Why Superoxide Imposes an Aromatic Amino Acid Auxotrophy on Escherichia coli

THE TRANSKETOLASE CONNECTION*

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The lack of superoxide dismutase and the consequent elevation of [O₂⁻⁻] imposes, on Escherichia coli, auxotrophies for branched chain, sulfur-containing, and aromatic amino acids. The former two classes of auxotrophies have already been explained, whereas the third is explained herein. Thus O₂⁻⁻ is known to interfere with the production of erythrose-4-phosphate, which is essential for the first step of the aromatic biosynthetic pathway. It does so by oxidizing the 1,2-dihydroxyethyldimethlythiazine pyrophosphate intermediate of transketolase and inactivating this enzyme.

Escherichia coli mutants, which are unable to express both the iron- and the manganese-containing superoxide dismutases, exhibit several phenotypic deficits, among which are oxygen-dependent auxotrophies for branched chain, sulfur-containing (1), and aromatic amino acids (2). The requirement for branched chain amino acids was explained on the basis of the oxidative inactivation of the dihydroxy acid dehydratase (3–6), which catalyzes the penultimate step in the relevant biosynthetic pathway. The requirement for sulfur-containing amino acids was attributed to leakage of sulfite from the cells (7, 8).

We have now investigated the aromatic acid auxotrophy of the sodA sodB strain and find an explanation quite different from that rationalized the other amino acid auxotrophies. Thus, the aromatic biosynthetic pathway begins with the condensation of erythro-4-phosphate (E-4-P) with phosphoenolpyruvate (PEP) to yield 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), and the production of E-4-P in turn is dependent on the sequential actions of transketolase (TK) and transaldolase. It has been shown that the intermediate of the TK reaction, which is 1,2-dihydroxyethyl thiazine pyrophosphate, is oxidized by O₂⁻⁻ (9, 10). We now report that this oxidation interferes with the production of E-4-P and thus accounts for the decrease of aromatic biosynthesis in aerobic sodA sodB E. coli.

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†The abbreviations used are: E-4-P, erythro-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; TK, transketolase; PEP, phosphoenolpyruvate; SOD, superoxide dismutase (Cu, Zn); sodA, the gene encoding the MnSOD; sodB, the gene encoding the FeSOD; LB, Luria-Bertani.

MATERIALS AND METHODS

The E. coli strains used were AB1157 parental: J1132 (11), sodA sodB; BJ502 (Hfr C str-stkt-2) (12, 13), which lacks the ability to produce transketolase A; and BJ502tk+, which has a functional tkA and DH52/pKD44B, prepared by insertion of a 5-kb BamHI fragment encoding transketolase A into the Te³ cosmid pLAFR3 (14). These strains have TK activities of 0.05, 0.17, and 1.5 units/mg, respectively (15).

Unless otherwise specified all strains were grown overnight in aerobic LB medium at 37 °C and were then diluted into the test media. When the test medium was one that contained 17 amino acids, but lacking Tyr, Trp, and Phe, the inoculum was taken from an overnight culture in anaerobic minimal medium to avoid transfer of aromatic amino acids from LB medium. The 17-amino acid medium contained M9 salts (16) supplemented with 100 mg/liter concentrations of the 20 amino acids commonly found in proteins except the three aromatics. In each case the defined medium also contained 0.2% glucose plus 3 mg/liter pantothenic acid and thiamine. Minimal medium contained the vitamins mentioned above plus 100 mg/liter Thr, Leu, His, Pro, and Arg and 0.2% glucose.

When enzyme activities were to be assayed, cultures were grown to A₁₀₀₀ of 0.5–0.6 and were washed twice in the chilled buffer to be used in the assay. The washed cells were disrupted in a French press, and the extracts were clarified by centrifugation before assay. The assays were performed as described in the published reports: thus DAHP synthase (17), 3-dehydroquinate synthase (18), 3-dehydroquinate dehydratase (19), shikimate dehydratase (20), TK (12, 21), and transaldolase (21).

The following substrates, needed for these assays, were prepared by published procedures as follows: DAHP (22), 3-dehydroquinate (23), and 5-dehydroshikimate (24). Metabolites were assayed in the cell extracts as follows: PEP (25), ATP (Sigma bioluminescent assay kit) and E-4-P (26). Culture fluids that had been centrifuged and then were passed through a 0.22-μm filter were assayed for DAHP (27) and 3-dehydroquinate (27) to check for leakage of these intermediates. Hypoxia was achieved in specifically fabricated stainless steel vessels, which were maintained at 37 °C after pressurization.

RESULTS

Shikimate Relieves the Aromatic Amino Acid Auxotrophy Imposed by Lack of SodA and SodB—As previously noted (2) the sodA sodB strain of E. coli exhibited an auxotrophy for Phe + Tyr + Trp. This auxotrophy is not attributable to leakage of metabolic intermediates, because it was not relieved by 0.25 M sucrose, which did relieve the Cys + Met auxotrophy (7, 8) (data not shown). To localize the point of blockage of aromatic biosynthesis, shikimate was tested and was found to replace the aromatic amino acid requirement. These results are shown in Fig. 1. Thus line 5 illustrates the very slow growth seen in the absence of the aromatics compared with the rapid growth when these were present (Fig. 1, line 1). Shikimate allowed growth almost as well as did the aromatics (Fig. 1, line 2). The shikimate was not a nonspecific stimulator of growth, because it did not augment the slow growth in the absence of Lys, Ser, and Gly (Fig. 1, lines 3 and 4).

Enzyme Activities—There are four enzymes on the pathway to the aromatic amino acids that precede shikimate; these...
are DAHP-synthase, 3-dehydroquinate synthase, 5-dehydroquinate dehydratase, and shikimate dehydrogenase. These were assayed in extracts of the parental and the sodA sodB strains using published procedures (12, 17–21) and preparing the necessary substrates as described (22–24). None of these activities was found to be much lower in the sodA sodB extracts than in the parental strain when the extracts were assayed promptly (data not shown). It appeared that none of these four enzymes is a target for O$_2^-$ and therefore that the effect of O$_2^-$ on aromatic biosynthesis was exerted at some still earlier point. Because the first step in this pathway involves the condensation of PEP with E-4-P, the extracts were assayed for PEP and ATP. The results showed that although ATP > PEP, there was no significant difference between the sodA sodB and parental strains (data not shown). These negative results focused our attention on E-4-P. E-4-P was not detectable in extracts of either AB1157 or JI132.

**Transketolase—**E-4-P is made by the action of transaldolase on sedoheptulose-7-phosphate plus glyceraldehyde-3-phosphate, and sedoheptulose-7-phosphate in turn is made by the action of TK on D-xylulose-5-phosphate plus D-ribose-5-phosphate. TK is thus essential for the production of E-4-P and was considered a likely target for O$_2^-$ both because of the previously reported oxidation of its 1,2-dihydroxyethyl thiamine pyrophosphate intermediate by O$_2^-$ (9, 10) and because TK-deficient mutants of *E. coli* have been reported (12) to be aromatic amino acid auxotrophs.

If O$_2^-$ was interfering with the TK reaction, then the sodA sodB strain should have difficulty using ribose as a carbon source (12), because ribose catabolism depends on the direct oxidative pathway that uses TK. Fig. 2 shows that the parental strain grew somewhat better on glucose than on ribose (compare lines 1 and 2), whereas the sodA sodB strain grew very much better on glucose than on ribose (lines 3 and 4). This result is in accord with expectations and with the reports that TK mutants do not grow on pentoses (12).

The ability of transketolase to scavenge O$_2^-$ in a manner dependent on its keto sugar phosphate substrates has been reported (9, 10) and has been attributed to a rapid oxidation of the 1,2-dihydroxyethyl thiamine pyrophosphate intermediate by O$_2^-$. In full accord with these reports we found that TK caused a dose-dependent inhibition of the reduction of cytochrome c by the aerobic xanthine oxidase reaction and that this was entirely dependent on the presence of either fructose-6-phosphate or xylulose-5-phosphate (data not shown).

**O$_2^-$ Inactivates Transketolase—**The first step in the oxidation by O$_2^-$ of the thiamine-bound glycol intermediate of the TK must be a univalent process yielding a bound glycol radical. The final production of glycolate, shown earlier (9, 10), must depend on additional steps. It appeared possible that the bound glycolate radical might, with some frequency, oxidize and inactivate the enzyme. In that case TK activity should be lower in the sodA sodB strain than in the parental strain. As shown in Fig. 3, TK activity was lower in the SOD null strain than it was in the parental strain. This was also true, but to a lesser degree, of transaldolase, whereas there were no differences in glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

**Effect of Paraquat—**If the relative paucity of TK in the sodA sodB strain had been attributable to inactivation of the enzyme by O$_2^-$, then paraquat should lower TK even in the parental strain. That this was the case is shown in Fig. 4. Thus line 1 presents the increase in TK activity, which accompanied the outgrowth of a stationary phase inoculum of AB1157 in LB medium. In contrast, the increase of TK activity was much less in the case of JI132 (Fig. 4, line 2). Paraquat at 20 $\mu$m suppressed the increase of TK in the parental strain (Fig. 4, line 3) and at 4 $\mu$m did so in the sodA sodB strain (Fig. 4, line 4). It should be noted that these low levels of paraquat did not significantly inhibit growth in this rich medium.

**Effect of Hyperoxia on Growth—**Paraquat increased O$_2^-$ production, but it did so by diversion of electron flow from useful pathways, and this can complicate interpretation of its effects. For this reason we tested the effect of hyperoxia, anticipating that it should inhibit growth more when the carbon source was ribose than when it was glucose. Fig. 5, line 1, represents the growth of AB1157 at 5.5 atm of O$_2$ on glucose, whereas line 2 shows the much slower growth on ribose. It should be noted that growth under ordinary atmospheric conditions was much less affected by switching from glucose to ribose (Fig. 2, lines 1 and 2).

**Effect of Hyperoxia on TK Activity—**If the relative paucity of growth on ribose compared with glucose, imposed by hyperoxia, was attributable to inactivation of TK by O$_2^-$, then we should be able to see this inactivation most clearly when *de novo* protein synthesis was inhibited, and we should be able to prevent the inactivation with a cell-permeant catalyst of the dissmutation of O$_2^-$ (28). The affirmation of these expectations is presented in Fig. 6. Thus bar 4 in Fig. 6A gives the activity of TK in midlog AB1157 treated with chloramphenicol and kept at 0 °C for 3.5 h. Bar 3 shows the decline in TK, which occurred when the...
cells were kept at 37.5 °C in the presence of chloramphenicol for that period. Bars 1 and 2 illustrate that the extent of TK inactivation under 3.2 atm of O₂ was made apparent only when protein synthesis was inhibited (Fig. 6A, bar 2). Fig. 6B presents the protection by the SOD mimic MnTM-2-PyP (28). Thus bar set 1 is a control showing that the mimic had no effect on TK activity in cells kept on ice in the presence of chloramphenicol. Bar set 2 shows that 25 μM MnTM-2-PyP diminished the loss of TK seen in air in the presence of chloramphenicol, whereas bar set 3 shows the profound inactivation of TK under 3.5 atm of O₂ and the striking protection provided by the SOD mimic. Hence the inactivation of TK was most likely attributable to O₂ and could be seen even in the SOD-replete strain when O₂ production was increased by raising pO₂.  

Effect of Overproducing TK—If inactivation of TK by O₂ was limiting growth under hyperoxia, then a strain overproducing TK; 10-fold, by virtue of a cosmid bearing the TK gene, should have a growth advantage under hyperoxia. In Fig. 7, comparison of lines 1 and 2 shows that this was the case. It should be noted that under normoxia the TK overproducer actually grew somewhat slower than its parental strain.  

Substrate-dependent Inactivation of TK by O₂—TK, either as the purified enzyme (Fig. 8A) or in an extract of JI132 (Fig. 8B), was inactivated when exposed to a photochemical flux of O₂ in the presence of fructose-6-phosphate (Fig. 8A, line 3), and this inactivation was diminished by 10 μg/ml SOD (Fig. 8A, line 2) and was dependent on the glycol-donating substrate (Fig. 8A, line 1). The TK activity in an extract of JI132 (Fig. 8B) was similarly inactivated (Fig. 8B, line 3) and was similarly protected by 10 μg/ml SOD (Fig. 8B, line 2). Fig. 8B, line 1, illustrates the stability of the TK activity in these extracts in the absence of the photochemical flux of O₂.  

Effect of Iron—We have previously noted (29) that enrich-
ment of growth media with iron relieved some of the phenotypic deficits of the sodA sodB strain. Iron enrichment elevated the levels of [4Fe-4S] containing dehydratases, and we surmised that the oxidative inactivation and reductive repair of these [4Fe-4S] clusters was providing a pathway for the scavenging of O2 and was thus protecting less repairable targets. The data in Fig. 9 show that TK is one of those less repairable targets of O2. It should be noted that growth in iron-enriched medium did not affect the activities of DAHP synthase, dehydroquinate synthase, 5-dehydroquinate dehydrogenase, or shikimate dehydrogenase (data not shown).

**DISCUSSION**

The blockade of aromatic amino acid biosynthesis imposed by O2 imposes an auxotrophy for these amino acids that was relieved by shikimate. Hence that blockade was in the early steps of the aromatic biosynthetic pathway. None of the enzymes catalyzing the early steps in this pathway was found to be O2 sensitive. Because this pathway begins with the condensation of PEP with E-4-P, and because an intermediate in the TK reaction was already known to be oxidized by O2 with a rate constant of 106 M−1 s−1 (10), it appeared possible that the problem might be imposed by this reaction.

The sodA sodB strain of E. coli did exhibit a relative deficiency in its ability to use ribose as a carbon source, whereas the ability of the SOD-replete strain to grow on ribose could be diminished by raising O2 production through application of paraquat or hyperoxia. TK activity was low in the sodA sodB strain, whereas there were no differences in glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase and only a marginally significant difference in transaldolase. Paraquat or hyperoxia lowered the TK activity in the bacteria, and a cell-permeant mimic of SOD activity protected. The inhibition of growth imposed by hyperoxia was lessened by over-
production of TK, and TK activity in either purified form or in crude extracts was inactivated by a photochemical flux of O$_2$ and was protected by SOD.

All of these data indicate that the synthesis of E-4-P was compromised by the O$_2$-TK reaction and that lack of E-4-P prevented the first step of the aromatic pathway. It should be noted that leakage of intermediates from the cells into the medium was tested for and was not observed (data not shown).

It is interesting that, although carefully sought after, E-4-P has never been detected in tissue extracts when specific assay methods were used (26, 30). The explanation offered for this failure has been the spontaneous dimerization of this sugar (26, 31). However, that dimerization has been seen to lower the concentration of E-4-P by only 75% in in vitro tests; so it would not be likely to entirely prevent detection of this sugar. Moreover, a dimerization reaction would proceed even less well when the concentration of the monomer was very low. We have noted a toxicity of short chain sugars and have related it to the reactivity of the exposed carbonyl group and to the formation of enediolate tautomers, which can oxidize to very toxic reactive species of the exposed carbonyl group and to the formation of toxicity of short chain sugars and have related it to the failure has been the spontaneous dimerization of this sugar (26, 31). However, that dimerization has been seen to lower the concentration of E-4-P by only 75% in in vitro tests; so it would not be likely to entirely prevent detection of this sugar. Moreover, a dimerization reaction would proceed even less well when the concentration of the monomer was very low. We have noted a toxicity of short chain sugars and have related it to the reactivity of the exposed carbonyl group and to the formation of enediolate tautomers, which can oxidize to very toxic a-β diketones (32). Hence short chain sugars should be kept at vanishingly low concentrations to prevent this. Tamarit et al. (33) have examined the proteins damaged in E. coli exposed to oxidative stresses. They identified these proteins by looking for protein carbonyls and did not find transketolase among them. Possibly transketolase escaped their notice because its oxidative inactivation does not generate a carbonyl group.

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