INTRODUCTION

Nitrophenols such as 2,4-dinitrophenol (2,4-DNP) and 2,4,6-trinitrophenol (picric acid) have been introduced into the environment through their use as explosives, synthetic intermediates, dyes and pesticides (Nishino et al., 2000; Spain, 2000). Although several bacteria exist which mineralize these compounds (Behrend & Heesche-Wagner, 1999; Blasco et al., 1999; Ebert et al., 1999; Hess et al., 1990; Lenke et al., 1992; Rajan et al., 1996), their general recalcitrance (Lenke et al., 1999; Hess et al., 1990) is a significant hindrance to their degradation. However, because of the difficulty in lysing the cells, the enzymes involved have, until now, remained unknown.

Nocardiooides simplex FJ2-1A was shown to contain a hydride transferase (HTES, or hydride transferring enzyme system) involved in the hydride transfer to the Meisenheimer complex (hydride Meisenheimer complex) is the first metabolite produced during mineralization of picric acid by Rhodococcus (opacus) erythropolis HL PM-1 (Lenke et al., 1992; Lenke & Knackmuss, 1992, 1996). The orange-red hydride σ-complex of picric acid (H−-PA, hydride σ-complex of picric acid; 2H−-PA, dihydride σ-complex of picric acid, 2,4-DNP, 2,4-dinitrophenol; H−-2,4-DNP, hydride σ-complex of 2,4-dinitrophenol; HTES, hydride transferring enzyme system).

The GenBank accession number for the sequence reported in this paper is AF435009.

Keywords: picric acid, two hydride transferases, NADPH-dependent F420 reductase

nbp gene functions of Rhodococcus (opacus) erythropolis HL PM-1 in the initial steps of 2,4,6-trinitrophenol degradation

Gesche Heiss,† Klaus W. Hofmann,† Natalie Trachtmann,† Dana M. Walters,‡ Pierre Rouvière‡ and Hans-Joachim Knackmuss†

Author for correspondence: Gesche Heiss, Tel: +49 711 685 5491, Fax: +49 711 685 5725.
e-mail: gesche.heiss@po.uni-stuttgart.de

Rhodococcus (opacus) erythropolis HL PM-1 grows on 2,4,6-trinitrophenol (picric acid) or 2,4-dinitrophenol (2,4-DNP) as sole nitrogen source. A gene cluster involved in picric acid degradation was recently identified. The functional assignment of three of its genes, npdC, npdG and npdI, and the tentative functional assignment of a fourth one, npdH, is reported. The genes were expressed in Escherichia coli as His-tag fusion proteins that were purified by Ni-affinity chromatography. The enzyme activity of each protein was determined by spectrophotometry and HPLC analyses. NpdI, a hydride transferase, catalyses a hydride transfer from reduced F420 to the aromatic ring of picric acid, generating the hydride σ-complex (hydride Meisenheimer complex) of picric acid (H−-PA). Similarly, NpdI also transformed 2,4-DNP to the hydride σ-complex of 2,4-DNP. A second hydride transferase, NpdC catalysed a subsequent hydride transfer to H−-PA, to produce a dihydride σ-complex of picric acid (2H−-PA). All three reactions required the activity of NpdG, an NADPH-dependent F420 reductase, for shuttling the hydride ions from NADPH to F420. NpdH converted 2H−-PA to a hitherto unknown product, X. The results show that npdC, npdG and npdI play a key role in the initial steps of picric acid degradation, and that npdH may prove to be important in the later stages.
aromatic ring of picric acid, giving rise to H−-PA (Ebert et al., 1999). The HTES also catalyses a second hydride transfer, converting H−-PA to the dihydride π-complex of picric acid (2H−-PA; Ebert et al., 2002). Both hydride transfer reactions require the NADPH-dependent F420 reductase (previously referred to as component A) as an electron shuttle from NADPH to F420.

Recently, a gene cluster of about 12.5 kb, involved in picric acid degradation, was identified in R. (opacus) erythropolis HL PM-1 using differential display and sequenced (Russ et al., 2000; Walters et al., 2001). We have named these genes npd for nitrophenol degradation. Our goal was to determine the function of npdC (previously ORF3), npdG (previously ORF7), npdH (previously undetected) and npdI (previously ORF8) by overexpressing the genes in Escherichia coli. Herewith, we have proven that the genes encode enzymes involved in picric acid catabolism.

METHODS

Bacterial strains, plasmids and bacterial culture. Strains and plasmids used are listed in Table 1. Recombinant E. coli strains were grown at 37 °C in Luria–Bertani (LB) medium supplemented with ampicillin (100 μg ml−1). For gene expression, overnight cultures of E. coli JM109(pNTG3), E. coli JM109(pNTG6), E. coli BL21 (DE3) (pNTG11) or E. coli TOP10 (pDMW10) were inoculated into LB and incubated for 1.5–3 h at 37 °C. The cultures were induced for 4–5 h at 30 °C with IPTG (1 mM) for E. coli TOP10 (pDMW10) and E. coli BL21 (DE3) (pNTG11), and with l-rhamnose (0.2% w/v) for E. coli JM109 (pNTG6) and E. coli JM109 (pNTG3). R. (opacus) erythropolis HL PM-1 was grown at 30 °C in 50 mM KH2PO4/NaHPO4 buffer (pH 7.4) containing 45 mM CaCl2, 50 mM acetate and mineral salts solution (Dorn et al., 1974), modified by adding 0.5 mM 2,4-DNP or 0.7 mM picric acid instead of (NH4)2SO4.

Molecular techniques. Standard protocols were used for manipulation of DNA (Ausubel et al., 2002; Sambrook et al., 1989). Plasmid DNA was isolated using the FlexiPrep Kit (Amersham Pharmacia Biotech). E. coli was transformed according to Inoue et al. (1990). Sequencing was performed by MWG Biotech AG (Ebersberg).

Fatty acid and mycolic acid analyses. Fatty acid analysis and analysis of mycolic acids were performed by the DSMZ GmbH (Braunschweig, Germany).

Sequence comparisons and database searches. Pairwise sequence comparisons and database searches were carried out using BLASTN, BLASTP, BLASTX and NCBI ORFfinder software (Altschul et al., 1990; Tatusova & Madden, 1999). Translations were achieved using the Translation Machine from the EMBL Outstation European Bioinformatics Institute. Conserved motif searches were performed using the programs CD-search, Pfam, Proscan and Block (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; http://www.sanger.ac.uk; http://ncpsb-phil.ibcp.fr/cgi-bin/pattern_prosite.pl; http://blocks.fhcrc.org/blocks-bin/blacks_search).

Table 1. Escherichia coli strains and plasmids used

<table>
<thead>
<tr>
<th>Bacterial strain/plasmid</th>
<th>Genotype/phenotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 recA1 gyrA96 thi hsdR17 (rK, mK) relA1 supE44 Δ(lac-proAB) (F’ traD36 proAB lacZΔM15) Δ</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>TOP10</td>
<td>F' mcrA Δ(mrr-34dRMS-mcrBC) o80dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)769 galU galK rpsL (strr) endA1 supG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>F' ompT hsdS (rK, mK) gal (Δcitzs857 ind1 sm7 nin5 lacUV5-T7 gene 1)</td>
<td>Calbiochem–Novabiochem</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTrcHis2-TOPO</td>
<td>Ap’, Pm, ColE1 origin; C-terminal tag containing six polyhistidine residues and myc epitope</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBAD/Thio-TOPO</td>
<td>Ap’, Pm&amp;BAD; ColE1 origin; C-terminal tag containing six polyhistidine residues and V5 epitope; His-Patch thioredoxin as an N-terminal fusion partner</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBW22</td>
<td>Ap’, Pm&amp;BAD; ColE1 origin</td>
<td>Wilms et al. (2001)</td>
</tr>
<tr>
<td>pQE-30</td>
<td>Ap’, phage T5 promoter; ColE1 origin; N-terminal tag containing six polyhistidine residues</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pET11a*</td>
<td>Ap’, phage T7 promoter; ColE1 origin; contains a polylinker with additional restriction sites: SmaI, KpnI, XhoI, SalI</td>
<td>Calbiochem–Novabiochem</td>
</tr>
<tr>
<td>pDMW10</td>
<td>PCR fragment containing npdG in pTrcHis2-TOPO</td>
<td>This work</td>
</tr>
<tr>
<td>pNTG3</td>
<td>Ndel/HindIII fragment containing npdI and His-tag from pQE-30 in pJoc2002</td>
<td>This work</td>
</tr>
<tr>
<td>pNTG6</td>
<td>Ndel/HindIII fragment containing npdC and His-tag from pBAD/Thio-TOPO in pJoc2002</td>
<td>This work</td>
</tr>
<tr>
<td>pNTG11</td>
<td>Ndel/HindIII fragment containing npdH and His-tag from pBW22 in pET11a</td>
<td>This work</td>
</tr>
</tbody>
</table>
Cloning and expression of npdC, npdG, npdH and npdI. npdA was amplified from R. (opacus) erythropolis HL PM-1 chromosomal DNA with specific primers. Primer P63 was designed with a BamHI site (underlined) (‘GGATCCCATGATCAAGGACAT-3’) and primer P68 was designed with a HindIII site (underlined) (‘GGGCTGAGTCCAGACATGCATG-3’). Following digestion with BamHI and HindIII, the resulting PCR product was ligated into the expression vector pQE-30 to yield plasmid pDMW4. npdC was amplified with primers P33 (‘ATGAGGTGGCTGAGATGGATG-3’) and P34 (‘GGCCGGTTCATGCTCGGGT-3’). The resulting PCR product was cloned into the pBAD/topo plasmid (Invitrogen) to yield pDMW7. npdG was amplified with primers P56 (‘ATGAGAGAGCCAGCA-AGATCCG-3’) and P57 (‘GCAGCTCAGTGATCATG-3’). The resulting PCR product was cloned into pTrcHis2-topo (Invitrogen) to yield pDMW10.

For expression, the npdC and npdI genes were amplified from pDMW7 and pDMW4, respectively. Primers were designed to incorporate Ndel and HindIII sites (underlined), and the Hsi-tag sequence at the 3’ end of npdC (‘TTTCTATATGGAT-CCGCAGATCCGATCCGATCC-3’) and 3’-TTGACATATCTGGATATCATGATG-3’) and the 3’ end of npdI (‘CTATTTGATCCAGAGGATATCATGATG-3’) and 3’-TTTGCGAGCTGGATATCATGATG-3’). Subsequently, npdH was cloned into pBW22 by amplification, the primers being designed to contain Ndel and HindIII sites (underlined) (5’-TTTCTATATGGATCCAGACATGCATG-3’ and 5’-TTTGCGAGCTGGATATCATGATG-3’). npdH was cloned into pBW22 by amplification, the primers being designed to contain Ndel and BamHI sites (underlined) (5’-TTTCTATATGGATCCAGACATGCATG-3’ and 5’-TTTGCGAGCTGGATATCATGATG-3’). The Hsi-tag was included in the expression vector pET11a (Ndel/Smal).

The PCR reaction mixtures contained 1 ng template DNA, 0.5 U Vent polymerase (New England Biolabs), 2 mM MgSO4, 4% DMSO, 0.2 mM each dNTP and 10 pmol each primer. Twenty-five amplification cycles were performed as follows: 95°C for 30 s, 55°C for 40 s, 72°C for 1 min 40 s. Primers were purchased from MWG Biotech. PCR fragments were eluted from agarose using an Easypure DNA purification kit (Biosym Diagnostics), restricted with appropriate restriction enzymes and ligated into the vector (Table 1).

Preparation of cell extracts and SDS-PAGE. Cells suspended in 50 mM KH2PO4/KHPO4 (pH 7.8) plus imidazole (5 mM) were lysed with a French press (Aminco) at 80 MPa. Cell debris was removed by centrifugation at 100000 g for 45 min at 4°C. The protein concentration was determined by the method of Bradford (1976) using a dye reagent concentrate (Bio-Rad protein assay). Bovine serum albumin served as the standard. SDS-PAGE was performed by the method of Laemmli (1970) on a Mini-PROTEAN II electrophoresis cell (Bio-Rad).

Purification of His-tag fusion proteins. His-tag fusion proteins were purified by Ni-NTA metal affinity chromatography (Qiagen), as described by the manufacturer. Fractions were tested for enzyme activity. Purified fractions were desalted by utilizing pD10 Desalting Columns (Amersham Pharmacia Biotech) and subsequently concentrated with a Vivaspin 2 ml concentrator (Sartorius).

Determination of the concentration of F420. F420, purified from Methanobacterium thermoautotrophicum using a QAE column, was obtained from Lacy Daniels (Peck, 1989). The lyophilized powder was dissolved in 50 mM Tris, pH 7.5, and the absorbance was determined at 420 nm. The concentration was calculated using an extinction coefficient of 41400 M⁻¹ cm⁻¹ (Purwantini & Daniels, 1996).

Enzyme assays and kinetic measurements. Enzyme assays and kinetic assays were performed with a Varian Cary 50 Biospectrophotometer controlled by Cary WinUV Bipackage software. All enzyme reactions were done in a total volume of 1 ml. The enzyme activity of NpdG in standard tests was measured in citrate-phosphate buffer (50 mM, pH 5.5) containing 10–100 µM NADPH, 10 µM F420 and 0.25 µg enzyme. The decrease in absorbance was monitored at 400 nm. The Km constant and Vmax were determined for NpdG only. Reaction rates were calculated by taking an extinction coefficient of 25700 M⁻¹ cm⁻¹ (Eirich et al., 1978). One unit of enzyme activity was taken as the amount of enzyme which converts 1 µmol F420 min⁻¹. For determination of the Km constant, the concentration of F420 was varied from 2 to 35 µM. The Km and Vmax values for F420 were calculated by using Sigma Plot for Windows, version 5.00 (SPSS).

Enzyme activities of NpdC, NpdH and NpdI were detected by repeated recording of UV-visible spectra (280–600 nm). Spectra were recorded for 6 min. Reactions with NpdC and NpdI were performed in 50 mM KH2PO4/KHPO4, pH 7.8, containing 125 µM NADPH, 11 µM F420, 100 µM substrate (picric acid, H3-PA or 2,4-DNP), 5 µg NpdG and 30 µg NpdI or NpdC. The first recording was performed in buffer containing F420 and NADPH only. After addition of NpdG, the second spectrum was recorded. The reaction was then commenced by the addition of NpdI or NpdC. Testing for enzyme activities in the absence of F420 or NADPH served as negative controls. Reactions with NpdH were performed in 50 mM KH2PO4/KHPO4, pH 7.8, containing 100 µM H3-PA and 30 µg NpdH.

HPLC analyses. Metabolites were detected by means of HPLC (Chromelac Chromatography Data Systems 4.30, equipped with a Dionex UV/Vis detector, UVD 1705/3405, a Dionex pump, P 380, and a Dionex autosampler, Gina50). Samples were resolved on Gromsil 100 Octyl-4 column (20 x 4 mm, particle size 5 µm; Grom) using a linear gradient of 0–100% (v/v) methanol, respectively, plus 5 mM PiCa (tetubutylammonium hydrogensulfate), pH 7.8, as the mobile phase. The precolumn was a Gromsil 100 Octyl-4 column (20 x 4 mm, particle size 5 µm; Grom). Enzyme reactions were terminated by freezing samples in liquid nitrogen prior to HPLC analysis.

Mass spectra. Liquid chromatography (LC) mass spectra were obtained by negative-mode electrospray ionization (ESI) on a high performance liquid chromatograph (Dionex P580A HPG) with a Dionex diode array detector, DAD 340, coupled to a TSQ 7000 mass spectrometer. Metabolites were resolved on an LC column [Luna RP18(2); Phenomenex; 3 µm, 150 mm x 2.1 mm] cooled to 15°C by a Dionex column oven, STH 585. The initial mobile phase comprised 80% (v/v) acetoni-
borate (345 mg; dissolved in 7 ml absolute acetonitrile) was added to the picric acid/acetonitrile solution under argon as a protective atmosphere at −20 °C. The precipitating dark powdery crystals were filtered through a sintered glass filter, followed by washing with ice-cold, absolute acetonitrile and finally dried in a vacuum (Christ Alpha 1-5). 2H−PA was prepared according to Severin & Schmitz (1962).

RESULTS

Reclassification of strain HL PM-1

Fatty acid and mycolic acid analyses (chain length of 48–54 carbon atoms) of R. erythropolis HL PM-1 suggest that it should be reclassified as Rhodococcus opacus. The almost complete 16S rDNA sequence of strain HL PM-1 (accession no. AF435009) was obtained and showed 99% sequence identity to the 16S rRNA gene of R. opacus strain 1CP and R. opacus strain GM-29. The first 500 bp revealed 99% sequence identity to R. opacus RB1 (Blasco et al., 1999) and the type strain R. opacus DSM 43205 (Klatte et al., 1994). Hence, we propose that R. erythropolis HL PM-1 should be reclassified as R. opacus HL PM-1.

Expression of npdC, npdG, npdH and npdI

All genes were expressed as His-tag fusion proteins (Table 1). npdG was cloned into pTrcHis2-TOPO and the resulting recombinant plasmid was designated pDMW10. In induced cultures of E. coli TOP10 (pDMW10), the enzyme constituted approximately 20% of the total cellular protein as estimated by SDS-PAGE. In non-induced cultures, no protein band of the expected size was detected. Hence, NpdG was purified from E. coli TOP10(pDMW10).

npdC and npdI were originally cloned into pBAD/Thio-TOPO and pQE-30, respectively. Recombinant cells carrying npdC or npdI in pBAD/Thio-TOPO or pQE-30 contained a substantial amount of inclusion bodies. Hence, npdC and npdI were amplified from the respective vectors and ligated with pJoc2702 to create pNTG6 and pNTG3, respectively. SDS-PAGE showed no enhanced protein bands of the expected sizes in cell extracts prepared from JM109(pNTG3) or JM109-(pNTG6), although the respective enzyme activities could be measured. Hence, the strains were used for subsequent protein production and purification.

npdH was first cloned into pBW22. The resulting recombinant cells showed very low levels of activity. npdH was then subcloned into pET11a+ to form pNTG11. Cell extracts of BL21 (DE3)(pNTG11) indicated that NpdH comprised 30% of the total cellular protein. Consequently, BL21 (DE3)(pNTG11) was used for protein purification.

Characterization of NpdC, NpdG, NpdH and NpdI

NpdG showed a 44% sequence similarity to an F<sub>120</sub>-dependent NADP reductase of Methanobacterium thermoautotrophicum in the database (Russ et al., 2000; Smith et al., 1997; Walters et al., 2001). Further, the first 27 aa of the translation product, NpdG, exhibited a 66% sequence identity to the amino-terminal region of the NADPH-dependent F<sub>120</sub> reductase of N. simplex FJ2-1A (Ebert et al., 1999). NpdC exhibited a sequence similarity of 42% to an N5,N10-methylene-tetrahydromethanopterin reductase of Methanococcus jannaschii (Bult et al., 1996; Russ et al., 2000) and NpdI possessed a sequence similarity of 46% to an F<sub>120</sub>-dependent N5,N10-methylene-tetrahydromethanopterin reductase from Archaeoglobus fulgidus (Klenk et al., 1997; Russ et al., 2000). In addition, the first 27 aa of NpdI exhibited a 77% sequence identity to the amino-terminal region of the hydride transferase of N. simplex FJ2-1A (Ebert et al., 1999).

Detailed sequence analyses of the picric acid degradation gene cluster revealed a short ORF (npdH) of 318 bp between npdG and npdI (Fig. 1). Database searches with NpdH showed no significant similarity to any known sequence.

No conserved motifs were detected using the programs CD-search, Pfam, Proscan or Block.

From SDS polyacrylamide gels, the molecular masses of NpdG, NpdC, NpdI and NpdH were calculated to be 28.8, 43.4, 41.7 and 13.8 kDa, respectively. The sizes of NpdG and NpdH were consistent with the predicted molecular masses of 27 and 12.9 kDa, respectively (taking fusion of the vector-encoded His-tag, myc and V5 epitopes into account). The predicted molecular masses for NpdC and NpdI were considerably smaller (35.9 and 32.9 kDa, respectively) than those calculated from SDS-PAGE. We have no explanation for this presently, except that the conditions used for SDS-PAGE (salts and SDS) may cause abnormal migration of the proteins.

NpdC, NpdG and NpdI were largely purified to complete homogeneity. NpdI could only be purified to 90%. NpdC, NpdG and NpdI were unstable in the presence of imidazole, a constituent of the elution buffer.
HPLC-ESI-MS proved the identity of the chemically methanol plus 5 mM PicA; 125 mm column. Coupled several weeks in 50 mM KH₂PO₄/K₂HPO₄ buffer (pH 7.8) plus 20% glycerol.

Reduction of F₄₂₀ by NpdG
Purified enzyme was incubated with NADPH and F₄₂₀ in citrate-phosphate buffer (50 mM, pH 5.5). A reduction in absorbance was observed at 400 nm, indicating the reduction of F₄₂₀ and the formation of F₄₂₀H⁺ (Purwantini et al., 1992). The Kₘ value was determined to be 9.68 ± 2.89 μM. The enzyme possessed a Vₘₐₓ of 17.27 ± 2.46 U mg⁻¹. The standard deviations were calculated from at least three repetitions. No activity was observed when F₄₂₀ was replaced by FAD (flavin adenine dinucleotide) or FMN (flavin mononucleotide).

NpdG catalyse a hydride transfer to picric acid or 2,4-DNP
To demonstrate that NpdG catalysed the conversion of picric acid to H⁻-PA, NpdG was incubated with picric acid, NADPH, F₄₂₀ and NpdG. No enzyme activity was detected in the absence of F₄₂₀. Enzyme activities were detected by repeated spectral scans. An increase in absorbance at 420 and 490 nm (λₘₐₓ of H⁻-PA at pH 7.8) was the same as described for H⁻-PA formation (Behrend & Heesche-Wagner 1999; Lenke et al., 1992). Furthermore, the spectral changes corresponded to a colour change from yellow to orange. The spectrum measured during HPLC analysis (λₘₐₓ 358, 406 and 440 nm) corresponded to the UV/Vis spectrum of the H⁻-PA and the molecular ion after elimination of HNO₂⁺, [M − HNO₂]⁻.

Fig. 2. Absorption spectrum showing the conversion of H⁻-PA to 2H⁻-PA by NpdG plus NpdC. The NpdG/NpdC assay contained F₂₅₀, NADPH and H⁻-PA in 50 mM KH₂PO₄/K₂HPO₄, pH 7.8. Scans were recorded every 60 s over a period of 10 min.

NpdC catalyses a hydride transfer to H⁻-PA
Spectroscopic scans were performed with NpdC plus H⁻-PA, NADPH, F₂₅₀ and NpdG. As for NpdG, no enzyme activity of NpdC was detected when omitting F₂₅₀ from the enzyme assay. Enzyme activity was detected as spectral changes exhibiting a decrease in absorbance at 420 and 490 nm (Fig. 2). This coincided with a colour change from orange to yellow. HPLC analysis showed that the new product possessed a retention volume of 2.6 ml [40% (v/v) methanol plus 5 mM PicA, pH 7.8; 250 mm column] or 2.4 ml [30% (v/v) methanol plus 5 mM PicA; 125 mm column]. The spectrum derived from HPLC analysis showed absorbance maxima at λₘₐₓ of 229 and 383 nm. These corresponded to 2H⁻-PA as described previously (Ebert et al., 2002) and to the chemically synthesized 2H⁻-PA. Coupled HPLC-ESI-MS of the latter confirmed the structure: 2H⁻-PA was detected at a retention volume of 0.9 ml. Two signals at m/z 232 and 185 were due to the molecular ion [M⁻]⁻ and the molecular ion after elimination of HNO₂⁺, [M − HNO₂]⁻.
NpdH converts 2H−-PA to product X

NpdH converted 2H−-PA to a new product, X. This was shown by repeated recording of spectroscopic scans; an increase in absorbance occurred at 389 nm (Fig. 3). HPLC analysis of product X showed a retention volume of 3.4 ml [40% (v/v) methanol plus 5 mM PicA, pH 7.8; 250 mm column] or 3.0 ml [30% (v/v) methanol plus 5 mM PicA; 125 mm column]. The spectrum obtained from HPLC analysis had absorbance maxima of 203, 236 and 390 nm. Coupled HPLC-ESI-MS showed a retention volume of 1.2 ml. The signal at m/z 232 corresponded to the molecular anion of the protonated 2H−-PA. From this ion, nitrite was eliminated, giving rise to the negative fragment ions at m/z 46 [NO2−] and m/z 185 [M−HNO2−], respectively. Since 2H−-PA was in equilibrium with product X, it was not completely converted to product X.

DISCUSSION

In the present study, NpdI, or hydride transferase II (plus NpdG, the NADPH-dependent F420 reductase), was shown to catalyse a hydride transfer to picric acid, forming H−-PA. NpdC, hydride transferase I (plus NpdG), was shown to catalyse hydride transfer to H−-PA, giving rise to 2H−-PA (Fig. 4). Based on in vivo transformations, 2H−-PA has, until recently, been believed to be a dead-end metabolite as part of a non-productive route of picric acid degradation (Lenke et al., 1992; Lenke & Knackmuss, 1992). Only recently was it shown that 2H−-PA is an intermediate of the productive route (Ebert et al., 2002). The authors showed that the purified HTES of N. simplex FJ2-1A (comprising a hydride transferase and an NADPH-dependent F420 reductase) not only converts picric acid to H−-PA, but also performs a second hydrogenation from H−-PA to 2H−-PA.

Since NpdC, NpdI and the hydride transferase of N. simplex FJ2-1A all catalyse hydride transfers, they are functionally alike. At the sequence level, no sequence similarity was detected between NpdC and the amino-terminal region of the hydride transferase of strain FJ2-1A. On the other hand, NpdI exhibited a sequence identity of 77% to the latter. Hence, we regarded NpdI as the immediate counterpart of the hydride transferase
of strain FJ2-1A, not NpdC. Functionally, both the hydride transferase of strain FJ2-1A and NpdI convert picric acid to H−PA, requiring F420H2 as the electron donor. Yet, the hydride transferase of strain FJ2-1A appears to possess a broader substrate specificity than NpdI: while the former has the ability to transfer a hydride ion to both picric acid and to H−PA, NpdI exhibited only minimal activity for H−PA. Instead, NpdC transferred hydride ions to H−PA, giving rise to 2H−PA (Fig. 4). This suggests that R. (opacus) erythropolis HL PM-1 has evolved an additional enzyme for this step. However, this does not exclude the possibility that N. simplex FJ2-1A may also harbour another hydride transferase.

Comparable to the hydride transferase of strain FJ2-1A, NpdI converted 2,4-DNP to H−2,4-DNP. Behrend & Heesche-Wagner (1999) initially described H−2,4-DNP as a metabolite of picric acid degradation by Nocardioides sp. CB 22-2. Furthermore, H−2,4-DNP would be expected to be the product of nitrite release from 2H−PA, as proposed for N. simplex FJ2-1A (Ebert et al., 2002). Indeed, cell-free extracts of R. (opacus) erythropolis HL PM-1 convert 2H−PA to H−2,4-DNP, suggesting it to be a true metabolite of picric acid degradation in the strain.

NpdG was shown to be essential for the hydride transferase reactions to take place by regenerating F420H2. An interesting aspect is that NpdG appears to only use F420 as a substrate. It cannot reduce flavins such as FAD or FMN. Enzymes playing a role in general metabolism, with F420 being involved, are known in mycobacteria, streptomycetes and Archaea (Berk & Thauer, 1997; Eker et al., 1989; Purwantini & Daniels, 1996). For this reason, even though specific for F420, NpdG may not necessarily be restricted to picric acid or 2,4-DNP degradation strains. Research is under way to test this hypothesis.

Although we cannot assign an explicit function to npdH, the gene product may well play an essential role in preparing 2H−PA for nitrite release. Since the molecular masses of the 2H−PA and product X were indistinguishable, while the retention volumes and the absorbance spectra differed, this suggests that product X may be a tautomeric form of protonated 2H−PA. This might be required to facilitate subsequent nitrite release. Vorbeck et al. (1998) made a similar observation in the course of TNT degradation, where the hydride complex of TNT (H−TNT) undergoes a second hydride attack, followed by protonation and subsequent tautomerization of the resulting dihydride complex (2H−TNT). Various other groups studying bacterial TNT degradation have detected several protonated forms of 2H−TNT (French et al., 1998; Pak et al., 2000). That the UV/Vis spectrum of product X (pH 7.8) was indistinguishable from that of 2H−PA (pH 12; at ≥280 nm) could be due to the same longer wavelength chromophore in both structures. Work is in progress to characterize product X, to show it to be part of a productive catabolic route of picric acid degradation and to assign an explicit function to NpdH.


Received 9 August 2001; revised 24 October 2001; accepted 1 November 2001.