The Mechanism of the Auxotrophy for Sulfur-containing Amino Acids Imposed upon Escherichia coli by Superoxide*

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Defects in both of the genes coding for the cytosolic superoxide dismutases (SODs) of Escherichia coli impose an oxygen-dependent nutritional requirement for cysteine. This is now seen to be a bradytroph, rather than an absolute auxotrophy, since lack of Cys merely imposed a growth lag and escape from this growth lag did not involve genetic reversion. This Cys bradytroph was not seen in the SOD-competent parental strain, and did not involve genetic reversion. This Cys bradytroph imposed a growth lag and escape from this growth lag than an absolute auxotrophy, since lack of Cys merely posed an oxygen-dependent nutritional requirement for cysteine biosynthesis. Medium conditioned by the aerobic growth of the SOD-defective strain relieved the growth lag. Bioassays with Cys mutants suggested that the conditioned medium contained SO₃²⁻ or its equivalent, and sulfite per se was able to eliminate the growth lag. However, some component of the conditioned medium reacted with added sulfite and interfered with attempts to assay for it colorimetrically.

These results suggest that the cell envelope of the SOD-defective strain was weakened, directly or indirectly, by O₂⁻ and then leaked sulfite. This prevents cysteine biosynthesis until sulfite accumulates in the medium.

An effective means of arriving at the function of any enzyme is to examine the phenotypic consequences of its mutational deletion. This approach has been used for the cytosolic SODs¹ of Escherichia coli. Thus, elimination of both the inducible Mn-SOD (SodA) and the constitutive Fe-SOD (SodB) yielded cells with dioxygen-dependent requirements for specific classes of amino acids (1) as well as an enhanced rate of spontaneous mutagenesis. The latter effect is understood to be due to DNA damage imposed by powerful oxidants secondarily engendered by O₂⁻ while the requirement for branched chain amino acids was explained on the basis of the direct inactivation of dihydroxy acid dehydratase by O₂⁻ (3–6). This enzyme catalyzes the penultimate step on the pathway of biosynthesis of branched chain amino acids.

It has been assumed that O₂⁻-sensitive enzymes would also account for the deficits in biosynthesis of aromatic and sulfur-containing amino acids, which is also characteristic of the sodA sodB strain (7). The investigations reported here indicate that the leakage of a metabolic intermediate, probably sulfite, caused the deficit in biosynthesis of the sulfur-containing amino acids.

MATERIALS AND METHODS

Sulfite was determined colorimetrically as described by Leinweber and Monty (8) and Grant (9). The Conway microdiffusion method was used. The effect of sulfite on the growth of E. coli J132 was explored using a slight modification of the procedure of Kredich (11). Thus, media were made up to contain 1.0 mM Na₂SO₃, and additional Na₂SO₃ was added to a concentration of 1.0 mM every 2 h, starting 4 h following inoculation. The Na₂SO₃ used was a fresh 100 mM filter-sterilized solution made up in water. When sulfite supplementation was being examined, the starting Na₂S concentration was 0.2 mM, and additional Na₂S was added to 0.2 mM every hour, starting 4 h following inoculation. When O-acetylselenine supplementation was studied, it was added to 1.0 mM.

Bacterial Strains—The strains of E. coli used in this work were J132, which bears insertional defects in sodA and sodB, and AB1157, which is the parental strain (12). The cysteine auxotrophs used cySCD159, cysA20, cysB8, and cysB403 were originally from the Salmonella Genetic Stock Centre, University of Calgary, Alberta, Canada. T2N 1N4, EC1801 (cysE trpE5 leu-6 thi hsdR hsdM) is a cysE derivative of the E. coli K12 strain J1A99 (see Table I). These strains were grown overnight at 37 °C in aerobic M9 medium (13) supplemented with 100 mg/liter of each of the 20 amino acids commonly found in proteins, 3 mg/liter of pantothenate and thiamin, and 0.2% glucose. The overnight cultures were then diluted 25-fold into fresh medium lacking Cys and Met. When conditioned medium was desired, J132 and AB1157 cultures were incubated aerobically or anaerobically to Asoc = 1.0, and the cells were removed by centrifugation. The spent medium was then filter-sterilized and supplemented with 50 mg/liter of all of the amino acids excepting only Cys and Met, 3 mg/liter of pantothenate and thiamin, and 0.2% glucose. This supplemented spent medium, in 5.0-ml aliquots, was inoculated with 25 μl of overnight cultures of the cysteine auxotrophs, and growth was followed turbidimetrically. LB medium contained (per liter) 10 g of Bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl. pH was adjusted to 7.0 with K₂HPO₄.

Anaerobic incubations were achieved either in a Coy chamber under 85% N₂, 10% H₂, and 5% CO₂, or in the BBL Gas Pack System. Aerobic incubations were performed in a shaking water bath at 37 °C and 200 rpm. To transfer cells from LB to restricted media, cultures were centrifuged and washed with M9 salts prior to dilution in the new medium. Growth was monitored by measuring the absorbance at 600 nm, or at 700 nm for the experiments with SOD minic MnTMPyP (Mn(III)-5,10,15,20-tetrakis(1-methyl-4-pyridyl)porphine) (14).

Enzyme Activities—Cells were collected by centrifugation and, after washing with cold 50 mM potassium phosphate at pH 7.4, were lysed by two passes through a French press. The cell lysate was clarified by centrifugation, and the supernatant fraction was assayed for protein (15), sulfite reductase (16, 17), O-acetylselenine sulfhydrylase (18), and fumarase C (19).

RESULTS

Auxotrophy for Sulfur-containing Amino Acids—The sodA sodB strain (J132) did not grow during 8 h of aerobic incubation in medium lacking Cys and Met, as shown by line 5 in Fig. 1, although it grew anaerobically in this medium (data not shown). Supplementation with 0.1 mg/100 ml Cys plus Met
allowed growth (line 1). Cys was clearly a better stimulant of growth (line 2) than was Met (line 4). Sulfite, an intermediate on the Cys pathway (20), stimulated growth (line 3), as did sulfide (data not shown). O-Acetylserine was without effect. An oxygen-dependent auxotrophy for Cys has been reported (21, 22); however, the case under investigation is distinct in that it was dependent on the sodA sodB genotype.

The cell-permeant SOD mimic MnTMPyP (14) allowed aerobic growth without Cys, whereas SOD or catalase were without effect, as shown in Fig. 2. The Cys auxotrophy of J1132 can thus be attributed to O2.

Bradytroph Rather than Auxotrophy—Lack of Cys imposed a lengthy lag rather than absolutely preventing the growth of J1132. Thus, as shown in Fig. 3, an inoculum of AB1157, in the medium lacking Cys and Met, started growing promptly, whereas J1132 lagged for ~15 h and then entered rapid growth. A similar growth pattern for J1132 has previously been reported (23). This was not due to outgrowth of a revertant or pseudorevertant, since an inoculum taken from midlog phase again exhibited a ~15-h lag in growth when diluted 200-fold into fresh medium.

It appeared possible that this bradytroph might be due to accumulation of some intermediate required for Cys biosynthesis. Fig. 4 presents a test of this hypothesis. The E. coli were grown to midlog phase in the -Cys-Met medium, and the conditioned medium was collected by centrifugation and filter-sterilized. It was then repleted with all components save only Cys and Met. J1 grew when diluted 200-fold into this enriched spent medium (line 1) but did not grow when similarly diluted into fresh medium (line 3) or into medium conditioned by the growth of AB1157 and then enriched (line 2). Evidently J132, but not AB1157, leaks into the medium some factor that facilitates growth in aerobic media lacking Cys and Met.

Enzyme Biosynthesis—Sulfite reductase and O-acetylserine sulfhydrylase are rapidly induced when E. coli are transferred into a medium whose sole sulfur source is SO4. The SOD-competent AB1157 behaved in this way, whereas the SOD-defective J132 failed to induce these enzymes. This was not due to instability of these activities in the presence of O2. Thus, the activities in extracts of anaerobically grown J132 were not affected by exposure to the O2 generated by the xanthine oxidase reaction (data not shown).

Osmolytes—We have previously noted that osmolytes partially relieved the amino acid requirements of aerobic J132 (24), and this was attributed to the effect on turgor pressure on leakage of metabolites through a damaged envelope. Fig. 5, panel A demonstrates that 0.4 M sucrose facilitated the growth of J1132 in medium lacking Cys + Met, and panel B shows that it also comparably hastened induction of sulfite reductase and of O-acetylserine sulfhydrylase.

Investigation of the Conditioned Medium—The nature of the
compound whose accumulation in the medium finally allowed aerobic growth of JI132 was investigated. As shown in Fig. 6A, medium conditioned by aerobic growth of JI132 allowed better growth of strains CD519 and A20 than did medium conditioned by AB1157. Fig. 6B demonstrates that this growth-facilitating factor was provided by JI132 more under aerobic than under anaerobic conditions. Table I indicates that the cys mutants CD519 and A20 required Cys, sulfite, or sulfide. However, as shown in Fig. 6, strain I68 did not benefit from the conditioned medium; hence, it did not contain sulfide. Sulfite, or some equivalent, is probably the component whose accumulation allowed growth of JI132 because the conditioned medium tested negative for sulfhydryl groups.

Sulfite could not be detected in the conditioned medium, using a colorimetric assay (8, 9). Moreover, sulfite, when added to this medium to 50 μM, could not be detected in the medium. Furthermore, acidification in a Conway microdiffusion chamber (10) did not drive SO₂ from the medium enriched with 50 μM sulfite into a trap of 1% KOH, 10% ethanol. In contrast, sulfite in water, or in fresh medium, was easily driven, by acidification, into this trapping solution. Aldehydes, such as acetaldehyde, prevented detection of sulfite colorimetrically, most probably due to the formation of the bisulfite-adduct, i.e. the α-hydroxy sulfonic acid. Even glucose, which exists predominantly in the pyranose form, was able to slow the rate of recovery of sulfite by Conway microdiffusion. Thus sulfite was recovered from water solution in 1 h by microdiffusion, whereas ...

**DISCUSSION**

Transfer of E. coli into media lacking Cys but containing sulfate ordinarily results in prompt induction of the Cys pathway enzymes (20). In the SOD-replete AB1157, this occurred under aerobic or anaerobic conditions, whereas in the SOD-deficient JI132 this occurred anaerobically but not aerobically. This failure on the part of JI132 could be relieved by a low molecular weight SOD mimic or by raising the osmolarity of the medium with sucrose.

Lack of cysteine, under aerobic conditions, imposed a long growth lag upon JI132; which was shortened by the SOD mimic and also by the osmolyte. This growth lag was also relieved by...
TABLE I

| Sulfur source                        | Growth of mutant<sup>a</sup> |
|--------------------------------------|-----------------------------|
|                                     | cysA20 (cysTWA)             |
| Sulfate                             | 0                           |
| Thiosulfate                         | 0                           |
| Sulfite or cysteine sulfonic acid    | 0                           |
| Sulfide                             | 0                           |
| O-Acetylserine                       | 0                           |
| + sulfate                            | +                           |
| + sulfide                           | +                           |
| Cysteine, cystine, glutathione, or djenolate | +        |
| Spent medium<sup>b</sup>             | 0                           |
|                                     | B403 (cysG)                 |
|                                     | 0                           |
|                                     | CDS19 (cysC,cysG,D)         |
|                                     | 0                           |
|                                     | 168 (cys)                   |
|                                     | 0                           |
|                                     | EC1801 (cysE)               |
|                                     | 0                           |

<sup>a</sup> Salmonella mutants with the exception of EC1801, which is E. coli.

<sup>b</sup> J1 was grown aerobically in M9 medium, supplemented with all amino acids (100 mg/liter each) except Cys and Met, 0.2% glucose, and vitamins, to a midlog phase (A<sub>600</sub> ~ 1). The spent medium was filter-sterilized and supplemented with 50 mg/liter of each amino acid except Cys and Met, 0.2% glucose, and vitamins.

some compound that gradually accumulated in the medium. When the identity of this compound was sought by bioassay with various cys mutants, it appeared to be sulfite. However no sulfite could be detected in the conditioned medium, either by direct colorimetric assay or by the microdiffusion method of Conway (10). Furthermore, sulfite added to the conditioned medium could not be recovered by these methods. Apparently the compound whose accumulation allows the growth of J132 without Cys is the nutritional equivalent of sulfite. Its identity remains a mystery.

The requirement of aerobic J132 for sulfur-containing amino acids can now be explained. O<sub>2</sub>, directly or indirectly, damages the cell envelope. When stressed by the unbalanced turgor pressure of the cell, the damaged envelope leaks small molecules. Leakage of sulfite or its nutritional equivalent limits the synthesis of sulfide by the action of sulfite reductase, and that in turn limits the synthesis of cysteine via the action of the O-acetylseryne sulfhydrylases. The lack of cysteine, and consequently also of methionine, limits all protein synthesis and hence growth. J132, which had commenced rapid growth following a ~15-h lag (Fig. 3), was seen to again lag for ~15 h when simply diluted 200-fold into fresh medium. This too is consistent with leakage of intermediates as the cause of Cys + Met bradytropy.

This explanation now seems obvious, in light of the previous demonstration that osmolytes facilitated the aerobic growth of J1132 (24). However, the finding of an O<sub>2</sub>-sensitive dehydratase on the pathway of biosynthesis of the branched chain amino acids (3) provided such a compelling explanation for this auxotrophy that a similar explanation was anticipated for the cysteine plus methionine auxotrophy. It will now be of interest to explore the extent to which leakage of metabolic intermediates contributes to the several auxotrophies exhibited by aerobic J1132.

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