

## Nitric Oxide Sensitivity of the Aconitases\*

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**Aconitases are important cellular targets of nitric oxide (NO<sup>•</sup>) toxicity, and NO<sup>•</sup>-derived species, rather than NO<sup>•</sup> per se, have been proposed to mediate their inactivation. NO<sup>•</sup>-mediated inactivation of the *Escherichia coli* aconitase and the porcine mitochondrial aconitase was investigated. In *E. coli*, aconitase activity decreased by ~70% during a 2-h exposure to an atmosphere containing 120 ppm NO<sup>•</sup> in N<sub>2</sub>. The NO<sup>•</sup>-inactivated aconitase reactivated poorly in *E. coli* under anaerobic or aerobic conditions. Elevated superoxide dismutase activity did not affect the aerobic inactivation of aconitase by NO<sup>•</sup>, thus indicating a limited role of the NO<sup>•</sup>- and superoxide-derived species peroxynitrite. Glutathione-deficient and glutathione-containing *E. coli* were comparably sensitive to NO<sup>•</sup>-mediated aconitase inactivation, thus excluding the participation of S-nitrosoglutathione or more oxidizing NO<sup>•</sup>-derived species. NO<sup>•</sup> progressively decreased aconitase activity in extracts in the presence of substrates, and inactivation was greatest at an acidic pH with *cis*-aconitate. The porcine mitochondrial aconitase was sensitive to NO<sup>•</sup> when exposed at pH 6.5, but not at pH 7.5, and irreversible inactivation occurred during catalysis. The requirement of an acidic pH or substrates for sensitivity may explain the reported resistance of aconitases to NO<sup>•</sup> *in vitro* (Castro, L., Rodriguez, M., and Radi, R. (1994) *J. Biol. Chem.* 269, 29409–29415; Hausladen, A., and Fridovich, I. (1994) *J. Biol. Chem.* 269, 29405–29408). An S-nitrosation of the aconitase [4Fe-4S] center catalyzed by the solvent-exposed electron withdrawing iron atom (Fe<sub>a</sub>) is proposed.**

Nitric oxide (NO<sup>•</sup>) is released as an intermediate of dissimilatory and assimilatory pathways of nitrite reduction in bacteria and fungi (1, 2). It is also produced by macrophages, neutrophils, endothelial cells, and epithelial cells to combat invading microorganisms and neoplastic tissue (3–5). In addition, NO<sup>•</sup> serves important functions as a second messenger for neurons and the vascular system (6, 7).

NO<sup>•</sup> is cytotoxic. Mechanisms of NO<sup>•</sup> toxicity are complex and may include its direct reaction with heme, non-heme iron, and copper proteins (8). Other NO<sup>•</sup>-derived species including nitroxyl anion, nitrosonium, peroxynitrite (ONOO<sup>-</sup>), nitrogen dioxide, and nitrosothiols may increase the spectrum of NO<sup>•</sup>-mediated damage to cells (9). The avid reactivity of NO<sup>•</sup> with superoxide radical (O<sub>2</sub><sup>-</sup>) to form the less discriminant oxidant ONOO<sup>-</sup> provides a potent mechanism for the deleterious actions of NO<sup>•</sup> (10, 11). Nevertheless, a thorough understanding

of NO<sup>•</sup> toxicity continues to demand a greater knowledge of the NO<sup>•</sup>-sensitive targets and the NO<sup>•</sup>-derived species involved.

The mammalian [4Fe-4S] cytoplasmic and mitochondrial aconitases are particularly sensitive to inactivation by NO<sup>•</sup> produced during various pathological conditions (12–18). In mammals, the cytoplasmic aconitase serves as an mRNA-binding regulator of iron homeostasis and may also participate with the mitochondrial aconitase as a catalyst of the energy-yielding reactions of the citric acid cycle (19, 20). In addition, there is a growing family of homologous labile [4Fe-4S] (de)hydratases serving important biosynthetic and energy-yielding functions (21, 22), which may account for the toxicity of NO<sup>•</sup> toward a variety of organisms under a variety of conditions. Yet, it remains unclear how NO<sup>•</sup> inactivates aconitases *in vivo*. The NO<sup>•</sup> resistance of various isolated aconitases has suggested the involvement of more reactive NO<sup>•</sup>-derived species, such as ONOO<sup>-</sup> or the nitrosothiol GSNO<sup>1</sup> (23, 24).

Here we describe the NO<sup>•</sup>-mediated inactivation of aconitase in *Escherichia coli* that occurs independently of ONOO<sup>-</sup> or GSNO formation and produces a poorly reactivatable aconitase. We also describe the effects of pH and substrates on the reaction of NO<sup>•</sup> with the *E. coli* and mitochondrial aconitases *in vitro*. The results may account for the apparent NO<sup>•</sup> resistance of aconitases (23, 24). Nitrosation of the aconitase [4Fe-4S] center catalyzed by the solvent-exposed iron atom (Fe<sub>a</sub>) is proposed.

### MATERIALS AND METHODS

**Cells and Reagents**—*E. coli* strain DH5α was from Life Technologies. The *E. coli* mutant JTG10 (gshA<sup>-</sup>), lacking glutathione, and its parental strain AB1157 were kindly provided by B. Dimple (25). DH5α was transformed with the multicopy plasmid pD11c (26) containing the *E. coli* *sodA* gene, and cultures of DH5αpD11c were maintained with 100 μg/ml ampicillin. The porcine mitochondrial aconitase expression vector pA was generously provided by H. Zalkin (27) and was introduced into *E. coli* strain BL21(DE3)LysS by standard methods. 1200 ppm (± 5%) NO<sup>•</sup> in ultrapure nitrogen, 99.998% N<sub>2</sub>, and 99.993% O<sub>2</sub> compressed gas cylinders were obtained from Praxair (Bethlehem, PA). Nitric oxide (98.5%) was from Aldrich. Porcine heart isocitrate dehydrogenase, barium (±)-fluorocitrate, MnCl<sub>2</sub>, sodium *cis*-aconitate, sodium citrate, sodium *trans*-aconitate, sodium tricarballate, NADP<sup>+</sup>, horse heart cytochrome c<sup>3+</sup>, xanthine, xanthine oxidase, phenazine methosulfate, porcine heart mitochondrial aconitase, chloramphenicol, bovine erythrocyte Cu,Zn-SOD (3500 units/mg), succinic acid, bovine serum albumin (fraction V), dithiothreitol, ferrous ammonium sulfate, Sephadex G25, reactive blue 2-Sepharose, Mes, Tris, Hepes, and KCl were from Sigma. Bacto-tryptone and yeast extract were obtained from Difco.

**Media, Growth of Bacteria, and Extract Preparation**—The minimal salts medium was made up with tap water and contained 60 mM K<sub>2</sub>HPO<sub>4</sub>, 33 mM KH<sub>2</sub>PO<sub>4</sub>, 7.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.7 mM sodium citrate, 1 mM MgSO<sub>4</sub>, 10 μM MnCl<sub>2</sub>, 10 μg/ml thiamine-Cl, 40 μg/ml L-arginine-Cl, and 10 mM sodium succinate. The 20 common L-amino acids were supplied at 40 μg/ml as indicated with cystine instead of cysteine. The

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<sup>1</sup> The abbreviations used are: GSNO, S-nitrosoglutathione; LB, Luria broth; SOD, superoxide dismutase; Mn-SOD, manganese-containing SOD; Cu,Zn-SOD, copper- and zinc-containing SOD; Mes, 4-morpholineethanesulfonic acid.

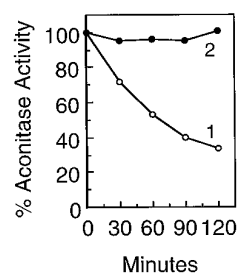
phosphate-buffered LB medium (28) contained 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of 66 mM K<sub>2</sub>HPO<sub>4</sub>, 33 mM KH<sub>2</sub>PO<sub>4</sub> plus 10 μM MnCl<sub>2</sub>. The pH of the LB and minimal salts media was adjusted to 7.0 with HCl or NaOH. Cultures were routinely grown aerobically in a rotary water bath at 37 °C with a medium:flask volume ratio of at most 1:5, and growth was monitored turbidometrically at 550 nm. To avoid disturbing the equilibration of gases, 1-ml samples were removed with a tuberculin syringe inserted through a rubber stopper-sealed 50-ml Erlenmeyer flask and connected via small tubing to the culture. Culture aliquots were transferred to 1.5-ml Eppendorf centrifuge tubes and were quickly centrifuged at 20,000 × g for 25 s, the supernatant was aspirated, and the cell pellet was frozen on dry ice. Bacteria were resuspended and lysed by sonicating the cell pellet in 0.1 ml of a buffer containing 50 mM Tris-Cl, pH 7.4, 0.6 mM MnCl<sub>2</sub>, and 20 μM (±)-fluorocitrate, and the lysate was frozen on dry-ice. The barium salt of fluorocitrate was solubilized by titrating a suspension with HCl. Fluorocitrate was included to stabilize aconitase in extracts (29). Carry-over of fluorocitrate (<1 μM) did not measurably affect subsequent measurements of aconitase activity in extracts. Cell pellets and lysates were stored at -70 °C for up to 2 weeks without loss of aconitase activity. Cell lysates were quickly thawed by incubating in a 25 °C water bath and clarified by centrifugation for 25 s at 20,000 × g immediately prior to the assay of aconitase activity. For the preparation of batch extracts, a 1-liter culture of DH5α, initiated with a 30% inoculum from an overnight culture grown in minimal salts medium, was grown to an absorbance at 550 nm of ~0.5 in minimal salts medium. Bacteria were harvested by centrifugation at 4000 × g for 30 min at 4 °C. Cell pellets were resuspended and lysed in approximately 5 volumes of ice-cold 100 mM Tris-Cl, pH 7.5, by sonicating with a microtip probe. Cell lysates were clarified by centrifugation at 20,000 × g, and aliquots were stored at -70 °C.

**Enzyme and Protein Assays**—Aconitase activity was routinely assayed in a reaction mixture containing 50 mM Tris-Cl, pH 7.4, 30 mM sodium citrate, 0.6 mM MnCl<sub>2</sub>, 0.2 mM NADP<sup>+</sup> and 1 unit/ml isocitrate dehydrogenase by following the absorbance increase at 340 nm at 25 °C for 5 min (30, 31). One milliunit of aconitase activity corresponds to 1 nmol of NADPH formed per min. Protein concentration was measured by the method of Bradford (32) using Coomassie Brilliant Blue protein staining reagent (Bio-Rad) and bovine serum albumin as the standard. SOD activity was measured by the competitive inhibition of cytochrome c<sup>3+</sup> reduction (33) in a 0.2-ml assay volume incubated at 25 °C, and SOD units were determined from a standard curve prepared with bovine Cu,Zn-SOD (3500 units/mg). A Molecular Devices SpectraMax 250 thermostatted microplate UV-visible spectrophotometer was routinely applied to the microassay of aconitase, SOD, and protein. A Beckman DU 640 UV-visible spectrophotometer equipped with a thermostatted oscillating multicuvette holder was used for some measurements.

**Gas Exposures**—A three-way gas proportioner (Cole-Parmer Instrument Co.) was used to achieve mixing and a constant flow rate of 30 ml/min for various mixtures of O<sub>2</sub>, N<sub>2</sub>, and NO<sup>•</sup>, and gas mixtures were passed through a trap containing sodium hydroxide pellets to remove NO<sub>2</sub> and higher oxides of nitrogen formed prior to entering reaction vessels. The proportioner was calibrated for flow rates by measuring the displacement of a saturated solution of NaCl at constant pressure and room temperature. Saturated NO<sup>•</sup> solutions (2 mM) were prepared by equilibrating water with 98.5% NO<sup>•</sup> that was first passed through 1 N NaOH. Aqueous NO<sup>•</sup> was diluted into N<sub>2</sub>-equilibrated buffer containing aconitase in a double septum-sealed tuberculin syringe, which allowed for variation in volume while maintaining zero gas head space. It was important to maintain zero gas head space to keep added NO<sup>•</sup> in the aqueous phase.

**Mitochondrial Aconitase Preparation**—Porcine mitochondrial aconitase was produced and isolated from *E. coli* strain BL21(DE3)LysSpA with minor modifications to published procedures (27). Affinity-purified recombinant aconitase had specific activities of 6–8 units/mg protein following reactivation and constituted >85% of the protein as judged from Coomassie-stained denaturing gels. Aconitase was reactivated in a reaction containing 0.5 mM ferrous ammonium sulfate and 5 mM dithiothreitol buffered with 100 mM Tris-Cl, pH 7.5, incubated under N<sub>2</sub> at 0 °C for 30 min (34). Reactivated aconitase was rapidly separated on Sephadex G-25 pre-equilibrated with N<sub>2</sub>-saturated 100 mM Tris-Cl, pH 7.5. For the investigations of the commercial aconitase buffered at pH 6.5, active aconitase was prepared in 100 mM K-Mes, pH 6.5. Aliquots of aconitase were stored under N<sub>2</sub> at -70 °C in vacutainer tubes (Becton-Dickinson, 47 × 10.25 mm).

**Data Analysis**—Results are representative of two or more independent experiments.



**Fig. 1. Inactivation of aconitase in *E. coli* exposed to NO<sup>•</sup> gas.** Cultures of DH5α were grown in a minimal succinate medium to an A<sub>550</sub> of ~0.50 and were treated with chloramphenicol (200 μg/ml) for 10 min under N<sub>2</sub>. Cultures were then exposed to an atmosphere containing 120 ppm NO<sup>•</sup> in N<sub>2</sub> (line 1) or N<sub>2</sub> (line 2) and were harvested for aconitase activity measurements at intervals as described under "Materials and Methods." Cultures were initiated with a 30% inoculum from an overnight culture grown in minimal succinate medium. 100% aconitase activity corresponds to 246 ± 4 milliunits/mg.

## RESULTS

**Aconitase Is Sensitive to NO<sup>•</sup> in *E. coli* in the Absence of O<sub>2</sub>**—The sensitivity of the aconitase activity in *E. coli* to NO<sup>•</sup> was measured by exposing vigorously shaking cell cultures to a constantly replenished atmosphere containing NO<sup>•</sup> balanced with N<sub>2</sub>. Aconitase activity loss was progressive during exposure of cell cultures to 120 ppm NO<sup>•</sup> gas (Fig. 1, line 1), while aconitase activity was stable during incubation under N<sub>2</sub> (line 2). Exposures were performed in the presence of the protein synthesis inhibitor chloramphenicol to preclude possible adaptation to NO<sup>•</sup>. The results clearly demonstrate a sensitivity of the *E. coli* aconitase to relatively low exposures of NO<sup>•</sup> and indicate that O<sub>2</sub>-dependent NO<sup>•</sup>-derived species, such as ONOO<sup>-</sup>, are not necessary for aconitase inactivation.

**NO<sup>•</sup> Inactivated Aconitase Is Slowly Reactivated in *E. coli* and O<sub>2</sub> Stimulates Reactivation**—The aconitase [4Fe-4S]<sup>2+</sup> center is in a dynamic state of cyclical inactivation-reativation in *E. coli* and mammals (14, 21, 30, 35). In this cycle, the solvent-exposed catalytic Fe<sub>a</sub> is oxidized and released by ambient O<sub>2</sub> resulting in aconitase activity loss. Activity is constantly regained by reduction of the [3Fe-4S]<sup>1+</sup> center and Fe<sup>2+</sup> insertion. In *E. coli*, reactivation of the O<sub>2</sub>-inactivated aconitase occurs with a t<sub>1/2</sub> of 3 min (30). We measured the reversibility of NO<sup>•</sup>-inactivated aconitase in *E. coli*. Exposure of cultures to an atmosphere containing 480 ppm NO<sup>•</sup> in N<sub>2</sub> for 60 min caused ~90% loss of aconitase activity (Fig. 2, line 1). Removal of NO<sup>•</sup> and replacement with nitrogen after the 60-min exposure allowed a very slow recovery of activity (line 1). Approximately 20% of the activity recovered after 90 min. Interestingly, the presence of 21% O<sub>2</sub> stimulated the reactivation of the NO<sup>•</sup>-inactivated aconitase (line 2); however, only ~50% of the activity was recoverable. The t<sub>1/2</sub> for the reactivation of the recoverable activity was ~15 min. By comparison, aconitase activity in *E. coli* was completely stable under N<sub>2</sub> (line 3) or during the transition from N<sub>2</sub> to 21% O<sub>2</sub> (line 4). It should be noted that nearly complete recovery of aconitase activity was observed when aconitase was oxidatively inactivated by O<sub>2</sub> (30, 35). The slow and incomplete reactivation of the NO<sup>•</sup>-inactivated aconitase suggests a mechanism of inactivation involving a less reversible modification by NO<sup>•</sup> than that observed with O<sub>2</sub>.

**Peroxyinitrite and GSNO Are Not Required for NO<sup>•</sup>-mediated Aconitase Inactivation**—Aconitases are rapidly inactivated by ONOO<sup>-</sup> and GSNO *in vitro* (23, 24), and roles for ONOO<sup>-</sup> or GSNO have been proposed to explain the NO<sup>•</sup>-mediated loss of aconitase activity *in vivo*. We investigated the effects of O<sub>2</sub> and GSH on the NO<sup>•</sup>-mediated inactivation of aconitase in *E. coli* to discern the possible participation of O<sub>2</sub>-derived ONOO<sup>-</sup> and GSH-derived GSNO in aconitase inactivation.

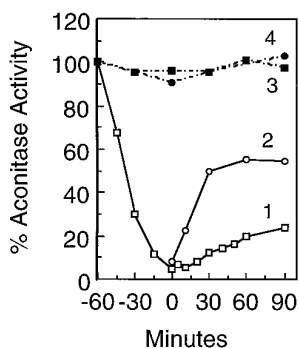


FIG. 2. **Reactivation of the NO<sup>•</sup>-inactivated aconitase in *E. coli*.** Cultures of DH5 $\alpha$  growing in minimal succinate medium ( $A_{550} = \sim 0.5$ ) were treated with chloramphenicol (200  $\mu\text{g}/\text{ml}$ ) for 10 min under N<sub>2</sub>, exposed to an atmosphere containing 480 ppm NO<sup>•</sup> in N<sub>2</sub> (lines 1 and 2) or N<sub>2</sub> (lines 3 and 4) for 60 min, and then flushed with N<sub>2</sub> (lines 1 and 3) or with N<sub>2</sub> containing 21% O<sub>2</sub> (lines 2 and 4). Cells were harvested at intervals, extracts were prepared, and aconitase activity and protein were assayed as described under "Materials and Methods." 100% aconitase activity corresponds to 225  $\pm$  25 milliunits/mg extract protein. Cultures were grown from a 30% inoculum of an overnight culture grown in minimal succinate medium, and cultures were treated at an  $A_{550}$  of  $\sim 0.5$ .

*E. coli* strain DH5 $\alpha$ pD11c overproducing the Mn-SOD  $\sim 15$ -fold (619  $\pm$  62 units/mg SOD) was compared with its parental strain DH5 $\alpha$  (39.8  $\pm$  0.9 units/mg SOD) for its sensitivity to NO<sup>•</sup>-mediated aconitase inactivation (Fig. 3A). Aconitase inactivation during NO<sup>•</sup> exposure was remarkably similar in DH5 $\alpha$  and DH5 $\alpha$ pD11c (compare lines 1 and 2), while aconitase activity was stable during control exposures to 21% O<sub>2</sub> in DH5 $\alpha$ pD11c (line 4) and in DH5 $\alpha$  (line 3). Aconitase activity was more resistant to the O<sub>2</sub><sup>-</sup>-generating agent phenazine methosulfate in DH5 $\alpha$ pD11c (Fig. 3B, line 2) than in DH5 $\alpha$  (line 1) as expected for a 15-fold elevation of SOD activity (30). The results demonstrate a limited role of O<sub>2</sub><sup>-</sup> and ONOO<sup>-</sup> in the mechanism of NO<sup>•</sup>-mediated aconitase inactivation.

The *E. coli* mutant JTG10, lacking glutathione, and its parental strain AB1157 (25) were used to measure the effects of cellular GSH on NO<sup>•</sup>-mediated aconitase inactivation. The absence of glutathione did not affect the NO<sup>•</sup>-mediated loss of aconitase activity in strain JTG10 relative to AB1157 under aerobic conditions (Fig. 4A, compare lines 1 and 2) or anaerobic conditions (Fig. 4B, lines 1 and 2). It should be noted that JTG10 contained a larger fraction of inactive aconitase and grew more slowly than AB1157 under aerobic growth conditions as previously reported (36); nevertheless, the relative loss of activity during NO<sup>•</sup> exposures was comparable in both strains. The failure of GSH to protect aconitase suggests a mechanism of inactivation independent of ONOO<sup>-</sup>, since the high concentrations of GSH (3.5–6.6 mM) in *E. coli* (37) would be expected to scavenge thiol oxidizing species such as ONOO<sup>-</sup> (38). Furthermore, the results do not support a buffering function of GSH against NO<sup>•</sup> (39) or the participation of GSNO in the mechanism of aconitase inactivation.

**NO<sup>•</sup> Inactivates Aconitases by Direct Attack of the Active-site Fe-S Center**—We explored the reaction of NO<sup>•</sup> with the aconitases *in vitro*. Aconitase was inactivated in extracts exposed to 1200 ppm NO<sup>•</sup> in N<sub>2</sub> (Fig. 5, line 2), and the virtual substrates fluorocitrate, *trans*-aconitate, and tricarballylate (29, 40, 41) completely blocked this inactivation (Fig. 5, compare lines 3–5 with lines 1 and 2). In addition, aconitase activity was slightly decreased by NO<sup>•</sup> exposure during catalysis with the substrate citrate ( $K_m = 30 \mu\text{M}$ ) (29) (Fig. 6, line 1). Increasing the ionic strength with 200 mM KCl increased the inhibitory effect of NO<sup>•</sup> (line 2), and lowering the pH of the reaction mixture from 7.7 to 6.8 increased the inactivation observed with citrate (compare

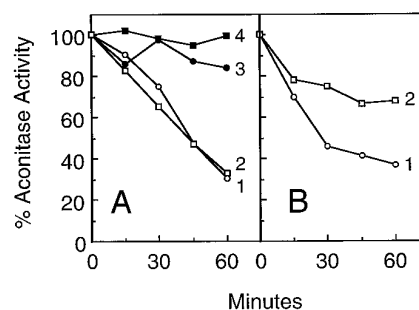


FIG. 3. **Effect of elevated Mn-SOD on NO<sup>•</sup>- and O<sub>2</sub><sup>-</sup>-mediated aconitase inactivation in *E. coli*.** Cultures of DH5 $\alpha$  (line 1) and DH5 $\alpha$ pD11c (line 2) were exposed to an atmosphere containing 960 ppm NO<sup>•</sup> in 21% O<sub>2</sub> (A) or 5  $\mu\text{M}$  phenazine methosulfate (B). Control cultures of DH5 $\alpha$  (line 3) and DH5 $\alpha$ pD11c (line 4) were exposed to 21% O<sub>2</sub> (A). Growth of cultures in phosphate-buffered LB was initiated with a 2% inocula from an overnight culture. Cultures were treated with chloramphenicol (200  $\mu\text{g}/\text{ml}$ ) at an  $A_{550}$  of  $\sim 0.5$  for 10 min prior to the gas exposures. Overnight growth of DH5 $\alpha$ pD11c was in the presence of 100  $\mu\text{g}/\text{ml}$  ampicillin. Cultures were harvested at intervals, extracts were prepared, and aconitase activity and protein were assayed as described under "Materials and Methods." 100% aconitase activity corresponds to 64  $\pm$  17 and 70  $\pm$  11 milliunits/mg protein for DH5 $\alpha$  and DH5 $\alpha$ pD11c, respectively.

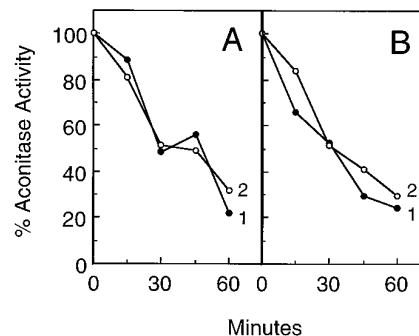
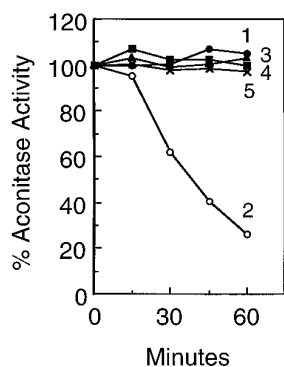


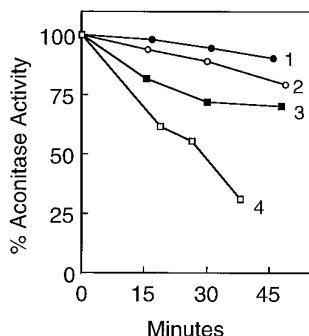
FIG. 4. **Effect of glutathione on NO<sup>•</sup>-mediated aconitase inactivation in *E. coli*.** Cultures of AB1157 (line 1) and JTG10 (line 2) were exposed to an atmosphere containing 480 ppm NO<sup>•</sup> in 21% O<sub>2</sub> (panel A) or 120 ppm NO<sup>•</sup> in N<sub>2</sub> (panel B). Cultures were grown in minimal succinate medium plus 20 L-amino acids with a 30% inoculum from an overnight culture grown in the same medium, and cultures were treated with chloramphenicol (200  $\mu\text{g}/\text{ml}$ ) at  $A_{550}$  of  $\sim 0.5$  for 10 min and equilibrated under the appropriate atmosphere prior to the exposures to NO<sup>•</sup>. Cultures were harvested at intervals, extracts were prepared, and aconitase activity and protein were assayed as described under "Materials and Methods." 100% aconitase activity was 193  $\pm$  10 and 107  $\pm$  4 milliunits/mg protein in aerobic AB1157 and JTG10 (panel A), respectively, while 100% activity was equivalent to 197  $\pm$  6 and 163  $\pm$  3 milliunits/mg extract protein in anaerobically incubated AB1157 and JTG10 (panel B), respectively.

lines 2 and 3). Further, the *E. coli* aconitase was more potently inhibited by NO<sup>•</sup> during catalysis with the substrate *cis*-aconitate than with citrate (compare lines 3 and 4). The results support a mechanism involving the reaction of NO<sup>•</sup> or an NO<sup>•</sup>-derived species, with the catalytic [4Fe-4S] center and suggest that the NO<sup>•</sup>-mediated inactivation observed *in vivo* occurs with the aconitase-substrate complex. Inactivation is favored by acidic conditions, and NO<sup>•</sup> appears to more favorably attack the *cis*-aconitate-aconitase intermediate.

The results did not exclude the possible involvement of a yet uncharacterized dioxygen-independent NO<sup>•</sup>-derived species in the mechanism of aconitase inactivation, since we were using extracts of *E. coli*. However, the effects of pH, salt, and substrates on the inactivation of the *E. coli* aconitase provided a valuable clue for understanding the previous failure of high concentrations of NO<sup>•</sup> to directly inactivate aconitases *in vitro* (23, 24). Thus, we supposed that acid pH or catalysis with *cis*-aconitate may facilitate the direct inactivation of aconitases

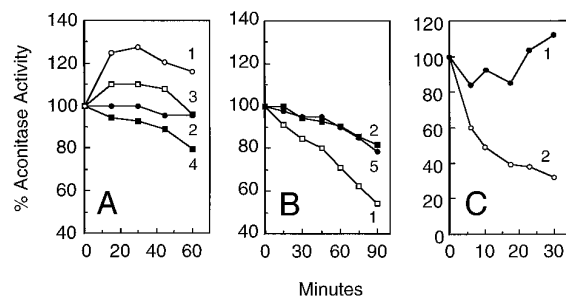


**FIG. 5. Effect of virtual substrates on NO<sup>•</sup>-mediated aconitase inactivation in extracts of *E. coli*.** Cell-free extracts of *E. coli* containing 12 mg/ml soluble protein in 100 mM Tris-HCl, pH 7.5, were vigorously stirred at 20 °C in a rubber septum-sealed 2.5-ml glass tube under a stream of N<sub>2</sub> (line 1) or N<sub>2</sub> plus 1200 ppm NO<sup>•</sup> with no addition (line 2), 20 mM *trans*-aconitate, pH 7.5 (line 3), 20 μM fluorocitrate (line 4), or 20 mM tricarallylate, pH 7.5 (line 5) in a total volume of 0.25 ml. At intervals, 5-μl aliquots were removed with a Hamilton syringe, diluted in 45 μl of 50 mM Tris-HCl, pH 7.4, containing 0.6 mM MnCl<sub>2</sub> and 20 μM fluorocitrate, and frozen on dry-ice. Aconitase activity was assayed as described under "Materials and Methods." Extracts contained 155 ± 9 milliunits of aconitase activity/mg of protein.



**FIG. 6. Inactivation of *E. coli* aconitase by NO<sup>•</sup> during catalysis.** Extracts of *E. coli* were added to a 5-ml reaction mixture pre-equilibrated under N<sub>2</sub> at 37 °C and containing either 30 mM citrate (lines 1-3) or 1.0 mM *cis*-aconitate (line 4), 0.4 mM NADP<sup>+</sup>, 0.6 mM MnCl<sub>2</sub>, 1 unit/ml isocitrate dehydrogenase, and 1 mg/ml bovine serum albumin. Reaction mixtures were buffered with either 50 mM Tris-HCl, pH 7.7 (lines 1 and 2) or 50 mM HEPES, pH 6.8 (lines 3 and 4) and were exposed under a 30 ml/min stream of 1200 ppm NO<sup>•</sup> plus N<sub>2</sub>, or N<sub>2</sub> only, in 50-ml stoppered flasks, and flasks were shaken at >200 rpm to facilitate gas equilibration. Reactions (lines 2-4) were performed in the presence of 200 mM KCl. At intervals, 0.5-ml aliquots were removed with a syringe and measured for absorbance at 340 nm. The percentage of aconitase activity was calculated from the rate of increase of absorbance at 340 nm under NO<sup>•</sup> relative to that measured for reactions incubated under N<sub>2</sub>. Reactions were initiated with 40 μg (lines 1 and 2), 600 μg (line 3), and 100 μg (line 4) of extract protein.

by NO<sup>•</sup> and that the NO<sup>•</sup> sensitivity of the porcine heart mitochondrial aconitase may also be more readily observed under these conditions. Consistent with previous reports, incubation of the commercial porcine mitochondrial aconitase preparation with NO<sup>•</sup> at pH 7.5 failed to inactivate aconitase during a 60-min exposure (Fig. 7A, compare lines 1 and 2) (24). Interestingly, NO<sup>•</sup> caused an apparent stimulation of aconitase activity at pH 7.5 either in the presence (compare lines 1 and 2) or absence (compare lines 3 and 4) of 200 mM KCl. However, at pH 6.5, exposure of the aconitase to an atmosphere of 1200 ppm NO<sup>•</sup> caused a loss of aconitase activity relative to the N<sub>2</sub> control (Fig. 7B, compare lines 1 and 2). Moreover, the activity loss was completely prevented by the virtual substrate fluorocitrate (20 μM) (Fig. 7B, line 5). A similar NO<sup>•</sup> resistance was observed with the purified recombinant porcine mitochondrial aconitase at pH 7.5 (Fig. 7C, line 1), and NO<sup>•</sup>-mediated inactivation was



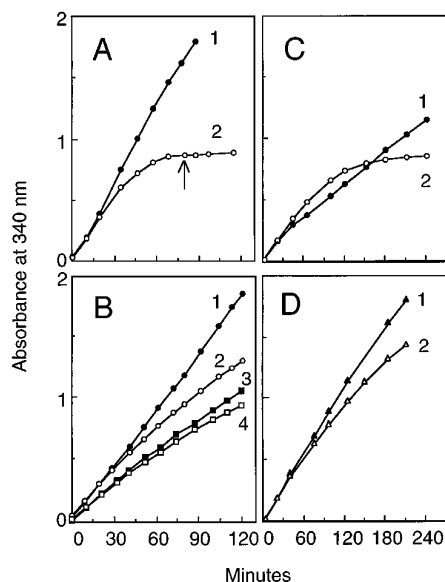
**FIG. 7. Effect of pH on the NO<sup>•</sup> sensitivity of the resting porcine mitochondrial aconitase.** Commercial porcine heart mitochondrial aconitase (0.25 mg) in a 0.1-ml volume buffered with 100 mM Tris-HCl, pH 7.5 (panel A) or 100 mM K-Mes, pH 6.5 (panel B) was vigorously stirred at 20 °C under a stream of N<sub>2</sub> plus 1200 ppm NO<sup>•</sup> (lines 1, 3, and 5) or N<sub>2</sub> (lines 2 and 4) at a flow rate of 30 ml/min in a 2.5-ml rubber septum-sealed glass tube either in the presence (lines 1, 2, and 5) or absence (lines 3 and 4) of 200 mM KCl. Aliquots were removed and assayed for aconitase activity at intervals. Fluorocitrate was added to 20 μM (line 5). Reaction mixtures were pre-equilibrated for 5 min with N<sub>2</sub>. C, purified recombinant porcine mitochondrial aconitase (5 μg) was exposed to 100 μM NO<sup>•</sup> at 25 °C in 0.2 ml of N<sub>2</sub>-equilibrated 100 mM Tris-HCl, pH 7.5 (line 1) or 100 mM K-Mes, pH 6.5 (line 2), and aliquots were removed and immediately assayed for aconitase activity. The percentage of aconitase activity was determined relative to the control activity during incubations under N<sub>2</sub>.

observed at pH 6.5 (line 2) during exposure of the enzyme to 100 μM NO<sup>•</sup>. Like the *E. coli* aconitase, both the relatively impure commercial preparation of porcine mitochondrial aconitase and the isolated recombinant enzyme were potentially inhibited/inactivated by NO<sup>•</sup> during catalysis with saturating concentrations of *cis*-aconitate (1 mM) at pH 6.8 (Fig. 8, A and C, respectively; compare lines 1 and 2). The inhibitory effect was not alleviated by flushing the reaction with N<sub>2</sub> to remove NO<sup>•</sup> (as indicated by the arrow, panel A). A lesser degree of inhibition/inactivation was observed with both preparations at pH 7.8 (Fig. 8, B and D; compare lines 1 and 2), and the absence of KCl further increased the resistance of the mitochondrial aconitase to NO<sup>•</sup> (Fig. 8B, compare lines 3 and 4 with lines 1 and 2). We conclude that NO<sup>•</sup> reacts directly and irreversibly with the aconitases and that inactivation occurs with the catalytically active aconitases.

#### DISCUSSION

Low levels of NO<sup>•</sup> gas exposure effectively inactivated the aconitase in *E. coli*. We can estimate the concentration of NO<sup>•</sup> required for aconitase inactivation to be <240 nM at an NO<sup>•</sup> gas concentration of 120 ppm if we simply assume a partitioning of NO<sup>•</sup> in the bacteria similar to that in aqueous solutions and apply the NO<sup>•</sup> saturation value for water of 2 mM at 1 atmosphere and 20 °C. A poor reactivability of the NO<sup>•</sup>-inactivated aconitase (Fig. 2) appears to contribute to the observed NO<sup>•</sup> sensitivity of the aconitase *in vivo*. Thus, a relatively slow inactivation of the aconitase by NO<sup>•</sup> coupled with a slow reactivation may have effects comparable with those of the fast inactivation and fast reactivation observed with O<sub>2</sub><sup>-</sup>, since the steady-state balance of active and inactive aconitase *in vivo* would be determined by the balance of inactivation and reactivation (21, 30).

ONOO<sup>-</sup>, GSNO, or other more oxidizing O<sub>2</sub>-dependent NO<sup>•</sup>-derived species do not appear to be involved in the mechanism of aconitase inactivation in *E. coli* as evidenced by the failure of SOD (Fig. 3A) or GSH (Fig. 4) to affect the NO<sup>•</sup>-mediated inactivation and by the dioxygen-independence of inactivation. Moreover, ambient O<sub>2</sub> and O<sub>2</sub><sup>-</sup> may protect aconitase by decreasing the steady-state levels of NO<sup>•</sup> through the formation of ONOO<sup>-</sup> and NO<sub>2</sub><sup>•</sup>. Interestingly, ONOO<sup>-</sup> would be expected to form at a high rate in *E. coli* under aerobic conditions and to



**FIG. 8. Inactivation of porcine mitochondrial aconitase by NO<sup>•</sup> during catalysis.** Porcine mitochondrial aconitase in a reaction mixture buffered with 50 mM Na-Hepes at pH 6.8 (panels A and C) or pH 7.8 (panels B and D) and containing 1.0 mM *cis*-aconitate, 0.4 mM NADP<sup>+</sup>, 0.6 mM MnCl<sub>2</sub>, 1 unit/ml isocitrate dehydrogenase, and 1 mg/ml bovine serum albumin was exposed to N<sub>2</sub> (lines 1 and 3) or 1200 ppm NO<sup>•</sup> in N<sub>2</sub> (lines 2 and 4) either in the presence (lines 1 and 2) or absence (lines 3 and 4) of 200 mM KCl at 37 °C. Commercial porcine heart aconitase (panels A and B) or purified recombinant porcine mitochondrial aconitase (panels C and D) were added at 4.8 and 0.125 μg/ml, respectively. Reaction mixtures (10 ml) were exposed under a gas stream (30 ml/min) in 50-ml stoppered flasks, and flasks were shaken on a rotary water bath at >200 rpm to facilitate gas equilibration. At intervals, aliquots were removed with a syringe and measured for absorbance at 340 nm. The arrow indicates the replacement of NO<sup>•</sup> in N<sub>2</sub> with N<sub>2</sub>.

inactivate the aconitase if we were simply to consider the high rate of metabolic O<sub>2</sub> production of 5 μM/s by *E. coli* (42), the high rate of reaction between NO<sup>•</sup> and O<sub>2</sub><sup>-</sup> of 6.7 × 10<sup>9</sup> M<sup>-1</sup> s<sup>-1</sup> (43), and the relatively high second order rate constant for the reaction of ONOO<sup>-</sup> with aconitase of ~10<sup>5</sup> M<sup>-1</sup> s<sup>-1</sup> (24). However, the scavenging of ONOO<sup>-</sup> by various oxidizable biomolecules or the binding of substrates to aconitase may adequately prevent the ONOO<sup>-</sup>-mediated oxidation of the aconitase [4Fe-4S]<sup>2+</sup> center *in vivo*. In contrast, NO<sup>•</sup> may have a selective affinity and reactivity with the substrate-bound solvent-exposed Fe<sub>a</sub> of the aconitase [4Fe-4S]<sup>2+</sup> center similar to that of O<sub>2</sub><sup>-</sup>. It should be mentioned in this regard that authentic NO<sup>•</sup> (18) and NO<sup>•</sup> donors (13) were reported to inactivate the mammalian cytoplasmic aconitase in extracts independently of O<sub>2</sub>/O<sub>2</sub><sup>-</sup> and thus ONOO<sup>-</sup> formation. However, in the case of NO<sup>•</sup> donors, the inactivation of aconitase via a transnitrosation was not excluded.

Catalytically active substrate-saturated aconitase was inhibited by NO<sup>•</sup> *in vitro*. The citrate-saturated aconitase was relatively more resistant than the *cis*-aconitate-saturated aconitase (Fig. 6), suggesting an effect of substrate ligands on the reactivity of NO<sup>•</sup> with the solvent-exposed Fe<sub>a</sub>. Citrate 3-OH oxygen binding to Fe<sub>a</sub> may decrease the NO<sup>•</sup> reactivity, or alternatively, *cis*-aconitate may increase the reactivity by altering the ligand field of Fe<sub>a</sub>. The citrate hydroxyl oxygen does alter the Fe<sub>a</sub> in the mitochondrial aconitase as indicated by ENDOR (electron nuclear double resonance), EPR, and Mössbauer spectroscopy (40, 41, 44). However, the ability of the hydroxylless *trans*-aconitate and tricarallylate to protect aconitase equally with that of the hydroxyl-containing inhibitor fluorocitrate (Fig. 5) suggests that an intermediate in the catalytic turnover of aconitase or a conformational state of the

aconitase active site affects the reactivity of NO<sup>•</sup> rather than solely hydroxyl oxygen liganding to Fe<sub>a</sub>. Interestingly, the resting isolated mitochondrial aconitase was relatively resistant to inactivation by NO<sup>•</sup> at pH 7.5 (Fig. 7). This result corroborates those of others in which the isolated mitochondrial, cytoplasmic, and *E. coli* aconitases were shown to be resistant to NO<sup>•</sup> at the high nonphysiological concentrations of 100–200 μM at pH 7.6 or 8.0 (23, 24). Importantly, we were able to demonstrate that the resting mitochondrial aconitase is inactivated at a more acidic pH during the exposure to near physiological concentrations of 1200 ppm NO<sup>•</sup> (~2 μM) (Fig. 7B) or even faster with 50-fold higher concentrations of NO<sup>•</sup> (Fig. 7C). Differences in the reactivity of NO<sup>•</sup> may be explained by the effects of pH on hydroxide liganding to the solvent-exposed Fe<sub>a</sub> of the substrate-free aconitase (40, 45) or may be due to effects on ionizable amino acid side chains in the active site (27, 46).

NO<sup>•</sup> has an affinity for heme and non-heme iron, and it appears likely that NO<sup>•</sup> binds to Fe<sub>a</sub> of the aconitase [4Fe-4S]<sup>2+</sup> center.<sup>2</sup> Further, the Lewis acid character of Fe<sub>a</sub> would be expected to withdraw electrons from NO<sup>•</sup> to produce an intermediate with nitrosonium (NO<sup>+</sup>)-like reactivity. NO<sup>+</sup> or NO<sup>δ+</sup> would then be able to attack nucleophilic sulfur in the Fe-S center. In this respect, the reaction of NO<sup>•</sup> at the active site of aconitase may be similar to the nitrosation of albumin catalyzed by dinitrosyl-iron complexes (47) with the exception that the reaction would be site-specific and autocatalytic. Factors affecting the electron withdrawing capacity of Fe<sub>a</sub>, such as Fe<sub>a</sub> ligands and substrates, may influence NO<sup>+</sup> or NO<sup>δ+</sup> formation and reactivity. A nitrosation of the Fe-S center may also explain the relatively poor reactivatability of the aconitase *in vivo* (Fig. 2).

It will be interesting to determine whether the NO<sup>•</sup> sensitivity of aconitase generally applies to members of the [4Fe-4S] (de)hydratase family or to other iron-sulfur enzymes. Also, it will be important to elucidate the NO<sup>•</sup>-mediated modification to the aconitase(s) that impedes its reactivation. Finally, it is hoped that the NO<sup>•</sup>-sensitive aconitases may provide insights into the mechanisms of NO<sup>•</sup> toxicity and the adaptive defenses against this toxicity. For example, the pH-dependent NO<sup>•</sup> sensitivity of the aconitases may increase the susceptibility of energy-dependent cell functions to the NO<sup>•</sup>- and acid-producing conditions of the intestine during endotoxic shock (48) or of the macrophage phagolysosome during the host immune response to infection (49).

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<sup>2</sup> Since the submission of this manuscript, we have learned of EPR studies demonstrating interactions of NO<sup>•</sup> with the Fe-S center of the beef mitochondrial aconitase that correlate with the loss of activity (50).

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