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Biotransformation of Explosives by the Old Yellow Enzyme Family of Flavoproteins

Richard E. Williams,[1]† Deborah A. Rathbone,[1]‡ Nigel S. Scrutton,[2] and Neil C. Bruce[1]*

Institute of Biotechnology, University of Cambridge, Cambridge CB2 1QT,[1] and Department of Biochemistry, University of Leicester, Leicester LE1 7RH,[2] United Kingdom

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Several independent studies of bacterial degradation of nitrate ester explosives have demonstrated the involvement of flavin-dependent oxidoreductases related to the old yellow enzyme (OYE) of yeast. Some of these enzymes also transform the nitroaromatic explosive 2,4,6-trinitrotoluene (TNT). In this work, catalytic capabilities of five members of the OYE family were compared, with a view to correlating structure and function. The activity profiles of the five enzymes differed substantially; no one compound proved to be a good substrate for all five enzymes. TNT is reduced, albeit slowly, by all five enzymes. The nature of the transformation products differed, with three of the five enzymes yielding products indicative of reduction of the aromatic ring. Our findings suggest two distinct pathways of TNT transformation, with the initial reduction of TNT being the key point of difference between the enzymes. Characterization of an active site mutant of one of the enzymes suggests a structural basis for this difference.

Large expanses of land and groundwater have been polluted through the manufacture, deployment, and disposal of explosives. Energetic compounds such as glycerol trinitrate (GTN), pentaerythritol tetranitrate (PETN), and 2,4,6-trinitrotoluene (TNT) are unique to nature, and their recalcitrance to biodegradation means they tend to persist in the environment. It is of significant interest to understand how certain microorganisms have evolved and adapted to utilize these toxic compounds as nitrogen sources for growth. Not surprisingly the enzymes mediating the microbial degradation of explosives have been the focus of much research for the remediation of contaminated land and groundwater.

A number of bacterial strains able to degrade the nitrate ester explosives GTN and PETN have been isolated from contaminated soil (5, 7, 34, 36). In each of these strains, a nicotinamide cofactor-dependent oxidoreductase catalyzes the reductive cleavage of the nitrate ester group to yield an alcohol and nitrite (6, 15, 29). The enzymes have similar amino acid sequences, and all contain flavin mononucleotide as a noncovalently bound cofactor. It has become apparent that some of these enzymes are also able to reduce TNT. Many flavoenzymes catalyze the reduction of nitro groups (8), and the products of TNT reduction by nitrate ester reductases include hydroxylamino-dinitrotoluenes (HADNTs) and amino-dinitrotoluenes (ADNTs). However, in addition, both PETN reductase from *Enterobacter cloacae* PB2 (14) and xenobiotic reductase B from *Pseudomonas fluorescens* I-C (25) catalyze reduction of the TNT aromatic ring by hydride addition, yielding hydride- and dihydride-Meisenheimer complexes (compounds 2 to 6, Fig. 1) and nitrite. Most current attempts at TNT bioremediation focus on nitro group reduction, where some of the resulting products bind tightly to soil (1, 2); unfortunately, it is difficult to demonstrate the long-term fate of such immobilized TNT derivatives. Nonaromatic transformation products, such as hydride- and dihydride-Meisenheimer complexes, have previously been reported to be endpoints of TNT biotransformation (25, 31), but offer a potential route to ring fission and eventual mineralization of TNT.

The nitrate ester reductases described to date have similar amino acid sequences (6, 15, 29) and are members of a family of enzymes related to yeast old yellow enzyme (OYE) (35). Genomic sequencing suggests that similar enzymes are present in a wide range of bacteria, yeast, and plants. The high degree of conservation of regions of primary and tertiary structure across the family would suggest that the enzymes are orthologous. If this is the case, the conserved functional role is still to be discovered.

In this study, we aim to determine whether other members of the OYE family share the catalytic activities reported for nitrate ester reductases. We compared the properties of PETN reductase from *E. cloacae* PB2 (14) with those of four related enzymes from the OYE family, focusing in particular on transformation of the explosives PETN, GTN, and TNT. By contrasting the behaviors of members of the family, we aim to better understand the mechanism of TNT transformation via hydride- and dihydride-Meisenheimer complex formation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Chemicals. TNT, PETN, and GTN were gifts of the Defence Science and Technology Laboratory of the Ministry of Defence and were used as supplied. Authentic standards of TNT, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2-hydroxylamino-4,6-dinitrotoluene, and 4-hydroxylamino-2,6-dinitrotoluene (Promochem), 2,4-dinitrotoluene and 2,6-dinitrotoluene (Aldrich) were used during analytical method development and for quantitation.

Cloning of PETN reductase from the type strain of *E. cloacae*. The type strain of *E. cloacae* (ATCC 13047/NCIMB10101) was obtained from NCIMB, Ltd., Aberdeen, United Kingdom. Southern hybridization of genomic DNA restriction

* Corresponding author. Present address: CNAP, University of York, P.O. Box 373, York YO10 5YW, United Kingdom. Phone: 44 1904 328777. Fax: 44 1904 328801. E-mail: ncb5@york.ac.uk.

† Present address: MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom.

‡ Present address: CNAP, University of York, York YO10 5YW, United Kingdom.

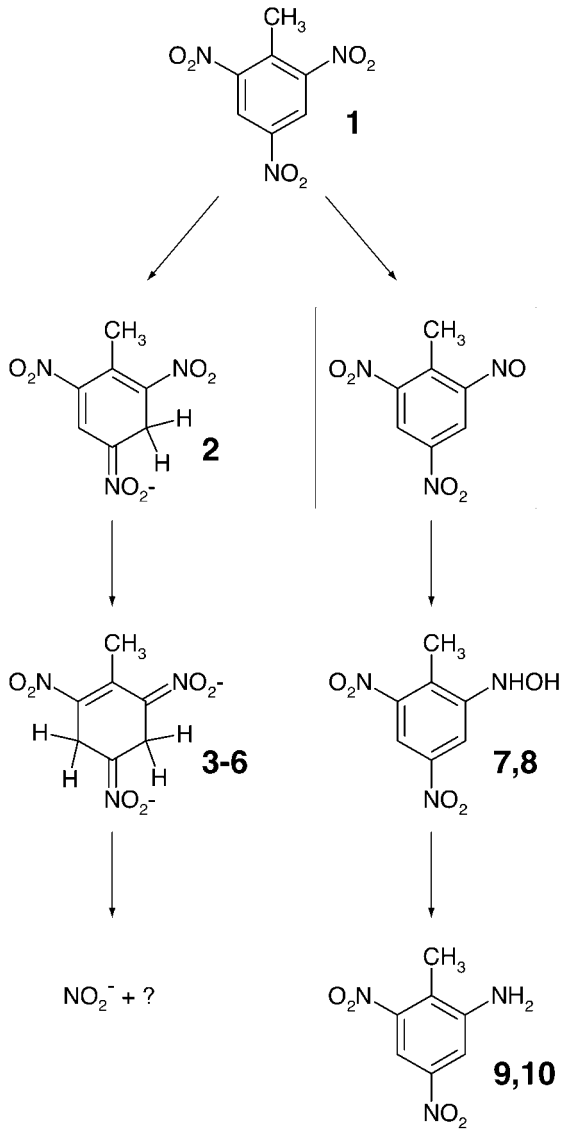

FIG. 1. Transformation of TNT by PETN reductase. Each step requires the oxidation of one equivalent of cofactor.

fragments was performed by standard methods (28), using a 950-bp EcoRV-HindIII fragment of the *E. cloacae* PB2 *onr* gene as a probe, and an ECL enhanced chemiluminescence random prime labeling and detection kit (Amersham Pharmacia). A 1,386-bp PstI-ClaI fragment of type strain *E. cloacae* genomic DNA, containing the entire *onr* gene, was cloned downstream of the *lac* promoter in pBluescript II-KS(+) (Stratagene).

Cloning of NEM reductase from *Escherichia coli* JM109. *N*-Ethylmaleimide (NEM) reductase was cloned from *E. coli* JM109 by using PCR primers based on the published gene sequence from *E. coli* W3110 (23). The PCR product was cloned into pCR2.1TOPO (Invitrogen) for sequencing. From this construct, the *nemA* gene was subcloned into pET11d (Novagen).

Expression and purification of enzymes. PB2 PETN reductase, type strain PETN reductase, and morphinone reductase were expressed in *E. coli* JM109, using SOB medium (28) supplemented with 50-μg/ml carbenicillin and 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), at 37°C overnight. NEM reductase and OYE were expressed in *E. coli* BLR(DE3)pLysS using Luria-Bertani (LB) medium supplemented with 50-μg/ml carbenicillin. Cells were grown at 37°C to early log phase (optical density at 600 nm of around 0.4), and then IPTG was added to a final concentration of 0.4 mM, and the incubation continued at 30°C for 3 h. Enzymes were purified by a succession of anion-exchange, Mimetic Orange 2 affinity, and high-resolution anion-exchange column chromatography steps, according to a modification of the protocol described previously for PB2 PETN reductase (15).

Substrate and cofactor preferences. Enzyme activity was assayed spectrophotometrically by monitoring the oxidation of NAD(P)H at 340 nm. Standard assay conditions were 100 μM substrate and 100 μM cofactor in 50 mM potassium phosphate buffer (pH 7.0) at 30°C, with 1 U defined as 1 μmol of cofactor oxidized per minute. Kinetic parameters were estimated by nonlinear regression to the Michaelis-Menten equation, using the GraFit software package (Erithacus Software, Staines, United Kingdom). The enzyme concentration was determined spectrophotometrically, using the flavin A_{462}. Extinction coefficients of proteins were calculated by quantitating the flavin mononucleotide released upon protein precipitation with trichloroacetic acid (21).

Transformation of TNT. All reactions were performed in 50 mM potassium phosphate buffer (pH 7.0) at 30°C. Reactions in time course experiments were performed in 2-ml high-performance liquid chromatography (HPLC) vials, in the thermostatically regulated autosampler chamber of a Waters Alliance HPLC system. Aliquots of 20 μl were automatically removed and analyzed every 20 min. Where stated, cofactors were continuously re-reduced by including 5-U/ml *Thermoanaerobium brockii* $NADP^+$-dependent secondary alcohol dehydrogenase (Sigma) and 2.5% (vol/vol) isopropanol in the reaction mixture. For reactions involving morphinone reductase, 20-U/ml yeast NAD^+-dependent primary alcohol dehydrogenase (Sigma) and 1% (vol/vol) ethanol were substituted.

Analytical techniques. HPLC separation was performed with a C_{18} column and a mobile phase of 50% acetonitrile and 50% 20 mM tetrabutylammonium phosphate (pH 7.0) in a modification of a method previously described (14). Retention times of analytes observed under these conditions are listed in Table 4. Concentrations of analytes were determined by integration of extracted chromatograms at 230 and 500 nm. Nitrite was determined by a modification of the

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli JM109	General cloning and expression strain	Stratagene
E. coli BLR(DE3)pLysS	Permits overexpression from T7 promoter	Novagen
E. cloacae NCIMB10101	Type strain of *E. cloacae* (ATCC 13047)	NCIMB, Aberdeen, United Kingdom
Plasmids		
pONR1	*onr* gene encoding *E. cloacae* PB2 PETN reductase downstream of *lac* promoter	15
pNONR2	*onr* gene encoding type strain *E. cloacae* PETN reductase downstream of *lac* promoter	This study
pET11d-*nem*A	*nemA* gene encoding *E. coli* NEM reductase (23) downstream of T7 promoter	This study
pMORB3	*morB* gene encoding *Pseudomonas putida* M10 morphinone reductase downstream of native promoter	13
pT7-OYE	OYE1 gene encoding *S. carlsbergensis* OYE (27), downstream of T7 promoter	Gift of Vincent Massey
pONR14	H184N mutant of *onr* gene encoding *E. cloacae* PB2 PETN reductase downstream of *lac* promoter	This study

TABLE 2. Cofactor preference of the five OYE family enzymes examined in this study

Cofactor	Sp act vs *trans*-2-hexenal (U mg^{-1})[a]				
	PB2 PETN reductase	Type strain PETN reductase	NEM reductase	Morphinone reductase[b]	OYE
NADPH	1.8 ± 0.0	2.6 ± 0.1	3.5 ± 0.1	0.1 ± 0.0	5.8 ± 0.2
NADH	0.3 ± 0.0	1.2 ± 0.1	1.5 ± 0.1	3.7 ± 0.1	0.6 ± 0.0
NADPH/NADH ratio	6	2	2	0.02	10

[a] The initial concentration of cofactor and substrate was 100 μM. Results are the mean ± standard error of three replicates. Background oxidase rate was not subtracted.
[b] For morphinone reductase, codeinone replaced *trans*-2-hexenal.

Griess method as described previously (5). Sodium nitrite was used as a standard, over the concentration range 0 to 100 μM.

Synthesis and purification of hydride adduct of TNT. TNT (50 mg) was reduced with sodium borohydride as previously described (30). The resulting mixture of TNT and H^--TNT was separated on a C_{18} solid-phase extraction column (Supelclean LC18; Supelco). H^--TNT was eluted with 1:1 (vol/vol) acetonitrile-water and stored at −20°C.

Site-directed mutagenesis of PB2 PETN reductase. An expression plasmid, pONR14, for the H184N mutant PB2 PETN reductase PB2 PETN reductase was produced from pONR1 by two-step PCR-based site-directed mutagenesis (16). Mutagenic oligonucleotide primers H184NF (5′-GGTTGAGCTTCACTCTGC GAACGGTTACC-3′) and H184NR (5′-GGTAACCGTTCGCAGAGTGAAG CTCAACC-3′) were paired with M13 forward and reverse primers. The success of mutagenesis was confirmed by DNA sequencing. The H184N mutant of PB2 PETN reductase was expressed and purified as described for the wild-type protein above.

Nucleotide sequence accession number. The nucleic acid sequence of the 1,386-bp PstI-ClaI fragment of type strain *E. cloacae* genomic DNA has been deposited in GenBank under accession no. AY388474.

RESULTS

PETN reductase from the type strain of *E. cloacae* degrades nitrate esters. Nitrate ester explosives have generally been regarded as environmentally recalcitrant, suggesting that most microbes lack the ability to degrade them. It is therefore possible that where isolates such as *E. cloacae* PB2 are found to be effective at nitrate ester cleavage, a specific adaptation has occurred. The type strain of *E. cloacae* (ATCC 13047) is from a clinical source and is not expected to have encountered nitrate ester compounds in any large concentration in the past. We have cloned and sequenced a gene from *E. cloacae* ATCC 13047 that encodes a homolog of PETN reductase from *E. cloacae* PB2. There are 15 amino acid differences between the enzymes from the two strains, the majority of which are conservative. The kinetics of GTN reduction by the purified type strain enzyme (apparent V_{max} = 43 ± 2 U mg^{-1}, apparent K_m = 50 ± 6 μM) are not significantly different from those reported for PB2 PETN reductase (apparent V_{max} = 12.1 U mg^{-1}, apparent K_m = 23 μM) (15).

Overlap of catalytic activities in the OYE family. For comparison with the *E. cloacae* enzymes, we selected three representative members of the OYE family: *E. coli* NEM reductase (23), *Pseudomonas putida* M10 morphinone reductase (13), and *Saccharomyces carlsbergensis* OYE (27). The pairwise amino acid identity across the five proteins ranges from 96 to 32%. The cofactor preferences of the five enzymes were assayed (Table 2). For all enzymes except morphinone reductase, NADPH was the most effective reductant, while morphinone reductase showed a strong preference for NADH. We then compared specific activities of the five enzymes towards a range of potential substrates (Fig. 2). In choosing compounds to assay, we included known substrates of PETN reductase (PETN, GTN, and TNT), morphinone reductase (codeinone), and OYE (cyclohexenone and *trans*-2-hexenal) together with two compounds related to TNT (1-nitrocyclohexene and 2-nitrobenzaldehyde). The specific activities observed for these substrates are shown in Table 3. Members of the OYE family catalyze a slow oxidation of nicotinamide cofactor in the absence of substrate—this background oxidase activity (0.4 to 1.6 U mg^{-1}) was determined and subtracted from the specific activities shown.

There is a considerable degree of overlap in the catalytic activities of the five enzymes, but there is no one substrate that is optimal for all enzymes. The *E. cloacae* PETN reductases and *E. coli* NEM reductase showed broadly similar activity profiles, with high activity against nitrate esters, reasonable activity towards α/β-unsaturated carbonyls, and transformation of TNT to yield orange products. In contrast, OYE and morphinone reductase show far less activity towards nitrate esters, and their transformation of TNT does not result in orange products. The substrate profile of morphinone reductase is particularly different from that of the other four enzymes, with only codeinone being a good substrate for the enzyme. None of the other enzymes showed any activity towards this compound under the assay conditions.

Transformation of TNT by members of the OYE family. All of the enzymes studied showed some activity towards TNT, although the nature of the products varied. As noted in Table 3, reduction of TNT by the *E. cloacae* and *E. coli* enzymes results in the formation of an orange product. This is not seen in the reactions catalyzed by morphinone reductase or OYE and suggests that reduction of TNT to form dihydride adducts ($2H^-$-TNT, compounds 3 to 6 of Fig. 1) is restricted to close relatives of PB2 PETN reductase. We therefore undertook a detailed comparison of the products formed during reduction of TNT by the five enzymes, with a view to understanding the molecular basis for the apparent difference between the enzymes. The reduction of TNT catalyzed by the five enzymes was followed over a time course. We wished to avoid acid quenching or extraction with solvent when taking time point samples, as neither of these procedures is ideally suited to the analysis of potentially unstable anionic species. We therefore incubated reactions in HPLC sample vials and monitored the reaction by repeated sampling and analysis by ion-paired HPLC (14, 31) in real time. Several equivalents of reduced cofactor are required for complete reduction of TNT. The reaction rate is extremely slow under the conditions used for

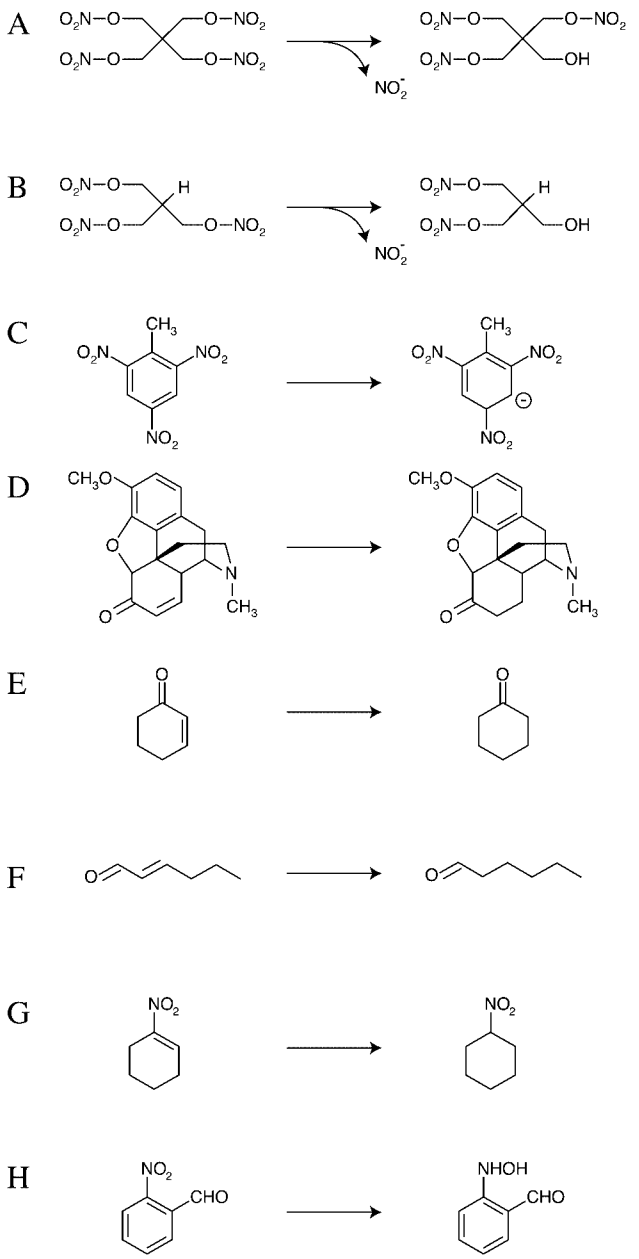

FIG. 2. Reactions catalyzed by enzymes from the OYE family. (A) Reductive denitration of PETN. (B) Reductive denitration of GTN. (C) Reduction of TNT (full pathway shown in Fig. 1). (D) Reduction of codeinone. (E) Reduction of cyclohexenone. (F) Reduction of *trans*-2-hexenal. (G) Reduction of 1-nitrocyclohexene. (H) Reduction of 2-nitrobenzaldehyde.

comparison of the five enzymes above (Table 3), being broadly comparable with the background oxidase rate of the enzymes. A cofactor recycling system was therefore added to the reaction mixtures to maintain a steady NAD(P)H concentration close to the initial value.

Concentrations of the metabolites listed in Table 4 were determined every 20 min for a total of 500 min. Under the reaction conditions used, TNT was completely consumed within 1 h. Time courses of TNT transformation by PB2 PETN reductase and OYE are shown in Fig. 3. Data from transformations with type strain PETN reductase and NEM reductase (not shown) were similar to those observed with PB2 PETN reductase, and data from transformations with morphinone reductase (not shown) were similar to those observed with OYE.

In all enzyme reactions, HADNTs (compounds 7/8) are produced rapidly and then consumed more slowly. During HADNT disappearance, the concentration of ADNTs (compounds 9/10) increases, but never exceeded 4% of the initial TNT concentration. A second, more polar product also appeared as the peak assigned to HADNT decreased. This peak was poorly resolved from NADPH and so proved impossible to quantitate reliably under the HPLC conditions employed. The UV/visible spectrum of this peak showed maxima at 217 and 340 nm and a shoulder at 250 nm. The spectrum and the short retention time are consistent with reported characteristics of 2,4-dihydroxylamino-6-nitrotoluene (32).

The maximum HADNT concentration in the *E. cloacae* and *E. coli* enzyme reactions is less than half of that observed during TNT transformation by OYE and morphinone reductase. A peak consistent with reported characteristics (19) of H^--TNT (compound 2) is seen transiently in the first samples of the time course. Four compounds, with spectra characteristic of dihydride adducts of TNT ($2H^-$-TNT), then appear and, more slowly, disappear. It was not possible to conclusively identify the precise nature of these species. Two forms of visible spectrum are observed, with the two HPLC peaks with shorter retention times, compounds 3 and 4, showing a broader absorbance peak than compounds 5 and 6. This suggests a difference in the conjugated dinitro system responsible for long-wavelength absorption. The peaks also differ in their UV absorption maxima, which could indicate differences in the isolated saturated nitro group.

Only traces of compounds 3 to 6 were by formed by OYE, and none were visible in incubations with morphinone reductase. The disappearance of compounds 3 to 6 was not accompanied by the appearance of any other metabolite detectable under the HPLC conditions used. The concentration of nitrite present in each transformation mixture at the end of each time course was assayed (Table 5). Only the *E. cloacae* and *E. coli* enzymes produced significant quantities of nitrite from TNT.

Nitroso and hydroxylamino metabolites of TNT have the potential to polymerize to yield azoxy dimers (32, 33). In addition, the possibility of abiotic condensation of $2H^-$-TNT isomers (compounds 3 to 6) and HADNTs (compounds 7/8) has been suggested (25). All these dimeric metabolites are strongly retained during reversed-phase HPLC.

The transformation of TNT by PB2 PETN reductase shown in Fig. 3 was repeated with a 10-fold-higher concentration of enzyme (data not shown). Under these conditions, the dihydride-TNT adducts (compounds 3 to 6) were consumed more rapidly and had been completely consumed by the end of the incubation. No long retention time peaks indicative of dimeric products were observed. Thus abiotic condensation is unlikely to play a significant role in the disappearance of compounds 3 to 6 during the transformation of TNT by PB2 PETN reductase under the reaction conditions used in our time course studies.

TABLE 3. Substrate preference of the five OYE family enzymes examined in this study

Substrate	Sp act (U mg^{-1})[a]				
	PB2 PETN reductase	Type strain PETN reductase	NEM reductase	Morphinone reductase[b]	OYE
PETN	21.4 ± 1.9	22.7 ± 3.0	31.7 ± 1.1	−0.3 ± 0.1	−0.5 ± 0.1
GTN	19.8 ± 0.2	35.5 ± 3.1	37.9 ± 5.3	−0.3 ± 0.0	0.5 ± 0.1
TNT	1.4 ± 0.3[c]	0.4 ± 0.1[c]	0.0 ± 0.3[c]	1.0 ± 0.1	3.2 ± 0.2
Codeinone	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.1	13.8 ± 0.9	−0.1 ± 0.1
2-Cyclohexenone	0.8 ± 0.1	2.7 ± 0.1	4.4 ± 0.4	−0.4 ± 0.1	5.1 ± 0.2
trans-2-Hexenal	1.5 ± 0.1	2.2 ± 0.1	2.7 ± 0.2	−0.2 ± 0.1	4.2 ± 0.2
1-Nitrocyclohexene	1.5 ± 0.1	7.5 ± 0.2	8.5 ± 0.1	0.5 ± 0.1	5.0 ± 0.3
2-Nitrobenzaldehyde	2.0 ± 0.1	5.2 ± 0.3	5.2 ± 0.5	0.5 ± 0.1	3.1 ± 0.2

[a] The initial concentration of both NADPH and substrate was 100 μM. Results are the mean ± standard error of three replicates. Background oxidase rate was determined and subtracted.
[b] For morphinone reductase, NADH replaced NADPH.
[c] Orange products observed.

Transformation of hydride adduct of TNT. Chemically synthesized H$^-$-TNT (compound 2) was used as a substrate for enzyme-catalyzed transformation. Both PB2 PETN reductase and OYE reduced H$^-$-TNT to form 2H$^-$-TNT isomers, which were then consumed to form unknown products (Fig. 4). No formation of products such as HADNTs or ADNTs was observed. In the case of PB2 PETN reductase, the time course of 2H$^-$-TNT formation from H$^-$-TNT and subsequent transformation (Fig. 4A) appears effectively identical to that observed with TNT as the initial substrate (Fig. 3C). OYE transforms H$^-$-TNT to 2H$^-$-TNT isomers more slowly than PB2 PETN reductase, and the balance of isomers formed is markedly different. Despite this, at the end of the transformations, roughly equal concentrations of nitrite were observed (data not shown).

Role of His 184. Comparison of the functional properties of five OYE family members identified clear contrasts between enzymes. Correlation of these differences with differences in the structures of OYE (12), morphinone reductase (3), and PB2 PETN reductase (4) suggested that two key substrate-binding residues might determine the behavior of the enzymes. A histidine residue at position 181 (of PETN reductase) is strongly conserved across the OYE family, while residue 184 varies between histidine and asparagine. In the *E. cloacae* and *E. coli* enzymes, this residue is histidine, while in OYE and morphinone reductase, the residue is asparagine. To explore the role of this residue in catalysis, we constructed the H184N mutant of PB2 PETN reductase.

Specific activities of the H184N mutant of PETN reductase were lower than those of the wild-type enzyme for all substrates (Table 6). TNT transformation by the H184N mutant was monitored under the same conditions used for the five enzymes above (Fig. 5). The activity of the H184N mutant towards TNT was low, with less than half the initial TNT concentration consumed after 600 min with an enzyme concentration of 0.4 μM. In an incubation with the same concentration of wild-type PETN reductase, all TNT was removed within 30 min (data not shown). The H184N mutant produced only trace levels of hydride addition products, even at the higher of the two enzyme concentrations tested. However, as with OYE, pure H$^-$-TNT is transformed by the H184N mutant to produce 2H$^-$-TNT isomers (Fig. 6).

TABLE 4. TNT metabolites observed in this study

Compound	Retention time (min)[a]	λ_{max} (nm)[a]	Quantitation wavelength (nm)
1, TNT[b]	10.3	230	230
2, H$^-$-TNT	14.3	478, 550	500
	3.6	258, 446	500
3–6, 2H$^-$-TNT ($^-$H$^+$/isomers)	4.8	282, 455	500
	5.6	272, 506	500
	6.4	237, 503	500
7, 2-Hydroxylamino-4,6-dinitrotoluene[b]	6.2	232, 350	230
8, 4-Hydroxylamino-4,6-dinitrotoluene[b]	6.2	232, 350	230
9, 2-Amino-4,6-dinitrotoluene[b]	8.6	220, 380	230
10, 4-Amino-2,6-dinitrotoluene[b]	8.7	230, 370	230

[a] As observed during ion-paired HPLC with a mobile phase of 50% (vol/vol) acetonitrile–50% (vol/vol) 20 mM tetrabutylammonium phosphate (pH 7.0).
[b] Validated by comparison with authentic standard.

DISCUSSION

Nitrate ester and nitroaromatic explosives have chemical structures with few parallels in natural products. It has been suggested that the xenobiotic-degrading abilities of organisms isolated from contaminated soil have evolved recently. Several pieces of evidence suggest this is the case for organisms degrading the explosive precursor 2,4-dinitrotoluene via a multienzyme pathway (18). Our findings show nitrate ester reductase activity is widespread across the OYE family and so, presumably, in the environment. It is therefore unlikely that this catalytic capability has arisen as a direct response to the release of nitrate esters into the environment.

Metabolism of TNT by the OYE family proceeds via two pathways (Fig. 1), the relative fluxes varying between enzymes. In this study, only PB2 PETN reductase and close relatives catalyzed the first pathway, involving sequential reduction of the aromatic ring by hydride addition. The second pathway, involving sequential reduction of one or more nitro groups of TNT, was catalyzed by all five enzymes. The two pathways are clearly separate: during transformation of purified H$^-$-TNT (compound 2), no TNT or intermediates from the second pathway were formed. Therefore, the regiospecificity of the initial reduction of TNT is crucial in determining the fate of the molecule. OYE's lack of significant ring reduction activity

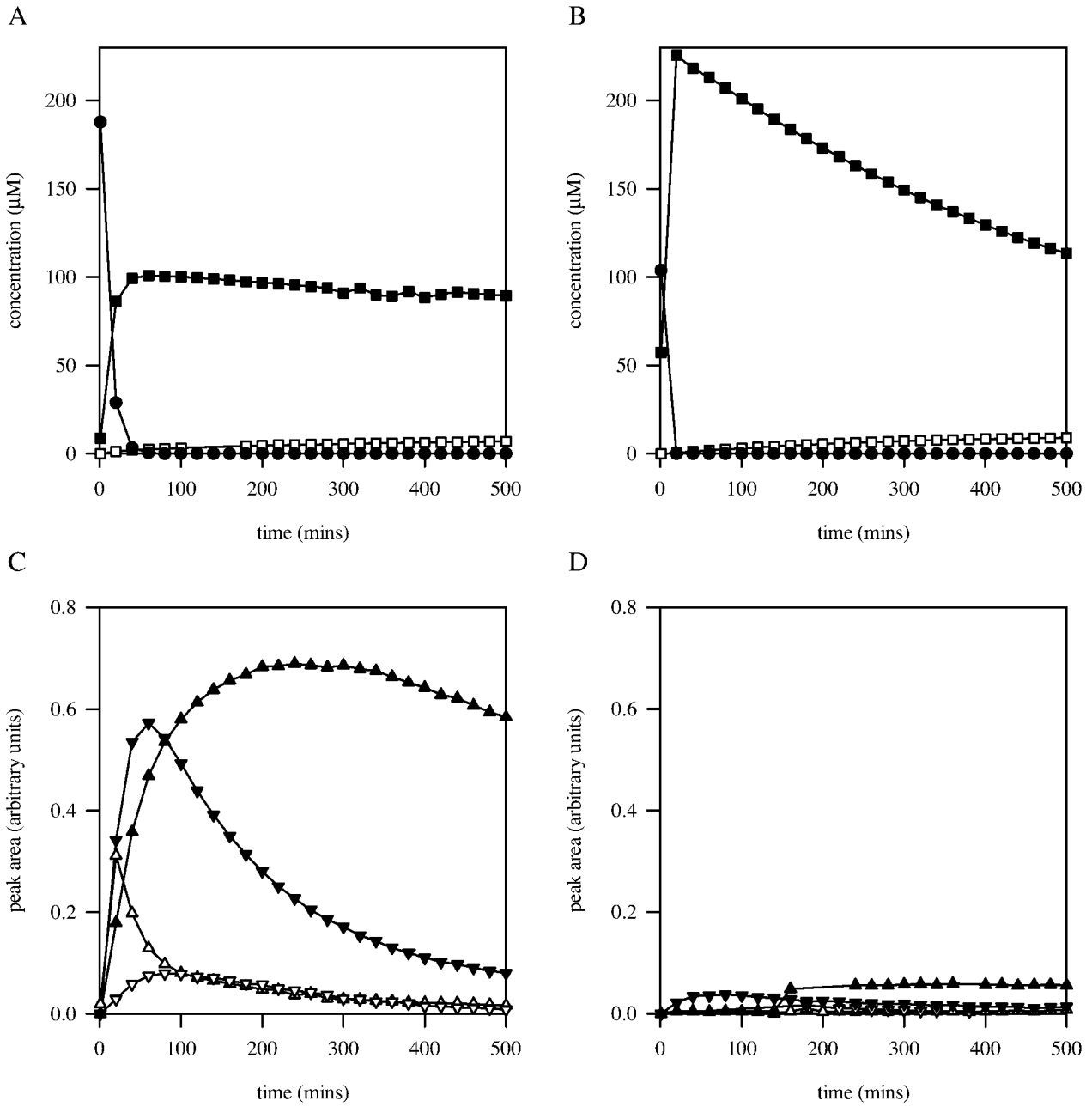

FIG. 3. Transformation of TNT by PB2 PETN reductase and OYE. PB2 PETN reductase (A and C) or OYE (B and D) at a concentration of 0.4 μM was incubated with 200 μM TNT, 200 μM NADPH, and a cofactor cycling system. Concentrations of TNT (compound 1, solid circles), HADNTs (compounds 7/8, solid squares), and ADNTs (compounds 9/10, open squares) are shown in panels A and B. Dihydride adduct products (compound 3, open triangles pointing up; compound 4, open triangles pointing down; compound 5, solid triangles pointing up; compound 6, solid triangles pointing down) are shown in panels C and D.

against TNT is at first sight surprising; H^--TNT formation is analogous to typical reactions of the OYE family, such as the saturation of α/β-unsaturated carbonyls and nitroalkenes (22). Indeed, in this study, OYE was found to reduce H^--TNT (compound 2) to $2H^-$-TNT isomers (compounds 3 to 6) as efficiently as PB2 PETN reductase. Hydride addition to the ring of TNT appears practically abolished in the H184N mutant of PB2 PETN reductase. H^--TNT is still an effective substrate for the enzyme, suggesting that it is the initial reduc-

TABLE 5. Nitrite concentrations at the end of TNT transformations

Enzyme	Nitrite conc (μM)	mol of nitrite released/mol of TNT added
PB2 PETN reductase	36	0.18
Type strain PETN reductase	40	0.20
NEM reductase	50	0.25
Morphinone reductase	6	0.03
OYE	6	0.03

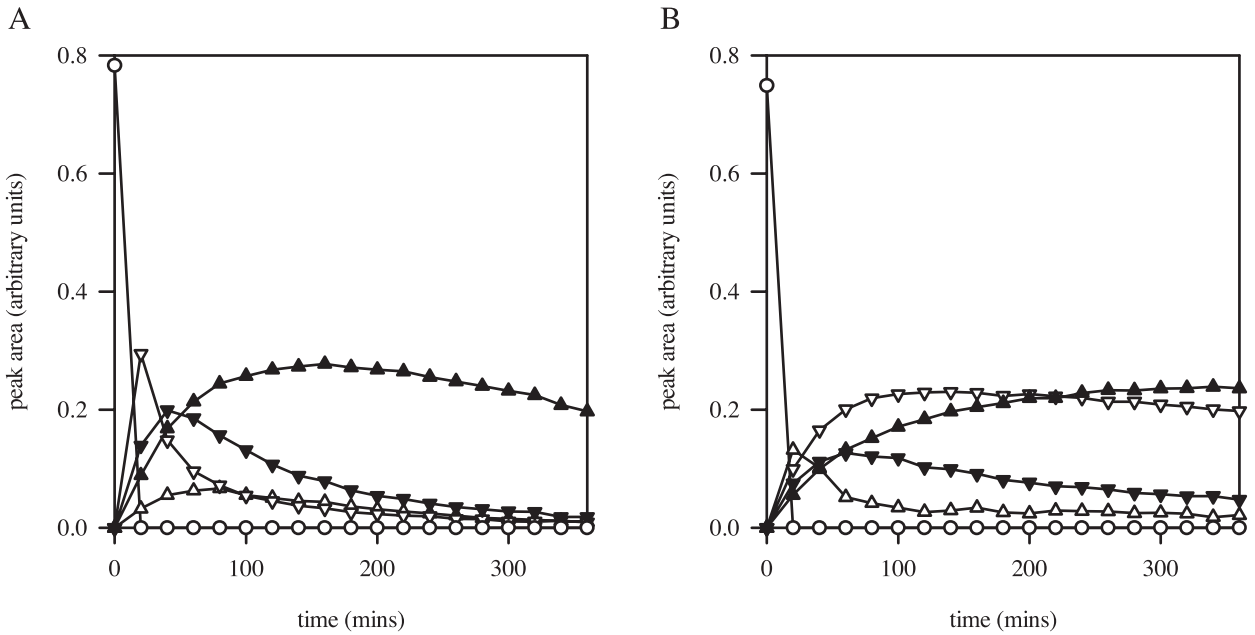

FIG. 4. Transformation of H^--TNT by PB2 PETN reductase and OYE. PB2 PETN reductase (A) or OYE (B) at a concentration of 0.4 μM was incubated with approximately 200 μM H^--TNT (compound 2, open circles), 200 μM NADPH and a cofactor cycling system. Dihydride adduct products (compound 3, open triangles pointing up; compound 4, open triangles pointing down; compound 5, solid triangles pointing up; compound 6, solid triangles pointing down) were monitored. No products of nitro group reduction (compounds 7 to 10) were observed.

tive step in TNT transformation that has been altered. The transformation of TNT by the H184N mutant is very similar to the activity of OYE, except for the substantially lower specific activity. Thus His/Asn 184 would seem to be critical in determining the outcome of the initial reduction of TNT, and so the eventual partition between ring-reduced and nitro-reduced products.

It has generally been found that the 4-nitro position of TNT is preferentially reduced (20) and that ADNTs are significant metabolites during aerobic microbial transformation (10, 26). However, in our experiments with pure enzymes, both isomers of HADNT were observed and ADNT appears not to be the major product (a maximum of 4% of TNT added, in the case of OYE) with more a polar metabolite instead being produced. The properties of this metabolite are consistent with those reported for 2,4-dihydroxylamino-6-nitrotoluene (32). The formation of dihydroxylamino derivatives of TNT has been reported under both aerobic (11) and anaerobic (17) conditions. Accumulation of hydroxylamino derivatives of TNT under aerobic conditions can lead to oxidative dimerization to yield azoxy products (9). Thus, during in vivo aerobic biotransformations involving nitro-reduction, substantial loss of mass balance to insoluble azoxy products is often observed. The time course experiments described here involved a system to maintain NAD(P)H levels despite the oxidase activity of the enzyme. The presence of NAD(P)H will preclude the accumulation of nitroso-dinitrotoluenes, preventing azoxy dimer formation. In effect, hydroxylamino- and dihydroxylamino derivatives are stabilized relative to their normal behavior in aerobic aqueous solution (33).

TABLE 6. Substrate preference of the H184N mutant of PB2 PETN reductase

Substrate	Sp act (U mg^{-1})[a]	
	Wild type	H184N mutant
PETN	21.4 ± 1.9	6.8 ± 0.4
GTN	19.8 ± 0.2	7.0 ± 0.4
TNT	1.4 ± 0.3	0.5 ± 0.0
Codeinone	0.1 ± 0.1	0.2 ± 0.1
2-Cyclohexenone	0.8 ± 0.1	0.0 ± 0.0
trans-2-Hexenal	1.5 ± 0.1	0.1 ± 0.0
1-Nitrocyclohexene	1.5 ± 0.1	0.3 ± 0.1
2-Nitrobenzaldehyde	2.0 ± 0.1	0.7 ± 0.0

[a] The initial concentration of both NADPH and substrate was 100 μM. The results are the mean ± standard error of three replicates. The background oxidase rate was determined and subtracted.

$2H^-$-TNT isomers accumulated during TNT transformation have previously been found to be dead end products (31) or to be consumed by an abiotic reaction with HADNT to give a product of $m/z = 376$ and the liberation of nitrite (25). In our time course studies of TNT transformation by PB2 PETN reductase, we have observed the disappearance of $2H^-$-TNT isomers 3 to 6 with a rate dependent on enzyme concentration. This process occurred in the presence and absence of HADNT and did not appear to lead to the formation of hydrophobic dimeric compounds. Eventually compounds 3 to 6 are completely consumed. The nature of the onward transformation is unclear; the resulting products lack visible absorbance, are probably highly polar (as no HPLC peak becomes apparent), and have lost, or can lose, nitrite. There are three possible roles for the OYE flavoenzymes in catalysis of $2H^-$-TNT disappearance: reduction, oxidation, and tautomerization. Tautomerization of $2H^-$-TNT to form a C-protonated nitroalkane form might in itself be sufficient to destabilize the compound.

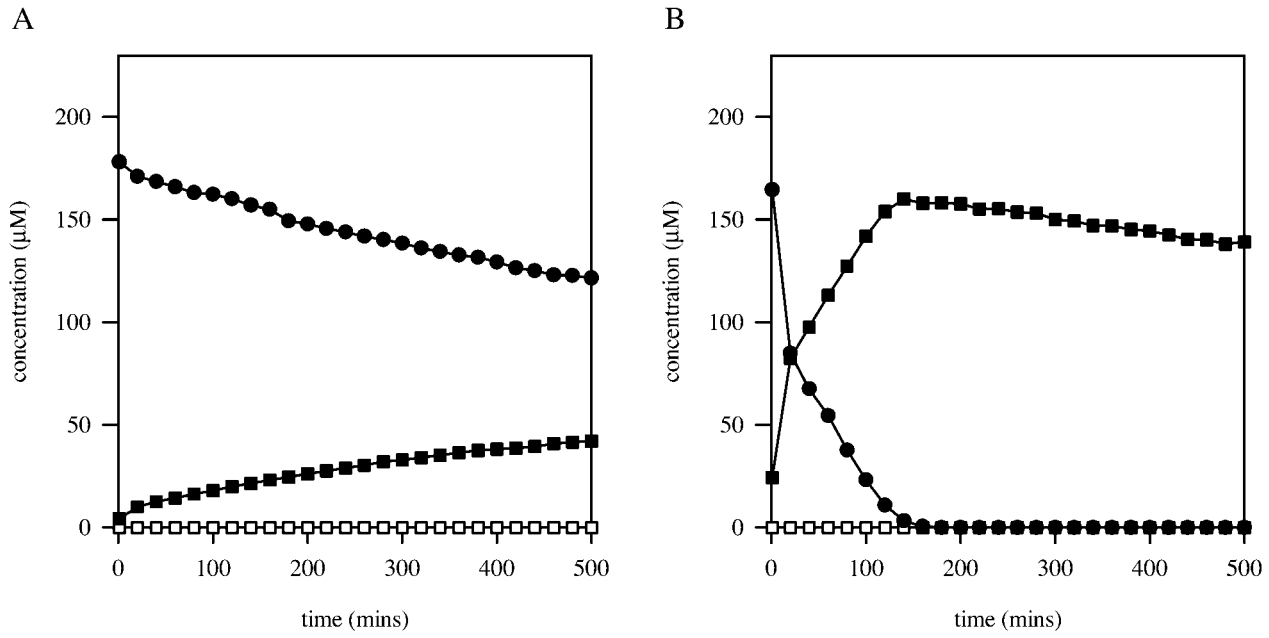

FIG. 5. Transformation of TNT by the H184N mutant of PB2 PETN reductase. The H184N mutant of PB2 PETN reductase, at a concentration of 0.4 μM (A) or 4 μM (B), was incubated with 200 μM TNT, 200 μM NADPH, and a cofactor cycling system. Concentrations of TNT (compound 1, solid circles), HADNTs (compounds 7/8, solid squares), and ADNTs (compounds 9/10, open squares) were monitored. Only traces of hydride adduct products (compounds 3 to 6) were observed (not shown).

Vorbeck et al. (31) report the rapid hydrolysis of a compound identified as C4-protonated $2H^-$-TNT, during nuclear magnetic resonance experiments. Protonation at this position (conversion of nitronate to nitro) is known to be slow (24), but OYE has been shown to catalyze the equilibration via the general acid function of Tyr 196 (22). Thus, certain members of the OYE family could add two hydride groups to TNT to yield $2H^-$-TNT and then selectively protonate the molecule. The protonated form would then either be hydrolyzed, to yield nitrite and an alcohol, or oxidized, to yield nitrite and a ketone.

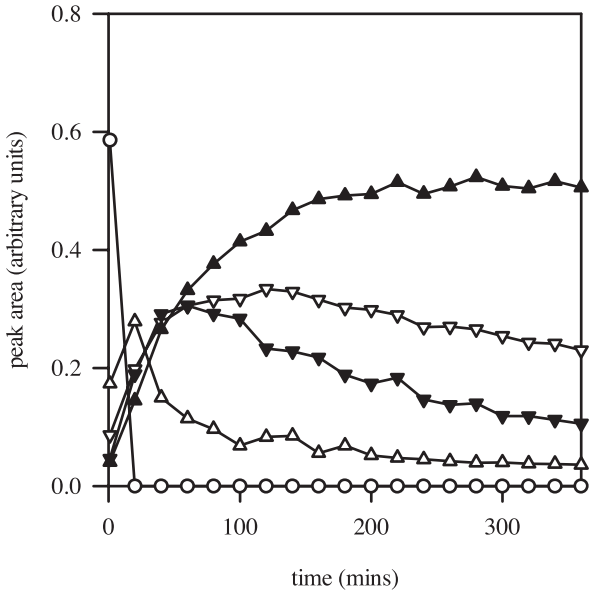

FIG. 6. Transformation of H^--TNT by the H184N mutant of PB2 PETN reductase. The H184N mutant of PB2 PETN reductase at a concentration of 0.4 μM was incubated with approximately 200 μM H^--TNT (compound 2, open circles), 200 μM NADPH, and a cofactor cycling system. Dihydride adduct products (3, open triangles pointing up; 4, open triangles pointing down; 5, solid triangles pointing up; 6, solid triangles pointing down) were monitored. No products of nitro group reduction (7 to 10) were observed.

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