The Role of α,β-Dicarbonyl Compounds in the Toxicity of Short Chain Sugars*

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The extent to which sugars serve as targets for superoxide was examined using glycolaldehyde as the simplest sugar and using superoxide dismutase (SOD)-replete and SOD-null strains growing under aerobic and anaerobic conditions. Glycolaldehyde was more toxic to the SOD-null strain than to its SOD-replete parent, and this differential effect was oxygen-dependent. The product, glyoxal, could be trapped in the medium by 1,2-diaminobenzene and assayed as quinoxaline. The SOD-null strain produced more glyoxal and eliminated it more slowly than the SOD-replete parent strain. Glyoxal was ~10 times more toxic than glycolaldehyde and was more toxic to the SOD-null strain than to the parental strain. 1,2-Diaminobenzene protected against the toxicity of glycolaldehyde. These Escherichia coli strains contained the glutathione-dependent glyoxalases I and II, as well as the glutathione-independent glyoxalase III. Of these enzymes, glyoxalase III was most abundant, and it was inactivated within the aerobic SOD-null strain and also in extracts when exposed to the flux of superoxide and hydrogen peroxide imposed by the xanthine oxidase reaction. Thus, it appears that short chain sugars are oxidized by superoxide yielding toxic dicarbonyls. Moreover, the defensive glyoxalase III is also inactivated by the oxidative stress imposed by the lack of SOD, thereby exacerbating the deleterious effect of sugar oxidation.

Sugars, in which carbon chain backbone is too short to permit conversion to cyclic hemiacetals, are prone to enolization and then to air oxidation. The superoxide is a product of air oxidation (1–3). Because the superoxide can also initiate the oxidation of such enediolates, free radical chain oxidations are possible (4). Fig. 1 presents a scheme for the tautomerism of the open chain such enediolates, free radical chain oxidations are possible (4). Fig. 1 presents a scheme for the tautomerism of the open chain enediolates (I) to the corresponding enediols (II) and for the sequential oxidations of the enediols to a monoradical (III) and then to a very unstable diradical (IV), which rearranges to the α,β-dicarboxyl (V). The one-electron oxidation can be caused slowly by dioxygen yielding superoxide or more rapidly by superoxide yielding hydrogen peroxide. We have previously noted that short chain sugars are toxic to Escherichia coli, aerobically but not anaerobically and that a scavenger of dicarbonyls, such as aminoguanidine, protected (5). Because a SOD-null strain was more prone to this toxicity than the parental strain, we concluded that superoxide was a factor in the oxidation of the short chain sugars and that α,β-dicarbonyls were the proximate toxic products of that oxidation. We did not then actually measure the α,β-dicarbonyls that were supposed to be the cause of the toxicity, nor did we consider the protective actions of glyoxalases that convert α,β-dicarbonyls to α-hydroxy acids. The data presented below fill those gaps and add to our understanding of sugars as sources of superoxide and as targets for that radical. We find that glyoxal is produced from glycolaldehyde more rapidly by a SOD-null strain than by the parental strain. It is also seen that the parental strain eliminates glyoxal more rapidly than the SOD-null strain. One particular α,β-diketone, i.e. methylglyoxal, can be made from dihydroxyacetone phosphate by a specific synthase that is widespread in bacteria and that has been cloned, sequenced, and overexpressed (6, 7). The α,β-dicarbonyl compounds, whether made by methylglyoxal synthase or as a result of autoxidation of short chain sugars, are potentially toxic because of their propensity to covalently modify both nucleic acids and proteins (8, 9). Our results suggest that under conditions of oxidative stress, such as stress imposed by a lack of SOD, the autoxidation of short chain sugars to dicarbonyls is more of a problem than is the activity of methylglyoxal synthase. Our results also indicate that the specificity of the defensive glyoxalases is broad enough to encompass glyoxal.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methylglyoxal, glyoxal, glycolaldehyde, m-glyceraldehyde, 1,2-diaminobenzene, aminoguanidine, S-lactoylglutathione, glutathione, ATP, and NADP were obtained from Sigma. D-Erythrose was from Fluka. 2,3-Dimethylxynoquinaline was from Aldrich. Xanthine oxidase, catalase, and glucose-6-phosphate dehydrogenase were from Roche Molecular Biochemicals. Yeast extract, Bacto-pectone, and casamino acids were from Difco.

**Cell Culture**—The E. coli strain used was AB1157, which was the parental strain for JI132 that was the ΔsodA ΔsodB mutant (10). Starter cultures were grown overnight in aerobic LB medium at 37 °C and were then diluted to 2 × 10⁶ cells/ml in M9CA medium. LB and M9CA media are as described previously (11). The anaerobic condition was achieved in a BBL Gas Pak anaerobic system (Becton Dickinson). When needed, extracts were prepared from 6-h cultures by centrifugation (washing the cells two times in 50 mM potassium phosphate, pH 7.8); then the cells, which had been resuspended in this buffer, were disrupted with a French press. The lysate was clarified by centrifugation.

**Glyoxal and Methylglyoxal Assay**—Glyoxal and methylglyoxal were assayed in the medium by using 1,2-diaminobenzene as derivatizing reagent by a modification of the protocol of Cordeiro and Ponces Freire (12). To a 1-ml sample containing glyoxal and/or methylglyoxal, we added 0.2 ml of 5 M HClO₄, 0.2 ml of 2,3-dimethylquinoloxaline as an internal standard, 0.2 ml of 10 mM 1,2-diaminobenzene, and water to a 2-ml final volume. After 1 h at 25 °C, high pressure liquid chromatography (HPLC) analysis was performed in a LKB-Bromma chromatograph. The column was a 5-μm, 250 × 4-mm RP-18 (Merck LiChrospher). The mobile phase was 40% (v/v) 25 mM ammonium formate buffer, pH 3.4, and 60% (v/v) methanol. A volume of 150 μl was...

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The abbreviations used are: SOD, superoxide dismutase; HPLC, high pressure liquid chromatography.
 injected. The flow rate was 1.6 ml/min and quinoxalines were detected at 315 nm.

**Enzymatic Assays—** Glyoxalase I was assayed by following an increase in A$_{340}$ because of S-α-lactoylglutathione formation (13). One unit of glyoxalase I is defined as the amount of enzyme required to form 1 μmol of S-α-lactoylglutathione/min. Glyoxalase II was assayed by monitoring the decrease in A$_{240}$ accompanying the conversion of S-α-lactoylglutathione to lactate plus GSH (14). One unit of glyoxalase II is defined as the amount of enzyme that hydrolyzes 1 μmol of S-α-lactoylglutathione/min. Glyoxalase III was assayed by a modification of the method of Misra et al. (15) using the HPLC assay for glyoxal and methylglyoxal as described above. One unit of glyoxalase III is defined as the amount of enzyme required to utilize 1 μmol of methylglyoxal or to form 1 μmol of l-lactate/min. 50 μM xanthine, 2 mM xanthine oxidase, 1 μg of Cu,Zn-SOD, 1 μg of catalase, or 25 mM mannitol were added to the cell extracts to explore the effects of reactive oxygen species.

ATP-dependent phosphorylation of glucose by glucokinase was assayed in cell extracts by monitoring the formation of NADPH and by assaying in cell extracts by monitoring the formation of NADPH and by monitoring the decrease in A$_{340}$ accompanying the conversion of S-α-lactoylglutathione to lactate plus GSH (14). One unit of glyoxalase II is defined as the amount of enzyme that hydrolyzes 1 μmol of S-α-lactoylglutathione/min. Glyoxalase III was assayed by a modification of the method of Misra et al. (15) using the HPLC assay for glyoxal and methylglyoxal as described above. One unit of glyoxalase III is defined as the amount of enzyme required to utilize 1 μmol of methylglyoxal or to form 1 μmol of l-lactate/min. 50 μM xanthine, 2 mM xanthine oxidase, 1 μg of Cu,Zn-SOD, 1 μg of catalase, or 25 mM mannitol were added to the cell extracts to explore the effects of reactive oxygen species.

**Results**

**Glycolaldehyde and Glyoxal: Effect of Superoxide, SOD, and 1,2-Diaminobenzene—** Glycolaldehyde did not inhibit aerobic growth of the SOD-replete AB1157 strain until its concentration exceeded 4.0 mM (Fig. 2A, line 1). In contrast, the growth of the SOD-null J1132 strain was suppressed in the lower range of 0–4.0 mM (Fig. 2A, line 2). This protective effect of endogenous SOD on the sensitivity to glycolaldehyde was not seen under anaerobic conditions (Fig. 2A, lines 3 and 4). When glyoxal was examined (Fig. 2B), a similar pattern was seen but at 10-fold lower concentrations. Thus, the anaerobic growth suppression became pronounced above 0.3 mM for both the J1132 and AB1157 strains (Fig. 2B, lines 3 and 4), and aerobically J1132 was more sensitive than AB1157 (Fig. 2B, lines 1 and 2). 1,2-Diaminobenzene converts α,β-dicarbonyl compounds to quinoxalines (12, 18), and if glyoxal is the cause of glycolaldehyde toxicity, 1,2-diaminobenzene should protect J1132. Comparison of lines 1 and 2 in Fig. 2C demonstrates that 2.0 mM glycolaldehyde slowed the aerobic growth of the J1132 strain, whereas lines 3 and 4 in Fig. 2C show that 1.0 mM 1,2-diaminobenzene significantly lessened the effect of glycolaldehyde. It should be noted that 1,2-diaminobenzene at 1.0 mM did not affect itself the growth of the J1132 strain in the absence of glycolaldehyde, although it was a growth inhibitor at a higher concentration (data not shown). It should also be recalled that 2.0 mM glycolaldehyde or 0.2 mM glyoxal were without effect on the anaerobic growth rates of AB1157 or J1132 (Fig. 2A, line 2, and lines 3 and 4). These data support the conclusion that glyoxal can be a cause of the aerobic toxicity of glycolaldehyde, superoxide plays a role in the conversion of the latter into the former, and somehow superoxide also increases the toxicity of glyoxal.

**Oxidation of Glycolaldehyde into Glyoxal—** Glycolaldehyde autoxidizes into glyoxal, and the extent of this autoxidation must be known so that corrections for it can be applied to results obtained with E. coli. Line 1 in Fig. 3A presents the accumulation of glyoxal from 2.0 mM glycolaldehyde in M9 medium. The rate was slower in M9CA medium (Fig. 3A, line 2) presumably because of consumption of glyoxal by reaction with the amino acids in the casein hydrolysate. Aminoguanidine completely eliminated the accumulation of glyoxal (Fig. 3A, line 3) by coupling with it to form an asymmetrical triazine (19). In accordance with these results, when the persistence of glyoxal was examined (Fig. 3B), glyoxal was seen to be stable in M9 medium (Fig. 3B, line 1) but less stable in M9CA medium (Fig. 3B, line 2). Arginine caused rapid consumption of glyoxal (Fig. 3B, line 4), whereas the scavenging of glyoxal by aminoguanidine was not pronounced (Fig. 3B, line 3).

The effects of 2.0 mM glycolaldehyde on the growth of E. coli and the concomitant accumulation and consumption of glyoxal were examined. Fig. 4A shows that both the SOD-replete AB1157 and the SOD-null J1132 strains grew at the same rates anaerobically (lines 2 and 4), whereas aerobically AB1157 grew much faster (line 1) than did J1132 (line 3). The glyoxal content of the medium was also followed as shown in Fig. 4B. Line 1 shows that aerobic AB1157 accumulated glyoxal to a maximum

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**Fig. 1. Oxidation of sugars to α,β-dicarbonyl compounds.** Compound 1 is an aldose in the open chain configuration. Tautomerism yields the enediol (II). Subsequent univalent oxidations by O$_2$ or O$_2^-$ yield the intermediates III and IV. The diradical IV rapidly collapses to the dicarbonyl (V).

**Fig. 2. Superoxide dependence of the toxicity of short chain sugars and the effect of 1,2-diaminobenzene.** A, toxicity of glycolaldehyde to aerobic and anaerobic E. coli. Line 1, AB1157 under aerobic conditions; line 2, J1132 under aerobic conditions; line 3, AB1157 under anaerobic conditions; line 4, J1132 under anaerobic conditions. B, toxicity of glyoxal to aerobic and anaerobic E. coli. Line 1, AB1157 under aerobic conditions; line 2, J1132 under aerobic conditions; line 3, AB1157 under anaerobic conditions; line 4, J1132 under anaerobic conditions. C, glycolaldehyde toxicity and protection by 1,2-diaminobenzene. Line 1, AB1157 + 2 mM glycolaldehyde; line 2, J1132 + 2 mM glycolaldehyde; line 3, AB1157 + 2 mM glycolaldehyde + 1 mM 1,2-diaminobenzene; line 4, J1132 + 2 mM glycolaldehyde + 1 mM 1,2-diaminobenzene. Overnight cultures of AB1157 and J1132 in LB medium were diluted to 2 × 10$^6$ cells/ml in M9CA medium. Cell growth was monitored at 600 nm after 8 h (A, B) of incubation at 37 °C.

**Fig. 3. Glyoxal contents in the medium without E. coli.** A, glyoxal production by glycolaldehyde. Line 1, M9 + 2 mM glycolaldehyde; line 2, M9CA + 2 mM glycolaldehyde; line 3, M9CA + 2 mM glycolaldehyde + 20 mM aminoguanidine. B, effects of aminoguanidine and arginine on glyoxal. Line 1, M9 + 200 μM glyoxal; line 2, M9CA + 200 μM glyoxal; line 3, M9CA + 200 μM glyoxal + 20 mM aminoguanidine; line 4, M9CA + 200 μM glyoxal + 20 mM arginine. M9 medium was enriched with 0.2% glucose plus 3 mg/ml pantothenic acid and thiamine. M9CA medium contained 0.2% casamino acids plus M9 medium. All media were incubated at 37 °C under aerobic conditions. Glyoxal contents of the medium were measured by HPLC as quinoxaline. 2,3-dimethylquinoxaline was used as an internal standard.
of 40 μM during the first 4 h of growth and then consumed it during subsequent growth. In contrast, aerobic JI132 continued to accumulate glyoxal to a maximum of 100 μM during the 10 h of observation (line 3). Under anaerobic conditions, there was no accumulation of glyoxal by either strain (lines 2 and 4). Line 5 shows the production of glyoxal by autoxidation in the aerobic M9CA medium without cells, whereas line 6 depicts an anaerobic control for the effect of medium alone. It again appears that JI132 produces glyoxal from glycolaldehyde more rapidly and eliminates it more slowly than AB1157. This is probably why its growth was more strongly inhibited by glycolaldehyde.

**Glyoxalase Activities**—*E. coli* is known to contain glyoxalases I, II, and III, although one report states that glyoxalase II was not detected (15). Because glyoxalases I and II cooperate in performing the GSH-dependent conversion of α,β-dicarbonyls to α-hydroxy acids, it would be expected that glyoxalase II would be present if glyoxalase I was present. Fig. 5 presents the glyoxalase activities in the AB1157 and JI132 strains grown under different conditions. The first point to be made is that glyoxalases I, II, and III are all present, although glyoxalase III > glyoxalase I > glyoxalase II > 0. Hence most of the glyoxalase activity in *E. coli* is because of the GSH-independent glyoxalase III. A comparison of Fig. 5, A and D, makes it clear that the AB1157 and JI132 strains have comparable glyoxalase activities when grown anaerobically but that JI132 has less glyoxalase III under aerobic conditions. There was no induction of glyoxalase III by growth in the presence of methylglyoxal (Fig. 5B). Paraquat, which can increase the aerobic production of superoxide, suppressed glyoxalase III in JI132 (Fig. 5C).

Inactivation of the abundant glyoxalase III by superoxide or by reactive species derived therefrom could explain the lower glyoxalase activity in the aerobic JI132 cells than in the AB1157 strain and could also explain the effect of paraquat; it could further clarify why glyoxal was more toxic to JI132 than to AB1157 and why this differential toxicity was oxygen-dependent. Hence, this possibility was explored.

**Inactivation of Glyoxalase III**—The effect of a flux of superoxide, produced by the xanthine oxidase reaction (20) on the glyoxalase III activity in extracts of *E. coli*, was examined. Fig. 6A shows that glyoxalase III activity was diminished in the case of AB1157 by exposure to the xanthine oxidase reaction, whereas Fig. 6B shows that the effect on JI132 extracts was greater. Because the SOD endogenous to AB1157 and present in the extract could have accounted for this difference, SOD was added to the extracts and was found to protect completely. Indeed, the added SOD raised the glyoxalase III activity in JI132 extracts to a level greater than that seen in extracts not exposed to the xanthine oxidase reaction. We suppose that this is explained by the inactivation of some glyoxalases III by endogenous superoxide production in the JI132 extracts before the sampling for assay. Superoxide can release Fe(II) from the [4Fe-4S] clusters of dehydratases, and that Fe(II) can reduce hydrogen peroxide, and mannitol should protect by scavenging the hydroxyl radical. Fig. 6 illustrates the protective effects of catalase and mannitol. It follows that glyoxalase III, the major glyoxalase in *E. coli*, is sensitive to inactivation by the pro-oxidant conditions created by the lack of SOD and that Fenton chemistry generates the proximate inactivator.

Glucokinase was examined in a similar way to see whether
the sensitivity of glyoxalase III to oxidation was unusual. Fig. 7 shows that glucokinase was not inactivated by the superoxide and hydrogen peroxide produced by the xanthine oxidase reaction. Thus, the sensitivity of glyoxalase III was special and might relate to the thiol group that is essential for its activity and possibly to the binding of iron adjacent to the active site thiol.

**Methylglyoxal—**While assaying for glyoxal in terms of the quinoxaline produced from the reaction with 1,2-diaminobenzene, we also measured methylglyoxal in terms of 2-methylquinoxaline. The primary reason for doing so was to gauge the extent to which methylglyoxal synthase was contributing to dicarbonyl production by its non-oxidative pathway. Fig. 8 indicates that the contribution of the methylglyoxal synthase to the total dicarbonyl production in cells exposed to glycolaldehyde was very small indeed. Thus, M9CA medium conditioned by the growth of JI132 underwent reaction with 1,2-diaminobenzene and then was subjected to HPLC in which quinoxaline, derived from glyoxal, eluted at 3 min, 35 s, and in which 2-methylquinoline, derived from methylglyoxal, eluted at 4 min, 20 s. There was no detectable methylglyoxal formed from the glucose present in this medium and in only traces of other dicarbonyls (Fig. 8A). When 200 μM glyoxal had been added to the culture (Fig. 8B), it was detected as quinoxaline at zero time eluted at 3 min, 35 s and was progressively consumed at longer times of incubation. Similarly, enriching the medium with 200 μM methylglyoxal gave 2-methylquinoline eluting at 4 min, 20 s at zero time and progressively less in samples drawn at longer times of incubation (Fig. 8C). The consumption of these dicarbonyls by JI132 largely reflects the activity of glyoxalases. When cultures enriched with glycolaldehyde (Fig. 8D) or glyceraldehyde (Fig. 8E) were examined, the major dicarbonyls detected were glyoxal and methylglyoxal, respectively. However, in the case of glycolaldehyde, the glyoxal was first generated and then consumed during the 24 h of incubation. In contrast, the glyceraldehyde was contaminated with methylglyoxal in the zero time sample. Erythrose (Fig. 8F) was contaminated by polar dicarbonyls, presumably erythrosine, in which quinoxaline products eluted at 2 min, 35 s. It also contained lesser amounts of glyoxal and methylglyoxal.

**DISCUSSION**

Short chain sugars, in which carbonyl functions cannot be blocked by the formation of furanose or pyranose rings, are prone to enolization followed by oxidation to toxic dicarbonyls. Thus, they express in an exaggerated way what can also occur with glucose by the slower process of non-enzymatic glycation and oxidations (24, 25). Glycolaldehyde, the simplest sugar, is more toxic to a SOD-null strain of *E. coli* (JI132) than to its SOD-replete parent (AB1157), and this extra toxicity is oxygen-dependent. The corresponding dicarbonyl, glyoxal, was ~10 times more toxic than glycolaldehyde, and JI132 was again more sensitive than AB1157 in an oxygen-dependent way. 1,2-Diaminobenzene protected JI132 against the toxicity of glycolaldehyde, presumably by converting glyoxal to the less toxic quinoxaline. We may infer that superoxide is an important cause of the oxidation of glycolaldehyde, and we may explain the greater and oxygen-dependent sensitivity of JI132 to glyoxal on the basis of an oxidative inactivation of glyoxalases. Glycolaldehyde itself becomes toxic at high concentrations, even anaerobically, probably by converting essential amino compounds to carbinolamines and to Schiff base salts.

When the SOD-replete AB1157 strain grew aerobically in the presence of glycolaldehyde, glyoxal first accumulated in the medium and was subsequently consumed. The SOD-null JI132, in contrast, accumulated glyoxal during the entire 10 h of incubation. Thus, it appeared that JI132 both converted glycolaldehyde to glyoxal more rapidly and disposed of it more slowly than did AB1157. Glyoxalases I, II, and III were all present in these strains, but the GSH-independent glyoxalase III was the most abundant and suppressed in the JI132 strain grown aerobically; it was further suppressed when JI132 grew in the presence of 1 μM paraquat. Thus, it appears that glyoxalase III was inactivated by the oxidative stress imposed by
lack of SOD activity and also by the presence of paraquat.

Exposure of bacterial extracts to the superoxide and hydrogen peroxide produced by the xanthine oxidase reaction caused a loss of glyoxalase III activity that was greater in the SOD-null extracts. This inactivation was prevented by SOD, catalase, or mannitol. Glucokinase in the extracts was not inactivated by the xanthine oxidase reaction. Glyoxalase III may be selectively inactivated by a flux of superoxide and hydrogen peroxide because it binds the Fe(II) released from the [4Fe-4S] clusters of dehydratases oxidized by superoxide. This bound Fe(II) would then react with hydrogen peroxide to yield Fe(II)–O, Fe(III)–OH, or hydroxyl radical, and these strong oxidants would preferentially attack the nearest target, which in this case is glyoxalase III.

When glucose was the carbon source, no dicarbonyls could be trapped by 1,2-diaminobenzene. This negative result is a measure of the degree of protection provided by blocking sugar carbonyls by hemiacetal ring closure. Moreover, the steady state concentrations of the triosephosphate intermediates of glycolysis must be very low because the equilibrium constant of the aldolase reaction greatly favors fructose 1,6-diphosphate. We conclude that dicarbonyl production from dihydroxyacetone phosphate, by the action of methylglyoxal synthase, must be insignificant and in full accord with the observation that 900-fold overexpression of this synthase did not cause observable detrimental effects (7). MacLean et al. (26) reported that glyoxalase III was the most abundant glyoxalase in E. coli but nevertheless concluded that glyoxalases I plus II were the most important route of methylglyoxal detoxification. This conclusion was based on the heightened sensitivity to methylglyoxal exhibited by a glyoxalase I-null mutant. However, this can be explained by the protective effect of lowering cytoplasmic pH (27) because of the activation of potassium efflux by the product of the glyoxalase I reaction, S-lactoylglutathione (26). Lowering the pH would slow the reaction of dicarbonyls with target amino or thiol compounds.

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