A Mechanism for Complementation of the sodA sodB Defect in Escherichia coli by Overproduction of the rbo Gene Product (Desulfoferrodoxin) from Desulfoarculus baarsii*

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Overexpression of rbo in Escherichia coli prevents the inactivation of the [4Fe-4S]-containing fumarases that otherwise occurs in the sodA sodB strain. It similarly protects against the increased sensitivity toward H₂O₂ which is imposed by the lack of SOD A and SOD B. These results would be explained on the basis of scavenging of O₂⁻ within the cells by RBO. This interpretation was supported by measurements of intracellular scavenging of O₂⁻ by the lucigenin luminescence method. Since SOD activity could not be detected in dilute extracts, of the RBO-overexpressing sodA sodB strain, we propose that RBO catalyzes the reduction of O₂⁻ at the expense of cellular reductants such as NAD(P)H. A similar mechanism may apply to other instances of complementation of SOD defects by non-SOD genes.

Superoxide dismutases catalyze the conversion of O₂⁻ into H₂O₂ plus O² and thereby protect aerobic cells against the toxicity of this radical (1). The potential deleterious actions of O₂⁻ are manifold and include oxidation of the [4Fe-4S] clusters of dehydratases such as aconitate. This interferes with metabolism by inactivating these dehydratases, and it has the additional consequence of causing release of iron from the oxidized clusters (1–3). The released iron is then available to participate in Fenton chemistry with hydroperoxides, resulting in the production of reactive hydroxyl or alkoxyl radicals or of strongly oxidizing iron-oxygen complexes (2, 3). When this Fenton chemistry is catalyzed by iron bound to DNA or to membranes, the result is apt to be a loss of viability (2–6). That this actually occurs in these cells has been both proposed (2, 3) and verified in Escherichia coli (4, 6).

Pianzzola et al. (7) have reported that insertion and expression of the rbo gene from Desulfoarculus baarsii or Desulfovibrio vulgaris into sodA¹ sodB E. coli corrects the phenotypic defects of this mutant. They achieved this by coupling the rbo gene to the IPTG-inducible ptac promoter. There are two ways in which the RBO protein (desulfoferrodoxin) might have achieved this complementation. One is by catalyzing the elimination of O₂⁻ and another is by increasing the rate of reconstitution of the oxidized iron sulfur clusters thereby increasing the activities of the corresponding enzymes and decreasing the level of “free” iron. Since SOD activity could not be detected in extracts of the sodA sodB + rbo strains, the second of these explanations was put forth (7).

We now report that expression of RBO in E. coli does provide for the elimination of O₂⁻ within these cells as judged from the luminescence of lucigenin, and we propose how this might be accomplished.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome c (III) and xanthine were from Sigma; glucose, H₂O₂, and salts were from Mallinckrodt Chemical Works; malate was from ICN; lucigenin from Aldrich; and yeast extract and bacteriostatone from Difco. Xanthine oxidase from bovine cream was prepared by R. Wiley as described by Waud et al. (8). SOD activity was assayed by the xanthine oxidase-cytochrome c method (9). Fumarase was assayed according to Hill and Bradshaw (10), and protein by the method of Lowry et al. (11). E. coli were grown, extracted, and assayed for fumarases A + B essentially as described previously (12).

The lethality of 2.5 mM H₂O₂ was explored by using 30 min of exposure followed by dilution and plating essentially as described by Carlizos and Touati (13). Lucigenin luminescence was used to measure the scavenging of O₂⁻ within E. coli as described previously (14). Controls demonstrated that this luminescence was dependent upon the simultaneous presence of lucigenin, the E. coli cells, and an electron source such as glucose. Lucigenin was added, to 0.1 mM, to samples taken from growing cultures, and the luminescence was measured and expressed per A₅₇₅nm. In some experiments 2.5% inocula were grown for 15 min and then for an additional 90 min ± 2.0 mM IPTG. The cells were then collected, washed once with 50 mM potassium phosphate, 0.1 mM EDTA at pH 7.8 and then resuspended in this buffer to A₅₇₅nm = 5.0. These cells were then used for luminescence measurements at A₅₇₅nm = 0.50 in this buffer plus 0.25% glucose.

Growth of Cells— Cultures were stored on sealed LB agar plates containing the selective antibiotics at 4 °C. Inocula taken from these plates were grown overnight at 37 °C in LB medium without antibiotics. Aeration was maintained by shaking at 200 rpm. Cultures intended for experimental manipulation were subcultured in LB medium without antibiotics. The strains used were: QC2509 = GC4468/pJF119EH; QC2510 = GC4468 ΔsodA sodB Δ2/pJF119EH; QC3235 = GC4468 ΔsodA sodB Δ2/pMJ25; QC3245 = GC4468 sodA::cat sodB Δ2/pJF119EH; QC3246 = GC4468 sodA::cat sodB Δ2/pMJ25. GC4468 was the parental strain, and pJF119EH was the control plasmid, whereas pMJ25 was the rbo-containing plasmid. These strains and the antibiotics used in maintaining them in culture have been described (17, 13).

RESULTS

Effect of RBO on Fumarase Activity—Fumarases A and B of E. coli are [4Fe-4S] dehydratases and are subject to oxidative inactivation by O₂⁻ both in vitro (15) and in vivo (16). Fig. 1 demonstrates that these fumarases are largely inactivated in the sodA sodB strain (compare bars 1 and 2). This was partially prevented when the rbo-bearing plasmid was present (bar 3) and was completely prevented when rbo expression was induced by IPTG (bar 4). A control demonstrated that IPTG had no effect on the fumarases A and B of the sodA sodB strain lacking rbo (data not shown). These results can be interpreted

¹ The abbreviations used are: sodA, the gene coding for the MnSOD; sodB, the gene coding for the FeSOD; SOD, superoxide dismutase; RBO, gene product of the rbo gene (desulfoferrodoxin); IPTG, isopropyl-1-thio-β-D-galactopyranosidase; LB, Luria-Bertani.

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either in terms of protection of the [4Fe-4S] clusters by lowering the steady-state $[O_2^-]$ or of increasing the rate of reconstitution of the oxidized clusters.

**Effect of RBO on the Lethality of $H_2O_2$**—The increased lethality of $H_2O_2$ toward the sodA sodB strain was observed by Carlioz and Touati (13) and verified by Imlay and Linn (17). Raising the concentration of free iron as a consequence of oxidative disassembly of [4Fe-4S] clusters by $O_2^-$ should increase the lethality of $H_2O_2$, as previously proposed (2, 3) and experimentally verified (4, 6). Fig. 2 demonstrates that 2.5 mM $H_2O_2$ caused greater lethality to the sodA sodB strain than to the SOD-competent parental strain (compare bars 1 and 2). The presence of rbo was partially protective (bar 3) and became fully protective when its expression was induced by IPTG (bar 4). These results indicate that the pool of free iron was lowered by RBO, and this could reflect either decreased $O_2^-$ attack on the susceptible [4Fe-4S] clusters or an increased rate of reductive reconstitution of those clusters.

**Effect of RBO on Lucigenin Luminescence**—Lucigenin, often used to detect and measure $[O_2^-]$, should not be used for this purpose because it can mediate $O_2^-$ production, much as does paraquat. Thus SOD-inhibitable lucigenin luminescence has been demonstrated even in systems not capable of producing $O_2^-$ in the absence of lucigenin (14, 18). It can, however, be used to measure the scavenging of $O_2^-$ by dismutation or other mechanisms; since a reaction of the lucigenin monocation radical with $O_2^-$ is on the pathway to luminescence, which is therefore dependent on $O_2^-$ (18, 19). Lucigenin luminescence has been used in this way to measure SOD activity in *E. coli* (14).

Fig. 3 demonstrates that expression of RBO increased the rate of scavenging of $O_2^-$ in *E. coli*. Thus the luminescence seen with the SOD-competent parent was much less than that seen with the sodA sodB mutant (compare bars 1 and 2). When rbo was present in the sodA sodB strain it decreased that luminescence (bar 3), and the increasing expression of rbo with IPTG decreased it further (bar 4). Comparison of bars 2 and 4 in Fig. 3 shows that IPTG had no effect in the absence of rbo. These experiments were done with cells in LB medium. They were then repeated with cells harvested from LB and then suspended in glucose plus buffer to $A_{600\,\text{nm}} = 0.5$. These results shown in Fig. 4 again demonstrate that the presence of rbo diminished lucigenin luminescence, and induction of rbo expression with IPTG did so to a greater extent. Results similar to those shown in Figs. 3 and 4 were obtained using QC3245 and QC3246 in place of QC2510 and QC3235 (data not shown). These results indicate that RBO somehow acted to scavenge $O_2^-$ in vivo, although SOD activity was not evident in extracts of the RBO producing sodA sodB cells (data not shown).

**DISCUSSION**

$O_2^-$ can be scavenged by dismutation, as catalyzed by SOD; and that activity can be observed in the absence of other reac-
tants. O$_2^-$ can also be scavenged by donating, or accepting, electrons in reactions with other molecules (1). In this case the “activity” would be dependent on the presence of the other molecule. An analogy would be the scavenging of H$_2$O$_2$ by GSH peroxidase, which is dependent upon the presence of GSH.

Another analogy is the scavenging of O$_2^-$ by the [4Fe-4S] containing dehydratases, such as aconitase. In this instance O$_2^-$ oxidizes the [4Fe-4S] clusters and the oxidized clusters can subsequently be reduced and reconstituted (2, 3, 15, 16). The overall process accomplishes the reduction of O$_2^-$ to H$_2$O$_2$ at the expense of cellular reductants. Our results indicate that RBO complements the absence of SOD by scavenging O$_2^-$ by this second mechanism; which can be efficient within the cell but would be hard to detect in extracts not enriched with the appropriate cellular reductant system.

Specifically, we are suggesting that reduced RBO can be rapidly oxidized by O$_2^-$ and then reduced again at the expense of cellular reductants, such as NADPH or GSH. There is a precedent for the scavenging of O$_2^-$ by this mechanism in the activity of the SOD mimic MnTMPyP (20) This manganic porphyrin was actively taken into E. coli where it was kept reduced by cellular reductants. It complemented the sodA sodB strain and diminished the luminescence of lucigenin even though its activity could not be seen in extracts (14, 20, 21).

It is possible that this sort of scavenging of O$_2^-$ by other than a dismutation reaction, may be used in organisms reportedly aerotolerant yet lacking in SOD. Neisseria gonorrhoea is one such organism (22). A similar explanation might apply to pseudo-reversions of SOD negative mutants of yeast (23–25) and of other organisms and to the complementation of the sodA sodB defect by ruberythrin (26). Clearly, lucigenin luminescence carefully applied may prove to be a useful tool for investigating the intracellular scavenging of O$_2^-$ in such instances.

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Note Added in Proof—RBO can increase the scavenging of O$_2^-$ even if it is not capable of directly reducing O$_2^-$ Thus RBO, by participating in the reductive reconstitution of iron-sulfur clusters that have been oxidized by O$_2^-$ can indirectly contribute to the net scavenging of O$_2^-$ Distinguishing between the proposed “direct” and “indirect” mechanisms of O$_2^-$ scavenging cannot be done on the basis of the results presented.

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