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Converging catabolism of 2,4,6-trinitrophenol (picric acid) and 2,4-dinitrophenol by *Nocardioides* simplex FJ2-1A

Sybille Ebert[1], Peter Fischer[2] & Hans-Joachim Knackmuss[1]*
[1]*Institut für Mikrobiologie and Institut für* [2]*Organische Chemie der Universität Stuttgart, Germany (* author for correspondence)*

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Abstract

Initial F_{420}-dependent hydrogenation of 2,4,6-trinitrophenol (picric acid) generated the hydride σ-complex of picrate and finally the dihydride complex. With 2,4-dinitrophenol the hydride σ-complex of 2,4-dinitrophenol is generated. The hydride transferring enzyme system showed activity against several substituted 2,4-dinitrophenols but not with mononitrophenols. A K_m-value of 0.06 mM of the hydride transfer for picrate as substrate was found. The pH optima of the NADPH-dependent F_{420} reductase and for the hydride transferase were 5.5 and 7.5, respectively. An enzymatic activity has been identified catalyzing the release of stoichometric amounts of 1 mol nitrite from 1 mol of the dihydride σ-complex of picrate. This complex was synthesized by chemical reduction of picrate and characterized by ^{1}H and ^{13}C NMR spectroscopy. The hydride σ-complex of 2,4-dinitrophenol has been identified as the denitration product. The nitrite-eliminating activity was enriched and clearly separated from the hydride transferring enzyme system by FPLC. 2,4-Dinitrophenol has been disproven as a metabolite of picrate (Ebert et al. 1999) and a convergent catabolic pathway for picrate and 2,4-dinitrophenol with the hydride σ-complex of 2,4-dinitrophenol as the common intermediate has been demonstrated.

Abbreviations: 2-Cl-4,6-DNP, 2-chloro-4,6-dinitrophenol; 4,6-DNH, 4,6-dinitrohexanoate; DNOC, 2-methyl-4,6-dinitrophenol; 2,4-DNP, 2,4-dinitrophenol; 2,6-DNP, 2,6-dinitrophenol; F_{420}, coenzyme F_{420}; $2H^-$-picrate, dihydride complex of picrate; H^--picrate, hydride σ-complex of picrate; HTES, hydride-transferring enzyme system; PCP, pentachlorophenol; 1,3,5-TNP, 1,3,5-trinitropentane; TNT, 2,4,6-trinitrotoluene.

Introduction

Nitrophenols are important building blocks for the synthesis of dyes, pesticides, and explosives. Because of their ubiquitous use nitrophenols occur as contaminants in industrial effluents and at low concentrations also in surface and groundwaters (Hallas & Alexander 1983). 2,4,6-Trinitrophenol (picric acid) and its ammonium or potassium salts (picrates) were used as explosives during the first and second world war. As a consequence they have been found as pollutants in ground water at former production and certain military sites (Wyman et al. 1992). The main source of dinitrophenols and picric acid in industrial wastewater is the production of nitrobenzene from benzene (Patil & Shinde 1989).

Because of the presence of the electron-withdrawing nitro groups as substituents dinitrophenols and trinitrophenols are not readily attacked by oxygenases as generally observed with aromatic xenobiotic compounds including *o*-nitrophenol (Zeyer & Kearney 1984) and p-nitrophenol (Hanne et al. 1993; Kadiyala & Spain 1998; Munnecke & Hsieh 1974; Spain et al. 1979). Consequently, initial biological reduction of the aromatic system is more likely than an oxidative attack (Rieger & Knackmuss 1995; Vorbeck et al. 1998). Up to now aerobic bacterial degradation of 2,4,6-trinitrophenol, 2,4,6-trinitrotoluene (TNT), or 2,4-dinitrophenol (2,4-DNP) through initial mono-

or dioxygenation is unknown. An exception is 2,6-dinitrophenol (2,6-DNP) which is subject to denitration by an initial dioxygenase (Ecker et al. 1992). Reduction occurs preferably at the nitro groups revealing unproductive dead end metabolites as products. Characteristically, gratuitous reduction of picric acid generates picramic acid as a dead end product (Tsukamura 1960). For picric acid, 2,4-DNP and TNT an unusual initial hydrogenation of the aromatic ring system is observed during *in vivo* transformations. Only for the nitrophenols was this hydrogenation identified as part of a complete catabolic sequence. For these compounds hydride σ-complexes have been identified as the initial metabolites (Lenke & Knackmuss 1992; Rieger et al. 1999; Vorbeck et al. 1998).

Following the OECD guidelines 2,4-DNP and picrate proved to be not biodegradable by activated sludge. Nevertheless, these compounds are utilized and mineralized aerobically by *Nocardioides simplex* FJ2-1A (Ebert et al. 1999) and several *Rhodococcus* strains (Blasco et al. 1999; Lenke & Knackmuss 1992). It is suggested that extensive ring hydrogenation results in a non-oxidative ring cleavage yielding 4,6-dinitrohexanoate (4,6-DNH). This metabolite was found in the supernatant of resting cell experiments with *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2 (Lenke et al. 1992). 3-Nitroadipate has been identified as a metabolite in the degradation of 2,4-DNP by *Rhodococcus* sp. strain RB1 which is proposed to be a product of an alternative pathway of oxidative ring cleavage and concomitant elimination of nitrite (Blasco et al. 1999).

We have described the hydride transfer to the aromatic ring system by a two-component enzyme system consisting of a NADPH-dependent F_{420} reductase and a hydride transferase. Coenzyme F_{420} functions as mediator for the hydride transfer and NADPH as the source of reduction equivalents (Ebert et al. 1999).

In the present paper we further characterize the hydride-transferring enzyme system (HTES) and its substrate specifity. We identify the hydrogenation products and an enzymatic denitration of the dihydride σ-complex of picrate ($2H^-$-picrate) to the hydride σ-complex of 2,4-DNP (H–2,4-DNP). These findings refute the former hypothesis that nitrite is eliminated from the σ-complex of picrate (H^--picrate) generating 2,4-DNP (Ebert et al. 1999; Rieger et al. 1999).

Materials and methods

Resting cell experiments with nitrophenols

Cells of *N. simplex* were grown as described earlier (Ebert et al. 1999) and harvested by centrifugation, resuspended in phosphate buffer (pH 7.4; 50 mM), and incubated on a water bath shaker at 30 °C. Turnover of nitrophenols was followed by HPLC analysis.

Purification of the NADPH-dependent F_{420} reductase, the hydride transferase and the nitrite eliminating activity

Preparation of cell-free extract by French press treatment and purification of the enzymes of the HTES to electrophoretic homogeneity were performed as described previously (Ebert et al. 1999). Nitrite eliminating activity was concentrated by fast-protein liquid chromatography (FPLC). Cell extract (74.1 mg of protein) was passed through an anion exchange column (Q Sepharose HP 16/10, Pharmacia, Uppsala, Sweden) equilibrated with 50 mM TRIS-HCl (pH 7.5). Nitrite eliminating activity was eluted from the column with a linear gradient (300 ml) from 0 to 1 M NaCl in 50 mM TRIS-HCl (pH 7.5). Protein concentrations were estimated by the method of Bradford (1976).

Purification of coenzyme F_{420}

For isolation and quantification of coenzyme F_{420} the strain was grown aerobically in batch culture in a 100 l stirred tank reactor (Bioengineering, Wald, Switzerland) at 30 °C and 550 rpm in liquid culture medium as described earlier (Ebert et al. 1999). The culture was aerated at a rate of 50 l per min. After consumption of picrate (decolorization of the medium) the cells were fed again with 0.35 mM picrate and harvested by centrifugation immediately after decolorization of the medium.

Coenzyme F_{420} was extracted with 70% ethanol out of 500 g wet cells of *N. simplex* as described by Lin and White (1986). Purification was performed according to Abken (1998). After the last purification step coenzyme F_{420} containing fractions were pooled and methanol was evaporated in vacuum at 40 °C bath temperature with a rotation evaporator. The residual aqueous solution of coenzyme F_{420} was lyophilized. The concentration of coenzyme F_{420} was calculated on the basis of a molar extinction coefficient at 420 nm of 41 400 M^{-1} cm^{-1} (pH 7.5) (Purwantini et al. 1992).

For recycling of the cofactor coenzyme F_{420} containing reaction mixtures were heated to 100 °C for 15 min. The precipitate was removed by centrifugation. The supernatant was diluted 1 : 1 with 50 mM TRIS-HCl (pH 8.5) and applied to an anion exchange column (Resource Q (6 ml), Pharmacia, Uppsala, Sweden) equilibrated with 50 mM TRIS-HCl (pH 8.5). The cofactor was eluted with 0.42 M NaCl.

Enzyme assay

The activity of the HTES towards picrate or 2,4-DNP as substrates was routinely assayed photometrically as described earlier (Ebert et al. 1999). Tests which were followed by HPLC analysis contained a higher NADPH concentration (1 mM) and substrate concentrations between 0.05 and 0.15 mM 2,4-DNP or picrate. Reactions for repeated recording of UV-visible spectra were started by addition of hydride transferase.

For determining the pH optimum of the NADPH-dependent F_{420} reductase the assay mixture contained buffer (citric acid – phosphate buffer for the pH range of 3 to 7 or 50 mM TRIS-HCl for pH 7 to 8.5), 0.5 mM NADPH, 0.044 mM coenzyme F_{420} and 0.3 μg per ml NADPH-dependent F_{420} reductase. Activity was followed by the decreasing absorption at 340 nm. The test was carried out anaerobically in microtiterplates with a microplate scanning spectrophotometer (Power $Wave_x$, Bio-tek® Instruments, Inc., Winooski, VT, USA) equipped with the KC4 software v. 2.5 (Bio-tek® Instruments, Inc., Winooski, VT, USA) under nitrogen at 30 °C and started by addition of substrate.

For determining the pH optimum of the hydride transferase the assay mixture contained buffer (100 mM succinic acid – NaOH buffer for the pH range of 3.5 to 5, 100 mM potassium phosphate buffer for pH 5 to 7.5, 100 mM TRIS-HCl for pH 7.5 to 10, 100 mM glycine – NaOH for pH = 10 to 12), 1 mM NADPH, 3.5 μM coenzyme F_{420}, 0.1 mM 2,4-DNP, 0.1 μg per ml NADPH-dependent F_{420} reductase and 78 ng per ml hydride transferase.

Transformation of $2H^-$-picrate was routinely monitored spectrophotometrically by following the decrease of absorption at 390 nm or by repeated recording of the UV-visible spectrum in the wavelength range between 250 and 600 nm. The reaction mixture contained the chemically synthesized dihydride complex of picrate and cell extract of *Nocardioides simplex* FJ2-1A or fractions of FPLC purification steps in 50 mM TRIS-HCl (pH 7.5). Reactions were started by the addition of protein.

Analytical methods

For quantification of substrates in stopped tests a reversed-phase high performance liquid chromatography (HPLC) system with a Gromsil 120 Oc4 column (125 × 4 mm, particle size 5 μm, Grom, Herrenberg, Germany) was used. The mobile phase consisted of 20% acetonitrile, 80% water and 0.26% H_3PO_4 (v/v/v). For determination of reduced nitroaromatic compounds an anion exchange column (Gromsil TSK- Gel Q-5PW, 125 × 4 mm, particle size 20 μm, Grom, Herrenberg, Germany) was used as a solid phase. The mobile phase consisted of 25 mM TRIS-HCl (pH 8). A linear gradient from 0.35 to 0.6 M NaCl in basic buffer was applied. Purity of the dihydride complex of picrate was examined by ion pair chromatography on the reversed-phase Gomsil column with an isocratic eluent consisting of 20% methanol-water and 5 mM tetrabutylammonium hydrogen sulfate adjusted to pH 12 with sodium hydroxide. Concentrations were determined at 210 nm, 240 nm, 260 nm and 300 nm. Products were identified by UV-visible spectra using a photodiode array detector (UVD 340S) from Gynkotek (Germering, Germany).

Nuclear magnetic resonance (NMR) spectra were recorded with an ARX 500 spectrometer (Bruker, Rheinstetten, Germany) at a nominal frequency of 500.14 MHz (^{1}H) and 125.77 MHz (^{13}C), respectively. Samples were dissolved in D_2O.

Materials

Synthesis of the dihydride σ-complex of picrate as authentic reference compound followed the procedure described by Severin and Schmitz (1962). The hydride σ-complex of picrate was synthesized as described by Rieger et al. (1999). According to Behrend and Heesche-Wagner (1999) an authentic standard of H^--2,4-DNP was prepared by adding sodium borohydride to an aequeous solution of 2,4-DNP. Immediately after chemical reduction the pH value of the reaction mix was adjusted to pH 7.5 with phosphoric acid. This H^--2,4-DNP standard in water was rather unstable and could be used only for up to 2 hours even if stored on ice.

Results

Growth of Nocardioides simplex *FJ2-1A and turnover of substrates by resting cells*

Nocardioides simplex FJ2-1A can grow with picrate as a nitrogen, carbon, and energy source. However, growth with the nitroaromatic compound in mineral medium is very slow ($t_d = 5d$). Therefore, acetate, yeast extract, peptone, and casamino acids were supplemented to grow the strain within 26 h to an optical density of 3.5 at 546 nm.

Content of coenzyme F_{420}

To quantify the amount of coenzyme F_{420} involved in the hydride transfer by the HTES, wet cells of *N. simplex* were extracted with 70% ethanol (Lin & White 1986) and the supernatant purified by FPLC (Abken 1998). This revealed 139 μmol of cofactor F_{420} per kg of wet cells. The content is lower than in *Methanobacterium thermoautotrophicum* (376 μmol per kg of wet cells) but nearly 16-fold higher than in *Streptomyces griseus* (8 μmol per kg of wet cells), *Streptomyces coelicolor* (9 μmol per kg of wet cells), or *Halobacterium cutirubrum* (9 μmol per kg of wet cells) (Eker et al. 1989).

Enzyme characterization

The NADPH-dependent F_{420} reductase and the hydride transferase are cytosolic enzymes. No activity was found in the membrane fraction. Activity of hydride transfer was maximal at pH 7.5. The NADPH-dependent F_{420} reductase had a maximum activity at pH 5.5. The HTES could be stimulated by ammonium sulfate at a concentration of 1 M. In order to function as a hydride donor for the reaction catalyzed by the hydride transferase coenzyme F_{420} must be reduced by the NADPH-dependent F_{420} reductase and NADPH (Ebert et al. 1999). FAD or FMN could not substitute for coenzyme F_{420}. Hence, the hydride transfer was investigated in a coupled test with an excess of the F_{420}-reductase and NADPH.

The affinity of the hydride transferase for picrate was determined by the coupled enzyme test. The reaction was stopped with phosphoric acid and substrate turnover quantified by HPLC. The apparent K_m for picrate was 0.06 mM. Substrate inhibition occurred at picrate concentrations above 0.125 mM. Turnover of 2,4-DNP did not follow Michaelis–Menten kinetics. H^--2,4-DNP was very unstable and spontaneously regenerated 2,4-DNP as detected by HPLC measurements. This effect has been described as a non-enzymatic disproportionation by Behrend (1999).

Several nitroaromatic compounds were tested as potential substrates for the HTES. Reactions were followed by repeated recording of UV-visible spectra and HPLC measurements. Mononitrophenols such as *o*-nitrophenol and *p*-nitrophenol and 2,6-DNP were not transformed by the HTES. As observed with whole cells DNOC was a poor substrate for the HTES (nearly 1% of activity with picrate). But increasing absorption during repeated recording of UV-visible spectra between 400 and 550 nm indicated formation of a hydride σ-complex. This is even more pronounced with 2-chloro-4,6-dinitrophenol (2-Cl-4,6-DNP) as substrate (approximately 90% of the activity with picrate).

Hydride transfer to picrate

Repeated scans of UV-visible spectra during picrate transformation with the HTES revealed a fast increase of absorption between 400 and 550 nm which is followed by a slow decrease in the same spectral range (Figure 1). The yellow color of picrate in the reaction assay mix turned quickly to orange and then slowly to pale yellow. The hydride σ-complex of picrate (H^--picrate) was identified as the orange metabolite by HPLC measurements. Therefore H^--picrate, as described by Rieger et. al. (1999), could not be the final product of the enzymatic reaction and further transformation occurred. The enzymatic reaction was stopped with phosphoric acid and the samples were analyzed with HPLC under acidic conditions. A sharp peak at a retention time of 7.6 min and a maximum absorption of 203 nm corresponded to the data measured for a standard of 1,3,5-TNP. As described by Severin and Adam (1963) 1,3,5-TNP is generated quantitatively by chemical decarboxylation of $2H^-$-picrate under acidic conditions. This indicates that two hydride ions were transferred by the HTES and $2H^-$-picrate was the product.

Synthesis of the authentic $2H^-$*-picrate and spectroscopic characterization*

$2H^-$-Picrate was synthesized by reducing picrate in alkaline solution with sodium borohydride (Severin & Schmitz 1962) and precipitated by addition of methanol. HPLC analysis under alkaline conditions gave a single peak (retention time 6.2 min). The UV-visible

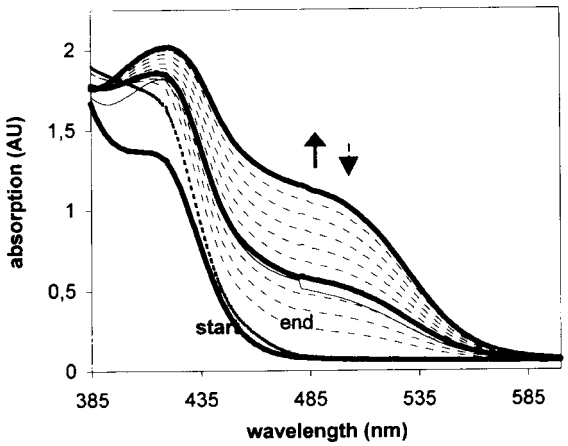

Figure 1. Repeated recording of UV-visible spectra at intervals of 35 seconds during an enzymatic transformation containing 0.1 mM picrate, NADPH-dependent F_{420} reductase and hydride transferase (11.5 μg/ml and 0.04 μg/ml protein, respectively), coenzyme F_{420} (11 μM), NADPH (1 mM), and TRIS-HCl (50 mM, pH 7.5). The spectra shown by heavy lines indicate increasing absorbance due to formation of the hydride σ- complex of picrate (0–105 sec) which subsequently declined (thin lines) corresponding to the formation of $2H^-$-picrate (105–455 sec).

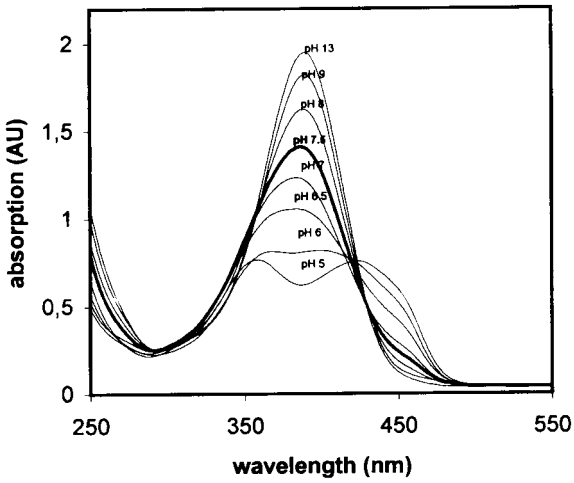

Figure 2. pH-Dependence of the UV-visible spectrum of $2H^-$-picrate (0.1 mM). The heavy line depicts the spectrum at pH 7.5, which was used for spectrophotometric assays.

spectrum in alkaline solution (pH 13) showed a maximum at 390 nm as described by Severin and Schmitz (1962) which changed characteristically both as to position and intensity with decreasing pH (Figure 2). A slight hypsochromic shift of the maximum from 390 to 385 nm and a considerable hypochromic effect was observed on lowering the pH to 7.5 (enzymatic test conditions). The increase of absorption around 450 nm is due to the increasing instability of $2H^-$-picrate at pH $\leq$ 6.5.

The ^1H and ^{13}C NMR spectra (D_2O, 300 K) unequivocally confirmed the structure of the dihydride σ-complex of picrate. The ^1H spectrum showed one singlet only, at δ = 4.04 ppm, for the four chemically equivalent protons in positions 3 and 5 of the cyclohexanoid ring system.

From the ^{13}C satellites a ^1J(C, H) coupling constant of 135 Mz is extracted which is characteristic for an sp^3 carbon atom with electronegative substituents. The ^{13}C NMR spectrum showed two signals at δ = 120.98 and 120.15 ppm with a relative intensity of 2 : 1 for the sp^2 carbon atoms bearing the nitro substituents (C-2, 6 and C-4). The quasi-carbonyl C-1 resonance was located at δ = 175.65 ppm, a value typical for the *ipso* carbon of a phenolate or the C-1 carbon of an enolate anion. An intensive singlet at δ = 30.41 ppm, i.e., in the region characteristic for aliphatic sp^3 carbon atoms, was assigned to C-3,5. The 1J connectivity between this 30 ppm ^{13}C and the 4.04 ppm proton resonance is established by a ^{13}C, ^1H COSY (C, H correlation). A ^1H test spectrum, taken after the time-consuming ^{13}C and correlation NMR experiments, clearly demonstrated the structural integrity of the $2H^-$-picrate complex even after 24 hours. Tests with the HTES unequivocally established the identity of the biological transformation and chemical reduction product of picrate and H^--picrate; HPLC retention times and UV-visible spectra were identical. Authentic $2H^-$-picrate was used to detect activity of its further transformation.

Identification of the product of the hydride transfer to 2,4-DNP

Intensive hydrogenation of 2,4-DNP yielding 2,4-dinitrocyclohexanone has been discussed in literature (Lenke et al. 1992). Therefore we analyzed whether one or two hydride ions were transferred to 2,4-DNP by the HTES of *N. simplex* FJ2-1A. During transformation of 2,4- DNP an increasing absorption between 400 and 550 nm and an isosbestic point at 405 nm was observed. H^--2,4-DNP as described by Behrend and Heesche-Wagner (1999) was formed. The UV-visible spectrum showed the characteristic absorption spectrum of the H^--2,4-DNP which was identical with that of an authentic standard (Behrend & Heesche-Wagner 1999). The reduction was not complete as analyzed in HPLC measurements. Since H^--2,4-DNP was highly unstable and spontaneously

disproportionated to 2,4-DNP, aminonitrophenol and minor unidentified products (Behrend 1999) a complete mass balance of 2,4-DNP hydrogenation could not be established. HPLC analysis using an anion exchanger column and UV-visible spectra recorded during an HPLC run verified the identity of the chemically synthesized H^--2,4-DNP and the enzymatic product.

Hydride transfer and subsequent nitrite elimination

H^--Picrate has been identified as the initial metabolite of picrate hydrogenation by its UV-visible spectrum during HPLC measurements. Repeated recording of UV-visible spectra clearly showed that the first hydride transfer is faster than the second one. Transformation of $2H^-$-picrate with concomitant nitrite elimination was catalyzed by crude extract as demonstrated by successive UV-visible spectral traces and the detection of nitrite in the reaction mix. Therefore the $2H^-$-picrate transforming activity was called denitrating activity.

All fractions obtained by FPLC separation of the crude extract were tested for activity of nitrite elimination from the chemically synthesized H^--picrate (Rieger et al. 1999) or from $2H^-$-picrate. As observed with picrate no H^--picrate transforming activity was detected in any single fraction. Only upon combination of fractions, containing the components of the HTES, H^--picrate was transformed with concomitant NADPH consumption but without nitrite release. Both σ-complexes H^-- and $2H^-$-picrate are stable under physiological conditions and nitrite was released neither spontaneously nor unspecifically by bovine serum albumin or denaturated enzyme. In contrast, nitrite-eliminating activity was observed in one protein fraction after the first enrichment step with $2H^-$-picrate as substrate, but not with H^--picrate.

Quantification of nitrite and the product of nitrite elimination

Like HTES the nitrite-eliminating activity was located in the cytosolic fraction of *N. simplex* cells and could be enriched by FPLC using an anion exchanger column. The activity eluted at a ionic strength of 0.28 M sodium chloride. This purification step allowed a separation from the components of the HTES which eluted at higher ionic strength (Ebert et al. 1999). Further purification efforts including hydrophobic chromatography, chromatography on a hydroxylapatite column, and strong anionic exchange column failed because of loss of activity. The buffers

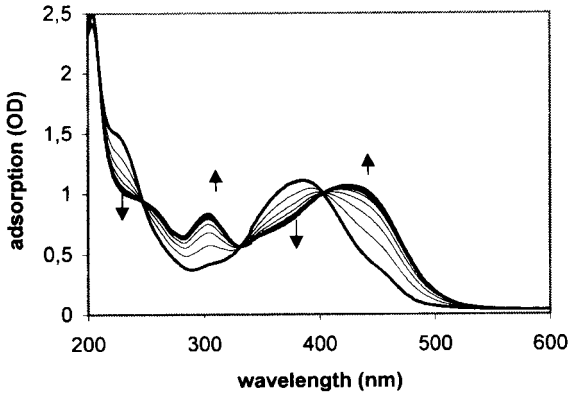

Figure 3. Successive UV-visible spectral traces (5 min intervals) during turnover of $2H^-$-picrate (heavy line) by enriched nitrite eliminating activity (0.024 mg protein in 50 mM TRIS-HCl (pH 7.5)).

TRIS-HCl (pH 7.5) and phosphate buffer did not influence activity. No positive effects were observed when metal ions such as Mg^{2+}, Ca^{2+}, Mn^{2n}, Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Zn^{2+}, K^+, or Na^+ were added to the reaction mix. Neither the presence of picrate nor 2,4-DNP affected the nitrite-eliminating activity. Addition of 0.1 M dithiothreitol, 1 M ammonium sulfate, or 1 M cyanide proved inhibitory.

The enzymatic reaction with $2H^-$-picrate was followed by repeated recording of UV-visible spectra (Figure 3). Isosbestic points indicated a linear correlation of changes in concentration of reacting species in the reaction mix and the final UV-visible spectrum suggested the formation of H^--2,4-DNP. The product of the reaction was unequivocally identified as H^--2,4-DNP by HPLC and its UV-visible spectrum measured *in situ*. A freshly synthesized authentic preparation of H^--2,4-DNP served as reference (Figure 4). Using an anion exchanger column H^--2,4-DNP eluted with a retention time of 8.2 min at a salt concentration of 0.471 M in a gradient of 0.35 to 0.6 M sodium chloride.

Nitrite release from picrate, H^--picrate, or $2H^-$-picrate as substrate was compared and quantified colorimetrically. Only with $2H^-$-picrate as substrate the nitrite eliminating protein could release nitrite which was formed in stoichiometric amount. A combination of the HTES and the nitrite-eliminating activity transformed both picrate and the H^--picrate to H^--2,4-DNP and nitrite as the only products. No nitrite elimination was observed when DNOC or 2-Cl-4,6-DNP was transformed by the combination of the HTES and the denitrating activity.

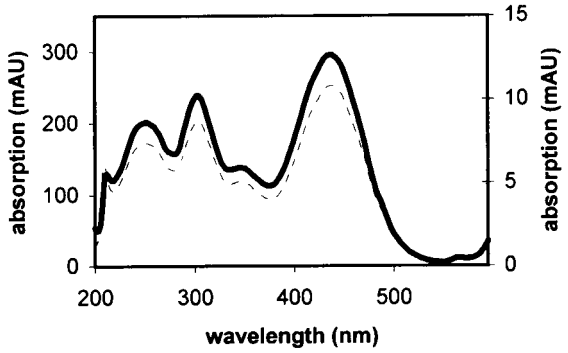

Figure 4. UV-visible spectra recorded during HPLC run of authentic H^--2,4-DNP (right *y* axis, bold line)and biologically formed H^--2,4-DNP during the reaction catalyzed by the nitrite eliminating activity with $2H^-$-picrate as substrate (left *y*-axis, thin line).

Discussion

The formerly described NADPH-dependent F_{420}-reductase and the hydride transferase in *N. simplex* FJ2-1A were further characterized. They were constitutively expressed which is in contrast to *Rhodococcus erythropolis* HL PM-1, another picrate degrading strain (Russ et al. 2000). Correspondingly, the concentration of cofactor F_{420} in *N. simplex* was constant irrespective of the presence of picrate in culture medium. Nevertheless, the enzymes of the two organisms are isofunctional and very similar. This is shown by the N-terminal sequence of the NADPH-dependent F_{420} reductase being almost identical and both enzymes are specific for NADPH as a hydride donor for coenzyme F_{420}. For other F_{420}-dependent NADPH reductases from actinomycetes or archaea the pH optima for the reduction of coenzyme F_{420} are in the same range of pH 5.5.

The hydride transferase of *N. simplex* also revealed high similarity to the corresponding enzyme, named F_{420}-dependent dehydrogenase (dh2), from *Rhodococcus erythropolis* HL PM-1 (Russ et al. 2000). Both enzymes catalyse the transfer of hydride to picrate or 2,4-DNP, the latter generating the same characteristic UV-visible spectrum of H^--2,4-DNP described by Behrend and Heesche-Wagner (1999). The color change from the bright yellow of picrate to the orange red of H^--picrate and finally to a pale yellow of $2H^-$-picrate can be rationalized in terms of a successive confinement of the delocalized π-electron system. The double hydride transfer into positions 3 and 5 of the carbon backbone creates two tetragonal centres which interrupt the original planar aromatic π-system of picrate.

The second hydrogenation step, reducing H^--picrate to $2H^-$-picrate, was hitherto considered as a reaction that misroutes the xenobiotic into an unproductive pathway. Such metabolic misrouting has been addressed by Vorbeck et al. (1998) as one of the catabolic reactions that prevent complete biodegradation of TNT. Formation of hydride and dihydride σ-complexes of TNT has also been observed with the picrate-utilizing *Rhodococcus erythropolis* strain HL PM-1, the 4-nitrotoluene-utilizing *Mycobacterium* sp. strain HL 4-NT-1 (Vorbeck et al. 1998), and the pentaerythritol tetranitrate or glycerol trinitrate-utilizing *Enterobacter cloacae* PB2 (Williams & Bruce 2000). Except for the latter organism these hydrogenations of TNT are gratuitous reactions that actually give rise to hydride complexes as dead end products. Slow growth of the *E. cloacae* strain on TNT as a nitrogen source under release of nitrite (Williams & Bruce 2000), however, indicates that hydride complex formation may allow partial utilization of TNT.

Enzymatic release of nitrite from H^--picrate and concomitant rearomatisation to 2,4-DNP were considered as the initial key reactions for productive breakdown of picrate (Ebert et al. 1999; Rieger et al. 1999). The present results, however, clearly indicate that cell free extract of *N. simplex* eliminated nitrite from $2H^-$-picrate. H^--2,4-DNP and stoichiometric amounts of nitrite were found by enzymatic transformation containing the enriched nitrite-eliminating activity and authentic $2H^-$-picrate as substrate. No such activity was found with H^--picrate as substrate.

Hydride transfer to picrate, H^--picrate, or 2,4-DNP takes place at position 3 of the aromatic ring. Picrate, 2,4-DNP, and other structurally related dinitrophenols, e.g., DNOC, 2-Cl-4,6-DNP, and 2,6-DNP, were tested as substrates for the HTES and the denitrating activity. Compared to whole cells specific activities of the partially purified HTES with picrate or 2,4-DNP as substrates (Ebert et al. 1999) were 2000 or 1000 fold higher, respectively. Purified HTES revealed specific activities of 45 U per mg of protein with picrate and 24 U per mg of protein with 2,4-DNP as substrate. Since 2,4-DNP is generated by disproportionation of H^--2,4-DNP an apparent lower value of activity with 2,4-DNP was observed. This is in contrast to the partially purified HTES which contained H^--2,4-DNP transforming activities.

DNOC is transformed only very slowly by HTES. Hydrogenation stops at the level of a hydride σ-complex of DNOC as indicated by characteristic

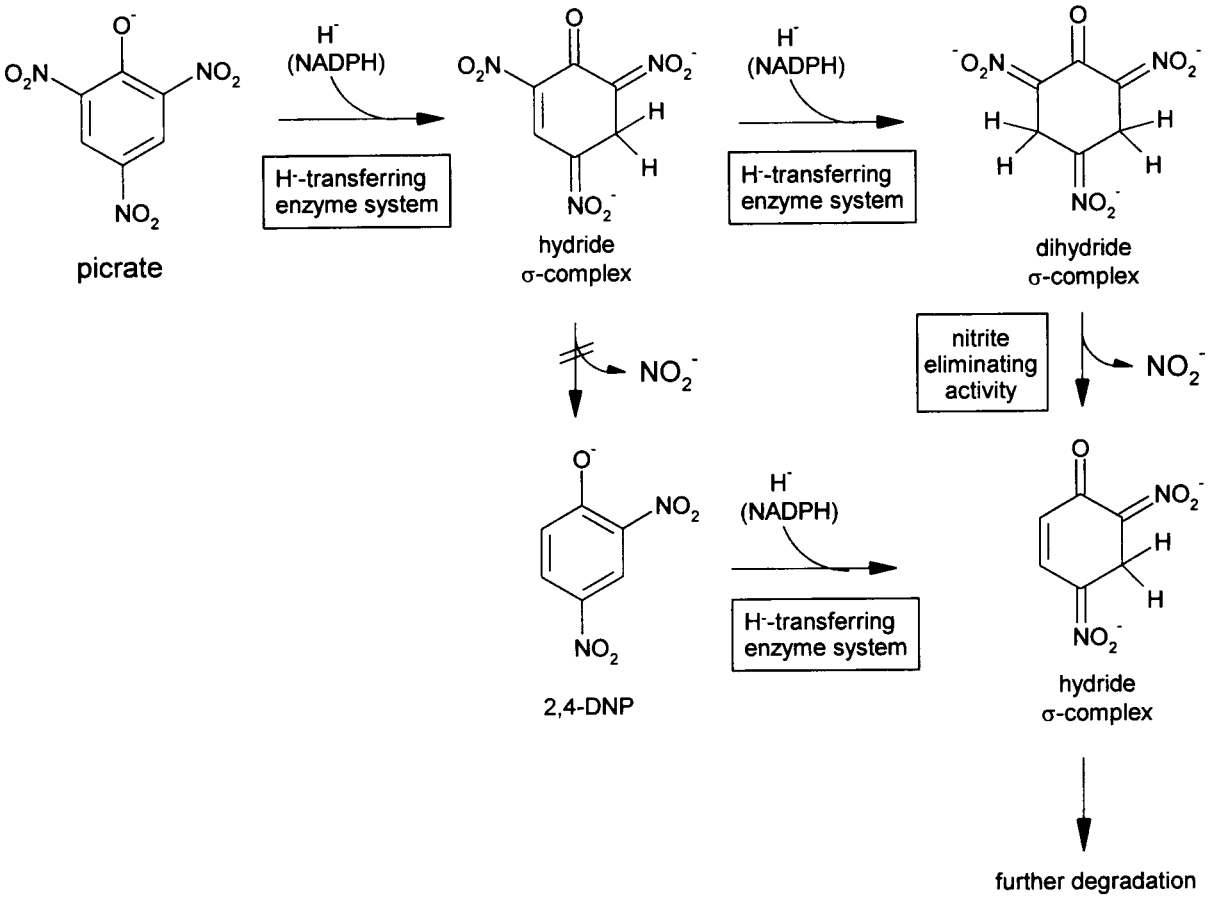

Figure 5. Convergent degradation of picrate and 2,4-DNP by *N. simplex*.

changes of the UV-visible spectra. Obviously, hydride is transferred only to dinitrophenols with nitro groups in 2 and 4 position. No nitrite was eliminated when enriched nitrite-eliminating activity was added to the reaction mixture. Under the same conditions 2-Cl-4,6-DNP was readily transformed by the HTES. UV-visible spectroscopy again indicated hydride σ-complex formation. However, nitrite was not eliminated when denitrating activity was added. Our observations with different substituted nitrophenols clearly identify two structural elements that are necessary for ring hydrogenation and subsequent nitrite elimination. (i) At least two nitro groups in 2- and 4-position must be present for hydride transfer. (ii) Nitrite elimination requires a double hydrogenation of the 2,4,6-trinitroaromatic ring. By conversion experiments and enzyme tests shown here it is evident that the electron withdrawing substituents of 2,4,6-substituted dinitrophenols increase the activity and electron donating groups such as methyl in DNOC decrease the activity of hydrogenation.

Accumulation of non-stoichiometric amounts of 4,6-DNH in the culture fluid is characterized by a fast increase and a slow decrease in concentration. Its occurrence and the formation of stoichiometric amounts of 2-methyl-4,6-dinitrohexanoate from DNOC by picrate grown cells of *Rhodococcus erythropolis* HL PM-1 (Lenke & Knackmuss 1996) indicate further hydrogenation of H^--2,4-DNP to a dinitrocyclohexanone and subsequent hydrolytic ring cleavage. Actually, H^--2,4-DNP is further converted by crude extract and NADPH stimulates this reaction (Behrend 1999). Because of the chemical instability of 4,6-DNH under physiological conditions ($t_{1/2}$ approximately 55 min at pH 7.2) its occurrence in culture fluid does not prove its role as a metabolite in the degradation pathway (Ebert et al. 1999). A NADPH-dependent monooxygenation in *para*-position has also been suggested for the degradation of H^--2,4-DNP

by Behrend and Heesche-Wagner (1999). Zablotowicz et al. (1999) proposed an initial attack at 2,4-DNP by a PCP 4-monooxygenase (pentachlorophenol 4-monooxygenase) causing the release of nitrite and concomitant hydroxylation in 4-position by a 2,4-DNP degrading *Sphingomonas* sp. UG 30 strain. To elucidate the lower pathway of 2,4-DNP and picrate degradation further investigations are necessary. Figure 5 depicts the upper pathway of picrate and 2,4-DNP catabolism.

The present data clearly identify $2H^-$-picrate as a key metabolite of picrate catabolism. Nitrite is eliminated enzymatically from this complex but not from H^--picrate. Therefore, 2,4-DNP cannot be a metabolite in the degradation of picrate by *N. simplex* as assumed for the degradation of picrate by *Rhodococcus erythropolis* HL PM-1 (Behrend & Heesche-Wagner 1999; Ebert et al. 1999; Lenke & Knackmuss 1992; Rieger et al. 1999). Consequently, 2,4-DNP and picrate are degraded via a convergent pathway with H^--2,4-DNP as a common metabolite.

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References

Abken H-J (1998) Reinigung und Chrakterisierung der F_{420}H2-Dehydrogenase aus *Methanosarcina mazei* Gö 1 und Identifizierung von Methanophenazin als neuartigen methanogenen Cofaktor. Mathematisch- naturwissentschaftliche Fakultät. Göttingen, Universität von Göttingen

Behrend C (1999) Pikrinsäuremetabolismus in *Nocardioides* sp. CB-22. Institut für Biochemie. Witten/Herdecke, Privatuniversität Witten/Herdecke

Behrend C & Heesche-Wagner K (1999) Formation of hydride-Meisenheimer complexes of picric acid (2,4,6-trinitrophenol) and 2,4-dinitrophenol during mineralization of picric acid by *Nocardioides* sp. strain CB 22-2. Appl. Environ. Microbiol. 65(4): 1372–1377 Blasco R, Moore E, Wray V, Pieper D, Timmis K & Castillo F (1999) 3-Nitroadipate, a metabolic intermediate for mineralization of 2,4-dinitrophenol by a new strain of *Rhodococcus* Species. J. Bacteriol. 181(1): 149–152

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72: 248–252

Ebert S, Rieger P-G & Knackmuss H-J (1999) Function of coenzyme F_{420} in aerobic catabolism of 2,4,6-trinitrophenol and 2,4-dinitrophenol by *Nocardioides simplex* FJ2-1A. J. Bacteriol. 181(9): 2669–2674

Ecker S, Widmann T, Lenke H, Dickel O, Fischer P, Bruhn C & Knackmuss H-J (1992) Catabolism of 2,6-dinitrophenol by *Alcaligenes eutrophus* JMP 134 and JMP 222. Arch. Microbiol. 158: 149-154

Eker APM, Hessels JKC & Meerwaldt R (1989) Characterization of an 8-hydroxy-5-deazaflavin: NADPH oxidoreductase from *Streptomyces griseus*. Biochim. Biophys. Acta 990: 80–86

Hallas LE & Alexander M (1983) Microbial transformation of nitroaromatic compounds in sewage effluent. Appl. Environ. Microbiol. 45(4): 1234–1241

Hanne LF, Kirk LL, Appel SM, Narayan AD & Bains KK (1993) Degradation and induction specifity in Actinomycetes that degrade *p*-nitrophenol. Appl. Environ. Microbiol. 59(10): 3503–3508

Kadiyala V & Spain JC (1998) A two-component monooxygenase catalyzes both the hydroxylation of *p*-nitrophenol and the oxidative release of nitrite from 4- nitrocatechol in *Bacillus sphaericus* JS905. Appl. Environ. Microbiol. 64(7): 2479–2484

Lenke H & Knackmuss HJ (1992) Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. Appl. Environ. Microbiol. 58(9): 2933–2937

Lenke H & Knackmuss H-J (1996) Initial hydrogenation and extensive reduction of substituted 2,4-dinitrophenol. Appl. Environ. Microbiol. 62(3): 784–790

Lenke H, Pieper DH, Bruhn C & Knackmuss H-J (1992) Degradation of 2,4-dinitrophenol by two *Rhodococcus erythropolis* strains, HL 24-1 and HL-24-2. Appl. Environ. Microbiol. 58(9): 2928–2932

Lin X-L & White RH (1986) Occurence of coenzyme F_{420} and its γ-monoglutamyl derivative in nonmethanogenic archaebacteria. J. Bacteriol. 168(1): 444–448

Munnecke DM & Hsieh DPH (1974) Microbial degradation of parathion and *p*-nitrophenol. Appl. Microbiol. 28(2): 212–217

Patil SS & Shinde VM (1989) Gas chromatographic studies on the biodegradation of nitrobenzene and 2,4-dinitrophenol. Environ. Pollut. 57: 235–250

Purwantini E, Mukhopadhyay B, Spencer RW & Daniels L (1992) Effect of temperature on the spectral properties of coenzyme F_{420} and related compounds. Anal. Biochem. 205: 342–350

Rajan J, Valli K, Perkins RE, Sariaslani FS, Barns SM, Reysenbach A-L, Rehm S & Ehringer M (1996) Mineralization of 2,4,6-trinitrophenol (picric acid): characterization and phylogenetic identification of microbial strains. J. Ind. Microbiol. 16: 319–324

Rieger P-G & Knackmuss H-J (1995). Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. In Spain JC (ed) Biodegradation of nitroaromatic compounds. Plenum Press, New York

Rieger P-G, Sinnwell V, Preuß A, Francke W & Knackmuss H-J (1999) Hydride-Meisenheimer complex formation and protonation as key reactions of 2,4,6-trinitrophenol biodegradation by *Rhodococcus erythropolis*. J. Bacteriol. 181(4): 1189–1195

Russ R, Walters DM, Knackmuss H-J & Rouvière P (2000). Identification of genes involved in picric acid and 2,4-dinitrophenol degradation by mRNA differential display. In Spain JC, Hughes JB & Knackmuss H-J (ed) Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers, Boca Raton

Severin T & Adam M (1963) Umsetzung mit Natriumborhydrid, II. Chem. Ber. 96: 448–452

Severin T & Schmitz R (1962) Umsetzung von Nitroaromaten mit Natriumborhydrid. Chem. Ber. 95: 1417–1419

Spain JC, Wyss O & Gibson DT (1979) Enzymatic oxidation of *p*-nitrophenol. Biochem. Biophys. Res. Commun. 88(2): 634–641.

Tsukamura M (1960) Enzymatic reduction of picric acid. J. Biochem. 48(5): 662–671

Vorbeck C, Lenke H, Fischer P, Spain JC & Knackmuss H-J (1998) Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. 64(1): 246–252

Williams RE & Bruce NC (2000). The role of nitrate ester reductase enzymes in the biodegradation of explosives. In Spain JC, Hughes JB & Knackmuss H-J (ed) Biodegradation of nitroaromatic compounds and explosives. Lewis Publisher, Boca Raton

Wyman JF, Serve MP, Hobson DW, Lee LH & Uddin D (1992) Acute toxicity, distribution, and metabolism of 2,4,6-trinitrophenol (picric acid) in Fischer 344 rats. J. Toxicol. Environ. Health 37: 313–327

Zablotowicz RM, Leung KT, Alber T, Cassidy MB, Trevors JT, Lee H, Veldhuis L & Hall JC (1999) Degradation of 2,4-dinitrophenol and selected nitroaromatic compounds by *Sphingomonas* sp. UG30. Can. J. Microbiol. 45: 840–848

Zeyer J & Kearney PC (1984) Degradation of *o*-nitrophenol and *m*-nitrophenol by a *Pseudomonas putida*. J. Agric. Food Chem. 32: 238–242