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Glycerol Metabolism in Superoxide Dismutase-deficient *Escherichia coli*

LUDMIL BENOV* and JAMEELA AL-IBRAHEEM

Department of Biochemistry, Faculty of Medicine, Kuwait University, P.O. Box 24923, Safat 13110, Kuwait

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Escherichia coli, which lacks cytoplasmic superoxide dismutases, exhibits various phenotypic deficits if grown aerobically. Here we report that *sodAsodB E. coli* cannot use glycerol under aerobic conditions. The reason is low activity of glycerol kinase (GK), the rate-limiting enzyme in glycerol metabolism. Superoxide does not inactivate GK, but makes it susceptible to inactivation by a heat-labile factor present in the cell-free extracts. This factor seems to be part of a proteolytic system, which recognizes and degrades oxidatively modified proteins.

Keywords: Glycerol kinase; Free radicals; Superoxide; Proteolytic susceptibility

Abbreviations: GK, glycerol kinase; G3PD, glycerol-3-phosphate dehydrogenase; G3P, glycerol-3-phosphate; PQ^{++}, paraquat; XO, xanthine oxidase

INTRODUCTION

Escherichia coli can utilize glycerol as a carbon source both aerobically and anaerobically. Glycerol metabolism requires the action of the glycerol facilitator, glycerol kinase and either the aerobic or the anaerobic glycerol-3-phosphate dehydrogenase.[1] *E. coli* mutants, which are unable to express both the iron and the manganese-containing superoxide dismutases, exhibit several phenotypic deficits, among which are oxygen-dependant auxotrophies for certain amino acids,[2,3] high rate of spontaneous mutations,[4] slow aerobic growth[2] and dead in stationary phase.[5]

We now report that in addition to the above-mentioned phenotypic deficits, *sodAsodB E. coli* strains are unable to utilize glycerol aerobically due to low activity of glycerol kinase, the rate-limiting enzyme in the metabolism of glycerol.[6]

MATERIALS AND METHODS

Glycerol kinase (EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) was obtained from

*Corresponding author. Tel.: +965-531-2300, ext. 6437. Fax: +965-533-8908. E-mail: lbenov@hsc.kuniv.edu.kw

Sigma and was additionally purified by gel-filtration. Bactotryptone, casamino acids, and yeast extract were from Difco.

M9CA medium was prepared according to Maniatis *et al.*[7] The strains of *E. coli* were GC4468 = parental,[8] DJ901 = *soxRS*,[9] QC1799 = *sodAsodB*, QCl817 = *sodAsodBsoxRS*.[10] All strains were provided by D. Touati. In parallel the same experiments were performed with AN387 = parental and AS237 = *sodAsodB* provided by J. Imlay.[11] The strains were grown aerobically overnight at 37°C in LB medium[7] and were then diluted 200-fold into M9 medium containing 20 mM glycerol instead of glucose and either 0.2 or 1% casaminoacids. Growth was followed by measuring $A_{600\,nm}$. For enzyme assays cultures were grown to a density of $A_{600\,nm}$ 0.5–0.8 in 1% casaminoacids+20 mM glycerol. The cells were washed three times and resuspended in the assay buffer. Cells were disrupted by a French Press and debris was removed by centrifugation. The crude extracts were immediately assayed for glycerol kinase[12] and glycerol 3-phosphate dehydrogenase.[13]

A xanthine oxidase system was used to generate superoxide. The reaction mixture contained, at final concentrations, 150 μM xanthine, ~100 units glycerol kinase, and xanthine oxidase. Xanthine oxidase activity was measured as superoxide dismutase inhibitable cytochrome c reduction at 550 nm and an amount yielding 4–6 μM superoxide per min was added to start the reaction. $FeSO_4$ was added to the xanthine oxidase system at final concentrations of 2 and 5 μM. Prior to use, glycerol kinase was passed through a Sephadex G25 column and was checked for SOD and catalase contaminations.

RESULTS

Glycerol significantly improved the growth of the parental strains (Fig. 1A) but had a negligible effect on the growth of the *sodAsodB E.coli* (Fig. 1B). Anaerobically, glycerol stimulated the growth of both, the mutant and the parent, equally well (Fig. 1C).

Aerobic metabolism of glycerol requires both GK and aerobic G3PD, where GK is the rate-limiting enzyme.[6] Cells missing aerobic G3PD grow on neither glycerol nor G3P in the presence of molecular oxygen. Furthermore, their growth on casein hydrolysate or succinate can be inhibited by glycerol or G3P.[1] Neither glycerol nor G3P, however, had inhibitory effect on the growth of *sodAsodB* cells in 1% casaminoacids (not shown). This suggested that G3PD was not responsible for the inability of SOD-deficient cells to grow on glycerol. Indeed, G3PD was found to be fully active in both the mutant and the parent. In contrast, GK activity was lower in the *sodAsodB* strains (Fig. 2). The activity of GK was not affected by deletion of the *soxRS* regulon, indicating that this enzyme is not a member of the regulon and its activity is not negatively controlled by products of the *soxRS*.

If elevated $[O_2^-]$ is responsible for the low activity of GK in *sodAsodB* strain, then redox-cycling agents, which increase O_2^- production, would similarly diminish GK in the parental cells. As seen, parental cells, incubated with 100 μM paraquat, showed half of the GK activity present in the controls (Fig. 3).

GK contains an essential –SH group in its active site and is easily inactivated by sulfhydryl reagents.[14] We assumed that O_2^- might be capable of inactivating GK by modifying the essential sulfhydryl group. The rate constant of O_2^- interaction with –SH is low,[15] but availability of "free iron" might catalyze –SH groups oxidation.[16] To test this possibility, purified *E. coli* GK was exposed to enzymatically generated superoxide. No significant decrease in GK activity was observed even after 60 min of incubation. In the presence of externally added Fe(II) (up to 5 μM) the activity dropped only by 11.2%±3.1 for 60 min. The obvious conclusion to be drawn from these results is that GK is not a superoxide-sensitive enzyme. At the same time, when cell-free extracts of *sodAsodB* cells were

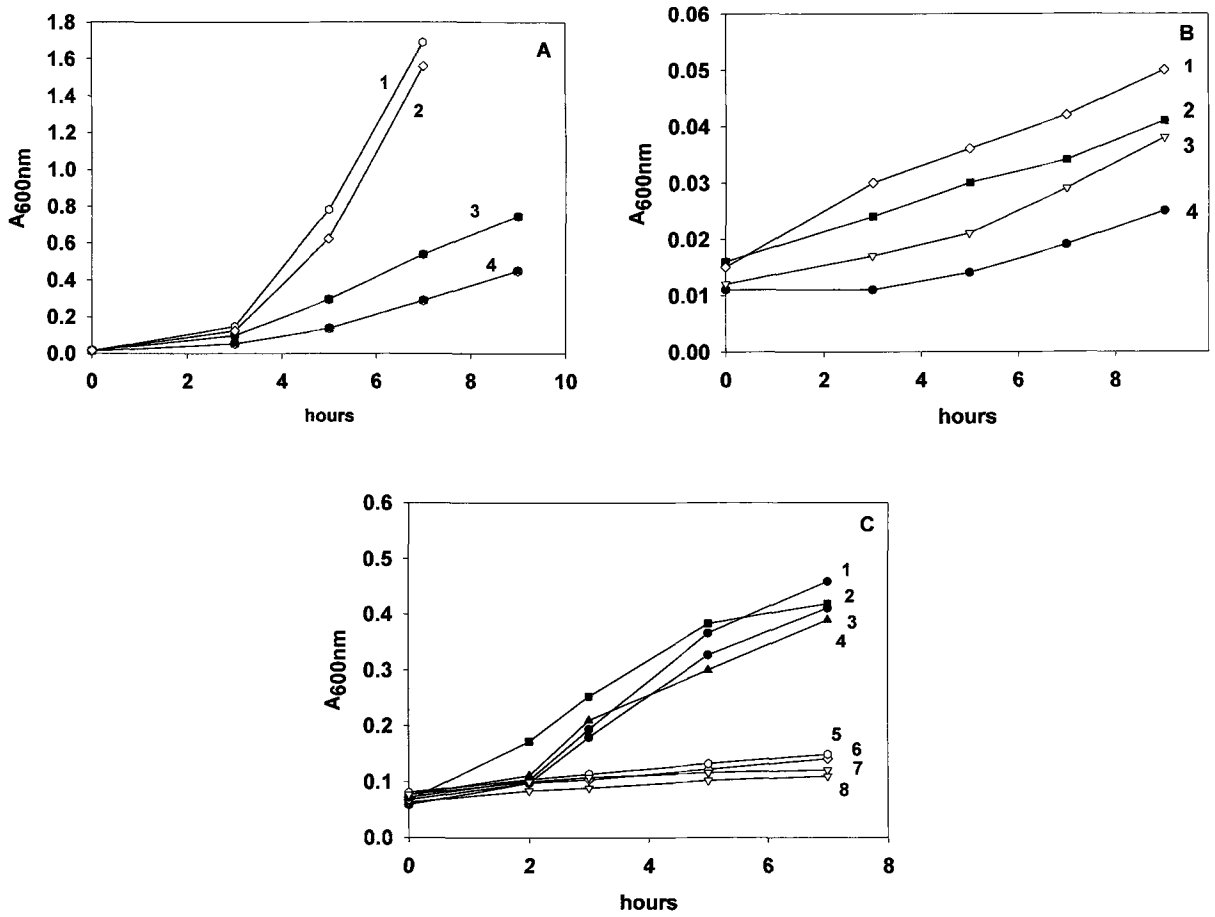

FIGURE 1 *SodAsodB E. coli* cannot grow aerobically on glycerol: 50 µl overnight aerobic LB cultures were transferred in 10 ml of M9 medium containing 0.2% casaminoacids with or without 20 mM glycerol. Growth was monitored as $A_{600\,nm}$. Panel A, aerobic growth of parental strains. Line 1, AN387+glycerol; line 2, GC4468+glycerol; line 3, GC4468; line 4, AN387; Panel B, aerobic growth of *sodAsodB* strains. Line 1, QC 1799+glycerol; line 2, QC1799; line 3, AS237+glycerol; line 4, AS237. Panel C, anaerobic growth. In addition to the above components the medium contained 25 mM $NaNO_3$. Line 1, QC1799+glycerol; line 2, AN387+glycerol; line 3, AS237+glycerol; line 4, GC4468+glycerol; line 5, GC4468; line 6, AS237; line 7, AN387; line 8, QC1799.

incubated at 37°C a progressive loss of GK activity was observed (Fig. 4). Keeping the extracts under nitrogen did not affect the rate of inactivation. Once inactivated, the enzyme could not be reactivated by mercaptoethanol or dithiothreitol.

Purified *E. coli* GK was also inactivated if pre-incubated with XO/xanthine, and then added to cell-free extracts (Fig. 5). The inactivation was much faster at 42°C than at 37°C. The cell-free extracts lost their ability to inactivate GK if incubated at 56°C for 15 min. Fractionation through various sizes Centricon filters showed that the inactivating factor(s) have molecular weight above 30,000. GK was not inactivated if pre-incubated with XO or xanthine alone.

DISCUSSION

The presence of superoxide dismutases in almost all aerobic organisms implies that their substrate, the superoxide radical is a potent cell-damaging agent. This idea remained controversial for a

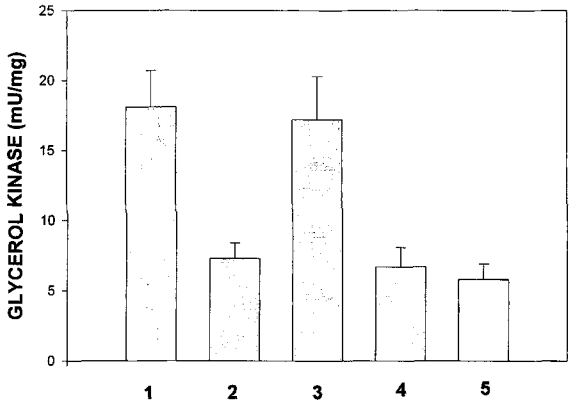

FIGURE 2 Glycerol kinase is low in *sodAsodB E. coli*:*E. coli* was grown in M9 medium containing 1% casaminoacids and 20 mM glycerol to a density of $A_{600\,nm}$ 0.5–0.8. The cells were washed and disrupted by a French press. Glycerol kinase was assayed in the crude extract as described in the "Materials and methods" section. Bar 1-AN387; bar 2-AS237; bar 3-GC4468; bar 4-QC1799; bar 5-QC 1817.

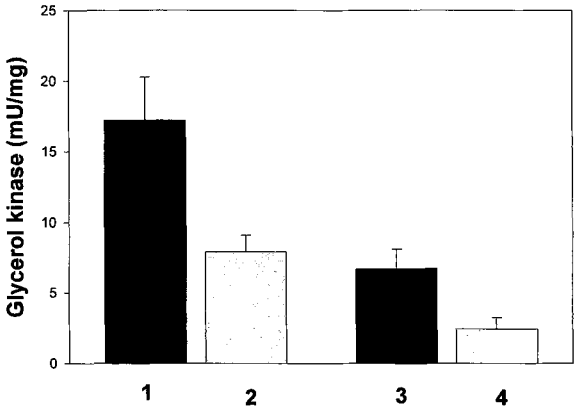

FIGURE 3 Paraquat decreases the activity of glycerol kinase: Parental and *sodAsodB* cells were grown in 1% casaminoacids+20 mM glycerol to a density of $A_{600\,nm}$ ~0.4. Paraquat was added to a final concentration of 50 μM for the *sodAsodB* and 100 μM for the parental, and the cultures were incubated for 3 more h. Bar 1, GC4468; bar 2, QC1799; bar 3, GC4468+PQ^{++}; bar 4, QC1799+PQ^{++}.

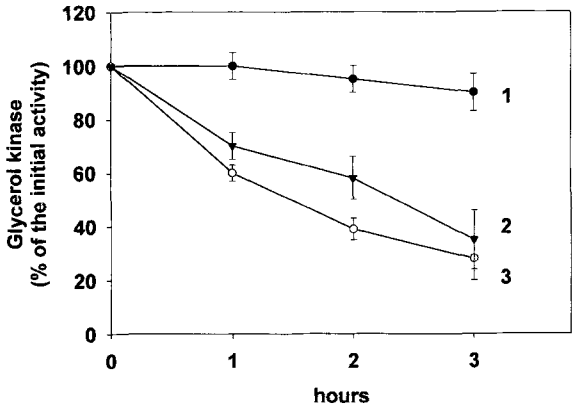

FIGURE 4 Glycerol kinase is inactivated faster in crude extracts of *sodAsodB* cells: Cell-free extracts of parental and *sodAsodB* cells were incubated at 37°C. Anaerobic incubation was achieved by blowing nitrogen over the crude extracts for 15 min. The anaerobic extracts were incubated in tightly sealed glass tubes and a separate tube was used for each time interval. Results are presented as a percentage of the initial enzyme activity. Line 1, GC4468; line 2, QC1799, anaerobic; line 3, QC1799, aerobic.

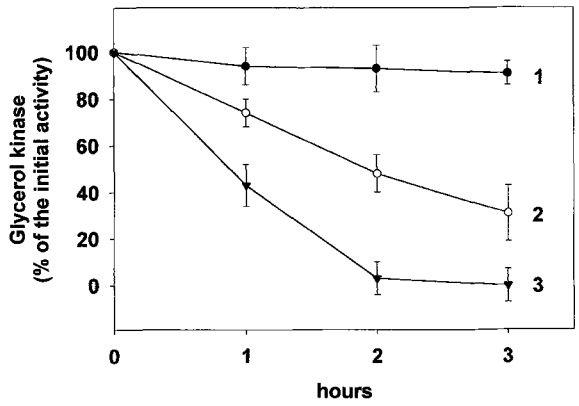

FIGURE 5 Inactivation of purified glycerol kinase: *E. coli* glycerol kinase (~100 units) was incubated 30 min with xanthine oxidase plus xanthine and aliquots were then added to 0.5 ml of *sodAsodB* cell-free extract containing 5 mg/ml protein. Line 1, GK+XO/xanthine, without cell-free extract; line 2, with cell-free extract at 37°C; line 3, with cell-free extract at 42°C.

long time due to the poor chemical reactivity of O_2^-. The first clear evidence, that *in vivo* superoxide is deleterious, was provided by creating *E. coli* mutants lacking cytoplasmic SODs.[2] These *sodAsodB* mutants grow well anaerobically, but exhibit various phenotypic deficits if grown aerobically. Among them are requirements for branched-chain, sulfur-containing, and aromatic amino acids, high rates of spontaneous mutations, low survival in stationary phase, high sensitivity towards hydrogen peroxide, and slow aerobic growth in all media. Each of these phenotypic deficits reflects damage to a specific intracellular target. Thus, stationary phase death, mutagenesis, and sensitivity towards hydrogen

peroxide are attributed to DNA damage; branched-chain amino acid auxotrophy, to inactivation of a [4Fe–4S] cluster-containing enzyme—dihydroxyacid acid dehydratase; aromatic amino acids auxotrophy, to inactivation of transketolase; and sulfur-containing amino acid auxotrophy, to damage of the cell envelope.

Here we describe another *sodAsodB* defect, inability to grow aerobically on glycerol. This defect appears to be due to low GK activity. In contrast to dihydroxyacid acid dehydratase and transketolase, GK was not inactivated by superoxide. The purified enzyme lost its activity only if pre-incubated with XO/xanthine and then added to cell-free extracts. The inactivation did not require oxygen and depended on a high molecular weight, heat-unstable factor(s). *E. coli* contains a soluble proteolytic system that can recognize and selectively degrade oxidatively modified proteins.[17,18] Our data suggest that in the *sodAsodB* cells as well as in the parental cells treated with paraquat, GK is oxidatively modified, without being inactivated. The modified enzyme, however, seems to be recognized and cleaved by protease(s). It has been demonstrated that $HO^{\cdot}-$ modified proteins are proteolytically degraded 50 times faster than the untreated ones.[19] Several lines of evidence [17–21] suggest that proteolysis is a common biological response to oxidative damage *in vivo*. It might appear that irrespective of its poor chemical reactivity, superoxide radical can modify various proteins and enhance their proteolytic degradation. Increased protein degradation together with decreased protein synthesis might be a reason for the slow aerobic growth of the *sodAsodB* mutants.

Acknowledgements

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