Pentaerythritol tetranitrate reductase: kinetic and structural basis of reactivity with NADPH, 2-cyclohexenone, nitroesters and nitroaromatic explosives.

Huma Khan‡, Richard J. Harris‡, Terez Barna‡, Daniel H. Craig‡*, Neil. C. Bruce§, Andrew W. Munro‡, Peter C. E. Moody‡ and Nigel S. Scrutton‡

‡ Department of Biochemistry and Centre for Chemical Biology, University of Leicester, University Road, Leicester LE1 7RH, UK
§ Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, UK

Running title: explosive degradation by PETN reductase

*Present address: Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh, Scotland, UK

Corresponding author: Professor N. S. Scrutton. Telephone +44 116 223 1337; Fax, +44 116 252 3369; email, nss4@le.ac.uk.

This work was funded by grants from the Biotechnology and Biological Sciences Research Council, the Wellcome Trust and the Lister Institute of Preventive Medicine. N.S.S. is a Lister Institute Research Professor. The first 3 authors contributed equally to the work.
ABBREVIATIONS AND FOOTNOTES

1 Abbreviations: PETN, pentaerythritol tetrinitrate; OYE, Old Yellow Enzyme; MR, morphinone reductase; EBP, estrogen binding protein; TNT, trinitrotoluene; GTN, glycerol trinitrate; 2,4-DNP, 2,4-dinitrophenol.
ABSTRACT
The reaction of pentaerythritol tetranitrate reductase with reducing and oxidising substrates has been studied by stopped-flow spectrophotometry, redox potentiometry and X-ray crystallography. We show in the reductive half-reaction of PETN reductase that NADPH binds to form an enzyme-NADPH charge-transfer intermediate prior to hydride transfer from the nicotinamide coenzyme to FMN. In the oxidative half-reaction, the 2 electron-reduced enzyme reacts with several substrates including nitroester explosives [glycerol trinitrate (GTN) and pentaerythritol tetranitrate (PETN)], nitroaromatic explosives [trinitrotoluene (TNT) and picric acid] and α,β unsaturated carbonyl compounds (2-cyclohexenone). Oxidation of the flavin by the nitroaromatic substrate TNT is kinetically indistinguishable from formation of its hydride-Meisenheimer complex, consistent with a mechanism involving direct nucleophilic attack by hydride from the flavin N5 at the electron-deficient aromatic nucleus of the substrate. Crystal structures of complexes of the oxidised enzyme bound to picric acid and TNT are consistent with direct hydride transfer from the reduced flavin to nitroaromatic substrates. The mode of binding the inhibitor 2,4 dinitrophenol (2,4 DNP) is similar to that observed with picric acid and TNT. In this position the aromatic nucleus, however, is not activated for hydride transfer from the flavin N5, thus accounting for the lack of reactivity with 2,4 DNP. Our work with PETN reductase establishes further a close relationship to the Old Yellow Enzyme family of proteins, but at the same time highlights important differences compared with the reactivity of OYE. Our studies provide a structural and mechanistic rationale for the ability of PETN reductase to react with the nitroaromatic explosive compounds TNT and picric acid, and for the inhibition of enzyme activity with 2,4 DNP.
INTRODUCTION

A large number of sites worldwide are contaminated with high explosives as a result of large-scale manufacturing and handling of these compounds. Bioremediation is an attractive means of decontaminating such sites (1), which has led to a search for enzymes capable of degrading high explosive compounds. We previously isolated a strain of Enterobacter cloacae (strain PB2) on the basis of its ability to utilise nitrate ester explosives such as pentaerythritol tetranitrate (PETN\textsuperscript{1}) and glycerol trinitrate (GTN) as sole nitrogen source (2). The ability of Enterobacter cloacae PB2 to utilise nitrate esters as a nitrogen source is conferred by the NADPH-dependent flavoenzyme PETN reductase (3). Sequence analysis of the cloned gene encoding PETN reductase has established a close evolutionary relationship with the flavoenzyme Old Yellow Enzyme (OYE) (4), and related enzymes such as bacterial morphinone reductase (MR) (5) and the estrogen-binding protein (EBP) of Candida albicans (6). These enzymes bind a variety of cyclic enones, including 2-cyclohexenone and steroids. Some steroids act as substrates whilst others are inhibitors of both PETN reductase and OYE. We have demonstrated that PETN reductase degrades all major classes of explosive including nitroaromatic compounds [e.g. trinitrotoluene (TNT); (7-9)] and cyclic triazine explosives [e.g. Royal Demolition Explosive (RDX)], making the enzyme attractive in phytoremediation of explosive contaminated land (10). Homologues of PETN reductase from strains of Pseudomonas (11) and Agrobacterium (12) have been isolated and these enzymes also show reactivity against explosive substrates. In the case of xenobiotic reductase from Pseudomonas fluorescens I-C, the products of TNT reduction have been identified and
shown to proceed either by hydride addition to the aromatic nucleus or by nitro group reduction (13).

The crystal structure of PETN reductase has been solved in both its oxidised and 2 electron-reduced forms (14). The structures of a number of complexed forms with both steroid substrates and inhibitors are also known (14). The enzyme is a conventional eight-fold β/α barrel protein that contains a single FMN redox centre, and overall resembles the structure of OYE (15). However, the mode of steroid binding to oxidized enzyme differs from that seen with OYE in that the reactive olefinic bond in the steroid is not positioned over the flavin N5 (14). Reactions performed with ‘A-side’ deuterated nicotinamide cofactor have shown that in 2 electron-reduced PETN reductase the steroid is ‘flipped’ compared with the mode of binding to oxidized enzyme (14). In this ‘flipped’ binding mode the reactive olefinic bond is aligned with the flavin N5 atom in a geometry that is compatible with hydride transfer to the steroid substrate. Deuterium labelling methods have enabled us to assign the reactive olefinic bond as the C1-C2 bond in 1,4-androstadiene-3,17-dione and prednisone, to elucidate the stereochemistry of bond reduction and to propose a mechanism for the reduction of cyclic enones (14). Our work on the stereochemistry of olefinic bond reduction by PETN reductase again establishes a close relationship with OYE. Vaz et al (16) have shown that reduction of α,β-unsaturated carbonyl compounds by OYE proceeds by hydride transfer from the flavin N5 to the β carbon followed by proton uptake at the α carbon — a finding that is consistent with our more recent determination of the stereochemistry of bond reduction catalysed by PETN reductase.
In this paper we report a detailed kinetic analysis of the reaction of PETN reductase with NADPH and the substrate 2-cyclohexenone, which is used widely as a ‘generic’ substrate of the OYE family of enzymes. We also report studies of enzyme oxidation by nitroester substrates (GTN and PETN) and the nitroaromatic explosives TNT and picric acid. The structures of PETN reductase complexed with picric acid, TNT, 2-cyclohexenone and the inhibitor 2,4 dinitrophenol (2,4-DNP) are also presented, which provide atomic insight into the mechanism of nitroaromatic reduction and the reduction of α,β unsaturated carbonyl compounds.
EXPERIMENTAL PROCEDURES

Chemicals and enzymes — Complex bacteriological media were from Unipath and all media were prepared as described by Sambrook et al (17). Mimetic Orange 2 affinity chromatography resin was from Affinity Chromatography Ltd. Q-sepharose resin was from Pharmacia. PETN reductase was prepared from E. coli JM109/pONR1 and purified as described (3), but also incorporating a final chromatographic step using Q-sepharose (14). NADPH, glucose 6-phosphate dehydrogenase, glucose 6-phosphate, benzyl viologen, methyl viologen, 2-hydroxy-1,4-naphthaquinone, phenazine methosulfate and 2,4 DNP were from Sigma. 2-cyclohexenone was from Acros Organics. Dr S Nicklin (UK Defence and Evaluation Research Agency) supplied TNT, GTN, PETN and picric acid. The following extinction coefficients were used to calculate the concentration of substrates and enzyme: NADPH ($\epsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$); PETN reductase ($\epsilon_{464} = 11.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$); 2-cyclohexenone ($\epsilon_{232} = 11.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Stock solutions of TNT (600 mM) were made up in acetone. Dilutions were then made into potassium phosphate buffer, pH 7.0, and the acetone concentration was maintained at 1 % (v/v). The presence of acetone in buffers at 1 % (v/v) was shown not to affect enzyme activity.

Redox potentiometry — Redox titrations were performed within a Belle Technology glove box under a nitrogen atmosphere (oxygen maintained at <5 ppm) in 50 mM potassium phosphate buffer, pH 7.0. Anaerobic titration buffer was prepared by flushing freshly prepared buffer with oxygen-free nitrogen. PETN reductase admitted to the glove box was de-oxygenated by passing through a Biorad 10DG column, with final dilution of the eluted protein to give a concentration of ~60 µM. Solutions of benzyl viologen,
methyl viologen, 2-hydroxy-1,4-naphthaquinone and phenazine methosulfate were added to a final concentration of 0.5 µM as redox mediators for the titrations. Absorption spectra (300 - 750 nm) were recorded on a Varian (Cary 50 probe) UV-visible spectrophotometer, and the electrochemical potential was monitored using a Hanna instruments pH/voltmeter coupled to a Russell Pt/calomel electrode. The electrode was calibrated using the Fe(II)/Fe(III)-EDTA couple (+108 mV) as a standard. The enzyme solution was titrated electrochemically using sodium dithionite as reductant and potassium ferricyanide as oxidant, as described by Dutton (18). After the addition of each aliquot of reductant, and allowing equilibration to occur (stabilization of the observed potential), the spectrum was recorded and the potential was noted. The process was repeated at several (typically ~ 40) different potentials. In this way, a set of spectra representing reductive and oxidative titrations was obtained. Small corrections were made for any drift in the baseline by correcting the absorbance at 750 nm to zero. The observed potentials were corrected to those for the standard hydrogen electrode (SHE) (Pt/calomel + 244 mV). Data manipulation and analysis were performed using Origin software (Microcal). Absorbance values at wavelengths of 468 nm (close to the oxidized flavin maximum) were plotted against potential. Data were fitted using Eq. 1, which represents a concerted 2-electron redox process derived by extension to the Nernst equation and the Beer-Lambert Law, as described previously (18):

\[
A_{468} = \frac{(a + b 10^{(E_{1/2} - E)/29.5})}{1 + 10^{(E_{1/2} - E)/29.5}}
\]

Eq. 1

where \(A_{468}\) is the absorbance value at 468 nm at the electrode potential \(E\), and \(a\) and \(b\) are the absorbance values of the fully oxidized and reduced enzyme, respectively, at 468 nm.
In using Eq. 1 to fit the absorbance-potential data the variables were unconstrained, and regression analysis provided values in close agreement to those of the initial estimates. Throughout the titration the enzyme remained soluble and corrections for turbidity were not required.

**Kinetic measurements**—Rapid reaction kinetic experiments were performed using an Applied Photophysics SF.17MV stopped-flow spectrophotometer contained within an anaerobic glove box (Belle Technology). Time-dependent reductions of PETN reductase with NADPH were performed by rapid-scanning stopped-flow spectroscopy using a photodiode array detector and X-SCAN software (Applied Photophysics). Spectral deconvolution was performed by global analysis and numerical integration methods using PROKIN software (Applied Photophysics). For single wavelength studies, data collected at 464 nm and 560 nm were analysed using nonlinear least squares regression analysis on an Acorn Risc PC microcomputer using Spectrakinetics software (Applied Photophysics). Experiments were performed by mixing PETN reductase in the appropriate buffer with an equal volume of NADPH in the same buffer at the desired concentration. For studies of the oxidative half-reaction, PETN reductase was titrated with sodium dithionite to the 2-electron level and then mixed with 2-cyclohexenone. In reductive and oxidative reactions, the concentration of substrate was always at least 10-fold greater than that of enzyme, thereby ensuring pseudo-first-order conditions. For each substrate concentration, at least five replica measurements were collected and averaged. Transients were generally recorded at 5 °C to maximise data capture for fast reaction rates. For slow oxidising substrates (*i.e.* TNT and 2-cyclohexenone), transients were recorded at 25° C.
Observed rate constants for flavin absorption changes accompanying (i) mixing of oxidized PETN reductase with NADPH or (ii) oxidation of reduced PETN reductase by oxidising substrates were obtained from fits of the data to a single exponential expression. Reductive transients at 464 nm are strictly biphasic (see Results), but the fast first phase (charge transfer formation) contributes only a very small absorption change, making analysis using a biphasic expression inappropriate. For this reason fitting using a single exponential expression was used and analysis was performed on the kinetic transient in which signal for the first 20 ms after the mixing event was truncated. In the reductive half-reaction, transients at 560 nm were analysed using Eq. 2:

\[
A_{600} = \frac{k_{\text{obs1}}}{k_{\text{obs2}} - k_{\text{obs1}}} C \left( e^{-k_{\text{obs1}}t} - e^{-k_{\text{obs2}}t} \right) + b
\]

where \(k_{\text{obs1}}\) and \(k_{\text{obs2}}\) are observed rate constants for the formation and decay of an oxidized enzyme-NADPH charge-transfer species, \(C\) is the amplitude term and \(b\) an offset value. Observed rates for the oxidative half-reaction were fitted using the rapid equilibrium formalism of Strickland et al [(19); Eq. 3] for the kinetic scheme (Eq. 4)

\[
k_{\text{obs}} = \frac{k_3 [S]}{K_a + [S]}
\]

\[
A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \overset{k_3}{\underset{k_4}{\rightleftharpoons}} D
\]

In Eq. 4, A is 2-electron reduced PETN reductase, B is oxidising substrate, C is the reduced enzyme-substrate complex and D is the oxidised enzyme-product complex. The lack of an ordinate intercept in plots of \(k_{\text{obs}}\) against substrate concentration indicates that substrate reduction is essentially irreversible (i.e. \(k_4 \sim 0\)).
**Ligand binding studies**—PETN reductase was titrated with stock solutions of picric acid, 2,4 DNP and TNT in 50 mM potassium phosphate buffer, pH 7.0. Spectroscopic titrations were performed using a Jasco double-beam V-550 spectrophotometer. Spectral changes resulting from the addition of ligand to PETN reductase indicated a 1:1 binding stoichiometry and the isosbestic points observed during the titration indicated a single step process. Absorption changes, \( \Delta A \), at 518 nm were plotted against ligand concentration. Data were fitted using Eq. 5 to obtain dissociation constants, \( K_d \), for the enzyme-ligand complex:

\[
\Delta A = \frac{\Delta A_{\text{max}}}{2E_T}\left[ (L_T + E_T + K_d) - \left( (L_T + E_T + K_d)^2 - 4L_T E_T \right)^{1/2} \right]
\]  

Eq. 5

where \( \Delta A_{\text{max}} \) is the maximum absorption change at 518 nm, \( L_T \) is the total ligand concentration and \( E_T \) the total enzyme concentration.

**Multiple turnover studies of PETN reductase with nitroaromatic substrates**—Multiple turnover studies were performed under anaerobic conditions and the reaction progress monitored by absorption spectroscopy. The reaction mix (total volume 1 mL) comprised 0.2 \( \mu \)M PETN reductase, 30 \( \mu \)M NADPH and 100 \( \mu \)M TNT contained in 50 mM potassium phosphate buffer, pH 7.0 and reactions were performed at 25 °C. An NADPH generating system comprising 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase was also included in the reaction mix. UV-visible spectra were recorded using a Jasco V530 spectrophotometer contained within a Belle Technology anaerobic glove box.
Crystallography — Crystals of PETN reductase-ligand complexes were prepared by co-crystallization in the manner described previously for PETN reductase-steroid complexes (14). The crystals have space group $P2_12_12_1$ with one molecule per asymmetric unit. Data were measured and reduced with the HKL suite (20), and electron density maps were calculated using the CCP4 suite (21) and displayed using XtalView (22). Refinement was carried out with CNS (23). Details of data collection and refinement are shown in Table 1. The data and coordinate files have been deposited with the RCS PDB, accession codes (1GVO, 1GVQ, 1GVR, and 1GVS).
RESULTS

Mid-point redox potential of the FMN — The titrations of enzyme with dithionite were from fully oxidised enzyme and proceeded gradually to the end-point of the titration by the addition of small aliquots of reductant, and then back again to oxidised enzyme by the addition of potassium ferricyanide. The observed spectral changes indicated the lack of turbidity during the course of titration and no hysteretic effects were observed. Spectra recorded at similar potentials in the reductive and oxidative phases of the titration were essentially identical. Representative spectra for the reductive phase are shown in Figure 1 and a plot of the absorbance at 468 nm versus potential is shown in the inset of Figure 1. Evidence for population of a semiquinone species during reductive and oxidative titrations was not obtained. A good fit of the data to Eq. 1 was observed. The spectral changes accompanying reduction of PETN reductase contrast with those seen for the photoreduction of OYE in which the anionic red semiquinone is populated (24), but are similar to comparable titrations performed with bacterial morphinone reductase (25). Eq. 1 describes a concerted 2-electron reduction of the enzyme and fitting the spectroelectrochemical data for PETN reductase produced a value for $E_{12}$ of $-193 \pm 5$ mV.

Reductive half-reaction of PETN reductase — The spectral changes accompanying reduction of PETN reductase by a stoichiometric concentration β-NADPH are illustrated in Figure 2A. Our previous studies with deuterated NADPD (A-side) have indicated that hydride transfer is from the A-side of the nicotinamide ring, consistent with the known stereospecificity of OYE (26). Analysis of the spectral changes accompanying flavin
reduction by numerical integration methods using a two-step model ($A \rightarrow B \rightarrow C$) revealed the presence of three enzyme forms. $A$ is oxidized PETN reductase, $B$ is an enzyme-NADPH charge-transfer intermediate characterised by a long wavelength absorption (550 to 700 nm) and $C$ is PETN reductase containing the dihydroflavin form of FMN. Residual absorption at ~460 nm indicates that reduction of the flavin is not complete, suggesting that hydride transfer is reversible. Reversibility will depend on the redox potentials of the FMN and NADPH in the enzyme-NADPH charge-transfer complex, and these may differ from the potentials of NADPH in solution (-320 mV) and unliganded PETN reductase (-193 mV). The kinetic scheme and observed spectral changes are similar to those described previously for OYE (26) and bacterial morphinone reductase (27), and is shown as a series of reversible reactions in Scheme 1.

Observed rate constants for the formation and decay of the enzyme-NADPH charge-transfer complex, and hydride transfer from NADPH to FMN, were obtained by performing rapid mixing experiments of PETN reductase with NADPH using single wavelength detection. The large absorption changes at 464 nm are suitable for monitoring flavin reduction (i.e. step $B \rightarrow C$), and a typical reaction transient is shown (Figure 3A). Charge-transfer formation and decay was monitored at 560 nm (Figure 3B). The rate of charge-transfer decay (560 nm) is identical to the rate of flavin reduction (464 nm), indicating that decay of the enzyme-NADPH charge-transfer complex is a direct consequence of flavin reduction. Formation of the charge-transfer complex is not readily observed at 464 nm, owing to the small accompanying absorption change and relatively large absorption change for flavin reduction at the same wavelength. However, a small deviation from the fit to a single exponential expression is seen in the very early time
domain of the transient (up to ~20 ms after mixing; not shown), which is likely attributed to formation of the NADPH-enzyme charge-transfer complex. Formation of the charge-transfer species is more readily observed at 560 nm (i.e. the ‘up’ phase of the kinetic transient) (Figure 3B). Eq. 2 describes the early phase of the kinetic transient reasonably well, but there is a small deviation from the fit perhaps suggesting more than one discrete charge-transfer species accumulates in the early time domain (Figure 3B, inset). Similar deviations (but more pronounced) have been seen with our work on the nicotinamide-dependent flavoprotein human cytochrome P450 reductase (28).

The dependence of the observed rates for formation of the charge-transfer species and flavin reduction (i.e. charge-transfer decay) on NADPH concentration is illustrated in Figure 4. Consistent with our kinetic scheme for the reductive half-reaction, the rate of formation of the charge-transfer species shows a linear dependence on NADPH concentration. The second order rate constant for formation of the charge-transfer complex is $0.95 \times 10^6 \pm 0.02 \times 10^6 \text{M}^{-1} \text{s}^{-1}$. For scheme 1, the value of the positive intercept of the ordinate axis ($32 \pm 7 \text{s}^{-1}$) approximates to $k_2 + k_{-1}$. Additionally, the observed rate of flavin reduction ($\sim12 \text{s}^{-1}$) measured at 464 nm is independent of NADPH concentration (Figure 4). An approximate value of $20 \text{s}^{-1}$ for $k_{-1}$ can therefore be estimated which gives rise to a value of about 20 $\mu$M for the enzyme-NADPH dissociation constant. Given that the rates of flavin reduction were measured at NADPH concentrations of 100 $\mu$M and above, this would account for the lack of an apparent dependence of the flavin reduction rate on NADPH concentration (Figure 4). In studies performed with EBP (29) and OYE (26) an additional intermediate has been proposed prior to formation of the charge-transfer complex. Incorporation of such an intermediate
into Scheme 1 for PETN reductase would still be consistent with the observed kinetic behaviour, but in the absence of direct evidence for such an intermediate we have omitted to show the presence of a pre charge-transfer species in the catalytic scheme.

*Oxidative half-reaction with 2-cyclohexenone and nitroester explosives* — PETN reductase uses a number of oxidising substrates including 2-cyclohexenone, the nitroesters GTN and PETN, and nitroaromatics picric acid and TNT. 2-cyclohexenone is a common oxidising substrate for the OYE family of enzymes (8). Studies of the oxidative half-reaction with 2-cyclohexenone were initiated by mixing 2-electron reduced PETN reductase, generated by titration with sodium dithionite, with substrate. Analysis of multiple wavelength data indicated that oxidation occurred without the development of visible charge-transfer intermediates or product release steps (not shown). The data were best described using a single-step model \( A \rightarrow B \) in which \( A \) is 2-electron reduced enzyme and \( B \) is oxidised enzyme. The rate of flavin oxidation was investigated as a function of 2-cyclohexenone concentration in single wavelength studies at 464 nm (Figure 5A). Observed rates were hyperbolically dependent on 2-cyclohexenone concentration and kinetic parameters were determined by fitting the data to Eq. 4. Fitting produced a limiting rate constant, \( k_{\text{lim}} \), for flavin oxidation of \( 33.3 \pm 1.5 \text{ s}^{-1} \) and an enzyme-substrate dissociation constant, \( K_d \), of \( 8.1 \pm 1.1 \text{ mM} \).

Oxidation of PETN reductase by the nitroester substrates GTN and PETN was found to occur rapidly. As with 2-cyclohexenone, multiple wavelength absorption studies indicated that enzyme oxidation occurred in a single kinetic phase. With GTN, observed rates were hyperbolically dependent on GTN concentration and fitting to the rapid
equilibrium formalism of Strickland et al (Eq. 4) yielded a limiting rate constant, \( k_{\text{lim}} \), for flavin oxidation of 518 ± 51 s\(^{-1}\) and reduced enzyme-GTN dissociation constant, \( K_d \), of 1.5 ± 0.3 mM (Figure 5B). Owing to the extreme insolubility of PETN, we were unable to analyse with confidence the dependence of the rate of flavin oxidation on PETN concentration. However, an observed rate of ~25 s\(^{-1}\) was measured at a single concentration of ~20 µM in reactions additionally containing 10 % ethanol (PETN is sparingly soluble in 10 % ethanol).

**Binding and reaction of PETN reductase with nitroaromatic explosives** — The binding of nitroaromatic compounds to oxidised PETN reductase results in perturbation of the electronic absorption spectrum of the enzyme-bound FMN (Figure 6). Optical titrations performed with picric acid and 2,4 DNP revealed clear isosbestic points at 506 nm. Plots of absorption change at 518 nm versus ligand concentration and fitting to Eq. 5 produced dissociation constants of 5.4 ± 1.1 µM and 1.0 ± 0.1 µM for picric acid and 2,4 DNP, respectively. Optical titrations performed with TNT over a similar concentration range failed to elicit perturbations in the flavin spectrum in the range 350 nm to 650 nm, suggesting relatively weak binding of this ligand and/or lack of electronic interaction with the flavin.

Single turnover stopped-flow studies of the oxidation of 2-electron reduced PETN reductase with TNT clearly indicate the development of spectral features between 520 nm and 700 nm) characteristic of the formation of a hydride-Meisenheimer complex [Figure 7A; (30)], and consistent with our previous studies (7). Prolonged incubation of the solution following formation of the hydride-Meisenheimer complex leads to further
spectral change, indicating further breakdown of the hydride-Meisenheimer complex (Figure 7B). The chemical identity of compounds generated by the reactions occurring after formation of the hydride-Meisenheimer complex have been investigated recently in studies with the PETN reductase homologue, xenobiotic reductase (13). The accumulation of different products after initial formation of the hydride-Meisenheimer complex is also apparent under multiple turnover conditions with PETN reductase. The spectra of the accumulated products are distinctly different from those observed in single turnover stopped-flow studies (Figure 7C). Single turnover stopped-flow studies performed at 464 nm (flavin oxidation and hydride-Meisenheimer complex formation) and 580 nm (hydride-Meisenheimer complex formation) produced monophasic absorption transients with identical kinetics, thus suggesting that hydride-Meisenheimer complex formation is kinetically indistinguishable from flavin oxidation. Plots of the concentration dependence of the rate of flavin oxidation and hydride-Meisenheimer complex formation (measured at 464 nm) versus TNT concentration are hyperbolic (Figure 7D) and fits to Eq. 3 produced values for the limiting rate of flavin oxidation, \( k_{\text{lim}} \), of \( 4.5 \pm 0.1 \) s\(^{-1}\) and the reduced enzyme-TNT dissociation constant, \( K_d \), of \( 88.9 \pm 12 \) \( \mu \)M.

Reduction of picric acid by 2-electron reduced PETN reductase occurs very slowly. Multiple-turnover studies performed under anaerobic conditions in a conventional spectrophotometer indicated the development of spectral signature between 430 nm to 600 nm suggesting formation of the picric acid hydride-Meisenheimer complex. Reduction, however, was very slow taking approximately 15 h and, unlike for TNT, the long-wavelength signature for the hydride-Mesienheimer was relatively stable over this period. Detailed chemical analysis of the breakdown products of picric acid and TNT
after formation of the hydride-Meisenheimer complex by PETN reductase is to be described elsewhere, and is beyond the scope of the present paper.

**Structures of PETN reductase in complex with nitroaromatic ligands and 2-cyclohexenone** — The structure of PETN reductase in the oxidised and reduced form, and in complex with steroid substrates and inhibitors, has been described (14). Herein, we describe the structure of oxidised PETN reductase in complex with TNT, picric acid, 2,4 DNP and 2-cyclohexenone. Each of these complexes shows positive difference density in the active site, and the refined interpretation (and electron density) for each enzyme-ligand complex is shown in Figure 8. The ligands are bound above the si-face of the isoalloxazine ring. The imidazole side-chains of the histidine pair (His-181 and His-184), previously shown to coordinate with the electronegative atoms in steroid ligands (14), makes a similar interaction with the carbonyl group of 2-cyclohexenone (Figure 8A). This binding mode positions the olefinic bond over the reactive flavin N5 atom to enable hydride transfer; as with steroid substrates (14), we infer Tyr-186 acts as proton donor during reduction of the olefinic bond. This role for Tyr-186 is consistent with the results of recent mutagenesis studies of the equivalent residue (Tyr-196) of OYE in reactions with α,β unsaturated carbonyl compounds (31). The His-181/His-184 pair also coordinates the hydroxy group of picric acid and 2,4 DNP (Figures 8B and 8C). Both nitroaromatics are located with the C5 carbon close to the flavin N5 in a position optimal for hydride transfer. With picric acid, the C5 position is activated for nucleophilic attack from the flavin N5 through resonance stabilisation; this is not the case with 2,4 DNP (see below). Despite the lack of opportunity for good interaction between the C1 methyl of
TNT and the His-181/His-184 pair, the difference density for TNT indicates that it is bound in a similar mode to picric acid and 2,4 DNP (Figure 8D). We infer, therefore, that reduction of the nitroaromatic nucleus of both TNT and picric acid occurs by similar mechanisms.

The shape of the electron density for 2-cyclohexenone is consistent with a second, minor, binding mode whereby the ligand is “flipped” by 180° degrees, thus pointing the carbonyl group of 2-cyclohexenone away from the histidine pair. The structures of PETN-reductase complexed with picric acid shows an apparent bond with unusual geometry between the nitro group at C6 and the indole ring of Trp-102. This may be the result of the superimposition of multiple, partially occupied conformations and is the subject of a separate high-resolution study. TNT is less than fully occupied and the 6-nitro group is clearly disordered and therefore does not show any density.
DISCUSSION

Our recent determination of the crystal structure of PETN reductase established a close relationship to OYE, confirming inferences drawn from gene sequencing studies. Both enzymes contain a single FMN cofactor, and the active sites of both enzymes are very similar. Despite this structural similarity, our solution studies of PETN reductase have established key differences in the reactivity of OYE and PETN reductase towards oxidising substrates. Unlike OYE, PETN reductase reduces the nitroaromatic compounds TNT and picric acid to form a hydride-Meisenheimer complex (8,9). Similar reactivity towards nitroaromatics has also been reported for the OYE homologue termed ‘xenobiotic reductase’ isolated from *Pseudomonas fluorescens* I-C (13). Reduction of nitroesters such as GTN appears to be a common feature of the OYE family of enzymes and has been demonstrated for the xenobiotic reductases of *Pseudomonas fluorescens* I-C and *Pseudomonas putida* II-B (32), PETN reductase (this work), *E.coli* N-ethyl maleimide reductase (8) and OYE (33). Recently, detailed stopped-flow studies of the GTN-catalysed re-oxidation of OYE were performed and the reaction was shown to involve the reductive liberation of nitrite (33). The oxidative half-reaction of both PETN reductase and OYE can be modelled using the rapid equilibrium formalism of Strickland *et al* (19). However, the limiting rate of flavin re-oxidation by GTN in OYE [40 s⁻¹ at 25 °C; (33)] is considerably less than that for PETN reductase (518 s⁻¹ at 5 °C); the reduced enzyme-GTN dissociation constants are similar (2.7 mM for OYE and 1.3 mM for PETN reductase). Further differences in the properties of PETN reductase and OYE can also be identified: OYE stabilise the red anionic semiquinone of FMN, but reductive titration of PETN reductase proceeds direct to the dihydroquinone. Thus, despite the similarities in
the overall active site structure of OYE and PETN reductase (14), key differences in the reactivity and redox properties of the enzymes are apparent.

Our studies of the reductive half-reaction of PETN reductase have established mechanistic similarities with OYE (26), MR (27) and EBP (34). In all cases, enzyme-NADPH charge-transfer complexes have been observed prior to flavin reduction by the nicotinamide coenzyme. In contrast, our stopped-flow and spectrophotometric studies of the oxidative half-reaction have established key differences between different members of the OYE family of enzymes. The oxidative half-reaction of PETN reductase with TNT and picric acid generates the hydride-Meisenheimer complexes of these substrates. In the case of TNT, the hydride-Meisenheimer complex then breaks down to form alternate products, the chemical identities of which are uncertain but have been studied in reactions catalysed by xenobiotic reductase from *Pseudomonas fluorescens* I-C (13). The crystal structures of the TNT-and picric acid-bound PETN reductase complexes indicate a plausible mechanism involving direct hydride transfer from the N5 atom of the flavin isoalloxazine ring to the C5 position of the aromatic nucleus of the substrate. Despite the lack of electronic interaction with the isoalloxazine ring, the crystal structure of the enzyme-TNT complex clearly indicates that TNT binds in a similar mode to picric acid. The loss of the key interactions with His-181 and His-184 seen in the enzyme-picric acid complex might provide a rationale for weaker binding of TNT in the active site (and thus loss of electronic interaction with the flavin). The binding mode of 2,4 DNP is also similar to that observed for picric acid. The question arises, therefore, as to why 2,4 DNP is an inhibitor, whereas TNT and picric acid (albeit poor) are substrates. The answer likely lies in the different resonance stabilised forms of 2,4 DNP: resonance stabilisation
of 2,4 DNP preferentially enhances the electrophilicity of the C3 atom in this inhibitor, but it is the C5 atom that is located above the flavin N5, and thus this geometry is not favourable for hydride transfer to the C3 of 2,4 DNP. In TNT and picric acid the electrophilicity of both the C3 and C5 atoms is enhanced through resonance stabilisation (see Figure 9 for TNT), thus enabling hydride transfer to the C5 atom. Given the relatively simple reaction for nitroaromatic reduction, a key question arising from our work is why OYE, and indeed other members of the OYE family, are not able to reduce TNT and picric acid to their hydride-Meisenheimer complexes. Careful structural comparisons coupled with mutagenesis studies should identify those residues that ‘switch on’ reductive attack of nitroaromatics – a line of inquiry we are currently pursuing.

REFERENCES


Scheme 1

\[
E_{\text{ox}} + \text{NADPH} \xrightleftharpoons[k_{-1}]{k_1} E_{\text{ox}}N\text{ADPH} \xrightleftharpoons[k_{-2}]{k_2} E_{\text{red}}N\text{ADP}^+ \xrightleftharpoons[k_{-3}]{k_3} E_{\text{red}} + \text{NADP}^+
\]
Table 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>2-cyclohexenone</th>
<th>Picric acid</th>
<th>2,4 DNP</th>
<th>TNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total reflections</td>
<td>79611</td>
<td>231227</td>
<td>301258</td>
<td>86939</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>22658</td>
<td>48899</td>
<td>69990</td>
<td>33719</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>2.0</td>
<td>1.55</td>
<td>1.38</td>
<td>1.7</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>94.4</td>
<td>95.8</td>
<td>97.8</td>
<td>97.2</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>4.1</td>
<td>3.2</td>
<td>3.8</td>
<td>3.0</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>28.5</td>
<td>34.7</td>
<td>32.7</td>
<td>24.6</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt; (R&lt;sub&gt;free&lt;/sub&gt;)</td>
<td>20.9 (26.5)</td>
<td>19.0 (22.5)</td>
<td>17.5 (20.0)</td>
<td>22.0 (23.7)</td>
</tr>
<tr>
<td>r.m.s deviations from ideal bond lengths (Å)</td>
<td>0.006</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 1. Data collection and refinement statistics for PETN reductase in complex with ligands. Data for the 2-cyclohexenone complex was collected at ESRF Grenoble using an ADSC Quantum-4 CCD detector and 0.7209Å radiation. The 2,4 DNP-complex data were collected at SRS-Daresbury using an ADSC Quantum-4 CCD detector and 0.87Å radiation. The data for the picric acid and TNT complexes were measured with an Raxis-4 Image-plate device and Cu Kα radiation (1.5418 Å).
FIGURE LEGENDS

Figure 1. Spectral changes accompanying the reductive titration of PETN reductase. Inset, plot of absorbance versus potential. Data are shown fitted to Eq. 1 ($E_{12} = -193 \pm 5$ mV).

Figure 2. Spectral changes observed during the reduction of PETN reductase (20 µM) with NADPH (20 µM). Panel A, time-dependent spectral changes for PETN reductase mixed with NADPH. The first spectrum was recorded at 1.28 ms after mixing. For clarity, only selected subsequent spectra are illustrated. Conditions: 50 mM potassium phosphate buffer, pH 7.0, 5 °C; the time-dependent dataset was recorded over a period of 1 s. Panel B, denconvoluted spectra of initial, intermediate and final forms of the enzyme obtained by global analysis using ProKin software. Spectrum 1 (solid line), oxidised enzyme; spectrum 2 (broken line), charge-transfer intermediate; spectrum 3 (dotted line), two electron-reduced enzyme.

Figure 3. Kinetic transients observed for the reductive half-reaction of PETN reductase. Panel A, transient observed at 464 nm; panel B, transient observed at 560 nm. The inset in panel B illustrates the same reaction recorded over 12 ms fitted to Eq. 2. Conditions: 50 mM potassium phosphate buffer, pH 7.0; reactions were performed using 20 µM PETN reductase and 200 µM NADPH at 5 °C.
Figure 4. Concentration dependence of the observed rates measured at 560 nm (charge-transfer formation) and 464 nm (hydride transfer). Conditions: 50 mM potassium phosphate buffer, pH 7.0 and 5 °C; reactions were performed using 20 μM PETN reductase. Filled squares, charge-transfer formation; open circles, hydride transfer.

Figure 5. Concentration dependence of the rate of flavin reoxidation measured at 464 nm for the reaction of dithionite-reduced PETN reductase with 2-cyclohexenone (panel A) and GTN (Panel B). Conditions: 50 mM potassium phosphate buffer, pH 7.0; reactions were performed using 20 μM PETN reductase, at 25 °C (2-cyclohexenone) and 5 °C (GTN). The fits shown are to Eq. 3.

Figure 6. Titrations of PETN reductase with picric acid and 2,4 DNP. Conditions, 50 mM potassium phosphate, pH 7.0, 25 °C; enzyme concentration 10 μM. Panel A, spectral changes observed on titrating PETN reductase with picric acid. Inset, detail for the region 500 nm to 525 nm. Panel B, plot of absorbance change versus picric acid concentration. Solid line indicates the fit to Eq. 5. Panels C and D as for panels A and B, respectively, but for 2,4, DNP.

Figure 7. Panel A, spectral changes occurring during the oxidation of 2-electron reduced PETN reductase (16 μM) following rapid mixing with TNT (16 μM). Arrows indicate direction of spectral change. The first spectrum is shown at 1.28 ms after mixing; for clarity, not all subsequent spectra are shown. The time period for acquisition of spectra is 5 s. Panel B, spectral changes following prolonged incubation of 2-electron reduced
PETN reductase with TNT, illustrating the decay of the hydride-Meisenheimer complex. Following formation of the hydride-Meisenheimer complex, subsequent spectra were recorded at 1.5 minute intervals. Arrows indicate direction of spectral change. Panel C, spectra observed during the multiple turnover of PETN reductase (0.2 µM) with TNT (100 µM). The reaction was performed over 50 minutes (each spectrum recorded after 1.5 minutes). Panel D, plot of observed rate of hydride-Meisenheimer complex formation and flavin reoxidation versus TNT concentration (data taken from stopped-flow studies performed at 464 nm). Solid line, fit to Eq. 4.

Figure 8. Difference electron density for each of the PETN reductase-ligand complexes. The contours are at 3σ. Panel A, the complex of oxidised enzyme and 2-cyclohexenone. Panel B, the complex of oxidised enzyme and picric acid; panel C, the complex of oxidised enzyme and 2,4 DNP; panel D, the complex of oxidised enzyme and TNT.

Figure 9. (A) The structure of TNT as the resonance hybrid of several canonical forms, illustrating the enhancement in the electrophilicity of C3 and C5, and (B) reduction of TNT to form the Meisenheimer-hydride complex.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A

B

C

D

Figure 8
Figure 9
Pentaerythritol tetranitrate reductase: kinetic and structural basis of reactivity with NADPH, 2-cyclohexenone, nitroesters and nitroaromatic explosives
Huma Khan, Richard J. Harris, Terez Barna, Daniel H. Craig, Neil C. Bruce, Andrew W. Munro, Peter C.E. Moody and Nigel S. Scrutton

J. Biol. Chem. published online March 28, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200637200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts