Growth in Iron-enriched Medium Partially Compensates *Escherichia coli* for the Lack of Manganese and Iron Superoxide Dismutase

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Enrichment of the growth medium with iron partially relieves the phenotypic deficits imposed on *Escherichia coli* by lack of both manganese and iron superoxide dismutases. Thus iron supplementation increased the aerobic growth rate, decreased the leakage of sulfite, and diminished sensitivity toward paraquat. Iron supplementation increased the activities of several [4Fe-4S]-containing dehydratases, and this was seen even in the presence of 50 μM/ml of rifampicin, an amount which completely inhibited growth. Assessing the O$_2^-$ scavenging activity by means of lucigenin luminescence indicated that the iron-enriched sodAsodB cells had gained some means of eliminating O$_2^-$ which was not detectable as superoxide dismutase activity in cell extracts. It is noteworthy that iron-enriched cells were not more sensitive toward the lethality of H$_2$O$_2$ despite having the usual amount of catalase activity. This indicates that iron taken into the cells from the medium is not available for Fenton chemistry, but is available for reconstitution of iron-sulfur clusters.

We suppose that oxidation of the [4Fe-4S] clusters of dehydratases by O$_2^-$ and their subsequent reductive reconstitution provides a mechanism for scavenging O$_2^-$ and that speeding this reductive reconstitution by iron enrichment both spared other targets from O$_2^-$ attack and maintained adequate levels of these enzymes to meet the metabolic needs of the cells.

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Among the targets susceptible to direct oxidation by O$_2^-$ are those dehydratases that contain [4Fe-4S] clusters. These enzymes, which include dihydroxy acid dehydratase (1–3), aconitase (4, 5), 6-phosphogluconate dehydratase (6–7), and fumarases A and B (8, 9), react with O$_2^-$ with rate constants of $-10^7$ M$^{-1}$ s$^{-1}$. Univalent oxidation of the [4Fe-4S] clusters by O$_2^-$ leads to loss of iron, leaving [3Fe-4S] clusters and an inactive form of the enzymes. The enzymes can subsequently be reactivated by reductive reconstitution (10, 11). Both inactivation and reactivation are ongoing processes in aerobic cells and their balance determines the fractional level of activity of these enzymes (12, 13).

One of the functions of superoxide dismutases is to protect these dehydratases against this inactivation by O$_2^-$, however, there are other means by which O$_2^-$ can cause damage, both directly and indirectly (14, 15). This leads to the possibility that consumption of O$_2^-$ by the reversible inactivation of the dehydratases may serve to prevent these other kinds of damage. In that case, increasing the rates of reactivation of the dehydratases by growing the cells in iron-enriched medium may partially reverse the phenotypic deficits of sodA sodB *Escherichia coli*. The results reported herein are consistent with that outcome.

**MATERIALS AND METHODS**

Bound sulfite was liberated with alkaline cyanide as described by Kunert (16) and used recently (17). The liberated sulfite was assayed colorimetrically (17, 18). The strains of *E. coli* used in these experiments were: AB1157, parental; JI132, sodA sodB (19); and pL16464 GS57, fumA fumC, and overproducing fumarase A by virtue of a multicopy plasmid bearing the fumA gene (20). Unless otherwise indicated starter cultures were grown overnight in LB and were then used to inoculate M9CA medium at a dilution of 1:200. LB and M9CA were prepared as described by Maniatis et al. (21). Growth in defined medium was achieved by washing cells taken from the overnight cultures 3 times in M9 followed by 200-fold dilution into M9 supplemented with 100 mg/liter of the 20 amino acids, except methionine and cysteine, commonly found in proteins (referred to as 18AA medium). In each case the defined medium also contained 0.2% glucose plus 3 mg/liter each of pantothentic acid and thiamin. Minimal medium contained the vitamins mentioned above plus 100 mg/liter of Thr, Leu, His, Pro, and Arg, and 0.2% glucose. The inocula for growth in minimal medium were taken from overnight anaerobic cultures in this medium. Iron was added as water solutions of FeSO$_4$ or FeCl$_2$ sterilized by filtration. Anaerobiosis was achieved in a Coy chamber. Aerobic growth was at 200 rpm. All growth was at 37 °C and was followed turbidimetrically at 600 nm. Iron was determined colorimetrically with a $n$-$n'$-dipyridyl applied to cell extracts (22). Fumarase activity was assayed according to Hill and Bradshaw (23) and catalase activity, as described by Beers and Sizer (24). Aconitase (4, 25), dihydroxy acid dehydratase (1), and lucigenin luminescence (26) were assayed as described previously.

**RESULTS**

*Iron Improves the Aerobic Growth of sodA sodB E. coli—Adding Fe(II) to the M9CA medium hastened the aerobic growth of the sodA sodB strain. Fe(II), 0.05–0.50 mM, caused a dose-dependent increase in growth. Thus the generation time, which was 84 min in the absence of added Fe(II), was shortened to 72 min by 0.1 mM Fe(II) and to 56 min by 0.5 mM Fe(II). The doubling time of the parental strain was 44 min without iron supplementation. The sodA sodB strain grew very slowly in medium lacking the aromatic, branched chain, and sulfur-containing amino acids, and 0.5 mM Fe(II) exerted a very substantial stimulatory effect. Thus the doubling time, which was 680 min without iron, was shortened to 112 min by 0.5 mM Fe(II). Supplementation with 0.5 mM Co(II) or Cu(II) was without effect, whereas 0.5 mM Mn(II) was beneficial but not as much as 0.5 mM Fe(II) (data not shown). Mn(II) has been reported to act as a functional replacement for superoxide dismutase in *Lactobacillus plantarum* (27) and in yeast (28).*
and mutations in yeast causing accumulation of Mn(II) complemented a lack of the CuZn-superoxide dismutase (29). This effect of Mn(II) can be attributed to the ability of Mn(II) and complexes thereof to catalyze the dismutation and/or the reduction of O$_2^-$ (30, 31). Mn(II) does not participate in Fenton chemistry (32, 33), whereas Fe(II) does. Hence a protective effect of Mn(II) is not surprising, but a protective effect by Fe(II) demands explanation.

Iron Diminishes the Leakage of Sulfite—The sodA sodB E. coli exhibits an oxygen-dependent requirement for sulfur-containing amino acids (34) and this has been attributed to the leakage of sulfite from the cells (17, 35). Fig. 1 shows that 0.5 mM Fe(II) in the 18AA medium diminished the accumulation of sulfite by the sodA sodB strain (compare bars 1 and 2). Incubation of the medium conditioned by the growth of the sodA sodB cells with 0.5 mM Fe(II) for 24 h did not significantly diminish recovery of sulfite, probably because the sulfite was present as a carbonyl-bisulfite adduct (17) and thus was not subject to rapid metal-catalyzed autoxidation. In any case the data in Fig. 1 have been corrected for the effect of Fe(II) on the recovery of sulfite. Hence the effect of Fe(II) was to decrease leakage of sulfite and not to diminish recovery of sulfite from the medium. As expected there was little leakage of sulfite from the parental strain growing aerobically (bar 4) or from the sodA sodB growing anaerobically (bar 3).

Fe(II) Supplementation Protects against Paraquat—The sodA sodB strain is ~100 times more sensitive to paraquat than the superoxide dismutase-replete parental strain (34). Fig. 2 shows the strong growth inhibition imposed by 2 and 4 μM paraquat on the sodA sodB strain (compare lines 4 and 5 with 3). Supplementation with Fe(II) eliminated the effect of paraquat and even allowed faster growth than that seen without paraquat (compare lines 1 and 2 with 3). These low micromolar levels of paraquat were without effect on the growth of the parental strain (data not shown).

Growth in iron-supplemented medium did result in greater cell content of iron and the sodA sodB strain accumulated more iron than did the parental strain. Thus both strains contained 2 μmol of iron/mg of protein when grown without added iron, but when grown in the presence of 0.5 mM Fe(II) the sodA sodB contained 16 and the parental strain 5.5 μmol of iron/mg of protein. The sodA sodB strain was more susceptible to the lethality of H$_2$O$_2$ than the parental strain as has been reported (34). However, growth in the presence of 0.5 mM Fe(II) did not increase the sensitivity of the sodA sodB strain to H$_2$O$_2$. Thus 30 min of exposure to 2.0 mM H$_2$O$_2$ at 37 °C caused ~75% loss of viability of washed cells suspended in M9 salts at A$_{600}$nm = 0.5 whether or not they had been grown in Fe(II)-supplemented medium.

Luminescence—Although the luminescence elicited from lucigenin is not a reliable measure of the concentration of O$_2^-$ because it can mediate production of O$_2^-$ it can be used to detect the scavenging of O$_2^-$ by superoxide dismutase or by other means (36, 37). Fig. 3 demonstrates that the sodA sodB cells grown in iron-supplemented medium luminesce less than when grown without such supplementation. The parental strain luminesced much less than the sodA sodB strain as previously noted (26). It thus appears that the sodA sodB cells gained some mechanism of scavenging O$_2^-$ when grown in iron-enriched medium. Washing the cells prior to addition of lucigenin increased luminescence, but the difference between the iron-rich and normal sodA sodB cells persisted, as did the striking difference between the sodA sodB and parental cells.

[4Fe-4S]-containing Dehydratases—Growth of the sodA sodB strain in iron-supplemented medium increased cell content of several [4Fe-4S]-containing dehydratase activities. Thus Fig. 4 demonstrates this for aconitase and for dihydroxy acid dehydratase, whereas Fig. 5 makes this point for fumarases A + B in the sodA sodB strain and for fumarase A in a FumA overproducing strain. The data in Figs. 4 and 5 were obtained with cells grown in M9CA medium with and without iron supplementation because that is the medium in which iron supplementation speeded the growth of the sodA sodB strain. It should be noted that the sodA sodB strain grew better in LB than in M9CA, contained ~10 times more aconitase, and did not show a further growth enhancement with iron supplementation.

Whether iron supplementation increased the activities of the [4Fe-4S]-containing dehydratases because of increased reactivation of the oxidatively inactivated enzymes or because it led to more de novo synthesis was explored with an inhibitor of transcription i.e. rifampicin. Iron, at 0.5 mM, increased the
Iron Complements sodA sodB E. coli

activity of aconitase in the sodA sodB strain by 3-fold. Rifampicin at 50 µg/ml, which completely inhibited growth, did not diminish the stimulatory effect of iron (Fig. 4). It follows that the effect of iron was due to reactivation rather than de novo synthesis. These experiments were performed by adding rifampicin to mid-log cultures in M9CA and then, after 15 min, adding Fe(II) to 0.5 mM to half of the cultures. After further incubation for 90 min, during which A$_{600}$ nm did not increase, cells were washed three times with 50 mM Tris at pH 7.6 and extracted and assayed for aconitase as described under “Materials and Methods.”

Because of its role in Fenton chemistry, one might anticipate that growing sodA sodB E. coli in iron-supplemented medium would make them more susceptible to oxidative stress. The data presented above show the opposite effect. Thus iron-supplemented cells grew faster in aerobic M9CA medium, leaked less sulfite, and were less susceptible to the growth inhibiting effect of paraquat than unsupplemented cells. The observation that less lucigenin luminescence was seen with the iron-supplemented cells indicates that they had an enhanced O$_2^-$-scavenging activity. However, no superoxide dismutase activity could be detected in extracts of the iron-supplemented cells.

One possibility for the seat of this activity within the cells would be an increased rate of reductive reconstitution of oxidatively inactivated [4Fe-4S]-containing dehydratases. The cycle of oxidative inactivation by O$_2^-$ followed by reductive reactivation of such clusters, could act as a sink for O$_2^-$. In keeping with this view, the iron-grown sodA sodB cells contained more aconitase, dihydroxy acid dehydratase, and fumarases A and B activities than cells grown without iron enrichment. Rifampicin, an inhibitor of transcription, did not eliminate this effect of iron on aconitase activity. It follows that the increase in aconitase, caused by iron supplementation, was mainly due to increased reactivation rather than de novo synthesis.

Increased reactivation would provide two benefits. One is to provide a sink for O$_2^-$ that could spare other, nonreactivatable targets of O$_2^-$ attack. A second benefit would be to maintain the activities of these essential dehydratases high enough to meet the metabolic needs of the cells. Of course such reductive scavenging of O$_2^-$ is inferior to that achieved by superoxide dismutase in that it produces one H$_2$O$_2$ per O$_2^-$ consumed and also consumes cellular reductants such as NADPH. In contrast, superoxide dismutase produces only 0.5 H$_2$O$_2$ per O$_2^-$ consumed and without consumption of reductants.

The failure of iron supplementation to increase the H$_2$O$_2$ sensitivity of E. coli is surprising in view of the demonstrated role of iron released from O$_2^-$-oxidized [4Fe-4S] clusters in exacerbating the lethality of H$_2$O$_2$ (38). It appears that iron taken into the cells from the medium is not available to participate in Fenton chemistry, although competent to reconstitute [4Fe-4S] clusters. The relatively trivial explanation that iron supple-
mentation increased catalase activity to a degree that offset increased toxicity was examined and eliminated. Thus the catalase activity of the sodA sodB cells grown with 0.5 mM Fe(II) was equal to that of the control cells (data not shown).

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