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Inactivation-Reactivation of Aconitase in *Escherichia coli*

A SENSITIVE MEASURE OF SUPEROXIDE RADICAL*

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The rapid inactivation of aconitase by O_2^-, previously seen to occur *in vitro*, was explored *in vivo*. A fraction of the aconitase in growing, aerobic, *Escherichia coli* is inactive at any instant but can be activated by imposition of anaerobic conditions. This reactivation occurred in the absence of protein synthesis and was inhibited by the ferrous chelator α,α'-dipyridyl. This fraction of inactive, but activatable, aconitase was increased by augmenting O_2^- production with paraquat, decreased by elevation of superoxide dismutase, and increased by inhibiting reactivation with α,α'-dipyridyl.

The balance between inactive and active aconitase thus represented a pseudoequilibrium between inactivation by O_2^- and reactivation by restoration of Fe(II), and it provided, for the first time, a measure of the steady-state concentration of O_2^- within *E. coli*. On this basis, $[O_2^-]$ was estimated to be ~20–40 pM in aerobic log phase *E. coli* containing wild type levels of superoxide dismutase and ~300 pM in a mutant strain lacking superoxide dismutase.

Superoxide radical (O_2^-) is a toxic by-product of normal aerobic metabolism (1), and aerobic organisms, with few known exceptions (2, 3), defend against O_2^- toxicity by expressing the O_2^--scavenging enzyme superoxide dismutase (SOD).[1] The vital role of SOD in protecting against O_2^- damage has been elegantly demonstrated through studies of SOD mutants of *Saccharomyces cerevisiae* (4, 5), *Escherichia coli* (6), and *Drosophila melanogaster* (7). Yet, a thorough understanding of the defensive role of SOD, and of the complex controls on SOD expression, requires a greater knowledge of both the critical cellular targets of O_2^- and the intracellular levels of O_2^- under a variety of conditions.

A number of O_2^--sensitive targets have been identified (8), and hydroxy acid dehydratases containing prosthetic iron-sulfur clusters were shown to be particularly sensitive to attack by O_2^- (9–12). The inactivating reaction between O_2^- and *E. coli* aconitase occurs at a nearly diffusion-limited rate in the absence of substrate, and this inactivation was only partially inhibited by virtual substrates (12).

We have investigated the inactivation-reactivation of aconitase and the relationship between aconitase activity and O_2^- levels within *E. coli*. We now report that O_2^- is a major determinant of aconitase activity under both normal and oxidative stress conditions and that aconitase can be used as a sensitive, albeit as yet imprecise, indicator of intracellular steady-state O_2^- levels. The logic of increasing SOD expression in response to iron starvation and oxidative metabolism is considered in light of this inactivation-reactivation of aconitase.

MATERIALS AND METHODS

E. coli K12 SOD mutants JI130 (sodA) and JI132 (sodAsodB), the catalase mutant UM1 (katEkatG), and the respective isogenic parental strains AB1157 and CSH7, obtained from J. A. Imlay (13), were used in these studies. Strains AB1157pBR322 and AB1157pDT1-16 were prepared by standard procedures (14) using pDT1-16 constructed by D. Touati (15) and pBR322 obtained from GIBCO/BRL.

Puromycin dihydrochloride, ampicillin, chloramphenicol, isopropyl β-thiogalactoside, manganous sulfate, L-amino acids, succinic acid, thiamine hydrochloride, α,α'-dipyridyl, paraquat, barium *dl*-fluorocitrate, $NADP^+$, porcine heart isocitrate dehydrogenase (type VI), and horse heart cytochrome *c* (type III) were obtained from Sigma. Beef liver catalase and bovine serum albumin (fraction V) were from Boehringer Mannheim. Bovine milk xanthine oxidase was kindly provided by K. V. Rajagopalan of this department.

Media and Growth of Bacteria—LB medium (14) contained 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 10 g of sodium chloride/liter. Minimal medium was made up with tap water and contained 60 mM K_2HPO_4, 33 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, 1 mM $MgSO_4$, $MnSO_4$ (as specified), 10 μg/ml thiamine hydrochloride, plus each of the 20 L-amino acids at 40 μg/ml with cystine instead of cysteine. Sodium succinate or glucose were supplied in the minimal medium at 0.4% (w/v) unless otherwise indicated. The pH of the LB and minimal salts medium was adjusted to 7.0 with NaOH or HCl. Cultures were routinely grown aerobically in a rotatory water bath at 37 °C with a medium volume to flask volume ratio of at most 1:5, and growth was monitored turbidimetrically at 550 nm.

In Vitro Stability of Aconitase—Extracts of anaerobically grown sodAsodB *E. coli*, which are devoid of SOD activity, were prepared as previously described (12). These extracts were divided into multiple small aliquots in vacutainer tubes under nitrogen and were rapidly frozen in a dry ice/ethanol bath and stored at −70 °C. Prior to use, these extracts were quickly thawed and transferred to a septum-sealed tube at 25 °C. This tube was kept anaerobic by a flow of 35 ml/min of N_2 while being stirred magnetically. The N_2 gas was freed of traces of O_2 by passage over hot copper foil. When aerobic conditions were desired, the flow of N_2 was replaced by a flow of air.

Preparation of Cell Lysates—Aliquots (3 ml) of log phase cultures were removed, without interrupting agitation, and were delivered into a pair of 1.5-ml Eppendorf tubes, which were immediately placed in a Fisher microcentrifuge and accelerated to 16,000 × *g* for ~10 s. Rapid deceleration was achieved by applying manual pressure to the rotor. The supernatant medium was then aspirated, and the pellets of cells were resuspended in 0.5 ml of ice-cold buffer (pH 7.4), hereafter called lysis buffer, containing 50 mM Tris-chloride, with 0.6 mM $MnCl_2$ and 20 μM fluorocitrate to stabilize aconitase (12). The cells were then sonicated in an ice bath for 10 s at 50 watts, using a

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[1] The abbreviations used are: SOD, superoxide dismutase; MnSOD, the manganese-containing superoxide dismutase; IPTG, isopropyl β-thiogalactoside; LB, Luria broth.

Branson Sonifier equipped with a microprobe. This lysate was clarified at 16,000 × *g* for 20 s, and the resultant extracts were frozen in dry ice/ethanol and stored at −70 °C until needed for assay of enzymatic activities. Speed was important, and the entire procedure, from sampling of the shaking, growing culture to freezing of the extracts, was completed within 2.5 min.

Anaerobic Reactivation of Aconitase—Vacutainer tubes (Becton-Dickinson, 47 × 10.25 mm) were filled to near capacity with ~2.6-ml aliquots of log phase aerobic cultures at $A_{550\ nm} > 0.5$ and were stoppered while allowing the excess volume to escape through a small syringe needle. Tubes were routinely incubated at 37 °C for 12 min or at shorter intervals where indicated to allow metabolic exhaustion of dissolved dioxygen and anaerobic reactivation of aconitase. Tubes were then briefly chilled in an ice slurry, and the cells were sedimented at 4 °C, at 5000 × *g*, for 1 min. The supernatant medium was then displaced with pressurized N_2, and the cell pellets were overlaid with 0.5 ml of ice-cold, N_2-saturated lysis buffer that was delivered from a gas-tight syringe. The tubes were unstoppered, and cell pellets were immediately sonicated for 10 s, which effectively resuspended and lysed the cells, and the lysates were clarified and frozen, as described above.

Enzyme and Protein Assays—Aconitase activity in cell-free extracts was assayed immediately after thawing by following absorbance at 340 nm at 22 °C in a 1.0-ml reaction mixture containing 50 mM Tris-Cl, pH 7.4, 30 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM $NADP^+$, and 1–2 units/ml of isocitrate dehydrogenase. Fluorocitrate was routinely present in the assay at ~1 μM, due to carry-over but did not measurably influence the expression of aconitase activity. One milliunit of aconitase activity catalyzed the formation of 1 nmol of isocitrate/min. SOD activity was assayed by the xanthine oxidase-cytochrome *c* method (16) with 0.5 mM EDTA to prevent Mn(II)-catalyzed consumption of O_2^-. Catalase was assayed by following the decrease in [H_2O_2] at 240 nm (17). Protein was assayed according to Bradford (18), with the Bio-Rad reagent and bovine serum albumin as the standard.

RESULTS

Inactivation-Reactivation in Vitro—Exposure of anaerobic extracts of sodAsodB *E. coli* to air caused rapid inactivation of aconitase. Subsequent exclusion of dioxygen resulted in a reactivation that was prevented by EDTA. These results, shown in Fig. 1, indicate that the *E. coli* aconitase, like its mitochondrial counterpart (19), is subject to oxidative attack on the [4Fe-4S] prosthetic group, with loss of iron, and that this was reversible by restoration and reincorporation of Fe(II). Previously reported results (12) demonstrated that SOD, but not catalase, largely protected against this inactivation, establishing that it was due to O_2^- generated within the cell extracts.

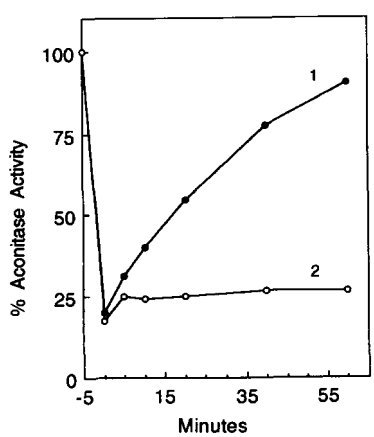

FIG. 1. **Inactivation and reactivation of aconitase *in vitro*.** Extracts of SOD-free *E. coli*, stirring under N_2 at 25 °C, were exposed to air for 5 min and then were again made anaerobic. *Line 1*, no EDTA; *line 2*, 1.0 mM EDTA. The concentration of extract protein was 25 mg/ml, and aconitase activity at time 0 was 9.1 milliunits/mg of protein.

Inactivation-Reactivation in Vivo—Aconitase activity was examined in cultures of *E. coli*, in the presence of chloramphenicol to block *de novo* protein synthesis and prevent growth. As shown by Fig. 2, *line 1*, the activity changed only minimally during 80 min of aerobic incubation. When dioxygen was excluded, the aconitase activity rapidly increased by ~15% and thereafter remained relatively constant (*line 2*). Addition of the O_2^--generating agent paraquat to 1.0 mM caused a progressive loss of aconitase activity (*line 3*), which was halted and reversed when dioxygen was excluded (*line 4*). It is clear that aconitase is inactivated by O_2^- *in vivo*, as it is *in vitro* (12), and that this is reversible. The half-time for the reactivation process was ~3 min.

Estimation of [O_2^-] within E. coli—If we assume that the citrate endogenous to *E. coli* (20) would slow the rate of inactivation of aconitase by O_2^-, much as fluorocitrate did *in vitro* (12), we can apply the rate constant of $2 \times 10^7\ M^{-1}\ s^{-1}$, which was determined *in vitro* (12), to the *in vivo* situation. This is done without attempting to compensate for the fact that the *in vitro* measurements were at 25 °C, whereas the *in vivo* temperature was 37 °C. The rate of reactivation, calculated from the pseudo-first order $t_{1/2} = 3$ min, is $0.004\ s^{-1}$.

Since the steady state of aconitase activity represents the balance between inactivation and reactivation, we can write $[\text{aconitase}_{act}]\,[O_2^-](2 \times 10^7\ M^{-1}\ s^{-1}) = [\text{aconitase}_{inact}](0.004\ s^{-1})$ and, therefore, $[O_2^-] = ([\text{aconitase}_{inact}]/[\text{aconitase}_{act}])(2 \times 10^{-10}\ M)$. This equation allowed calculation of the percent of the total aconitase that would be active within *E. coli* at any given [O_2^-]. This relationship, which is shown in Fig. 3, indicates that ~90% of the enzyme would be active at 2×10^{-11} M O_2^-, ~50% at 2×10^{-10} M O_2^-, and ~10% at 2×10^{-9} M O_2^-. Since

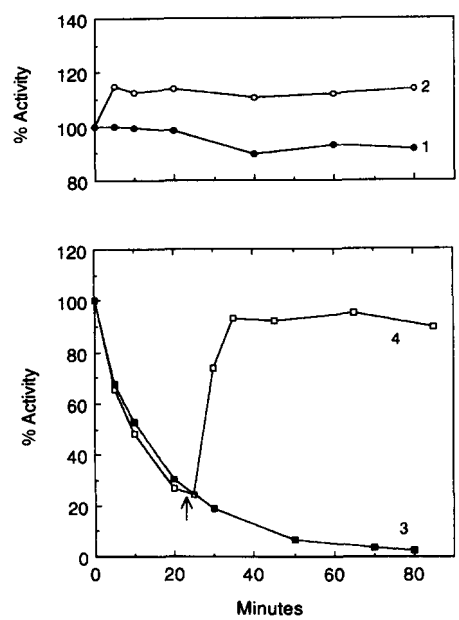

FIG. 2. **Inactivation and reactivation of aconitase *in vivo*.** Growth of AB1157 in aerobic LB medium was initiated with 2% inocula taken from overnight cultures. During log phase growth ($A_{550\ nm} \simeq 2.6$) chloramphenicol was added to 200 μg/ml, and incubation was continued for 15 min, at which point paraquat was, or was not, added to 1.0 mM and anaerobiosis was, or was not, imposed; samples were subsequently removed at intervals for assay of the fraction of active aconitase. *Line 1*, no paraquat and aerobic; *line 2*, no paraquat and anaerobic; *line 3*, paraquat and aerobic; *line 4*, paraquat and made anaerobic at the *arrow*. The aconitase activity at $t = 0$ was 192 ± 11 milliunits/mg.

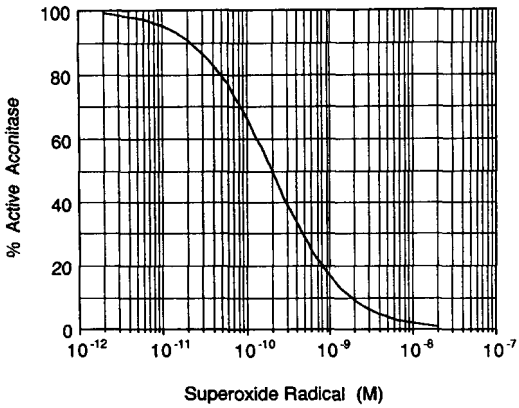

FIG. 3. **Percent active aconitase as a function of [O_2^-].** The percent of total aconitase that would be active at any given [O_2^-] was calculated on the basis of a $k_{inact} = 2 \times 10^7$ M^{-1} s^{-1} and a $k_{act} = 0.004$ s^{-1}.

the data (Fig. 2, *line 2*) showed that ~87% of the aconitase was active in aerobic *E. coli*, we estimate a steady-state [O_2^-] of ~30 pM.

Effects of [SOD] and Metabolic Emphasis on [O_2^-]—Strains of *E. coli*, which accumulate different levels of SOD, were grown aerobically in LB medium and sampled at intervals for assay of turbidity, aconitase activity, and SOD activity. As shown by Fig. 4*A*, the SOD-proficient AB1157 grew somewhat more rapidly (*line 1*) than did JI130 (*line 2*), which was defective only in sodA, whereas JI132, which was defective in both sodA and sodB, grew very slowly (*line 3*). The relative aconitase activities in these strains, at any time, paralleled their abilities to grow in this aerobic medium, as shown in *panel B*, whereas *panel C* shows that AB1157 contained more total SOD than did JI130 both early and late in the growth of these cultures but not at intermediate times. The increase in specific activity of aconitase with age of the culture, which was seen with all strains (*panel B*), probably reflects induction of aconitase in response to changes in the medium. Aconitase is known to be induced in response to aerobiosis and to changes in carbon source (21).

Additional data derived from these cultures are shown in Table I. When [SOD] was ~16 units/mg of protein, as was the case for AB1157, aconitase was ~90% active and [O_2^-] was ~20 pM; whereas when SOD activity was not detectable, as was the case with JI132, the aconitase was only ~40% active and the corresponding [O_2^-] was ~300 pM. Mn(II) enrichment was explored because it can elevate total SOD activity, especially in the case of SOD overproducers (22). However, with these strains it exerted no significant effects.

Earlier work had indicated that growth of *E. coli* on glucose was associated with modest down-regulation of SOD (23). This led to the postulate that a predominantly glycolytic energy supply would decrease production of O_2^-. The responsiveness of aconitase to [O_2^-] allowed an examination of this supposition. Thus, cultures of *E. coli* AB1157 and JI130 were grown in a salts medium supplemented with amino acids and with either glucose or succinate as the carbon source. At midlog phase, cells were treated with the protein synthesis inhibitor puromycin, since JI130 is resistant to chloramphenicol, and extracts were prepared and assayed for SOD and aconitase activity, and [O_2^-] was calculated from the fraction of the total aconitase that was active. The results in Table II show that, as expected, total aconitase was 3–4-fold greater in succinate-grown than in glucose-grown cells and, as previously reported (23), that growth on glucose diminished total

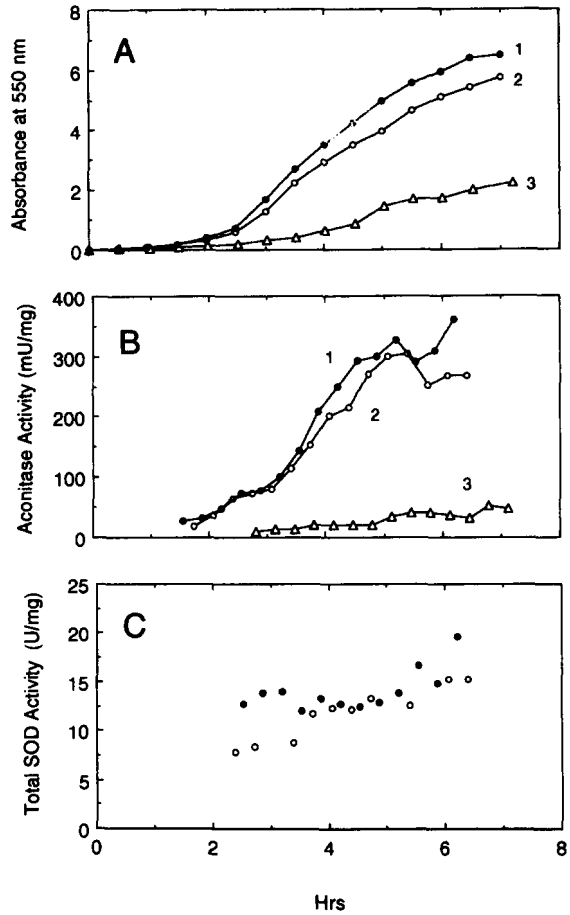

FIG. 4. **Aconitase and [SOD] in SOD-proficient and SOD-deficient strains.** Growth of AB1157 (*line 1*), JI130 (*line 2*), and JI132 (*line 3*) in aerobic LB medium was initiated with 2% inocula from aerobic log phase ($A_{550\,nm} = 0.65$) cultures. Growth was monitored turbidimetrically (*panel A*), and extracts were prepared at intervals and assayed for aconitase activity (*panel B*) or for SOD activity (*panel C*). In *panel C*, ● denotes AB1157 and ○ denotes JI130. JI132 did not contain detectable SOD and is not indicated in *panel C*.

SOD activity by ~1.4-fold. However, the fraction of aconitase that was active and the [O_2^-] calculated therefrom, were not significantly affected by the carbon source. JI130, containing less SOD than AB1157, did exhibit a correspondingly higher [O_2^-] on both carbon sources, as expected.

Since lowering [SOD] did correlate with a decrease in the fraction of active aconitase, we anticipated that elevation of [SOD] would have the opposite effect. We examined this expectation even though the asymptotic approach to 100% active aconitase at high [SOD] would limit the magnitude of changes that might be observed. *E. coli* AB1157pDT1-16, in which sodA is under control of the IPTG-inducible *tac* promoter (15), was used, whereas a strain carrying pBR322 served as the control. As shown in Table III, the strain carrying pDT1-16 contained 8–9 times more SOD than did the control, which correlated with an increase in active aconitase from ~89% to ~97% of total aconitase.

Effects of Dipyridyl and Paraquat—Since EDTA could prevent reactivation of O_2^--inactivated aconitase *in vitro*, it appeared likely that a membrane-permeable hydrophobic ferrous chelator might do so *in vivo*. Eliminating, or slowing, the back reaction in this way would unmask the rate of inactivation of aconitase by O_2^-. As shown by the data in Fig. 5, α,α'-dipyridyl at an optimal concentration of 250 μM increased the

TABLE I

Active aconitase and SOD levels in SOD mutants grown on LB medium

Aerobic cultures of *E. coli* SOD mutants JI132 (sodAsodB), JI130 (sodA), and the isogenic parental strain AB1157 were initiated with 1% inocula from static overnight cultures and were grown with vigorous aeration at 37 °C in LB medium with or without added $MnSO_4$. Puromycin (250 μg/ml) was added during log phase growth, and cultures were incubated aerobically for 15 min. Cells were harvested, extracts were rapidly prepared, and aconitase and superoxide dismutase activity were assayed as described under "Materials and Methods." Total aconitase activity was determined by measuring the aconitase activity under extract prepared from cultures after *in vivo* reactivation, as described under "Materials and Methods." Superoxide radical concentrations were calculated using the estimated rate constants for inactivation ($k_{inact} \approx 2 \times 10^7$ M^{-1} s^{-1}) and reactivation ($k_{act} \approx 0.004$ s^{-1}) of aconitase. ND, not detectable.

Strain	$MnSO_4$	Harvest A_{550nm}	Total SOD	Aerobic aconitase	Total aconitase	Active aconitase	Average theoretical $[O_2^-]$
	μM		*units/mg*	*milliunits/mg*	*milliunits/mg*	%	*pM*
AB1157		2.58	15.8	147.5	157.2	93.8	18
AB1157	10	2.45	16.7	127.3	142.2	89.5	
JI130		2.10	10.0	137.0	158.0	86.7	29
JI130	10	2.10	7.9	137.2	155.3	88.3	
JI132		1.13	ND	21.6	55.0	39.3	318
JI132	10	1.25	ND	21.9	57.6	38.0	

TABLE II

Active aconitase and SOD levels in SOD mutants grown on minimal salts medium

Aerobic cultures of *E. coli* SOD mutant JI130 (sodA) and the isogenic parental strain AB1157 were initiated with 1% inocula from static overnight LB medium cultures in minimal salts medium plus 0.4% (w/v) succinate or glucose with all 20 L-amino acids (40 μg/ml), thiamine HCl (10 μg/ml), and manganous sulfate (10 μM). Cultures were grown with vigorous aeration at 37 °C, and puromycin (250 μg/ml) was added during log phase growth. After 15 min of incubation with puromycin, cells were harvested, extracts were prepared, and aconitase and superoxide dismutase activities were assayed as described under "Materials and Methods." Total aconitase activity was determined by measuring the aconitase activity in extracts after *in vivo* reactivation. Superoxide radical concentrations were calculated using the estimated rate constants for inactivation ($k_{inact} \approx 2 \times 10^7$ M^{-1} s^{-1}) and reactivation ($k_{act} \approx 0.004$ s^{-1}).

Strain	Carbon source	Harvest A_{550nm}	Total SOD	Aerobic aconitase	Total aconitase	Active aconitase	Average theoretical $[O_2^-]$
			units/mg	*milliunits/mg*	*milliunits/mg*	%	*pM*
AB1157	Succinate	0.72	30.3	163.2	188.6	86.5	30
AB1157	Succinate	0.68	25.2	165.9	197.5	84.0	
JI130	Succinate	0.75	11.6	112.5	162.0	69.4	85
JI130	Succinate	0.74	10.2	114.4	161.2	71.0	
AB1157	Glucose	0.90	17.9	51.3	62.9	81.6	42
AB1157	Glucose	0.75	22.8	46.9	56.0	83.8	
JI130	Glucose	0.78	9.4	35.1	44.6	78.7	59
JI130	Glucose	0.54	8.4	36.5	48.2	75.7	

TABLE III

Active aconitase and SOD levels in a MnSOD overproducer

Aerobic cultures of the MnSOD overproducer strain, AB1157pDT1-16, and the control strain, AB1157pBR322, were initiated with 1% inocula from static overnight LB medium cultures into minimal salts medium plus 0.4% (w/v) succinate with all 20 L-amino acids (40 μg/ml), thiamine HCl (10 μg/ml), ampicillin (50 μg/ml), IPTG (100 μg/ml), and manganous sulfate (100 μM). Cultures were grown with vigorous aeration at 37 °C, and puromycin (250 μg/ml) was added during log phase growth. After 15 min of incubation with puromycin, cells were harvested, extracts were prepared, and aconitase and superoxide dismutase activities were assayed as described under "Materials and Methods." Total aconitase activity was determined by measuring the aconitase activity in extracts after *in vivo* reactivation. Superoxide radical concentrations were calculated using the estimated rate constants for inactivation ($k_{inact} \approx 2 \times 10^7$ M^{-1} s^{-1}) and reactivation ($k_{act} \approx 0.004$ s^{-1}).

Strain	Harvest A_{550nm}	Total SOD	Aerobic aconitase	Total aconitase	Active aconitase	Average theoretical $[O_2^-]$
		units/mg	*milliunits/mg*	*milliunits/mg*	%	*pM*
AB1157pBR322	0.72	23.1	173.5	186.6	93.0	25
AB1157pBR322	0.72	22.4	166.4	190.2	87.5	
AB1157pBR322	0.75	25.3	178.6	208.5	85.7	
AB1157pDT1-16	0.72	202.6	179.5	182.0	98.6	6
AB1157pDT1-16	0.72	202.6	164.5	170.9	96.3	
AB1157pDT1-16	0.75	206.8	178.6	185.1	96.5	

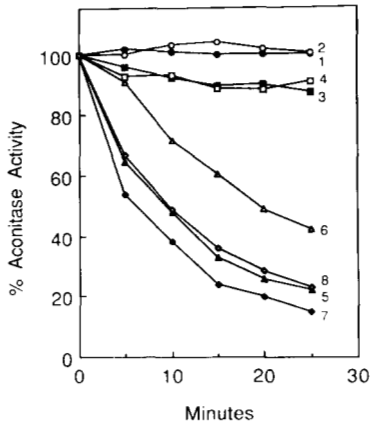

FIG. 5. **Effects of dipyridyl on aconitase.** Growth of cultures in aerobic minimal medium, containing all 20 amino acids at 40 μg/ml each, 100 μg/ml IPTG, 100 μM $MnSO_4$, 50 μg/ml ampicillin, and 0.4% (w/v) sodium succinate, was initiated with 1% inocula taken from overnight static cultures grown in LB medium with 50 μg/ml ampicillin. At midlog phase growth ($A_{550\,nm}$ = 0.65), chloramphenicol was added to 200 μg/ml from a 100 mg/ml stock in ethanol and incubation was continued for 15 min, at which point α,α′-dipyridyl dissolved in ethanol, ethanol only, or water was added to cultures of the control strain, AB1157pBR322 (*lines 1, 3, 5,* and *7*), or the MnSOD-overproducer, AB1157pDT1-16 (*lines 2, 4, 6,* and *8*). Samples were taken at intervals for extract preparation and assays. *Lines 1* and *2*, water to 0.6% (v/v); *lines 3* and *4*, ethanol to 0.6% (v/v); *lines 5* and *6*, α,α′-dipyridyl to 250 μM with 0.6% (v/v) ethanol as solvent; *lines 7* and *8*, α,α′-dipyridyl to 300 μM plus 0.6% (v/v) ethanol. At time 0, aconitase was 167 ± 11 milliunits/mg in extracts from AB1157pBR322 and 177 ± 15 milliunits/mg in AB1157pDT1-16.

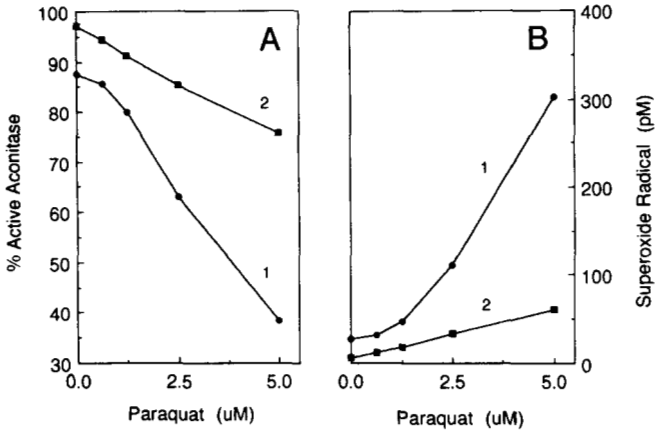

FIG. 6. **Effects of paraquat on aconitase and $[O_2^-]$.** Growth in aerobic minimal salts medium containing all 20 amino acids at 40 μg/ml, 0.4% (w/v) succinate, 100 μM $MnSO_4$, 50 μg/ml ampicillin, and 100 μg/ml IPTG was initiated with 1% inocula from static overnight cultures in LB. During log phase ($A_{550\,nm}$ = 0.65), puromycin was added to 250 μg/ml, and incubation was continued for 10 min, at which point paraquat was added to the indicated concentrations and 15 min later extracts were prepared for determination of the fraction of active aconitase (*panel A*), from which $[O_2^-]$ was calculated (*panel B*). Aeration was continuous until the samples were taken for lysis. *Line 1*, AB1157pBR322; *line 2*, AB1157pDT1-16. 100% aconitase activity measured for 0 μM paraquat was 209 and 185 milliunits/mg for AB1157pBR322 and AB1157pDT1-16, respectively.

apparent rate of inactivation in the control strain (*line 5*), whereas it exerted a diminished effect in the strain that overproduced MnSOD (*line 6*). At 300 μM α,α′-dipyridyl (*lines 7* and *8*), the difference in aconitase stability became less obvious, presumably because of O_2^--independent inactivation by the chelator. It is thus clear that steady-state aconitase

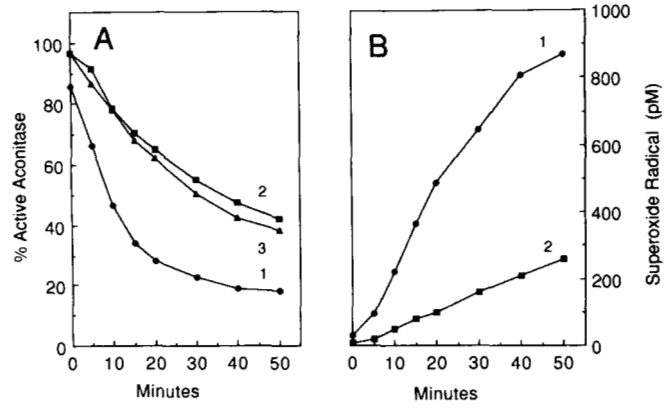

FIG. 7. **Kinetics of the paraquat effect.** Conditions were as described in the legend to Fig. 6, except that, following addition of paraquat at 5 μM, samples were taken at intervals for lysis and determination of the fraction of aconitase that was active. *Panel A*, *line 1*, AB1157pBR322; *line 2*, AB1157pDT1-16; *line 3*, AB1157pDT1-16 plus 44 units/ml of bovine liver catalase. *Panel B* presents $[O_2^-]$ calculated from the percent active aconitase.

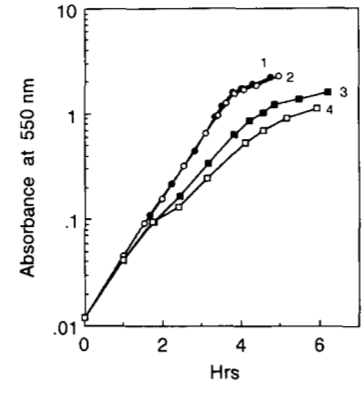

FIG. 8. **Effect of catalase on growth.** Aerobic growth was initiated with 1% inocula taken from aerobic log phase cultures ($A_{550\,nm}$ = 0.45) growing on 0.1% glucose. Growth in salts plus 0.4% (w/v) carbon source was followed as $A_{550\,nm}$. *Line 1*, strain CSH7 (control) growing on glucose; *line 2*, UM1 (katEkatG) on glucose; *line 3*, CSH7 on succinate; *line 4*, UM1 on succinate.

activity is the reflection of a dynamic balance between inactivation by O_2^- and reactivation by restoration of Fe(II). It is also clear that lowering $[O_2^-]$ by overproduction of [SOD] decreased that rate of inactivation.

Another way to expose the opposing processes underlying the steady state of aconitase activity within *E. coli* is to increase the production of O_2^- with paraquat. Since *E. coli* respond adaptively to increased O_2^- by induction of MnSOD, these experiments were done in the presence of puromycin to block protein synthesis. Fig. 6*A*, *line 1*, shows a dose-dependent depression of aconitase activity by paraquat. This inactivation of aconitase by paraquat was indeed due to O_2^-, since it was diminished in the strain overproducing SOD by 8–9-fold (Fig. 6*A*, *line 2*). Intracellular $[O_2^-]$, calculated from the data in Fig. 6*A*, are shown in Fig. 6*B*. It is apparent that paraquat elevated intracellular $[O_2^-]$ in a dose-dependent fashion and did so to a greater degree when [SOD] was normal (*line 1*) than when it was elevated (*line 2*).

H_2O_2, produced by O_2^- dismutation, did not contribute measurably to the inactivation of aconitase in these catalase-proficient strains. This was shown by adding catalase to the culture to 44 units/ml, a level approximately 50-fold greater than that endogenous to the cells. Exogenous catalase did not significantly decrease inactivation of aconitase in the presence

TABLE IV

Active aconitase levels in catalase-deficient E. coli

Aerobic cultures of *E. coli* catalase mutant UM1 (katEkatG) and the parental strain CSH7 were initiated with 1% inocula from aerobic log phase cells (A_{550nm} = 0.4–0.5) in a minimal salts medium plus 0.4% (w/v) glucose or sodium succinate with all 20 L-amino acids (40 μg/ml), thiamine HCl (10 μg/ml), and manganous sulfate (10 μM). Cultures were grown to midlog phase with vigorous aeration at 37 °C and treated with puromycin (250 μg/ml) for 15 min. Cultures were harvested and extracts were prepared immediately to determine aerobic aconitase or after reactivation in the presence of beef liver catalase (44 units/ml) for measurement of total aconitase. Enzyme assays were performed as described under "Materials and Methods."

Strain	Carbon source	Harvest A_{550nm}	Catalase activity	Total SOD	Aerobic aconitase	Total aconitase	Active aconitase
			units/mg	*units/mg*	*milliunits/mg*	*milliunits/mg*	%
CSH7	Glucose	0.67	6.1	22.4	37.7	45.8	82.3
UM1	Glucose	0.65	<0.4[a]	21.4	26.4	42.7	61.8
CSH7	Succinate	0.55	12.4	27.7	158.0	173.5	91.1
UM1	Succinate	0.69	<1.2[a]	26.7	139.2	172.3	80.8

[a] Although strain UM1 contains no catalase activity, a slow rate of H_2O_2 removal by extracts, as measured by the decrease in absorbance at 240 nm, was detected.

of 5 μM paraquat (Fig. 7*A*, *lines 2* and *3*). It should be noted that H_2O_2 does readily cross the cell envelope. Comparison of *line 1* with *lines 2* and *3* in Fig. 7*A* again demonstrates that overproduction of SOD protected aconitase against inactivation in these paraquat-containing cultures. Fig. 7*B* presents the concentrations of O_2^-, which were calculated from the fraction of active aconitase. It is interesting to note that the very high levels of $[O_2^-]$ imposed by paraquat in the SOD normal strain (*line 1*) appeared to approach a limit, as a function of time of exposure to paraquat, whereas the increase of $[O_2^-]$ was linear over the 50 min of observation in the SOD overproducer. These increases in $[O_2^-]$ with time probably reflect uptake and accumulation of paraquat. In the SOD normal cells, damage imposed by O_2^- could limit the supply of NAD(P)H and of ATP or of membrane potential. The former would diminish the rate of reduction of paraquat and therefore lower its ability to generate O_2^-, whereas the latter would slow the active uptake of paraquat. These effects of O_2^- might account for the tendency of $[O_2^-]$ to approach a limit in the SOD normal but not in the SOD overproducer.

Catalase Deficiency—Aconitase, *in vitro*, was much more susceptible to inactivation by O_2^- than by H_2O_2 (12). It remained to be seen whether H_2O_2 could contribute to the inactivation of aconitase in *E. coli*, especially in light of the recent report of the H_2O_2 sensitivity of plant mitochondrial aconitase (24). As shown in Fig. 8, a catalase-deficient strain (UM1) grew somewhat slower on succinate than did a control strain (compare *line 4* with *line 3*), whereas there was no discernible difference on glucose (*lines 1* and *2*). These growth data suggested some *in vivo* protection of aconitase by catalase and therefore some contribution of H_2O_2 to the inactivation of aconitase in catalase-deficient cells. Enzyme assays performed on extracts of these cells verified this deduction, as shown in Table IV. It is interesting to note that catalase, like SOD, was up-regulated when *E. coli* were grown with carbon sources, such as succinate, that require tricarboxylic acid cycle enzymes such as the O_2^-/H_2O_2-sensitive aconitase (Table IV and Ref. 25).

DISCUSSION

O_2^- is generated within log phase aerobic *E. coli* at a rate of 5 μM/s (26), and similar rates have been estimated for actively respiring mitochondria (27–29). SOD, by reacting with O_2^- with a rate constant >2 × 10^9 M^{-1} s^{-1}, partially protects susceptible targets by competing with them for the high flux of O_2^-. Aconitase is a particularly sensitive target, and we have now seen that 10–17% of the aconitase in aerobic log phase SOD-proficient *E. coli* is inactive, at any instant, due to the effect of O_2^-. Increasing the generation of O_2^- with paraquat increased the fraction of total aconitase that was inactive. Conversely, decreasing $[O_2^-]$ by overproduction of SOD had the opposite effect.

Inactivation of aconitase by O_2^- was opposed by its reactivation by incorporation of Fe(II). Thus, this reactivation, which was seen when O_2^- production was halted by exclusion of dioxygen, was prevented *in vitro* by EDTA and was inhibited *in vivo* by α,α′-dipyridyl. The steady-state ratio of active to inactive aconitase is the result of a dynamic balance between inactivation by O_2^- and reactivation by insertion of Fe(II), and it could provide a way to measure $[O_2^-]$ within *E. coli*.

Application of this measure depends upon knowledge of the rate of reactivation under ambient conditions and of the rate constant for the inactivation of aconitase by O_2^-. The rate of reactivation within the cells was measured and was found to be ~0.004 s^{-1}. The rate constant for the O_2^--aconitase reaction was taken to be 2 × 10^7 M^{-1} s^{-1} from *in vitro* studies of the inactivation of fluorocitrate-saturated aconitase (12). It is reasonable to assume that citrate and fluorocitrate would protect the aconitase active site against O_2^- to a similar degree and that the aconitase within *E. coli* would be saturated with citrate. The latter statement derives from measurements that show that [citrate] within *E. coli* greatly exceeds the K_m for citrate (12, 20). It must be noted that the *in vitro* measurements of the rate of inactivation of aconitase by O_2^- were performed at 25 °C, whereas the cells were grown at 37 °C. The rate constant determined *in vitro* may thus be too low by a factor of ~2 for application to the cells. An increase in this rate constant will proportionally decrease the estimates of $[O_2^-]$. In addition, we have assumed that the SOD added to SOD-free extracts would be the major route of O_2^- elimination. However, were this not the case, SOD-independent pathways could also affect the *in vitro* estimate of the rate of inactivation of aconitase by O_2^-. Future studies will be directed toward refinement of these numbers.

Although as yet imperfect, the aconitase method allows, for the first time, measurement of $[O_2^-]$ *in vivo*. When applied to log phase, aerobic, SOD-proficient cells, it yielded $[O_2^-] \approx 20$–40 pM, whereas for SOD-deficient cells, the corresponding value was ~300 pM. The $[O_2^-]$ that was previously projected (26) for SOD-proficient *E. coli* was ~200 pM. This figure was based upon the rate of production of O_2^- by the cells and its rate of removal by endogenous SOD, and its accuracy depended upon several assumptions. For instance, it did not account for removal of O_2^- by sensitive and restorable targets,

such as aconitase, and it may thereby have erred on the high side. This effect of restorable targets for O_2^- of lowering the steady-state $[O_2^-]$ would become greater the lower the [SOD] is, and this effect may explain why $[O_2^-]$ was ~3 orders of magnitude less than that previously predicted (26) for the SOD-deficient cells.

Any decrease in intracellular [Fe(II)] would slow the reactivation of O_2^--inactivated aconitase, much as did addition of α,α'-dipyridyl. This would lead to a decrease in the level of active aconitase, which could only be offset by decreasing the rate of inactivation by O_2^-, and this could be achieved by up-regulation of SOD. What has just been considered for aconitase applies to all [4Fe-4S]-containing targets for O_2^-, such as the dihydroxy acid dehydratase (9, 10) and the 6-phosphogluconate dehydratase (11). This may explain why it is adaptive for *E. coli* to induce MnSOD in response to iron starvation (15, 30–33) and thus provide a rationale for the involvement of the Fur (ferric uptake regulation) protein in the transcriptional control of sodA (32, 33).

The sensitivity of aconitase to O_2^- may also explain the down-regulation of SOD in *E. coli* growing on glucose (23). Thus, aconitase is most essential to *E. coli* utilizing nonfermentable carbon sources and least essential on a fermentable carbon source. The cell can therefore tolerate a greater $[O_2^-]$ and make do with lower [SOD] when meeting its energy needs by fermentation (12). This reasoning may also be applied to explain the transcriptional regulation of sodA by the Arc (aerobic respiration control) system (33). Thus, the Arc system regulates the enzymes of the citric acid cycle (34), including aconitase. When aconitase is up-regulated, more SOD will be needed to compete with it for the O_2^-.

We anticipate that further exploration of the inactivation of aconitase and of other sensitive targets by O_2^- will provide additional clarifications of previously puzzling phenomena.

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