Hydrogen Peroxide Sensitivity of Catechol-2,3-Dioxygenase: a Cautionary Note on Use of xylE Reporter Fusions under Aerobic Conditions

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Catechol-2,3-dioxygenase (C23O) of Pseudomonas putida, encoded by the xylE gene, was found to be sensitive to hydrogen peroxide (H2O2) when used as a reporter in gene fusion constructs. Exposure of Pseudomonas aeruginosa katA or katA katB mutants harboring katA- or katB-lacZ (encoding β-galactosidase) or xylE-lacZ fusion plasmids to H2O2-stimulated β-galactosidase activity, while there was little or no detectable C23O activity in these strains. More than 95% of C23O activity was lost after a 5-min exposure to equimolar H2O2, while a 10,000-fold excess was required for similar inhibition of β-galactosidase. Electron paramagnetic resonance spectra of the nitrosyl complexes of C23O showed that H2O2 nearly stoichiometrically oxidized the essential active-site ferrous ion, thus accounting for the loss of activity. Our results suggest using caution in interpreting data derived from xylE reporter fusions under aerobic conditions, especially where oxidative stress is present or when catalase-deficient strains are used.

The use of gene reporter cassettes, including those encoding β-galactosidase (lacZ), luciferase (luxAB), and catechol-2,3-dioxygenase (C23O) (xylE), has been a tremendous benefit to scientists studying gene regulation. The lacZ gene is the most widely used reporter in bacteria, while others that include luxAB, gfp (green fluorescent protein), phoA (alkaline phosphatase), and xylE fusions are used less often. The use of lacZ fusions in bacteria has proven to be the best tool with which to accurately assess promoter activity under conditions of oxidative stress. For example, in Escherichia coli, lacZ fusions were used to show that the transcriptional activity of oxidant-regulated genes including sodA (encoding Mn-superoxide dismutase), katE (encoding catalase/hydroperoxidase II), and katG (encoding catalase/hydroperoxidase I), among others, is markedly increased upon exposure to either O2- or H2O2-generating agents (16, 29, 34).

Unlike the reliable lacZ reporters, other gene fusions have some drawbacks. For example, the use of the Vibrio harveyi luxAB genes (encoding luciferase) in transcriptional gene fusions was cautioned against, because it generates O2- (12). Fusions linked to phoA are limited to proteins expressed in the periplasm (21), while those linked to gfp are only semiquantitative (24). The use of xylE fusion constructs does not appear to have any of these disadvantages (32).

The product of the xylE gene of Pseudomonas putida, C23O, is an important component in the degradation pathways of toluene and xylenes and catalyzes the dioxygenolytic cleavage of the aromatic ring (8, 11, 18, 25). Interestingly, it has been observed that optimal toluene degradation occurs under reduced oxygen tension with some C23O enzymes (17). This suggests a sensitivity to oxygen or one of its reduced forms, despite the fact that C23O requires oxygen for activity. Oxygen sensitivity may be derived from the fact that each of four C23O subunits contains an essential iron atom which must be in the Fe(II) oxidation state for activity in extradiol-type aromatic dioxygenases (3, 4, 25). Oxidation of this iron would inactivate the enzyme. Indeed, early studies showed that C23O is inactivated by low levels of the oxidizing reagent H2O2 in vitro (25). Our later studies of other Fe(II)-containing dioxygenases showed that H2O2 treatment resulted in enzyme inactivation and the appearance of electron paramagnetic resonance (EPR) signals attributable to ferric ion in most but not all cases (3, 23, 37). Several other iron-containing enzymes including the antioxidants iron superoxide dismutase and catalase are also sensitive to elevated levels of H2O2 (7, 9). In this study, we describe the sensitivity of C23O to H2O2 measured both in vivo using isogenic catalase mutants (Table 1) of the aerobic gram-negative bacterium Pseudomonas aeruginosa and in vitro using purified enzyme. We suggest caution in interpreting data obtained using xylE reporter fusions under aerobic conditions.

Sensitivity of C23O but not β-galactosidase to H2O2 in vivo using P. aeruginosa catalase gene lacZ and xylE fusions. The katA gene of P. aeruginosa encodes the major, constitutively expressed catalase, KatA (13, 20). In contrast, KatB activity is only detected upon exposure to paraquat, a redox-cycling agent paraquat (6). As shown in Fig. 1A, XylE reporter activity in the katA mutant, where catalase activity is virtually undetectable (20), was reduced 93%. The KatA-XylE reporter activity in the katA katB double mutant was reduced ~70%. Since H2O2 is required to activate the katB gene (6), it is not surprising that KatB reporter activity was very low or undetectable. In fact, KatB-LacZ reporter activity was reduced ~5,000-fold relative to KatA-LacZ reporter activity (Fig. 1B).

Treatment with H2O2 stimulates catalase gene lacZ reporter activity but inhibits xylE reporter activity in catalase-deficient strains. To test the hypothesis that H2O2 increases katA::lacZ and katB::lacZ but not katA::xylE or katB::xylE activity, aero-
bic, mid-logarithmic-phase organisms were exposed to a sublethal 1 mM dose of H$_2$O$_2$ for 1 h, a condition which markedly increases KatB activity and, to a far lesser extent, KatA activity (6). Figure 2A shows that KatA-LacZ activity was only slightly increased in wild-type bacteria but was increased 1.4-fold following H$_2$O$_2$ treatment in the katA katB mutant. KatA-XylE activity was also increased upon exposure to H$_2$O$_2$ in wild-type bacteria but was dramatically inhibited in the control and H$_2$O$_2$-treated katA katB strains (Fig. 2B). In contrast to KatA activity, KatB activity is stimulated upon exposure to H$_2$O$_2$ (6). This is reflected in the KatB-LacZ results shown in Fig. 2B. Wild-type and katA katB mutant KatB-LacZ activity increased 10.7- and 35-fold upon exposure to H$_2$O$_2$. In contrast, KatB-XylE activity in the H$_2$O$_2$-treated katA katB mutant was reduced ~15-fold and was not detectable in control bacteria.

**Sensitivity of purified C23O but not of β-galactosidase to H$_2$O$_2$.** The in vivo results suggest that H$_2$O$_2$ is much more damaging to C23O than to β-galactosidase. To compare the

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**TABLE 1. Strains and plasmids used in this study**

| Strain or plasmid | Genotype or characteristics$^a$ | Source or reference |
|-------------------|---------------------------------|---------------------|
| **P. aeruginosa** |                                 |                     |
| PAO1              | Prototrophic, wild-type strain  |                     |
| PAO1 katA         | katA::Gmr$^+$                   | 14                  |
| PAO1 katB         | katB::Gmr$^+$                   | 13                  |
| PAO1 katA katB    | katA::Gmr$^+$ katB::Tc$^+$      | 13                  |
| Plasmid           |                                 |                     |
| pBluescript KS(−/+) | Extended polylinker pUC derivative, Ap$^r$ | Stratagene |
| pEX100T           | sacB Ap$^r$ oriT mob             |                     |
| pSJ1918G          | pUC1918 + 2.3-kb xylE and aacC1 (Gmr$^+$) cassette | 31 |
| pSMB3             | Ap$^r$, pNOT19 with a 2.7-kb EcoRI-SmaI fragment from P. aeruginosa containing katB | 6 |
| pDH501            | pSMB3 with a 3.2-kb lacZ cassette within EcoRV site of katB | This study |
| pDH502            | pDH501 with a 5.8-kb oriT sacB fragment of pMOB3 | This study |
| pDH503            | pSMB3 with a 1.4-kb xylE cassette within EcoRV site of katB | This study |
| pDH504            | pDH503 with a 5.8-kb oriT sacB fragment of pMOB3 | This study |
| pZP30             | Broad-host-range translational lacZ fusion plasmid | 30 |
| pkata::lacZ       | pZP30 containing a 756-bp katA promoter fragment with the first 33 codons fused to lacZ | 20 |
| pkatB::lacZ       | pZP30 containing a 475-bp katB promoter fragment with the first 59 codons fused to lacZ | 26 |
| pkata::xylE       | Same as pkata::lacZ but with lacZ replaced by a 2.3-kb xylE-aaC1 fragment from pX1918G | This study |
| pkatB::xylE       | Same as pkatB::lacZ but with lacZ replaced by a 2.3-kb xylE-aaC1 fragment from pX1918G | This study |

$^a$ Abbreviations used for genetic markers were as described by Holloway et al. (15). mob, mobilization site (ColE1); oriT, origin of transfer (RK2); Ap$^r$, ampicillin resistance; Gmr$^+$, gentamicin resistance; Tc$^+$, tetracycline resistance. Plasmids pDH501 to pDH504 were used as “feeder” plasmids for the lacZ and xylE fusion plasmids used in this study.

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**FIG. 1.** Quantification of catalase gene reporter activity in wild-type and catalase mutant bacteria. *P. aeruginosa* PAO1 katA, katB, and katA katB strains (13) harboring plasmids containing katA- or katB-lacZ or -xylE transcriptionsal fusions were grown aerobically for 24 h in L broth containing carbenicillin (0.4 mg/ml) for plasmid maintenance. Bacteria were washed twice in either ice-cold 50 mM potassium phosphate buffer (pH 7.5) (for C23O assays) or Z buffer (for β-galactosidase assays) (22) containing 39 mM 2-mercaptoethanol and were sonicated in an ice water bath for 10 s with a Heat-Systems, Inc. (Farmington, N.Y.), model W-225 sonicator at setting 5. Cell extracts were assayed for C23O and β-galactosidase activities as previously described (19, 28). The results are expressed as the means ± standard errors of the means of three replicates. (A) Lane 1, PAO1/pkatA::xylE; lane 2, PAO1/pkatB::xylE; lane 3, katA mutant/pkatA::xylE; lane 4, katA mutant/pkatB::xylE; lane 5, katB mutant/pkatA::xylE; lane 6, katB mutant/pkatB::xylE; lane 7, katA katB mutant/pkatA::xylE; lane 8, katA katB mutant/pkatB::xylE. (B) Lanes are identical to those in panel A except that the fusions are to lacZ. The values in panel A are all statistically significantly different from one another ($P < 0.01$, Student’s $t$ test). The differences between lanes 1, 3, 5, and 7 of panel B are not statistically significant.
FIG. 2. H₂O₂-mediated activation of katA and katB gene transcription: demonstration of increased β-galactosidase activity but not C23O activity in catalase-deficient bacteria. Bacteria were grown aerobically to mid-exponential phase (optical density at 600 nm = 0.6) in L broth and were allowed to grow an additional hour in the presence (lanes 2 and 4) or absence (lane 1 and 3) of 1 mM H₂O₂. Cell extracts were then assayed for β-galactosidase (A and C) and C23O (B and D) activity.

The results are expressed as the percentage of reporter activity in uninduced wild-type bacteria (n = 3). Lane 1, PAO1; lane 2, PAO1 + H₂O₂; lane 3, katA katB; lane 4, katA katB + H₂O₂. In panel A, values in lanes 1, 2, and 4 are not statistically significant, yet that in lane 3 is significantly reduced (P < 0.05). All remaining values are statistically significantly different at P values of < 0.01.

FIG. 3. Relative sensitivity of purified P. putida C23O (A) and β-galactosidase (B) to H₂O₂. C23O was isolated from P. putida mt-2 cells maintained on m-toluate and grown in 10-liter batch cultures on benzoate as the sole carbon source from 1-liter liquid starter cultures containing 1:2 m-toluate-benzoate as carbon sources. The total culture time on benzoate was 12 h, and the extradiol catechol oxidation activity was approximately 10⁵ U/100 g (wet weight) of cells. The purification was as previously described (4), except that 50 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0) was used in place of potassium phosphate buffer in all steps. Samples with a specific activity greater than 150 U/mg were pooled, concentrated, and stored at −20°C in small aliquots until use. Purified P. putida C23O (10 U) and E. coli β-galactosidase (10 U; Sigma) were incubated with increasing concentrations of H₂O₂ at room temperature for 5 min. C23O and β-galactosidase activities were then assayed as previously described (19, 28) and expressed as the percentage of the control without H₂O₂. The bars indicate the means ± standard errors of the means of three replicates. Concentrations of H₂O₂ added follow. (A) Lane 1, control; lane 2, 1 μM; lane 3, 10 μM; lane 4, 100 μM. (B) Lane 1, control; lane 2, 1 mM; lane 3, 10 mM; lane 4, 100 mM; lane 5, 200 mM; lane 6, 300 mM; lane 7, 400 mM; lane 8, 500 mM.
FIG. 4. EPR spectra of the Fe-nitrosyl complex of purified \textit{P. putida} C23O show that loss of activity correlates with oxidation of the active-site Fe(II). Purified \textit{P. putida} C23O (160 \textmu M) was incubated on ice with 0 \textmu M (---), 300 \textmu M (--), or 30 mM (---) \textit{H}_2\text{O}_2 for 30 min at pH 7. Then 7.5 U of bovine liver catalase was added to destroy any residual \textit{H}_2\text{O}_2, and the enzyme activity was determined (this concentration of catalase cannot be detected by EPR). The samples were then transferred by a gastight syringe to an EPR tube under argon. Nitric oxide (NO) was added by slowly bubbling the gas through the sample under argon flow. Trace oxygen was removed from the argon gas by passage over a BASF copper catalyst at 160°C. Samples were flushed with argon after NO addition to remove excess NO from the headspace. The samples were frozen by slow immersion in liquid \textit{N}_2, and the EPR spectra were recorded. The enzyme activity of the EPR samples after the measurement was determined by first thawing them under argon. The samples were transferred by a gastight syringe to a serum-stoppered vial under argon. Subsequently, the NO was removed by cycles of evacuation and flushing with Ar. Activity measurements of the samples were approximately unchanged from those determined before exposure to NO.

The loss of signal in the 4-g region is proportional to the oxidation of Fe(II) to Fe(III) in the sample. At least three slightly different S 3/2 species are present with resonance pairs at g values of 4.18 and 3.82, 4.11 and 3.91, and 4.02 and 3.98. Multiple species are usually seen for nitrosyl complexes of Fe(II) dioxygenases. EPR measurement conditions using a Bruker E500 spectrometer equipped with an Oxford ESR-910 liquid helium cryostat follow: temperature, 2 K; modulation amplitude, 10 G; modulation frequency, 100 kHz; microwave power, 200 \textmu W; and microwave frequency, 9.63 GHz. Data were digitally recorded and analyzed as previously reported (10). Spin quantitations were performed by single or double integration of the first derivative spectra (1) using an Fe(II)-NO-EDTA complex as a standard. EPR spectra of S 3/2 and S 5/2 complexes were analyzed as previously reported (23, 37). Iron content was quantified by atomic absorption.

sensitivity of C23O and \beta-galactosidase to \textit{H}_2\text{O}_2, aliquots of the purified enzymes were incubated at room temperature with increasing concentrations of \textit{H}_2\text{O}_2. As shown in Fig. 3A, the C23O activity loss was directly correlated with the nearly stoichiometric titration of the active-site Fe(II) in the sample with \textit{H}_2\text{O}_2. Most of the activity was lost after a 1:2 titration, and there was no detectable C23O activity after the enzyme was incubated with a 20-fold excess of \textit{H}_2\text{O}_2. In contrast, 100 mM \textit{H}_2\text{O}_2 (a 10,000-fold excess) was required for \textasciitilde 50% inhibition of \beta-galactosidase activity (Fig. 3B).

EPR analysis of \textit{H}_2\text{O}_2-treated C23O. The effect of \textit{H}_2\text{O}_2 on the active-site iron of C23O can be directly evaluated by EPR spectroscopy. Our past studies have shown that complexing of the enzyme with NO converts an EPR-silent metal center into an EPR-active species with two of the three resonances symmetrically distributed around g values of 4 (3, 4). This is the characteristic spectrum of a spin (S) 3/2 species that is formed by transferring one electron from the iron to the NO and antiferromagnetic coupling between the resulting species (5). This species is easily distinguished from that resulting from oxidation of the Fe(II) to Fe(III), which yields an S 5/2 EPR spectrum with g values near 4.3. As shown in Fig. 4, untreated C23O yielded an intense S 3/2 EPR spectrum from the active-site Fe(II)-NO complex. Quantitation of the spectra showed that all of the iron in the sample formed a complex. After a 30-min incubation with a twofold excess of \textit{H}_2\text{O}_2 on ice, a dramatic decrease in the S 3/2 spectrum from Fe(II)-NO occurred, accompanied by a 96% (\pm 5%) loss of activity and the appearance of a broad signal from Fe(III) (g = 4.3). Quantitation of the S 3/2 signal showed that 87% (\pm 5%) of the Fe(II) was oxidized. Incubation of an identical sample with a 200-fold excess of \textit{H}_2\text{O}_2 resulted in a complete loss of the S 3/2 signal and activity as well as a further increase in the S 5/2 signal from Fe(III). These results show directly for the first time that the origin of the loss of activity of C23O following \textit{H}_2\text{O}_2 treatment is oxidation of the active-site iron in accord with previous indirect experiments (25). Many studies have indicated that the mechanism of extradiol dioxygenases requires Fe(II), and thus its oxidation would inhibit the enzyme (2, 33). Because the Fe(III) EPR signal of the inactivated C23O is very broad, it is likely either that the iron is lost from the enzyme following oxidation or that the environment of the iron becomes highly disordered.

Conclusions. In this study, we demonstrate that C23O is very sensitive to approximately stoichiometric levels of \textit{H}_2\text{O}_2 both when it is present as a gene fusion product in vivo and as the purified enzyme in vitro. Arguably, the attractiveness of the \textit{xyI}E reporter system in monitoring gene-promoter activity is based upon its rapid spectrophotometric assay that is zero-order with respect to its substrate catechol. However, this study raises concern over the interpretation of data obtained under highly aerobic conditions. Recently we have cloned, overexpressed, and characterized another extradiol dioxygenase similar to C23O that catalyzes the cleavage of 3,4-dihydroxyphenyl acetic acid (homoprotocatechuate) between the 2- and 3-ring carbons to yield a yellow product analogous to the product of the C23O-catalyzed reaction (23, 35). Homoprotocatechuate-2,3-dioxygenase (H23O, encoded by \textit{hpcd}) is unique among Fe(II)-containing dioxygenases in that it exhibits very low sensitivity to \textit{H}_2\text{O}_2. Moreover, a homologous H23O containing Mn(II) rather than Fe(II) has also been cloned and characterized and was also found to be insensitive to \textit{H}_2\text{O}_2 (27, 36). Thus, gene fusions to either of these enzymes could offer an alternative to C23O when experiments require highly aerobic and/or oxidative stress conditions. Such experiments are in progress.

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