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Sequence and Properties of Pentaerythritol Tetranitrate Reductase from *Enterobacter cloacae* PB2

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Pentaerythritol tetranitrate reductase, which reductively liberates nitrite from nitrate esters, is related to old yellow enzyme. Pentaerythritol tetranitrate reductase follows a ping-pong mechanism with competitive substrate inhibition by NADPH, is strongly inhibited by steroids, and is capable of reducing the unsaturated bond of 2-cyclohexen-1-one.

We previously reported the isolation of *Enterobacter cloacae* PB2 on the basis of its ability to use nitrate esters such as pentaerythritol tetranitrate (PETN) and glycerol trinitrate (GTN) as nitrogen sources. *E. cloacae* PB2 possesses a soluble PETN reductase capable of reductively liberating nitrite from nitrate esters with oxidation of NADPH (1) (Fig. 1). PETN reductase is a monomeric flavoprotein of M_r 40 000. Recently, White et al. (20) reported the isolation of a strain of *Agrobacterium radiobacter* capable of growth with GTN as the sole nitrogen source and showed that cell extracts from this organism liberated nitrite from GTN and PETN with oxidation of NADH, suggesting the activity of a similar enzyme.

Nitrate esters, though produced in large amounts for use as explosives and vasodilators (13), are rare in nature (7, 12), and multiply substituted nitrate esters are not known to occur naturally. The origin of enzymes apparently specialized for their breakdown is therefore of interest. To investigate this question, the structural gene encoding PETN reductase, designated *onr* (for organic nitrate reductase), was cloned using degenerate oligonucleotide probes.

Cloning and sequence analysis of *onr*. The N-terminal sequence of PETN reductase purified from *E. cloacae* PB2 as previously described (1) was found to be SAEKLFTPLKV GAVTAPNRVFMAPLT. On the basis of *E. cloacae* typical codon usage, the following oligonucleotide probes were designed, based on residues 2 to 11 and 18 to 26, respectively: (i) AC-TTT-(G/C)AG-(G/C)GG-(G/C)GT-GAA-(G/C)AG-TTT-TTC-(G/C)GC; (iii) GT-(G/C)AG-(G/C)GG-(G/C)GC-CAT-GAA-(G/C)AC-(G/C)CG-GTT. Southern blots using standard procedures (15) showed that both probes bound to the same region of PB2 genomic DNA. A 1,525-bp *Nco*I-*Cla*I genomic DNA fragment was cloned in pBluescript SK+ (Stratagene) to give pONR1. Sequencing indicated the presence of an open reading frame beginning with codons matching the known N-terminal sequence of PETN reductase. The sequence of *onr* and deduced amino acid sequence of PETN reductase are shown in Fig. 2. The sequence predicts a protein of 364 residues with an M_r of 39,358 excluding the N-terminal methionine, consistent with the M_r of approximately 40,000 estimated for PETN reductase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1). A putative ribosome-binding site and transcription termination sequence

were detected. No obvious σ^{70}-like promoter sequence was present, but three regions between a putative upstream termination sequence and the ribosome-binding site have significant homology to *Escherichia coli* σ^S-dependent promoters (21).

The deduced amino acid sequence of PETN reductase was compared to sequences in protein and nucleic acid databases using the BLAST program of the GCG package (6). The most similar proteins found were members of the old yellow enzyme family of α/β-barrel flavoprotein oxidoreductases (17). These include old yellow enzyme of *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* (11, 14, 18), homologs from the yeast *Kluyveromyces lactis* (10) and the protozoan *Trypanosoma cruzii* (OWL:U31282), a steroid-binding protein from the yeast *Candida albicans* (9), and morphinone reductase from the bacterium *Pseudomonas putida* M10 (4, 5). Expressed sequence tags from the plants *Arabidopsis thaliana*, *Oryza sativa*, and *Brassica campestris* suggest the presence of a related enzyme in plants. The most similar enzyme is morphinone reductase of *Pseudomonas putida* M10, the only other known bacterial member of this subgroup. PETN reductase and morphinone
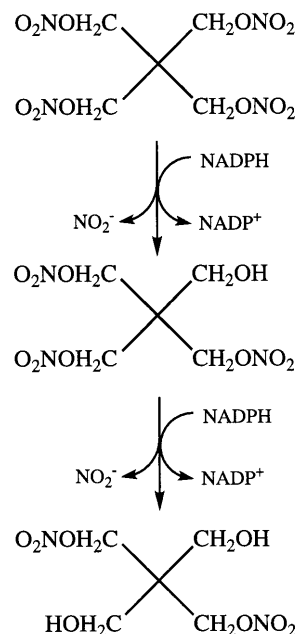

FIG. 1. Transformation of PETN by PETN reductase.

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```
NcoI(-279)      possible termination sequence
ccatggataa aggagccagc ggcgtgattg ccctgttggc tcaggcgctg -230

                                                -35
gagagcgggc gcaatgaaaa aacgctctcc ttctccggcg atccgctcac -180

          -10
gcaggcacag gtgctctatt ccctctggtt aggcgccaac ctgcaagcaa -130

                                           -35
aaatgtctcg cagcgccgtg ccgctcgaaa gcgcgctggc gcatgtgaaa -80

    -10     -35                     -10
aactgtatta ccgcgcctgg cgtgtagccg gcgttttttat ttacccttttt -30

    SalI(-25)          rbs
actagtcgac tggtctactc aggagccgtt atgtccgctg aaaagctgtt +20
                                M   S   A   E   K   L   F

taccccactg aaagtgggtg ccgttactgc cccaaaccgc gtgtttatgg +70
 T   P   L   K   V   G   A   V   T   A   P   N   R   V   F   M

                              EcoRV(106)
ccccacttac ccgtctgcgc agcatcgagc cgggcgatat cccaacgcca +120
A   P   L   T   R   L   R   S   I   E   P   G   D   I   P   T   P

ttgatgggtg agtattaccg ccagcgcgcc agcgcgggcc tgattatctc +170
L   M   G   E   Y   Y   R   Q   R   A   S   A   G   L   I   I   S

cgaagccacg cagatttctg ctcaggcaaa aggctacgcc ggtgcaccgg +220
 E   A   T   Q   I   S   A   Q   A   K   G   Y   A   G   A   P

gtctgcacag cccggaacag atcgccgcgt ggaaaaaaat caccgcaggc +270
G   L   H   S   P   E   Q   I   A   A   W   K   K   I   T   A   G

gtgcatgctg aagatggccg tattgcggtt cagctgtggc acaccggtcg +320
V   H   A   E   D   G   R   I   A   V   Q   L   W   H   T   G   R

tatctcacac agcagcatcc agcctggcgg tcaggcgccg gtttctgcct +370
 I   S   H   S   S   I   Q   P   G   G   Q   A   P   V   S   A

ctgccctgaa cgccaatacc cgcacttccc tgcgcgatga aaacggtaat +420
S   A   L   N   A   N   T   R   T   S   L   R   D   E   N   G   N

    SalI(430)
gcgatccgcg tcgacaccac cacgccacgc gcgctggagc tggacgagat +470
A   I   R   V   D   T   T   T   P   R   A   L   E   L   D   E   I
```

```
SmaI(471)                               SmaI(512)
cccgggtatc gtgaatgatt tccgtcaggc cgtcgccaac gcccgggaag +520
 P   G   I   V   N   D   F   R   Q   A   V   A   N   A   R   E

                              BstEII(556)
cgggcttcga cctggttgag cttcactctg cgcacggtta cctgctgcat +570
A   G   F   D   L   V   E   L   H   S   A   H   G   Y   L   L   H

cagttcctgt ccccgtcttc caaccagcgt accgaccagt acggcggcag +620
Q   F   L   S   P   S   S   N   Q   R   T   D   Q   Y   G   G   S

cgttgaaaac cgcgcgcgtc tggtgcttga agtggtggat gctgtctgta +670
V   E   N   R   A   R   L   V   L   E   V   V   D   A   V   C

atgagtggag cgcagaccgc attggtattc gtgtctcccc gatcggtact +720
N   E   W   S   A   D   R   I   G   I   R   V   S   P   I   G   T

    SalI(730)
ttccagaacg tcgacaacgg tccgaacgaa gaagcagacg cgctgtatct +770
F   Q   N   V   D   N   G   P   N   E   E   A   D   A   L   Y   L

gattgaagag ctggccgaaac gcggtatcgc ctatctgcac atgtccgaga +820
I   E   E   L   A   K   R   G   I   A   Y   L   H   M   S   E

cggacttggc aggcggcaag ccttacagtg aagccttccg tcagaaagtg +870
T   D   L   A   G   G   K   P   Y   S   E   A   F   R   Q   K   V

cgcgagcgct tccacggcgt gattatcggg gcgggtgcgt atacggcaga +920
R   E   R   F   H   G   V   I   I   G   A   G   A   Y   T   A   E

                              StuI(945)
aaaagccgag gatttgatcg gtaaaggcct gatcgacgcc gtggcctttg +970
K   A   E   D   L   I   G   K   G   L   I   D   A   V   A   F

gccgtgacta cattgctaac ccggatctgg ttgcccgttt gcagaaaaaa +1020
G   R   D   Y   I   A   N   P   D   L   V   A   R   L   Q   K   K

                              HindIII(1047)
gccgaactga acccgcagcg tcctgaaagc ttctatggcg gcggcgcgga +1070
A   E   L   N   P   Q   R   P   E   S   F   Y   G   G   G   A   E

                                        -35 term-
aggttatacc gactacccttt cactgtaatc ccgctttgta cattgatagc +1120
 G   Y   T   D   Y   P   S   L   *

ination sequence  -10
ggcgaccttt cgccgctata ctaaaacatc gtttctgttc aaaaagataa +1170

                              rbs
tccattcgac tggttaatga ggaaattatg cgcctacttc acaccatgct +1220
                                M   R   L   L   H   T   M   L

                    ClaI(1246)
gcgcgttggc gacctgcaac gttccatcga t
R   V   G   D   L   Q   R   S   I   D
```
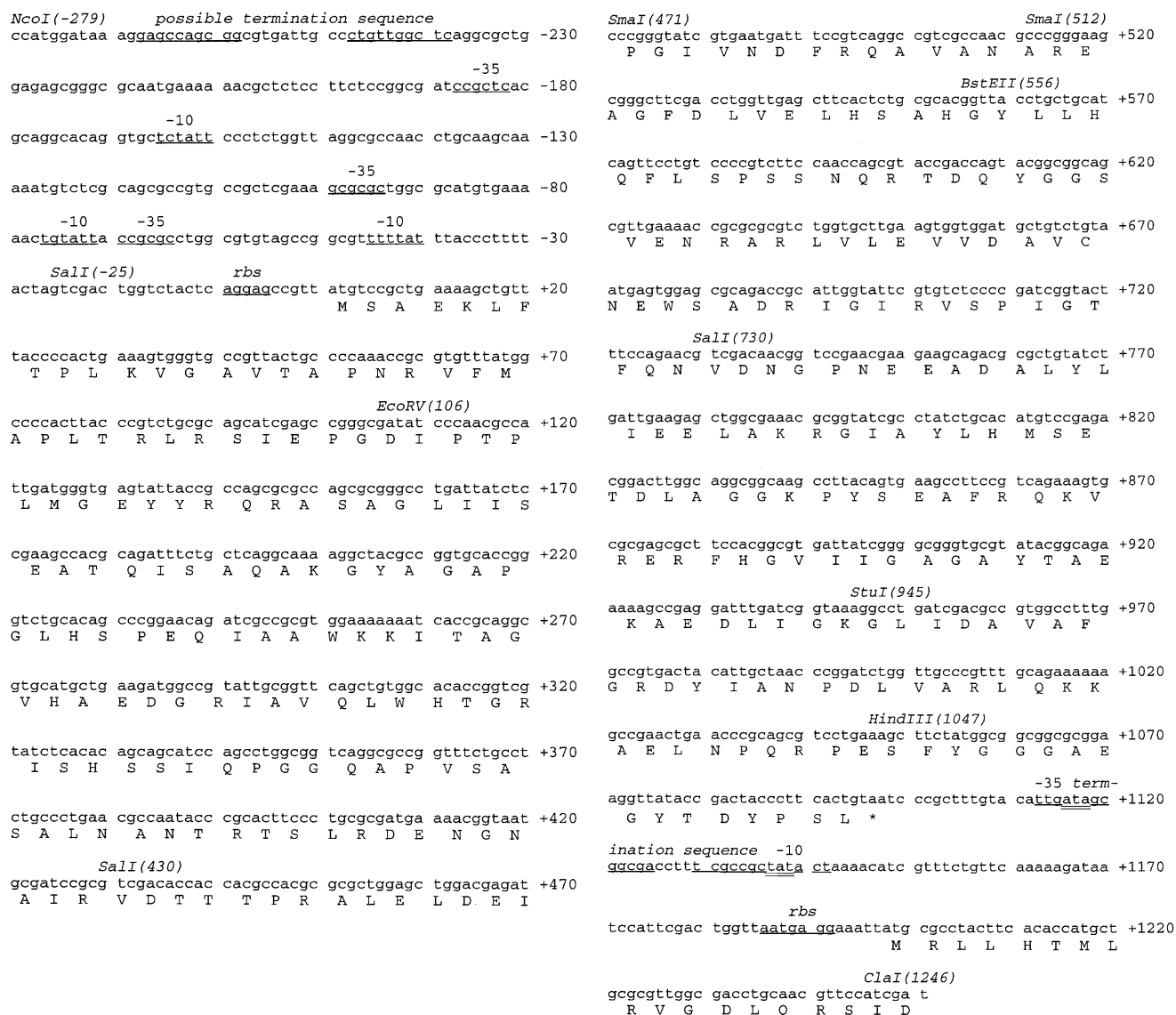
FIG. 2. Sequence of *onr* and deduced amino acid sequence of PETN reductase. Nucleotides are numbered with 1 being the A of the ATG initiating the gene. Putative ribosome-binding sites (*rbs*), transcription termination sequences, and the −35 and −10 regions of possible promoters are indicated.

reductase share 53% sequence identity and 71% sequence similarity as determined by the GAP program of the GCG package. An alignment of the deduced amino acid sequences of PETN reductase, morphinone reductase, and old yellow enzyme is shown in Fig. 3.

The physical properties of PETN reductase are consistent with a close relationship to the old yellow enzyme family. Like all of the known members of this group, it is a simple flavoprotein with flavin mononucleotide as a prosthetic group, its subunit M_r is approximately 40,000, and it is an oxidoreductase using a reduced pyridine nucleotide cofactor as an electron donor. However, all of these enzymes are active as homodimers, whereas the elution position of PETN reductase in gel filtration, as well as its significant passage through ultrafiltration membranes with a nominal M_r cutoff of 10,000 under high-salt conditions, suggest that PETN reductase is monomeric (1). Since PETN reductase is retained by ultrafiltration membranes under low-salt conditions, it is conceivable that

dimers form under such conditions; this possibility is under investigation.

The structure of old yellow enzyme has been shown to be an eight-stranded α/β-barrel (3). The flavin is hydrogen bonded by side chains from residues T-37, Q-114, R-243, and R-348 (3). All of these residues are conserved in both morphinone reductase and PETN reductase (Fig. 3). Of the residues which hydrogen bond with the flavin through the peptide backbone in old yellow enzyme, G-324 and G-347 are conserved, while several others are replaced by conservative substitutions such as glycine to alanine. Residues F-374 and Y-375, which provide a hydrophobic pocket for the dimethyl-benzene ring of the flavin, are also conserved, although F-296 is not.

Overexpression and purification of recombinant PETN reductase. The insert of pONR-1 is immediately downstream of the *lac* promoter of pBluescript SK+. It was found that *E. coli* JM109/pONR-1 grown in a rich medium such as SOB (15) in the presence of 0.4 mM isopropyl-β-D-thiogalactopyr-

```
MorB    1          PDT SFSNPGLFTP LQLGSLSLPN RVIMAPLT.. RSRTPDSVPG  41
 Onr    1              SAEKLFTP LKVGAVTAPN RVFMAPLTRL RSIEPGDIPT  38
OYE1    1    SFVKDFKPQ ALGDTNLFKP IKIGNNELLH RAVIPPLTRM RALHPGNIPN  49
                                 SA        SB        S1

MorB   42   R.LQQIYYGQ RA..SAGLII SEATNISPTA RGYVYTPGIW TDAQEAGWKG  88
 Onr   39   P.LMGEYYRQ RA..SAGLII SEATQISAQA KGYAGAPGLH SPEQIAAWKK  85
OYE1   50   RDWAVEYYTQ RAQRPGTMII TEGAFISPQA GGYDNAPGVW SEEQMVEWTK  99
                H1                S2                            H2

MorB   89   VVEAVHAKGG RIALQLWHVG RVS.HELVQP DGQQPVAPSA LKAEGAECFV 137
 Onr   86   ITAGVHAEDG RIAVQLWHTG RIS.HSSIQP GGQAPVSASA LNANTRTSLR 134
OYE1  100   IFNAIHEKKS FVWVQLWVLG WAAFPDNLAR DGLRYDSASD .......NVF 142
                S3                HA        SC

MorB  138   EFEDGTAGLH PTSTP.RALE TDEIPGIVED YRQAAQRAKR AGFDMVEVHA 186
 Onr  135   D.ENGNAIRV DTTTP.RALE LDEIPGIVND FRQAVANARE AGFDLVELHS 182
OYE1  143   MDAEQEAKAK KANNPQHSLT KDEIKQYIKE YVQAAKNSIA AGADGVEIHS 192
                HB        SD              H3                    S4

MorB  187   ANACLPNQFL ATGTNRRTDQ YGGSIENRAR FPLEVVDAVA EVFGPERVGI 236
 Onr  183   AHGYLLHQFL SPSSNQRTDQ YGGSVENRAR LVLEVVDAVC NEWSADRIGI 232
OYE1  193   ANGYLLNQFL DPHSNTRTDE YGGSIENRAR FTLEVVDALV EAIGHEKVGL 242
                HC                            H4                    S5

MorB  237   RLTPFLELFG LTDD.EPEAM AF..YLAGEL DRRG.....L AYLHFNEPDW 278
 Onr  233   RVSPIGTFQN VDNGPNEEAD AL..YLIEEL AKRG.....I AYLHMSETDL 275
OYE1  243   RLSPYGVFNS MSGGAETGIV AQYAYVAGEL EKRAKAGKRL AFVHLVEPRV 292
                                      H5                    S6

MorB  279   .....IGGDI TYPEGFREQM RQRFKGGLIY CGNYDAGRAQ ARLDDNTADA 323
 Onr  276   .....AGGK. PYSEAFRQKV RERFHGVIIG AGAYTAEKAE DLIGKGLIDA 319
OYE1  293   TNPFLTEGEG EYEGGSNDFV YSIWKGPVIR AGNFALHP.. .EVVREEVKD 339
                          H6              S7              H7

MorB  324   ....VAFGRP FIANPDLPER FRLGAALNEP DPSTFYGGAE VGYTDYPFLD 369
 Onr  320   ....VAFGRD YIANPDLVAR LQKKAELNPQ RPESFYGGGA EGYTDYPSL  364
OYE1  340   KRTLIGYGRF FISNPDLVDR LEKGLPLNKY DRDTFYQMSA HGYIDYPTYE 389
                S8        HD        H8                            HE

MorB  370   NGHDRLG 376
 Onr
OYE1  390   EALKLGWDKK 399
```

FIG. 3. Alignment of the deduced amino acid sequences of PETN reductase (Onr), morphinone reductase (MorB), and old yellow enzyme (OYE1). The alignment was generated by the PILEUP program of the GCG package. Structural elements from OYE1 shown are taken from reference 6. The α-helices and β-strands forming the eight-stranded α/β-barrel are shown as H1 to H8 and S1 to S8, respectively; extra-barrel helices are shown as HA to HE, and extra-barrel strands are shown as SA to SD.

anoside (IPTG) expressed high levels of PETN reductase activity, suggesting that PETN reductase formed 30 to 50% of soluble cell protein. Assays of enzyme activity were performed as described previously (1). In the absence of IPTG, expression was approximately half of that observed with IPTG, suggesting that expression is driven both by the *lac* promoter of the vector and by a promoter in the insert. Recombinant PETN reductase was purified by affinity chromatography. Cells from 1 liter of stationary-phase culture of *E. coli* JM109/pONR1 grown in SOB medium were disrupted using a French press, and clarified extract was loaded onto a column with a 5-cm diameter packed with 70 ml of Mimetic Orange 2 A6XL (Affinity Chromatography Ltd., Freeport, Ballasalla, Isle of Man, United Kingdom) (packed height 3.6 cm) at a flow rate of 4 ml/min. The column was washed with 300 ml of 10 mM phosphate buffer, pH 7.0. Bound PETN reductase was then eluted with 60 mM ammonium acetate. The most active fractions, totalling 60 ml, were pooled and freeze-dried. The freeze-dried powder was stored at −20°C and made up as required in 50 mM phosphate buffer, pH 7.0. The crude extract contained 2,250 U

of activity at a specific activity of 6.1 U/mg, and the product contained 1,100 U at 13.6 U/mg, a yield of 49% at a purification factor of 2.2. The purified enzyme appeared homogeneous by SDS-PAGE and comigrated with PETN reductase purified from *E. cloacae* PB2. The N-terminal 10 amino acid residues of the recombinant enzyme were determined and were found to be identical to those of the enzyme purified from *E. cloacae* PB2.

Kinetic mechanism. To investigate the kinetic mechanism of PETN reductase, activities were measured at various concentrations of GTN and NADPH. GTN was used as a substrate rather than PETN because its greater solubility allows variation of the substrate concentration over a much wider range. GTN concentration was varied between 10 and 100 μM and NADPH concentration was varied between 40 and 150 μM. In all cases, GTN was added from a 1,000× stock solution in ethanol. Addition of ethanol at several times this level had no effect on the observed rate. Apparent V_{max} and K_m values were determined at each NADPH concentration by nonlinear regression using the GraFit software package (8). Figure 4A
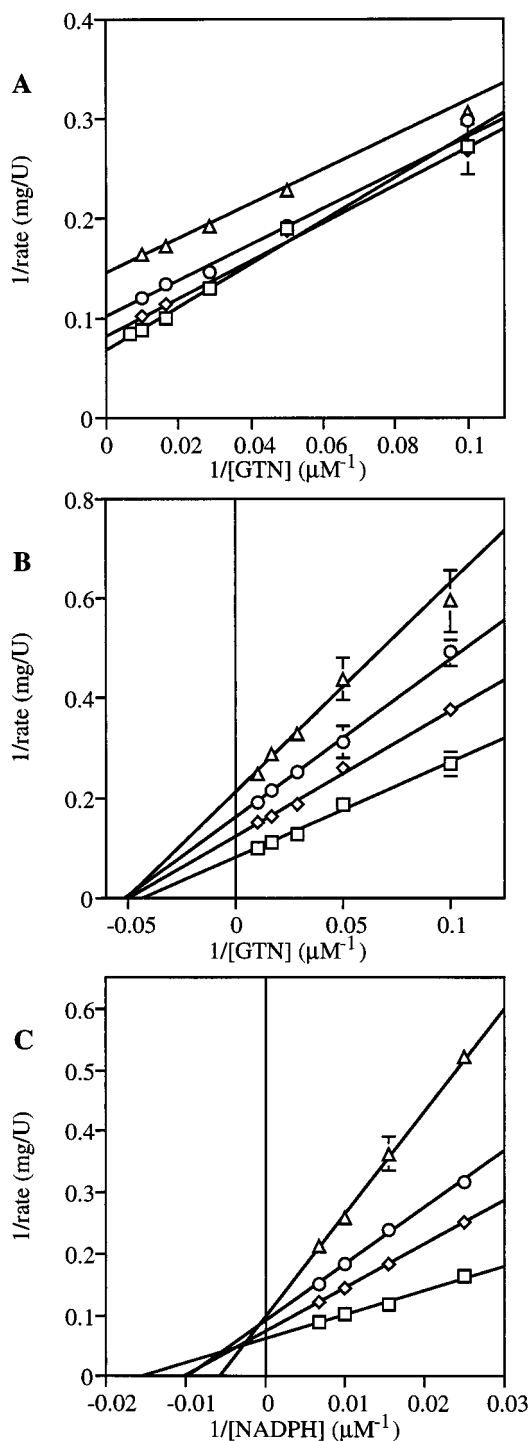
FIG. 4. (A) Double-reciprocal plot showing PETN reductase activity at various levels of GTN and NADPH. Lines shown were fitted separately to each data set by nonlinear regression using the GraFit software package (8). Each point shown is the mean of three independent assays. Error bars indicate one standard error. Triangles, 40 μM NADPH; circles, 65 μM NADPH; diamonds, 100 μM NADPH; squares, 150 μM NADPH. (B and C) Double-reciprocal plots showing inhibition of PETN reductase activity by testosterone. (B) NADPH concentration fixed at 100 μM and GTN concentration varied between 10 and 100 μM. Squares, no inhibitor; diamonds, 0.05 μM testosterone; circles, 0.1 μM testosterone; triangles, 0.2 μM testosterone. (C) GTN concentration fixed at 100 μM and NADPH concentration varied between 40 and 150 μM. Squares, no inhibitor; diamonds, 0.05 μM testosterone; circles, 0.1 μM testosterone; triangles, 0.2 μM testosterone.

shows a double-reciprocal plot including the lines fitted by nonlinear regression. Convergence occurs to the right of the y axis. This is characteristic of a ping-pong mechanism with competitive inhibition by the fixed substrate, in this case NADPH (2). In physical terms this implies that NADPH is capable of binding unproductively to the reduced form of the enzyme. The general equation for such behavior is

$$v = \frac{V_{\max}[A][B]}{[A][B] + K_B[A](1+[A]/K_{AR}) + K_A[B]}$$

where $[A]$ is the concentration of NADPH, $[B]$ is the concentration of GTN, v is the observed rate, $V_{\max}$ is the theoretical rate at saturating substrate concentrations ignoring substrate inhibition, K_A is the apparent K_m for NADPH at saturating levels of GTN, K_B is the apparent K_m for GTN at saturating levels of NADPH and K_{AR} is the dissociation constant for unproductive binding of NADPH to the reduced form of the enzyme.

Parameters for this equation were estimated by linear regression analysis of the slopes and intercepts of the lines shown in Fig. 4A. The following values were obtained: $V_{\max} = 25.2 \pm 1.5$ U/mg; $k_{cat} = 16.6 \pm 1.0$ s^{-1}; $K_A = 107 \pm 10$ μM; $K_B = 39.3 \pm 3.5$ μM; and $K_{AR} = 400 \pm 58$ μM. Errors indicated are one standard error based on the error of the linear regression.

A partial F-test was performed to determine whether the substrate inhibition term was statistically significant in describing the data. The F statistic was calculated as 30.1 with 1 numerator and 68 denominator degrees of freedom. This value is significant at the 1% level of confidence.

Similar kinetic behavior has been described for morphinone reductase, in which substrate inhibition was observed at high levels of NADH, and product inhibition patterns also suggested that the reduced product hydrocodone could bind unproductively to the reduced form of the enzyme (4, 5).

It has been found that NADP(H) is bound in an unusual manner in old yellow enzyme, such that the adenine portion is unbound and binding is primarily due to the nicotinamide ring and the nicotinamide ribose moiety (3). In this regard it is interesting that PETN reductase, like old yellow enzyme, is NADPH-dependent, whereas morphinone reductase, which shares 53% sequence identity with PETN reductase, is NADH dependent and does not appear to be significantly active with NADPH (4). In studies of the crystal structure of old yellow enzyme, it was necessary to use an NADP analog, since oxidized cofactor does not bind to oxidized enzyme, nor does reduced cofactor bind to reduced enzyme (3); by contrast, the reduced forms of both morphinone reductase and PETN reductase appear to show significant binding to the respective reduced cofactors. Further study of these enzymes may therefore cast light upon the unusual mode of cofactor binding in this growing enzyme family.

Interaction with steroids. Old yellow enzyme, estrogen-binding protein and, morphinone reductase are all known to bind steroids and to reduce the double bond of 2-cyclohexen-1-one (4, 9, 11, 16, 19). Several steroids were tested as inhibitors of PETN reductase and were found to be potent inhibitors of GTN reduction. The concentrations of progesterone, testosterone, and cortisone required to halve the observed rate at 100 μM GTN and 100 μM NADPH were approximately 0.022, 0.12, and 0.31 μM, respectively.

Inhibition by testosterone was investigated in more detail. Assays were performed with testosterone concentrations ranging from 0.05 to 0.20 μM, either with NADPH fixed at 100 μM and GTN varied between 10 μM and 100 μM or with GTN fixed at 100 μM and NADPH varied between 40 μM and 150

TABLE 1. Apparent kinetic parameters for substrates
of PETN reductase[a]

Substrate	K_m (mM)	V_{max} (U/mg)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\ mM^{-1}$)
GTN	0.023 ± 0.002	12.1 ± 0.5	480 ± 20	$21,000 \pm 3,000$
EGDN[b]	2.4 ± 0.3	9.5 ± 0.5	380 ± 20	160 ± 30
2-cyclohexen-1-one	0.74 ± 0.06	3.2 ± 0.1	125 ± 3	170 ± 20

[a] Apparent kinetic parameters were measured at 0.1 mM NADPH. Kinetic parameters were estimated by nonlinear regression using the GraFit software package (8). Errors shown are one standard error.
[b] EGDN, ethylene glycol dinitrate.

μM. Apparent V_{max} and K_m for each testosterone concentration were determined by nonlinear regression using the GraFit software package (8). Double-reciprocal plots showing data and fitted lines are shown in Fig. 4B and C. Convergence to the left of the y axis occurs with both GTN and NADPH as varied substrates, implying that testosterone binds to both the oxidized and the reduced forms of the enzyme. The general equation describing this behavior is

$$v = \frac{V_{max}[A][B]}{[A][B] + K_B[A](1+[A]/K_{AR}+[I]/K_{IR}) + K_A[B](1+[I]/K_{IO})}$$

where K_{IO} is the dissociation constant for the binding of the inhibitor to the oxidized form of the enzyme, K_{IR} is the dissociation constant for the binding of the inhibitor to the reduced form of the enzyme, and other parameters are as previously described.

Using the values for V_{max}, K_A, K_B, and K_{FA} determined previously, K_{IO} and K_{IR} were estimated from linear slope replots of the lines fitted for each inhibitor concentration. K_{IO} was estimated as 70 ± 6 nM and K_{IR} was estimated as 136 ± 7 nM. Errors indicated are one standard error based on the error of the linear regression in each case. By contrast, apparent K_m values for GTN were in the order of 10 to 30 μM, depending on the concentration of NADPH.

The α/β-unsaturated ketones 2-cyclohexen-1-one and codeinone were investigated as substrates for PETN reductase. Activity was detected with 2-cyclohexen-1-one. Apparent kinetic parameters measured in the presence of 0.1 mM NADPH are shown in Table 1, together with the corresponding values for the known substrates GTN and ethylene glycol dinitrate. Kinetic parameters for PETN could not be estimated due to its poor solubility in aqueous solutions (1). No activity was detected with codeinone, the best known substrate for morphinone reductase.

The observations that both morphinone reductase and PETN reductase bind steroids more strongly than their known substrates and that they are most closely related to eukaryotic enzymes suggest that they may be descended from a eukaryotic steroid reductase.

Nucleotide sequence accession number. The nucleotide sequence of *onr* has been submitted to GenBank and has been assigned the accession number U68759.

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