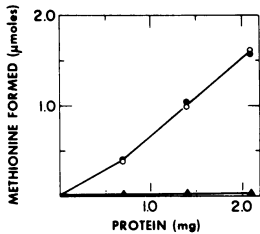

FIG. 2. *Effect of protein concentration on the conversion of L-methionine-dl-sulfoxide, L-methionine-l-sulfoxide, and L-methionine sulfone to methionine. The incubation conditions and assay are described in the text. The reaction mixtures contained 2.5 μmol of each substrate. L-Methionine-l-sulfoxide was prepared from the racemic mixture as previously described (21). (O) L-Methionine-dl-sulfoxide; (●) L-methionine-l-sulfoxide; (▲) L-methionine-sulfone.*

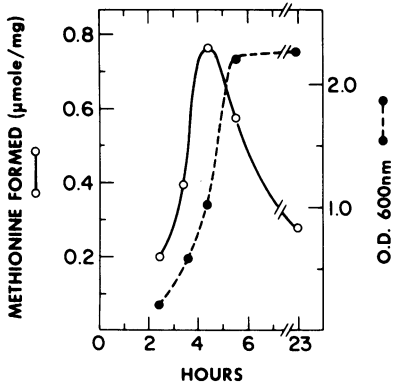FIG. 3. *Effect of growth on the specific activity of the enzyme system that converts methionine sulfoxide to methionine. E. coli B was grown in a glucose salts medium to different cell densities, and the cells were harvested, extracts were prepared, and enzymatic activity was measured as described in the text.*

The present study shows that *E. coli* contains an enzymatic system capable of reducing methionine sulfoxide to methionine. In vivo, the rate of reduction is sufficiently rapid so that methionine sulfoxide can support the growth of a methionine auxotroph at a rate comparable to that observed with methionine. In contrast to methionine sulfoxide, methionine sulfone could not support the growth of the auxotroph. The reduction of methionine sulfoxide to methionine required the presence of an NADPH-generating system. These requirements are similar to those previously found for the yeast system (2). Although the enzyme was found to be present at all states of growth of the organism, there were significant changes in the specific activity of the enzyme during the growth cycle.

Although this enzymatic system reduces free methionine sulfoxide to methionine, it is not clear that the free amino acid is the natural or only substrate. It is interesting to speculate that the functional substrate could be methionine sulfoxide residues in proteins which have arisen as a result of the in situ oxidation of methionine. Thus, it is possible that the changes in specific activity of the methionine sulfoxide reductase system during growth are related to the active metabolic production of oxidizing agents, such as the superoxide and hydroxyl radicals and hydrogen peroxide, during the log phase of growth. All of these reagents could cause the oxidation of protein-bound methionine residues, and this enzymatic reducing system might provide the cell with a mechanism to insure that methionine sulfoxide residues in proteins do not accumulate.

Another sulfoxide-reducing system that has been reported in *E. coli* utilizes biotin sulfoxide as the substrate (7). It has been shown (10) that four closely linked gene products are involved in converting biotin sulfoxide to biotin, and that methionine sulfoxide is not a substrate. In another study (5), it has been suggested that protein-bound biotin sulfoxide could be the true substrate. It would appear from both of these studies that complex enzymatic systems are present in *E. coli* to maintain biotin and methionine in the reduced state. Work is in progress to ascertain whether a protein-bound methionine sulfoxide can serve as a substrate for this reaction.

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