A Novel [2Fe-2S] Ferredoxin from *Pseudomonas putida* mt2 Promotes the Reductive Reactivation of Catechol 2,3-Dioxygenase*

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Catechol 2,3-dioxygenase (XylE) is a component of the TOL plasmid-encoded pathway for the degradation of toluene and xylenes and catalyzes the dioxygenolytic cleavage of the aromatic ring. Purified XylE is oxygen-sensitive and unstable in vitro, particularly in the presence of substituted catechol substrates, but it is stabilized in vivo by another protein, XylT, encoded by the *xylT* gene located just upstream of *xylE*. In this study, we have purified to homogeneity the XylT product from a recombinant *Escherichia coli* strain containing a hyper-expressible *xylT* gene and characterized it as a novel [2Fe-2S] ferredoxin. It is the first example of a soluble ferredoxin with a net positive charge at neutral pH. The EPR signal of the iron sulfur cluster has rhombic symmetry as is the case for plant-type ferredoxins, but the XylT absorbance spectrum resembles more closely that of adenodoxin. The midpoint redox potential was determined to be $-373 \pm 6$ mV, at pH 8.5. XylT was unusually unstable for a [2Fe-2S] ferredoxin, with half-lives of 69 min at 25 °C in air and 70 min at 37 °C in argon. With photochemically reduced 5-deazaflavin for the controlled generation of reductant, it was demonstrated that XylT mediates the rapid reactivation of purified inactive catechol 2,3-dioxygenase in vitro. Inactivation of XylE by 4-methylcatechol resulted in oxidation of the active site iron to a high spin ferric state that was detectable by EPR. Spectroscopic evidence presented here demonstrates that XylT reactsivate XylE through reduction of the iron atom in the active site of the enzyme. It is the first instance of a ferredoxin-mediated reactivation of an enzyme. The level of expression of XylT in *Pseudomonas putida* mt2 cells is low and the calculated XylT/XylE molar ratio is consistent with the proposal that XylE reactivation involves catalytic nonstoichiometric amounts of XylT.

*Pseudomonas putida* mt2 carries the TOL plasmid pWW0, which encodes a set of enzymes responsible for the transformation of toluene and xylene to central pathway intermediates (1).

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The catabolic (*xyl*) genes are organized in two operons, the so-called upper and meta-operons. The enzymes encoded by the upper operon catalyze the sequential oxidation of toluene to benzoate, whereas the enzymes of the meta-operon convert benzoate to Krebs cycle intermediates (2). In the meta-pathway, the enzyme catechol 2,3-dioxygenase encoded by *xylE* catalyzes the extradiol cleavage of the aromatic ring. This reaction has been studied in detail, and a general mechanism for the oxidative cleavage of catechol by extradiol dioxygenases has been proposed (3). The two atoms of the oxygen molecule are incorporated in the catechol substrate on two adjacent carbon atoms of the aromatic ring, one of which already carries a hydroxyl substituent of the diol and the other of which is unsubstituted. At the active site, the enzyme possesses a single iron atom that binds the substrate and oxygen and participates in the catalytic cycle.

Structural determination of the related biphenyl 2,3-dioxygenase revealed that the iron atom is bound to the polypeptide through covalent linkages with the side chains of three residues, histidines 146 and 210 and aspartate 260 (4), residues that are conserved among extradiol dioxygenases (5). In the active enzyme, the iron atom is in the ferrous state and is assumed to remain steady throughout the catalytic cycle. However, in the presence of certain substrates, such as 4-methylcatechol and chlorocatechols, oxidation of the iron atom occurs, accompanied by inactivation of the enzyme (6). Slow but significant inactivation also takes place during enzyme turnover with catechol as substrate (7).

In a recent study, Polissi and Harayama (8) found that a mechanism exists in vivo that hinders irreversible inactivation of catechol dioxygenase. Mutants lacking a functional *xylT* gene lost the ability to grow on *p*-xylene and *p*-toluate as carbon sources. In addition, it was found that 4-methylcatechol, which is an intermediate in the degradation of *p*-xylene and *p*-toluate, irreversibly inactivated catechol 2,3-dioxygenase in the *xylT* mutants, while the enzyme remained active in wild-type *P. putida*. It was therefore envisioned that the *xylT* gene product might participate in a mechanism of protection or reactivation of the catechol 2,3-dioxygenase (8). The *xylT* gene lies immediately upstream of *xylE*, and its sequence suggests that it may code for a ferredoxin (9).

In this study, we have purified the *xylT* gene product and tested whether it can reactivate catechol 2,3-dioxygenase in vitro. The *xylT* gene was hyperexpressed in *Escherichia coli* yielding a red protein that was purified and characterized as a novel [2Fe-2S] ferredoxin. An experimental system was developed to monitor XylT-dependent reactivation of the catechol 2,3-dioxygenase. It was demonstrated that XylT alone can reactivate catechol 2,3-dioxygenase in a reaction that is rapid and specific and requires reductant. The low amount of XylT de-
tected in wild-type *P. putida* cells suggests that it acts catalytically rather than stoichiometrically.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—*E. coli* strain DH5α (Life Technologies, Inc.) was used as a host for general DNA manipulations, as well as for *xyE* hyperexpression. Strain BL21(DE3) (Novagen) was employed for hyperexpression of the *xyT* gene. Cultures were grown on LB medium containing appropriate antibiotics. For *XylT* hyperproduction, cultures were grown at 28 °C in 10-liter fermentors supplemented with 50 μM FeSO₄. For *XylE* production, *E. coli*, DH5α (pAW31) (10), was grown overnight at 37 °C in 2-liter flasks containing 0.8 liter of LB medium containing 0.5 mM EDTA, 10% isopropyl alcohol (v/v) and 5 mM MgCl₂ and then stored at −20 °C.

**Cloning and Hyperexpression of the *xylT* Gene in *E. coli*—**The *xylT* gene was amplified by polymerase chain reaction using T7 polymerase (Promega), plasmid pAW31 (10), as a template and the following primers: 5′-tcgagagcgaGAACAGTGCCGGCTACG-3′; 3′-GTCACCTCTTCGCACTTCCTC-5′. Plasmid pV72 (5′-ggaattcCTGACGTCACCTCTTC-3′; BamHI sites were included) was purified, and *xylT* was inserted into plasmid pET11a. The resulting plasmid called pET91 was introduced into the *E. coli* strain BL21(DE3) (Novagen). Induction of *xylT* expression was initiated with 1 mM isopropyl β-D-thiogalactoside when the bacterial density reached an *A₅₇₀* of 1.8. Bacteria were harvested 6 h later.

**Purification of *XylT*—**The purification procedure was performed at 4 °C under argon using Tris-HCl buffer, pH 9.0, containing 2 mM dithiothreitol. Bacterial cells from 20 liters of culture were resuspended in 120 ml of 50 mM Tris-HCl, pH 9.0, containing 5 mM EDTA and subjected to lysozyme treatment (0.2 mg/ml) for 15 min. The bacterial extract was sonicated for 30 s and then diluted to 190 ml with buffer containing 10 mM Tris-HCl, pH 8.0, and 5 μM benzyl-viologen. Ammonium sulfate was then added to 1.1 final concentration, and the protein extract was centrifuged at 20,000 × g for 25 min. The supernatant fraction was applied to a 30-ml phenyl-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 1 mM ammonium sulfate in Tris-HCl, pH 8.0, and then stored at 20 °C.

**Enzyme Assays**—*XylT* activity was assayed by slab gel electrophoresis under denaturing conditions (SDS-PAGE) according to Jouanneau et al. (13). Protein concentrations were determined using the BCA assay (Pierce).

**Measurement of Redox Potential—**Redox titration of *XylT* was performed spectrophotometrically using 5-deazaflavin as a photoreductant and benzyl-viologen as a dye indicator (5′-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.

**Immunochemical Analysis of *XylT* in *P. putida* Extracts—**The affinity antibody against purified recombinant *XylT* was used. Recombinant *XylT* was purified from *P. putida* XylG was also immobilized on protein A-Sepharose (Sigma). A sample equivalent to 0.15 of packed resin was incubated with 0.4 ml of antiimmunogen in two Spin-X centrifuge filter units (Costar) for 1 h at room temperature under constant agitation. The resin was washed three times with 0.5 ml of 0.2 M sodium borate, pH 9. Bound IgG antibodies were eluted by treatment for 30 min with 20 mM ethylmethylimidazolide (Sigma) in 0.5 ml of borate buffer. The wash buffer and wash solution was incubated for 2 h in 0.2 M ethanamine, pH 8.0, and finally stored in 10 ml phosphate, 140 mM NaCl, 2.7 mM KCl, 0.5% Tween 20, pH 7.4.

**Immunoblotting**—Proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech). The membranes were blocked with 0.1% Tween 20 and then incubated with 1:15,000 × g x in a Beckman TL-100 ultracentrifuge. Samples of the high speed supernatant fractions, equivalent to 3.4 mg of protein, were incubated with immobilized anti-*XylT* IgG (20 or 40 μl) in a volume of 0.3 ml. Incubation was carried out at 4 °C in Spin-X centrifuge filter units (Costar) maintained under constant agitation (Eppendorf thermomixer). The beads of immobilized IgG-Sepharose were washed three times with 0.5 ml of 10 mM potassium phosphate, 0.15 M NaCl, 0.1% Triton X-100, pH 7.5. Bound proteins were then eluted by treatment for 5 min at 95 °C in 25 μl of SDS mixture. Samples of the eluted proteins were separated by electrophoresis on a 16% polyacrylamide gel containing 13% glycerol in a Tris-Tricine buffer system (14). Proteins were electroblotted on a nitrocellulose membrane as described previously (15), which was then developed with anti-*XylT* IgG (Costar) at a 1:250 dilution and a goat anti-rabbit peroxidase conjugate (1:10,000 dilution; Sigma) as primary and secondary antibodies, respectively. The blot was revealed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

**Analytical Methods—**UV-visible absorption spectrophotometry, EPR spectroscopy, and estimation of protein-bound iron were performed as described previously (16). For preparation of the nitroso complex of the dioxygenase, enzyme samples were made anaerobic under an argon phase, transferred into EPR tubes, and slowly bubbled with NO gas for 3 min.

**Measurement of Redox Potential—**Redox titration of *XylT* was performed spectrophotometrically using 5-deazaflavin as a photoreductant and benzyl-viologen as a dye indicator (5′-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.

**Purification of *XylE*—**Bacterial cells were rinsed with 25 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, pH 7.5. Bacteria were disrupted by sonication and centrifuged for 30 min at 100,000 × g, and the supernatant fraction containing *XylT* was adjusted to 1 M ammonium sulfate and then applied to a 40-ml DEAE-cellulose column (DE52). The XylT fraction was eluted with Tris-HCl, pH 9.0, and then diluted 10-fold in this buffer. After the reduction was determined using the BCA assay (Pierce).

**Immunoblotting**—Proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech). The membranes were blocked with 0.1% Tween 20 and then incubated with 1:15,000 × g x in a Beckman TL-100 ultracentrifuge. Samples of the high speed supernatant fractions, equivalent to 3.4 mg of protein, were incubated with immobilized anti-*XylT* IgG (20 or 40 μl) in a volume of 0.3 ml. Incubation was carried out at 4 °C in Spin-X centrifuge filter units (Costar) maintained under constant agitation (Eppendorf thermomixer). The beads of immobilized IgG-Sepharose were washed three times with 0.5 ml of 10 mM potassium phosphate, 0.15 M NaCl, 0.1% Triton X-100, pH 7.5. Bound proteins were then eluted by treatment for 5 min at 95 °C in 25 μl of SDS mixture. Samples of the eluted proteins were separated by electrophoresis on a 16% polyacrylamide gel containing 13% glycerol in a Tris-Tricine buffer system (14). Proteins were electroblotted on a nitrocellulose membrane as described previously (15), which was then developed with anti-*XylT* IgG (Costar) at a 1:250 dilution and a goat anti-rabbit peroxidase conjugate (1:10,000 dilution; Sigma) as primary and secondary antibodies, respectively. The blot was revealed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

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m potassium phosphate, pH 7.5, containing 1% isopropyl alcohol and incubated with 400 μM 4-methylcatechol for 30 min at room temperature. In control experiments, an equivalent amount of enzyme was treated similarly, except that 4-methylcatechol was omitted. After extensive dialysis twice against 1000 volumes of 0.1 m potassium phosphate, pH 7.5, containing 10% isopropyl alcohol, the enzyme samples were made anaerobic in 4-ml glass vials and kept under argon during reactivation.

Chemical reactivation of XyIE was carried out in the presence of 1 mM ferrous iron and 1 mM cysteine (17). XyI-dependent reactivation was performed under argon in 4-ml vials containing 50 mM inactivated XyIE, 1 mM 5-deazaflavin, 1 mM glycine, 0.1 mM potassium phosphate, pH 7.5, and a variable concentration of XyIT in a final volume of 200 μl.

Reaction vials were kept at 20 °C in a thermostatted water bath and were illuminated by the light of a slide projector for defined time periods. After light exposure, the samples were allowed to equilibrate for 30 min in the dark before measuring XyIE activity. Assays were done in triplicate on 10 μl of reaction mixture. Ferredoxins other than XyIT were tested under the same conditions.

For EPR measurements, XyIE was inactivated with 100 mM or 200 mM 4-methylcatechol, dialyzed extensively, and then concentrated to a final concentration ranging from 105 to 126 μM. Part of the preparation was subjected to reactivation for 5–10 min in the light in the presence of 10 mM glycine, 2.5 mM 5-deazaflavin, and XyIT, as indicated. Another part, used as a control, was incubated under identical conditions except that XyIT was omitted.

RESULTS

To facilitate isolation of the xylT gene product, its coding sequence was cloned in the expression vector pET9a under the transcriptional control of the strong T7 promoter. While verifying the DNA sequence of xylT, we found the first two bases of codon 76 to be inverted compared with the previously reported sequence (GC instead of CG) (9). This inversion would result in an alanine-to-glycine change in the deduced polypeptide sequence. We observed the same difference upon sequencing the xylT region of plasmid pAWS31. The new xylT sequence resembles more closely those of four homologous genes currently in the EMBL/DDJB/GenBankTM data base.

Expression of xylT in the E. coli strain BL21(DE3) gave rise to the production of a polypeptide of the expected size (approximately 15 kDa) that was visualized as a prominent band on SDS-PAGE with an apparent molecular mass of approximately 15 kDa. The molecular mass of the XylT polypeptide as deduced from its polynucleotide sequence is 12,034 Da. The molecular mass of the native XylT protein estimated by gel filtration was about 12 kDa, indicating that it is monomeric. The XylT polypeptide is characterized by a predominance of basic over acidic residues (9) and has a theoretical isoelectric point of 8.27 (Table I). Although this value was not confirmed experimentally, the basic character of XylT was consistent with its lack of binding to DEAE-cellulose in the 7–9 pH range and its retention on a strong anion exchange column only at pH 9.0 (Fig. 1, lane 2).

The XylT protein is relatively unstable due to its sensitivity to oxygen and temperature. The protein denatures when exposed to air, resulting in both a loss of its absorbance in the visible region and its precipitation. From the decrease in its absorbance at 416 nm, which followed first order kinetics, the half-life of XylT in air was estimated to be 69 min at ambient temperature. Based on the same criterion, XyIT appeared to be unstable at 37 °C (Table I), consistent with the finding that it was mainly present in apoform in E. coli grown at this temperature.

Spectroscopic Properties—The UV-visible absorption spectra of the oxidized and reduced forms of XyIT are presented in Fig. 2. Protein purified under reducing conditions spontaneously oxidized upon removal of dithionite by anaerobic gel filtration, probably because of residual oxygen present in the argon (approximately 200 ppm). The spectrum of oxidized XyIT exhibited three maxima due to the chromophore at 336, 416, and 456 nm, as well as a shoulder near 540 nm. The absorption of the polypeptide at 278 nm is not very pronounced, consistent with the absence of tryptophan and the low content of aromatic residues in XylT sequence. Upon reduction, a general decrease of the absorption in the visible region occurred, and a peak appeared at 540 nm (Fig. 2). Based on protein determination using the BCA assay, and given the molecular mass of XyIT (12 kDa), the absorption coefficient of the oxidized form was calculated to be 9.52 mM⁻¹cm⁻¹ at 416 nm. All of these properties are similar to those of other [2Fe-2S] ferredoxins (18). Determination of the iron content of XyIT indicates the presence of one [2Fe-2S] cluster per polypeptide chain (Table I).

EPR analysis of purified XyIT in the reduced form gave a signal centered at g = 1.94 with general rhombic symmetry that was detectable at temperatures as low as 40 K. This signal may be attributed to a [2Fe-2S] cluster with an S = 1/2 spin state (Fig. 3). Double integration of the EPR signal of spectrum II in Fig. 3 and comparison with a standard, i.e., the [2Fe-2S] ferredoxin from Rhodobacter capsulatus (19), gave a value of about 0.9 spin/mol of protein, thus providing further evidence that XyIT contains one [2Fe-2S] cluster.

The line shape of the EPR signal was, however, unusual for this type of cluster in that the resonance lines were relatively broad, and, as a consequence, the gυ component of the tensor

![Fig. 1. Purification of recombinant XyIT.](Image)
By gel filtration ($M_r$),
By SDS-PAGE ($M_r$),
From the sequence
Isoelectric point (theoretical)
Iron content (Fe atom/mol)
Midpoint redox potential
Absorption coefficient

A$_{416}$ (oxidized) 9.52 mm$^{-1}$·cm$^{-1}$
A$_{278}$ (oxidized) 12.5 mm$^{-1}$·cm$^{-1}$
Stability$^a$
In air at 25 °C 69
In air at 4 °C 3270
In air at 20 °C 372
In air at 30 °C 295
In air at 37 °C 70

$^a$ Expressed as half-life (min) under given conditions. Values were calculated from the kinetics of the absorbance decrease at 416 nm.

**FIG. 3.** EPR spectroscopic properties of XylT. Samples were reduced with excess dithionite (2 mM) in 50 mM Tris-HCl, pH 9. Spectrum I, Crude extract containing 45 µM XylT; spectrum II, 28 µM of purified XylT; spectrum III, 22 µM of purified XylT with 16% glycerol. EPR spectra were recorded at 10 K with the following conditions: microwave frequency, 9.220 GHz; modulation frequency, 100 kHz; modulation amplitude, 1 millitesla; microwave power, 0.1 milliwatt. Relevant $g$ values are indicated.

**TABLE I**

| Parameter                                | Value   |
|------------------------------------------|---------|
| Molecular mass                           | 12,000  |
| By gel filtration ($M_r$)                 | 12,034 Da |
| By SDS-PAGE ($M_r$)                       | 15,000  |
| From the sequence                         | 12,034 Da |
| Isoelectric point (theoretical)           | 8.27    |
| Iron content (Fe atom/mol)                | 1.88 ± 0.13 |
| Midpoint redox potential                  | −373 ± 6 mV |
| Absorption coefficient                    |         |
| $A_{416}$ (oxidized)                      | 9.52 mm$^{-1}$·cm$^{-1}$ |
| $A_{278}$ (oxidized)                      | 12.5 mm$^{-1}$·cm$^{-1}$ |
| Stability$^a$                             |         |
| In air at 25 °C                           | 69      |
| In air at 4 °C                            | 3270    |
| In air at 20 °C                           | 372     |
| In air at 30 °C                           | 295     |
| In air at 37 °C                           | 70      |

$^a$ Expressed as half-life (min) under given conditions. Values were calculated from the kinetics of the absorbance decrease at 416 nm.

**FIG. 2.** UV-visible absorbance of XylT. Spectra of XylT-50 µM in 50 mM Tris-HCl, pH 9.0, in the oxidized and reduced states were recorded on a Hewlett-Packard diode array spectrophotometer.

was not well resolved (Fig. 3, spectrum II). The broadening might reflect some microheterogeneity of the protein preparation in the vicinity of the Fe-S cluster. Considering the relative instability of XylT, the question arose whether the anomalies detected by EPR spectroscopy resulted from conformational changes in the protein during the purification. This hypothesis was assessed by recording EPR spectra in the E. coli soluble extract (Fig. 3, spectrum I) as well as at the subsequent steps of the purification (data not shown). The signal observed did show small differences compared with that of purified XylT (Fig. 3, spectrum II), indicating that XylT may have undergone minor changes during isolation. The EPR spectrum of the protein in the cell extract (Fig. 3, spectrum I) exhibited a greater apparent complexity, which was primarily due to the contribution of E. coli proteins. Interestingly, the addition of glycerol to purified XylT protein before freezing the EPR tube caused a significant change in the high field region of the spectrum (Fig. 3, spectrum III), reflecting a more pronounced rhombic symmetry with $g_{x,y,z} = 1.88, 1.94$, and 2.04. Hence, the EPR data give clear evidence that XylT can adopt at least two slightly different conformations at low temperature. It is unknown whether the heterogeneity detected in the frozen XylT sample reflects that of a sample at ambient temperature. The addition of glycerol to XylT had no detectable effect on the absorbance spectrum of the oxidized form. It is nevertheless plausible that the two signals detected by EPR with purified XylT (Fig. 3, spectra II and III) correspond to two conformations that could have been trapped upon freezing. These properties seem to denote an unusual flexibility of the protein around the cluster binding site.

To further characterize the [2Fe-2S] cluster of XylT, its redox potential was determined by spectrophotometric titration using benzyl-viologen as potential indicator. The calculated midpoint redox potential was $−373 ± 6$ mV at pH 8.5 and 25 °C (Fig. 4). The dioxygenase preparation thus obtained could be fully reactivated by subsequent incubation with ferrous iron and cysteine, conditions that are known to promote enzyme recovery in vitro (7, 17). The rate of chemical reactivation is rather slow and takes about 1 h for completion. A slow reactivation was also obtained by incubation of the inactive dioxygenase with 5-deazaflavin, which generates strong reductants when exposed to light. In contrast, incubation of the dioxygenase with both 5-deazaflavin and stoichiometric amounts of XylT promoted rapid reactivation of the enzyme (Fig. 5). The rate as well as the extent of the reactivation was dependent on the XylT concentration. In control experiments, it was found that the dioxygenase remained inactive when incubated with XylT in the dark or with heat-denatured XylT (Table II). These results indicate that the XylT-dependent reactivation did not merely result from substitution of the ferric iron in the active site of the enzyme by ferrous iron originating from the Fe-S cluster of XylT. Rather, the requirement of a source of reductant (the photoactivated deazaflavin) suggested that reactivation of the dioxygenase involved an electron transfer from XylT.
to the active site of the enzyme. When XylT was replaced by either spinach ferredoxin or a *R. capsulatus* ferredoxin (FdVI) at concentrations 10-fold higher than that of the dioxygenase, no significant reactivation was detected (data not shown). Spinach ferredoxin and *R. capsulatus* FdVI are representative members of two major subgroups of [2Fe-2S] ferredoxins, those found in plants, on the one hand (22), and those similar to adrenodoxin, on the other (23, 24). Since neither of the two ferredoxins was competent in reactivating XylE in *vivo*, it may be concluded that XylT catalyzes a specific reactivation.

**XylT-mediated Reduction of the Active Site of the Dioxygenase**—The experiments described above demonstrated that XylE reactivation requires a source of reductant and XylT as mediator, suggesting that enzyme recovery occurs through reduction of the iron atom in the catalytic site. To demonstrate that such a reduction takes place when the dioxygenase is reactivated by XylT, we have monitored the redox state of XylE in *vivo* by EPR spectroscopy. The active site of the native enzyme contains a high spin Fe(II), which is certainly due to contaminating Cu²⁺. The feature around *g* = 2 noted by an asterisk is due to free Fe(III). The feature around *g* = 2 noted by an asterisk is due to contaminating Cu²⁺.

Thus, the signal shown in Fig. 6 might denote the presence of a ferric iron with an *S* = ½ ground state (Fig. 6a). In control experiments, it was shown that the signal observed actually came from the enzyme-bound iron and not from a chelate of free iron with 4-methylcatechol or its product. The EPR signal of the 4-methylcatechol-inactivated dioxygenase was clearly distinct from the signal arising from a nonspecific oxidation of the active site iron, provoked, for example, by H₂O₂ treatment (H₂O₂-inactivated enzyme gave an EPR signal centered near *g* = 4.3; data not shown). Thus, the signal shown in Fig. 6a might denote the presence of a ligand molecule resulting from the oxidation of 4-methylcatechol remaining bound to the enzyme catalytic site, despite the extensive dialysis step that preceded the EPR analysis. A preparation of inactivated dioxygenase, having about 7% residual activity, was subjected to reactivation by XylT at a XylT/XylE molar ratio of 1:10. The specific activity of the enzyme increased up to 50% of the initial activity within 5 min. EPR analysis of the reactivated sample (Fig. 6b) revealed a complete disappearance of the enzyme-bound Fe(III) signal. This result suggests that XylT reactivates XylE through reduction of Fe(III) to Fe(II) in the enzyme active site. To assess this as-
FIG. 7. EPR analysis of the nitrosyl complexes of inactivated and reactivated forms of XylE. The two XylE samples (inactivated and reactivated) already analyzed as indicated in Fig. 6, were equilibrated with NO gas. Spectrum a, nitrosyl complex of inactivated XylE; spectrum b, nitrosyl complex of XylT-reactivated XylE; spectrum d, nitrosyl derivative of untreated XylE. Trace c was obtained by subtracting spectrum a from spectrum b after the two spectra were normalized to the same XylE concentration. Recording conditions were as in Fig. 6. Gain was 5-fold greater in a and b than in d.

First, we checked whether the extent of Fe(III) reduction as estimated by EPR analysis correlated with the extent of enzyme activity recovery. A sample of inactivated dioxygenase showing 14.3% residual activity (25 units/mg protein) was subjected to EPR analysis and spin quantitation. For this purpose, the low field component of the signal at \( g = 7.54 \) was integrated and compared with the corresponding signal of the beef liver catalase used as a reference (25). This estimation gave a minimal value of 0.38 Fe(III)/tetramer. By chemical assay, the enzyme sample was found to contain 0.75 Fe atom/mol, suggesting that part of the iron was in the reduced Fe(II) form. After reactivation, the enzyme activity reached 85 units/mg protein, which corresponds to reactivating about one-third of the inactivated enzyme molecules. Thus, there is a good correlation between the amount of Fe(III)-containing dioxygenase and the extent of XylT-mediated reactivation achieved. We noticed that XylE inactivation was always accompanied by a variable but significant loss of iron. For example, in the experiment described above, iron loss accounted for 56% of the initial iron content of the enzyme. Since the apoenzyme was not reactivated in the XylT-dependent reaction described in this study, it follows that only a fraction of the inactive dioxygenase can be reactivated in the in vitro system employed here.

Second, experiments were performed to monitor the reduction status of the iron atom at the dioxygenase active site upon XylT-dependent reactivation. Nitric oxide binds the Fe(II) atom of dioxygenase to form a nitrosyl complex with an electronic spin of \( S = \frac{1}{2} \) (26), a property that was exploited to monitor the changes in the nitrosyl-Fe(II) complex signal intensity upon reactivation of XylE. The same samples that gave the spectra shown in Fig. 6 as well as the unactivated enzyme were equilibrated with NO gas and analyzed by EPR (Fig. 7). The uninaivated enzyme sample exhibited a two-component signal with \( g_s = 4.16 \) and \( g_A = 3.91 \), hereafter referred as signal A (Fig. 7d). The enzyme inactivated with 4-methylcatechol gave a signal (Fig. 7a) composed of a signal similar to signal A and a minor signal with \( g_s = 4.11 \) and \( g_A = 3.99 \) (signal B). Several \( S = \frac{1}{2} \) species had already been observed for the NO complex of catechol 2,3-dioxygenase (26). Analysis of another inactivated enzyme sample, which had completely lost dioxygenase activity (residual activity <1%), yielded no detectable Fe(II)-NO signal, suggesting that the signal shown in Fig. 7a arose from the fraction of enzyme that was still active. After XylT-dependent reactivation, the signal of the nitrosyl complex globally increased in intensity (Fig. 7b) compared with that of the inactive sample, indicating that XylT mediated reduction of Fe(III) to Fe(II) at the enzyme active site. This result extends our observation that the Fe(III) signal of the inactivated enzyme disappeared upon reactivation and provides further evidence that enzyme reactivation occurred through reduction of the iron atom at the active site. The calculated difference in intensity of spectrum a and spectrum b, given as trace c in Fig. 7, may be seen as the superposition of three signals: a major signal B, a minor signal A, and a new signal (signal C) with \( g_s = 4.17 \) and \( g_A = 3.88 \). Some of the signals we observed might result from the binding at the XylE active site of a ligand resulting from oxidation of 4-methylcatechol. Existence of such a ligand was already suggested by the analysis of the Fe(III) signal of the inactive enzyme (Fig. 6a).

In addition, we have considered the possibility that XylT might reactivate XylE by transferring Fe(II) atoms, instead of electrons, to the inactive enzyme. We have measured the EPR signal intensity of the dithionite-reduced XylT cluster before and after XylE reactivation and found it to be unaltered by the XylE reactivation procedure. This rules out the possibility that the ferredoxin functions by transferring iron atoms to the enzyme active site.

Estimation of XylT Content of \( P. \) putida mt2—Preliminary attempts to directly detect XylT in crude extracts of \( P. \) putida mt2 by Western blot analysis were unsuccessful, because the sensitivity of the method (about 5 ng/lane on a polyacrylamide slab gel) was not adequate to visualize the very low level of XylT in the extract. It was therefore necessary to specifically concentrate XylT by immunoaffinity capture from the soluble protein fraction on immobilized anti-XylT IgG (see “Experimental Procedures”), followed by Western blot analysis. Material recovered in this way from 3.4 mg of soluble protein from \( P. \) putida mt2 gave a detectable XylT-specific signal (Fig. 8, lanes 4 and 5). No signal was observed in the case of the control strain KT 2442, which lacks the pWWO plasmid. The level of the XylT protein in \( P. \) putida mt2 was tentatively estimated from the signal intensity observed on the Western blot. Given that about 15 mg of XylT was recovered from a sample containing 0.7 mg of soluble protein (Fig. 7, lane 5), XylT would account for 0.0021% of the soluble protein. There was also interest to determine the XylT/XylE molar ratio in \( P. \) putida protein extracts. The amount of XylE was estimated indirectly from activity assays, based on the assumption of an average specific activity of 300 units/mg for the purified enzyme (26). The XylE activity in the \( P. \) putida soluble extract was 0.85 ± 0.09 units/mg (\( n = 3 \)), from which we calculated a XylT/XylE ratio.

FIG. 8. Immunodetection of XylT in \( P. \) putida cell extracts. Cell extracts were prepared from benzoate-grown \( P. \) putida bacteria by ultrasonication and subsequent high speed centrifugation, and XylT in the extracts was captured with immobilized anti-XylT IgG as described under “Experimental Procedures.” Affinity-concentrated material from 0.7 mg of soluble protein was analyzed by Western blotting. Lanes 1–3, purified XylT (50, 20, and 10 ng, respectively); lane 4, \( P. \) putida mt2 proteins captured with 20 \( \mu \)l of immobilized IgG; lane 5, mt2 proteins captured with 40 \( \mu \)l of immobilized IgG; lane 6, KT 2442 proteins captured with 20 \( \mu \)l of immobilized IgG; lane 7, 50 ng of purified XylT.
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ratio of 0.091. This figure should be considered only approximately, since both values are minimum estimates; on the one hand, the yield of XylT capture by immunoaffinity may be less than 100%, on the other hand, not all of the Xyle molecules in the P. putida extract may be fully active.

**DISCUSSION**

Hyperexpression of the xylT gene of the TOL plasmid pWW0 in the heterologous E. coli host allowed us to purify and characterize the XylT protein as a novel [2Fe-2S] ferredoxin. Based on UV-visible absorbance and EPR spectroscopy, XylT exhibited properties grossly similar to plant-type ferredoxins (27). These similarities reflect the common nature of their prothetic group, a [2Fe-2S] cluster with rhombic EPR signal. As revealed by resolution of the crystal structure of model plant-type ferredoxins, the cluster is bound to the polypeptide chain through four cysteinyl ligands, which have a defined and conserved spacing in the sequence (28, 29). The XylT sequence contains six cysteines, four of which, located at positions 41, 46, 49, and 51, conform to the conserved spacing and would therefore serve as potential ligands of the cluster (9). However, the possibility that cysteine 36 serves as an alternative ligand cannot a priori be ruled out.

On the other hand, XylT differs from other [2Fe-2S] ferredoxins in many respects. Its overall amino acid sequence shows little similarity with known ferredoxins. A unique feature of the XylT polypeptide is the predominance of basic over acidic residues, which confers on the protein a net positive charge at neutral pH. This is the first known example of a ferredoxin with a basic character, and this property might be related to its specific function. We have shown that representative members of the two major classes of [2Fe-2S] ferredoxins failed to substitute for XylT in Xyle reactivation. In the case of R. capsulatus FdVI, an analogue of the Pseudomonas putidaredoxin (24), the high value of its midpoint redox potential ($E_0^{'} = -255$ mV)$^2$ compared with that of XylT could explain its lack of activity. On the other hand, the $E_0'$ value of the spinach ferredoxin ($-420$ mV; Ref. 30) is close to that of XylT and thus cannot account for its absence of reactivity toward Xyle. This plant ferredoxin is, however, known for its very acidic isoelectric point (pI = 5.4) and a highly negative net charge, which might hinder productive interaction with Xyle. It may thus be inferred that the positive net charge of the XylT protein is a prerequisite for its molecular interaction with Xyle. This assumption is currently being evaluated through cross-linking experiments between XylT and Xyle. The unusual basic character of XylT, and its specificity with respect to Xyle reactivation, could also explain why in P. putida, which most likely contains other ferredoxins, XylT is so essential for maintenance of Xyle activity and hence bacterial growth on para-substituted derivatives of toluene (8).

XylT exhibits a number of other distinctive properties. Although its cluster resembles that of plant-type ferredoxins, in that it gives a rhombic EPR signal, XylT absorbance in the visible region of the spectrum is closer to that of adrenodoxin (31) and putidaredoxin (32), which instead yield a nearly axial EPR signal (33). The EPR analysis revealed that XylT has intermediate properties, which might reflect greater flexibility of the protein around the cluster. In addition, the very strong effect of glycerol on the EPR signal suggests that the [2Fe-2S] cluster is exposed to the solvent. In accord with this assumption, and in contrast to most [2Fe-2S] ferredoxins, XylT is a relatively unstable protein exhibiting a considerable propensity to lose its cluster, especially when exposed to air. This property might appear somewhat paradoxical for a protein involved in the repair of an enzyme that has sustained oxidative damage. However, the intracellular microenvironment of aerobic microorganisms is characterized by a fairly low oxygen tension due to the membrane-borne respiratory chain, and bacterial cells might thus afford conditions conducive to XylT stability. In this respect, aerobically grown E. coli bacteria turned out to be a convenient system for the biosynthesis of XylT and its purification. The protein yield, about 1 mg/liter of culture, was, however, relatively low compared with the heterologous expression of more stable [2Fe-2S] ferredoxins like human ferredoxin (34), Anabaena ferredoxin (35), R. capsulatus FdIV and FdV (19, 36), or FdxI from Sphingomonas sp. RW1 (37).

XylT is also novel in its function. We have demonstrated that XylT specifically mediates the reduction of the active site iron in inactive catechol 2,3-dioxygenase, which results in the reactivation of the enzyme. This result confirms and extends the prediction made by Polissi and Harayama (8) on XylT function, based on the characterization of xylT deletion mutants.

In the majority of cases, ferredoxins function as electron carriers in association with oxidoreductases and participate in this way in the oxidation or reduction of various substrates or metabolites (27). XylT therefore represents a rare example of a ferredoxin that does not provide electrons for the reduction of a substrate but rather for the reactivation of an enzyme. An analogous case of ferredoxin-mediated enzyme protection has been described for Azotobacter vinelandii (38, 39). A [2Fe-2S] ferredoxin in this bacterium was found to confer protection against oxygen-mediated inactivation upon nitrogenase. Although the mechanism of this protection is not known in detail, it was shown that A. vinelandii [2Fe-2S] ferredoxin forms a tight complex with nitrogenase that is more oxygen-stable than the free enzyme (39). Hence, in contrast to XylT, the A. vinelandii ferredoxin does not reanimate an enzyme (nitrogenase) but rather prevents its inactivation. XylT therefore appears to be the first ferredoxin reported to catalyze the reactivation of an enzyme in a reaction that shows a relatively narrow specificity. It may thus be considered to be an activase.

The extent of XylT-dependent reactivation of various preparations of Xyle was somewhat variable. This is assumed to reflect the variable degree of irreversible inactivation of different enzyme preparations through loss of iron. We indeed showed that the relatively harsh conditions employed to obtain inactivation in vitro caused release of iron from a substantial proportion of enzyme molecules. We also showed that XylT can only reanimate the fraction of inactive enzyme that retains iron. Another plausible explanation of variable dioxygenase inactivation would be a tight binding of the product or an intermediate of 4-methylecatechol oxidation at the enzyme active site, which might hinder reactivation by XylT.

Evidence that XylT acts as the Xyle-specific activating protein in the natural host is provided by immunochemical demonstration of the XylT product in P. putida extracts. The level detected was extremely low. However, given that catalytic amounts of XylT were sufficient to reanimate Xyle in vitro, it seems reasonable to predict that XylT should efficiently reanimate Xyle in vivo. It remains to be seen whether increasing the in vivo level of XylT, by hyperexpression of its structural gene, might improve the biodegradation of toluene or analogous molecules by P. putida.

Since the discovery of the xylT gene (9), a number of related genes in operons encoding biodegradation of aromatic compounds like naphthalene (nahT; Ref. 40), phenol (phhQ; Ref. 41), substituted phenols (dmpQ; Ref. 42), and toluene (thbW; Ref. 43) have been identified. The putative polypeptide sequences deduced from the genes have comparable sizes (101–

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113 residues) and share a high degree of similarity with XylT. In particular, the XyIT analogues are predicted to contain the four conserved cysteines that are potential ligands of the [2Fe-2S] cluster, as well as two additional conserved cysteines at positions equivalent to Cys\(^{19}\) and Cys\(^{36}\) in XylT. This suggests that the latter two cysteines might form a disulfide bond. In addition, their sequences indicate that the XyIT analogues have basic isoelectric points, located between 7.5 and 8.4. This information, combined with the fact that the \(\text{xyIT}\) gene homologues are located upstream of extradiol dioxygenase-encoding genes, indicates that these analogues have a similar function. The XyIT-like proteins appear as a new family of ferredoxins that might have practical relevance if they can be used to engineer microorganisms with improved capabilities in bioremediation.

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