ENZYME, GENE UND MECHANISMEN DES OBEREN ABBAUWEGES VON PIKRINSÄURE UND 2,4-DINITROPHENOL DURCH NOCARDIOIDES SIMPLEX FJ2-1A

Von der Fakultät für Geo- und Biowissenschaften
der Universität Stuttgart
zur Erlangung der Würde eines
Doktors der Naturwissenschaften (Dr. rer. nat.)
genehmigte Abhandlung

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Tag der mündlichen Prüfung: 2. März 2001

Institut für Mikrobiologie der Universität Stuttgart
2001

Die Untersuchungen der vorliegenden Arbeiten wurden am Institut für Mikrobiologie der Universität Stuttgart durchgeführt.

Für meine Eltern

Inhaltsverzeichnis

Abkürzungen

1,3,5-TNP	1,3,5-Trinitropentane
2,4-DNP	2,4-Dinitrophenol
2-Cl-4,6-DNP	2-Chlor-4,6-dinitrophenol
$2H^-$-Pikrat	Dihydrid-σ-Komplex von Pikrat
$2H^-$-TNT	Dihydrid-σ-Komplex von TNT
4,6-DNH	4,6-Dinitrohexanoat
Dinoseb	2-*sec*-Butyl-4,6-dinitrophenol
DNOC	2-methyl-4,6-dinitrophenol, Dinitro-*ortho*-cresol
DOC	Dissolved Organic Carbon
F_{420}	Koenzyme F_{420}
FAD	Flavinadenindinucleotid
FMN	Flavinadeninmononucleotid
FPLC	Fast-Protein Liquid Chromatography
H^--2,4-DNP	Hydrid-σ-Komplex von 2,4-DNP
H^--Pikrat	Hydrid-σ-Komplex von Pikrat
H^--TNT	Hydrid-σ-Komplex von TNT
H_4MPT	N^5,N^{10}-Tetrahydromethanopterin
HPLC	High Pressure Liquid Chromatography
HTES	Hydrid-transferierendes Enzymsystem
NAD/NADH	Nicotinadenindinucleotid, oxidierte/reduzierte Form
ORF	open reading frame
PCR	Polymerase chain reaction
PDA-Detektor	Photodiodenarray-Detektor
TAT	2,4,6-Triaminotoluol
TNT	2,4,6-Trinitrotoluol

Tris	Tris-(hydroxymethyl)-aminomethan
SDS	Natriumdodecylsulfat
UV/Vis	Ultraviolett/Visible

Kurzfassung

Der Bakterienstamm *Nocardioides simplex* FJ2-1A, welcher aus industriellem Abwasser isoliert wurde, ist in der Lage, 2,4-Dinitrophenol (2,4-DNP) und Pikrinsäure unter aeroben Bedingungen vollständig abzubauen. Im ersten Schritt wird dabei Hydrid auf den aromatischen Ring des elektronenarmen π-Systems der Nitroaromaten übertragen. Dabei entsteht der jeweilige Hydrid-σ-Komplex der Verbindung als Produkt. Diese initiale Reduktion wird von einem Enzymsystem, das sich aus mehreren Komponenten zusammensetzt, katalysiert. Die beteiligten Enzyme und Kofaktoren wurden detektiert, gereinigt, ihre Funktion identifiziert und das Enzymsystem charakterisiert. Kofaktor F_{420}, welcher als typischer methanogener Kofaktor bekannt ist, wurde als Mediator der Hydridübertragung identifiziert. Von NADPH, dem Donor der Reduktionsäquivalente, wird Hydrid mittels einer NADPH-abhängigen F_{420}-Reduktase auf Koenzym F_{420} übertragen. Der sich anschließende Hydridtransfer auf das aromatische System des Nitroaromaten wird durch eine Hydridtransferase katalysiert.

Die N-terminalen Sequenzen der mit FPLC gereinigten Proteine wurden bestimmt und dienten zur Ableitung von Oligonukleotiden für die Herstellung einer Sonde mittels PCR. Ein 7.2 kb großes DNA Fragment, das die Gene der beiden Enzyme beinhaltet, wurde isoliert und sequenziert. Bei Datenbankvergleichen zeigte die F_{420}-Reduktase Ähnlichkeiten zu verschiedenen F_{420}-abhängigen $NADP^+$-Reduktasen aus Archaea und Streptomyceten. Das pH-Optimum der untersuchten F_{420}-Reduktase und homologer Enzyme liegen im Bereich von pH=5. Die Hydridtransferase ist N^5,N^{10}-Methylen-tetrahydro-methanopterin-Reduktasen ähnlich.

Der Dihydrid-σ-Komplex von Pikrat wurde als Produkt einer zweifachen Hydridübertragung auf das aromatische Ringsystem von Pikrat identifiziert. Dieser zweite Transfer von Hydrid wird ebenfalls durch das charakterisierte Enzymsystem katalysiert. Der Dihydrid Komplex des Pikrats wurde chemisch synthetisiert und ^{1}H- und ^{13}C-NMR spektroskopisch untersucht. Er wird enzymatisch unter Elimination von Nitrit und der Bildung des Hydrid-σ-Komplexes von 2,4-DNP umgesetzt. Die Nitrit eliminierende Aktivität wurde mit FPLC angereichert und charakterisiert. Diese Aktivität zeigt eine hohe Substratspezifität. Als Produkt der Reduktion von 2,4-DNP katalysiert durch das Hydrid übertragende Enzymsystem entsteht der korrespondierende Hydrid-σ-Komplex. Es findet keine weitere Hydrierung statt.

Voraussetzungen für die Struktur eines möglichen Substrates der Hydridtransferase sind Nitrosubstituenten in 2- und 4-Stellung am aromatischen Ring von Phenol. Eine Nitritfreisetzung erfolgt lediglich aus dem zweifach hydrierten Dihydrid-σ-Komplex von Pikrat.

Als Folge dieser Untersuchungen ergibt sich für *Nocardioides simplex* FJ2-1A ein konvergenter Abbauweg von Pikrat und 2,4-DNP mit dem Hydrid-σ-Komplex des 2,4-DNP als gemeinsamem Metaboliten.

Abstract

Nocardioides simplex FJ2-1A isolated from industrial waste water is able to mineralize picrate and 2,4-dinitrophenol aerobically. The first step of degradation is the transfer of hydride to the aromatic ring system of the π-electron deficient nitroaromatic compounds. The reaction products are the corresponding hydride σ-complexes. A multi component enzyme system catalyzes this reduction. Involved proteins and cofactors were detected, purified, their function was identified, and the system characterized. Cofactor F_{420}, a typical methanogenic coenzyme, was identified as a mediator for the hydride transferring process. Hydride is funneled from NADPH as hydride donor by a NADPH-dependent F_{420}-reductase to coenzyme F_{420} and transferred then to the aromatic system of the nitro compound by a hydride transferase.

N-terminal sequences of the enzymes were determined and oligonucleotides for synthesis of probes with PCR were deduced. A 7.2 kb DNA fragment containing the corresponding structural genes was detected and cloned. The F_{420}-reductase is similar to several F_{420}-dependent $NADP^+$-reductases of archaea and *Streptomyces* species when compared to databases. The pH-optima of the investigated and homologous enzymes are similar. The hydride transferase shows homologies to N^5,N^{10}-methylene-tetrahydromethanopterin-reductases.

A dihydride σ-complex of picrate ($2H^-$-picrate) was identified as product of a twofold hydride transfer to the aromatic ring system catalysed by the enzyme system. This $2H^-$-complex was synthesized chemically and investigated by ^{1}H- and ^{13}C-NMR. It is transformed enzymatically under nitrite elimination and formation of the hydride σ-complex of 2,4-dinitrophenol. The nitrite eliminating activity was enriched by FPLC and characterized. The activity shows high substrate specifity. The product of 2,4-dinitrophenol reduction by the enzyme system was identified as its hydride σ-complex (H^--2,4-DNP). No further hydrogenation occurs.

Structural requirements for the hydride transfer are nitro substituents in 2 and 4 position of the aromatic ring of the phenolic compound. Nitrite elimination occurs solely from the dihydride σ-complex of picrate.

In conclusion picrate and 2,4-dinitrophenol are degraded via convergent pathways with the H^--2,4-DNP as a key metabolite by *Nocardioides simplex* FJ2-1A.

1. Einführung

Pikrinsäure (2,4,6-Trinitrophenol) wird bereits seit ca. 150 Jahren praktisch eingesetzt. Johann Rudolf Glauber (1604-1670) erwähnte erstmals in seinen alchimistischen Schriften das Kaliumpikrat (Priesner und Figala 1998). Im Jahre 1771 beobachtete Woulfe Pikrinsäure bei der Oxidation von Indigo mit Salpetersäure (Falbe und Regitz 1998), (Elvers et al. 1991). 72 Jahre später klärte Laurent die Strukturformel von Pikrinsäure auf (Elvers et al. 1991).

Pikrinsäure gilt als ältester „Nitrofarbstoff" und wurde ab Mitte des 19. Jahrhunderts zum Gelbfärben von Seide und Wolle eingesetzt. Erst im Jahr 1885 entdeckte Turpin den Sprengstoffcharakter dieser Substanz (Falbe und Regitz 1998). Im Ersten Weltkrieg wurde sie in großem Maße bei Frankreich als Füllung von Brisanzgranaten eingesetzt. Doch aufgrund ihrer hygroskopischen Eigenschaft, welche zur Korrosion der Munitionshülsen führte, verlor Pikrinsäure nach dem Ersten Weltkrieg mehr und mehr an Wichtigkeit und wurde von TNT (2,4,6-Trinitrotoluol) verdrängt. Die Eigenschaft mit Metallen der Munitionshülsen extrem stoßempfindliche Pikrate zu bilden, macht Kampfstoffe wie Granaten des Ersten Weltkrieges zu sehr gefährlichen Altlasten.

In beiden Weltkriegen wurden TNT und Pikrinsäure als Explosivstoffe eingesetzt. Die Böden, auf welchen die industriellen Produktionsstätten standen, sind zum großen Teil kontaminiert. Aufgrund der guten Wasserlöslichkeit von Pikrinsäure (11 g/l bei 20°C) ist diese fast vollständig aus den Böden ausgewaschen und so ins Grundwasser gelangt. Die als Altlasten zurückgebliebenen schwerlöslichen Nitrotoluole und mit Pikrinsäure verunreinigtes Grundwasser stellen heute ein Entsorgungsproblem dar.

Nitroaromaten wie Dinitro-*ortho*-cresol (2-Methyl-4,6-dinitrophenol, DNOC) oder 2-*sec*-Butyl-4,6-dinitrophenol (Dinoseb) wurden als Herbizide eingesetzt. Dieser Einsatz ging einher mit Untersuchungen zur Langlebigkeit dieser Verbindungen bei ihrem Eintrag in die Natur: „As the butyl-derivate of dinitrophenol does not appear to be attacked by microorganisms (no breakdown has been observed in several enrichment experiments) it is possible that this compound will preferably be used as a more stable herbicide than dinitro-*ortho*-cresol." (Gundersen und Jensen 1956). Trotz der Wahl resistenter Verbindungen für den Einsatz als Herbizide sind Mikroorganismen angereichert worden, welche diese Verbindungen umsetzen.

Gundersen und Jensen (Gundersen und Jensen 1956) isolierten einen *Corynebacterium simplex* Stamm, der DNOC als Kohlenstoff- und Stickstoffquelle verwertete. Die Nitrogruppen wurden als Nitrit eliminiert und im Kulturmedium nachgewiesen. Ein *Pseudomonas* Isolat reduzierte zuerst die Nitrogruppen zu Aminogruppen, welche anschließend eliminiert wurden (Twefik und Evans 1966). Beide Abbauwege konvergieren auf der Stufe des 2,3,5-Trihydroxytoluol als gemeinsamem Metaboliten. Weitere Mikroorganismen, die DNOC unter Nitrit-Freisetzung metabolisierten, beschrieben Jensen und Lautrup-Larsen (Jensen und Lautrup-Larsen 1967). Dinoseb hingegen wurde unter aeroben Verhältnissen lediglich zu reduzierten Produkten umgesetzt. Von anaeroben Konsortien wurde es abgebaut (Stevens et al. 1991; Kaake et al. 1992)

Pikrinsäure sowie 2,4-Dinitrophenol (2,4-DNP) finden sich in Abwässern der Nitrobenzol produzierenden Industrie. Beide Stoffe entstehen zu 0,1% als Nebenprodukte bei der Synthese und stellen bedingt durch ihre Persistenz in Klärprozessen ein Problem dar (Patil und Shinde 1989).

Aus Nitroaromaten werden bedeutende Produkte wie Farbstoffe, Pestizide, Insektizide und Sprengstoffe chemisch synthetisiert. Besonders hervorzuheben ist dabei Nitrobenzol. Diese Substanz ist Synthon für Intermediate zur Produktion vieler wichtiger Verbindungen. Im Jahr 1989 dienten in Deutschland 93% als Ausgangsstoff für Anilin (BUA-Stoffbericht 1991). Die verbleibenden 7% wurden für die Synthese von Dinitrobenzolen, m-Chlornitrobenzolen, Nitrobenzolsulfonsäuren und als Lösungsmittel eingesetzt. Nach Zahlen des Beratergremiums für umweltrelevante Altstoffe (BUA-Stoffbericht 1991) wurden in der Bundesrepublik Deutschland im Jahr 1989 ca. 200 000 t dieses Stoffes produziert. Im Vergleich dazu betrug die Produktion in ganz Westeuropa 540 000 t.

Die bei diesem Prozess anfallenden Nitrophenole gelangen in die Kläranlage und färben nach Angaben der BASF das Wasser orangerot (BASF 1997). Abwasser, das aus diesem Verfahren resultiert, wird in manchen Unternehmen einer thermolytischen Behandlung direkt vor Ort unterzogen. Unter Druck und bei Temperaturen von ca. 280°C zerfallen die problematischen Nitrophenolate in Ameisensäure oder Oxalsäure, welche kein Problem für die Mikroorganismen einer Kläranlage darstellen. Es können so große Mengen an Abwasser (17 000 l/h) entsorgt werden. Trotz dieser Möglichkeit der Aufarbeitung besteht von Seiten der

Industrie ein Interesse an der Entwicklung eines biologischen Verfahrens zur direkten Beseitigung dieser Problemstoffe. Um einen technischen Prozess zur Mineralisierung von 2,4-DNP und Pikrinsäure entwickeln zu können, ist die Kenntnis der dahinter stehenden Biochemie, der beteiligten Enzyme und der dazugehörigen Gene essentiell.

Die meisten in der Natur vorzufindenden Nitroaromaten sind anthropogenen Ursprungs. Natürlich synthetisierte Di- oder Trinitroverbindungen sind bisher nicht bekannt. In der Natur vorkommende Mononitroverbindungen wie Chloramphenicol (Chlormycetin) (Raistrick 1949), Aristolochiasäuren, Aureothin (Buckingham 1994) und Pyrrolnitrin (van Pée et al. 1981) werden von Pflanzen und Bakterien gebildet. Sie dienen als Antibiotika und Fraßschutzstoffe.

Nitroaromaten sind, wie aus zahlreichen Untersuchungen hervorgeht, toxische Verbindungen. 200 mg 2,4-DNP/kg Körpergewicht bzw. 5 mg Pikrinsäure/kg Körpergewicht führen beim Menschen zum Tod. Eine chronische Belastung durch diese Stoffe hat in beiden Fällen eine Methämoglobinbildung, Cyanose und Nierenschäden zu Folge.

2,4-DNP ist bekannt als protonophorer Entkoppler der oxidativen Phosphorylierung (Loomis und Lipmann 1948; Pinchot 1967; Illvicky und Casida 1969; Terada 1981). Es werden sowohl monomolekulare als auch bimolekulare Modelle der entkoppelnden Wirkung in der Literatur beschrieben. Gemeinsam ist allen, dass Protonen über die Membran ins Cytosol transportiert werden und somit der zur ATP-Synthese nötige pH-Gradient zerstört wird. Charakteristisch für protonophore Entkoppler ist, dass es sich im Allgemeinen um schwache Säuren mit einer Säurestärke (pK_a-Wert) im Bereich von 5-7 handelt. Die negative Ladung des Anions ist, bedingt durch elektronenziehende Substituenten, über das Molekül delokalisiert. Dies führt zu einer Minimierung der elektrischen Feldstärke und somit zu einer Löslichkeit des Anions in einer unpolaren Membran (Terada 1981).

Versuche mit Hühnern zeigten, dass bei gleichzeitiger Gabe von 2,4-DNP zum Futter der Austausch von Sauerstoff und Kohlendioxid, und damit die metabolische Rate, um 40-70% erhöht wurde. Das Körperfett ging dabei verloren (Dominguez et al. 1993).

Der Metabolismus von Pikrat bei Ratten beschränkt sich auf die Reduktion der Nitrogruppen zu Aminogruppen. 60% werden über den Harn sogar untransformiert ausgeschieden (Wyman et al. 1992).

Leuenberger (Leuenberger et al. 1988) identifizierte Nitrophenole als sekundäre Schadstoffe, die durch die Reaktion von Stickoxiden mit den, aus Abgasen stammenden, aromatischen Verbindungen entstehen. 2-Nitrophenol (2-NP), DNOC, 2,4-DNP und Dinoseb ließen sich im Regenwasser nachweisen, wobei aber nur das Auftreten von Dinoseb jahreszeitabhängig war und mit dem Einsatz als Herbizid korrelierte.

Nitroaromatische Verbindungen sind in der Umwelt sehr resistent gegenüber einem mikrobiellen Angriff. Dennoch sind Mikroorganismen und Abbauwege bekannt, welche in einigen Fällen zur Mineralisierung führen. Es sind sowohl monooxygenolytisch katalysierte initiale Abbauschritte wie dioxygenolytisch katalysierte beschrieben. Abbildung 1 gibt einen schematischen Überblick über den Nitroaromatenabbau.

Eine Dioxygenierung unter Nitriteliminierung wurde bei Nitrobenzol (Nishino und Spain 1995), 1,3-Dinitrobenzol (Dickel und Knackmuss 1991), 3-Nitrobenzoat (Nadeau und Spain 1995), 2-Nitrotoluol (Haigler et al. 1994) und 2,6-Dinitrophenol (Ecker et al. 1992) beobachtet.

Eine initiale Monooxygenierung ist für 2-Nitrophenol (Zeyer und Kearney 1984) und 4-Nitrophenol (Spain und Gibson 1991; Kadiyala und Spain 1998), 4-Chlor-2-nitrophenol (Bruhn et al. 1988) und 4-Nitroanisol (Schäfer et al. 1996) beschrieben worden.

Die Nitrogruppe besitzt einen elektrophilen Charakter, was sie für eine reduktive Umsetzung durch Mikroorganismen prädestiniert. Sie wird dabei, über die Nitrosoverbindung als Zwischenstufe, zur Hydroxylaminoverbindung reduziert.

Dieser Metabolit kann, katalysiert durch eine Mutase, intramolekular, ähnlich einer Bambergerumlagerung, zur 2-Aminophenolverbindung umgelagert werden. Beispiele dafür sind Nitrobenzol (Nishino und Spain 1993), 3-Nitrophenol (Schenzle et al. 1997), 2-Chlor-5-nitrophenol (Schenzle et al. 1999) und 4-Chlornitrobenzol (Katsivela et al. 1999).
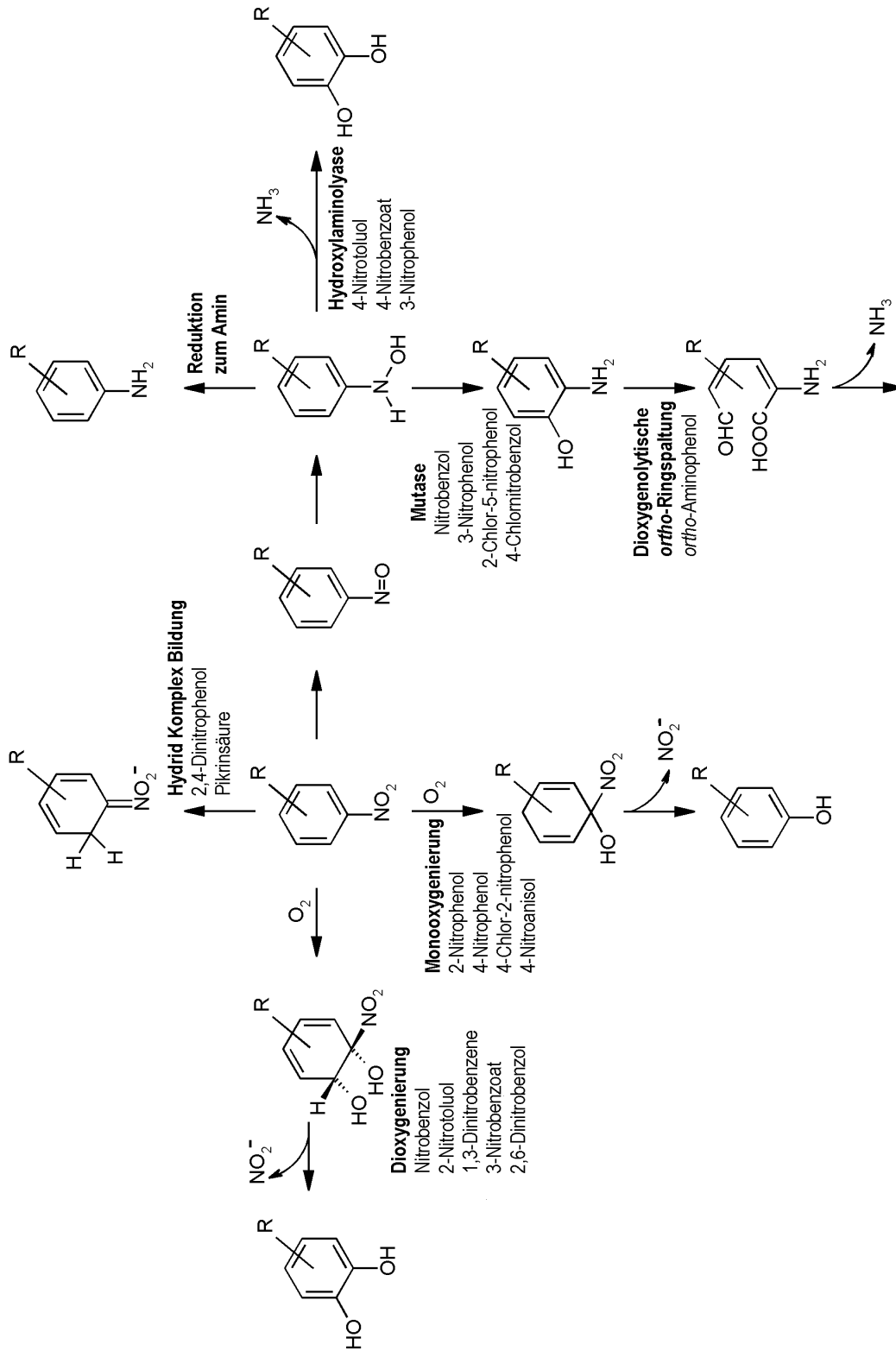
Abbildung 1. Übersicht über den Abbau von Nitroaromaten (Nishino, 2000).

Eine andere beobachtete Möglichkeit ist die durch eine Hydroxylaminolyase katalysierte Freisetzung von Ammoniak. Die korrespondierenden Hydroxylamine von 4-Nitrotoluol (Haigler und Spain 1993; Rhys-Williams et al. 1993), 4-Nitrobenzoat (Groenewegen et al. 1992; Groenewegen und deBont 1992) und 3-Nitrophenol (Meulenberg et al. 1996) reagieren weiter zu Brenzkatechinen (Pyrocatecholen).

Unter anaeroben Bedingungen oder bei einer unproduktiven Umsetzung wird die Nitrogruppe unselektiv über die Nitroso- und Hydroxylgruppe bis zur Aminogruppe reduziert. Dies wurde im Zusammenhang mit der unproduktiven Reduktion von Pikrinsäure zu Pikraminsäure (Tsukamura 1960) und bei der anaeroben Umsetzung von 2,4-Dinitrophenol (2,4-DNP) beobachtet (Donlon et al. 1996; Uberoi und Bhattacharya 1997). Eine wichtige Rolle spielt diese Reduktion bei der anaeroben Behandlung von TNT-kontaminierten Böden. Im anaeroben Milieu werden, mit einem leicht vergärbaren Substrat wie Glucose, die drei Nitrogruppen am aromatischen Ring dieser Verbindung mikrobiell, über alle möglichen Zwischenstufen, die sich aus den Intermediaten mit Nitroso-, Hydroxylamino- und Aminogruppen ergeben (Rieger und Knackmuss 1995), zu 2,4,6-Triaminotoluol (TAT) reduziert. Eine aerobe Nachbehandlung beseitigt die gebildeten Fermentationsprodukte und ermöglicht eine irreversible Chemisorption des TAT an die Huminstoffe des Bodens. Bei dieser Prozessführung findet zwar keine Mineralisierung des Schadstoffes statt, aber eine Immobilisierung und Humifizierung. Diese Sanierungstechnik unterbindet eine Ausbreitung ins Grundwasser. Sie kann als biologisches Sanierungsverfahren für TNT-kontaminierte Böden eingesetzt werden (Achtnich et al. 1999; Achtnich et al. 1999; Achtnich et al. 1999; Achtnich et al. 2000).

Pikrinsäure und 2,4-DNP wurden von *Corynebacterium simplex* (Gundersen und Jensen 1956), verschiedenen *Pseudomonaden* (Germanier und Wuhrmann 1963; Jensen und Lautrup-Larsen 1967), *Nocardia alba* (Germanier und Wuhrmann 1963) und *Arthrobacter*-Stämmen (Jensen und Lautrup-Larsen 1967) unter Freisetzung von Nitrit transformiert. Ein Mechanismus des Abbaus oder Metaboliten sind nicht beschrieben.

Die Herabsetzung der Elektronendichte im aromatischen Ring, bedingt durch den negativen mesomeren und induktiven Effekt der Nitrogruppen, gestattet einen nukleophilen Angriff am Ring des Aromaten. Eine hierdurch bedingte, äußerst interessante, mikrobielle Umsetzung wurde bei TNT, Pikrinsäure und 2,4-DNP

beobachtet. An das Ringsystem wird chemoselektiv Hydrid addiert und als Produkt ein Hydrid-Meisenheimer-Komplex (Hydrid-σ-Komplex) gebildet.

Erstmals beobachtet wurde der Mechanismus von Lenke und Knackmuss im Jahr 1992 (Lenke und Knackmuss 1992). *Rhodococcus erythropolis* HL 24-1 und HL 24-2 wurden mit 2,4-DNP als Stickstoffquelle isoliert (Lenke et al. 1992). Beide Stämme konnten Pikrat umsetzen, wenn sie zuvor mit 2,4-DNP induziert wurden. Eine spontane Mutante des Stamms HL 24-2, HL PM-1 konnte Pikrat als Stickstoffquelle verwerten und wie später erkannt wurde, auch mit Pikrat als Stickstoff-, Kohlenstoff- und Energiequelle wachsen (Rieger et al. 1999). Eine beobachtete Orangefärbung des Mediums war nicht auf Pikraminsäure zurückzuführen. Weitere Untersuchungen legten die Vermutung nahe, dass es sich um einen Hydrid-Meisenheimer-Komplex des Pikrates handelt. Die bisherigen Erkenntnisse zum Pikrinsäureabbau sind in Abbildung 2 zusammengefasst.

Rieger (Rieger et al. 1999) identifizierte und synthetisierte diesen Komplex chemisch durch Reduktion der Pikrinsäure mit Natriumborhydrid. Da eine Bildung des σ-Komplexes kinetisch bestimmt ist, galt es, die Reaktion im richtigen Moment abzustoppen, um eine zweifache Hydrierung zu vermeiden. Ein zellfreier Extrakt von *R. erythropolis* setzte diesen Hydrid-Komplex um. Theoretische Überlegungen ließen eine Nitritfreisetzung aus der protonierten Form dieses Komplexes, unter Bildung von 2,4-DNP, erwarten. Der initiale reduktive Angriff des aromatischen Systems wurde damit als neuartiger Mechanismus des Abbaus von nitroaromatischen Verbindungen durch aerobe Bakterien erkannt.

Der Meisenheimer-Komplex des Pikrats wird durch Ruhezellen u.a. weiter zu einem Dihydrid-σ-Komplex, dem Dianion des 2,4,6-Trinitrocyclohexanons, reduziert (Lenke und Knackmuss 1992). Der Nachweis wurde indirekt, über die Identifizierung von 1,3,5-Trinitropentan, erbracht (Lenke und Knackmuss 1992), welches durch saure Hydrolyse und Decarboxylierung aus dem Dihydridkomplex entsteht (Severin und Adam 1963). In anaeroben Umsätzen wurden nur kleine Mengen 2,4-DNP nachgewiesen. Diese Beobachtungen führten zu dem Schluss, dass der Hydrid-Meisenheimer-Komplex unter Nitriteliminierung eventuell spontan rearomatisiert und der beobachtete Dihydrid-σ-Komplex ein Dead-end-Metabolit einer Nebenreaktion ist (Lenke und Knackmuss 1992).

Die Umsetzung von 2,4-DNP wurde an *Rhodococcus erythropolis* HL 24-1 und HL 24-2 (Lenke et al. 1992), *Rhodococcus* sp. Stamm RB1 (Blasco et al. 1999) und *Nocardioides* sp. Stamm CB 22-2 (Behrend und Heesche-Wagner 1999) eingehend untersucht.
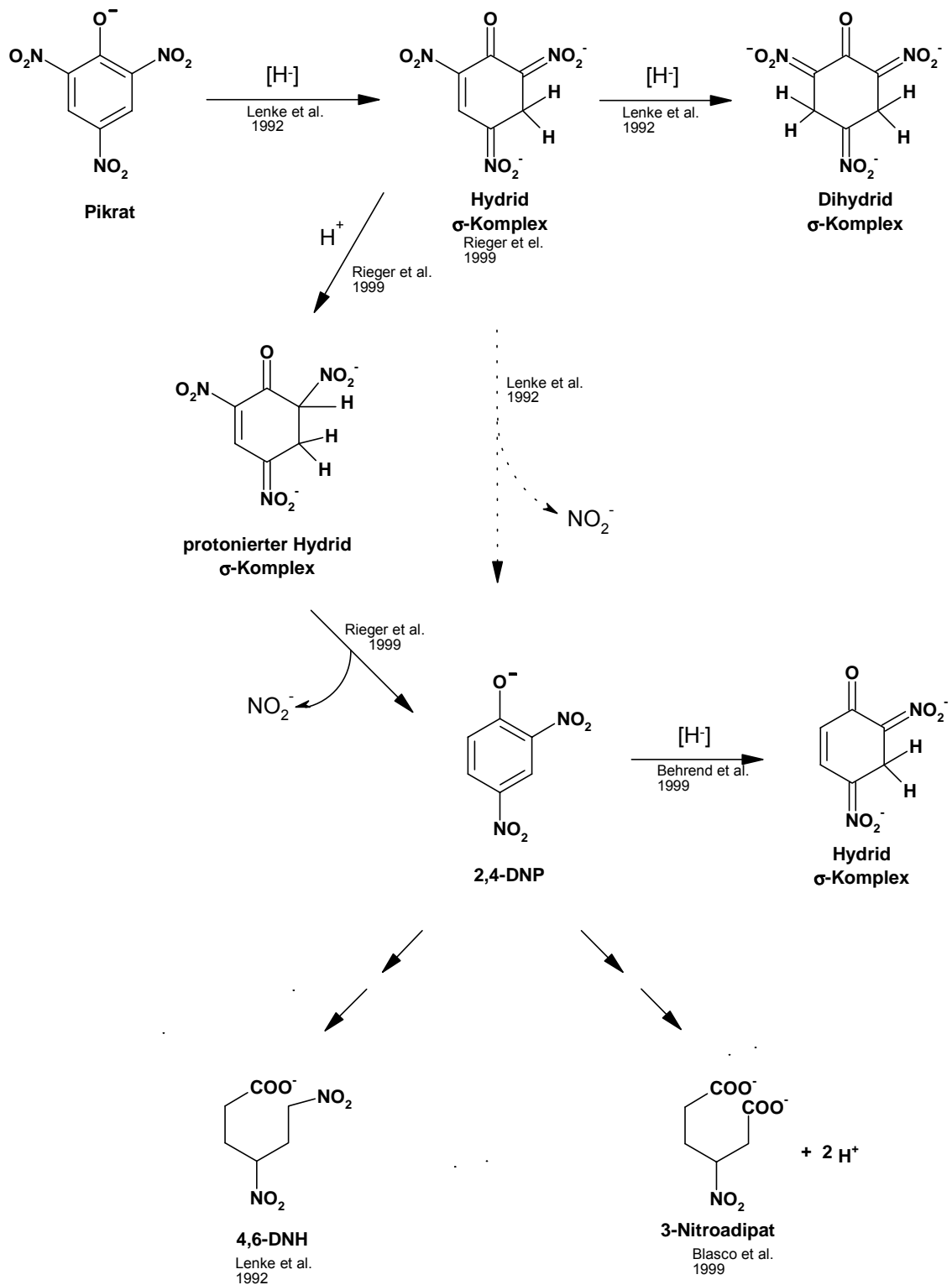

Abbildung 2. Bisher beschriebener Abbauweg von Pikrinsäure und 2,4-DNP.

Aus Überständen von Ruhezellumsätzen mit 2,4-DNP als Substrat wurde 4,6-Dinitrohexanoat (4,6-DNH) isoliert und identifiziert. Auch beim Wachstum der Stämme HL 24-1 und HL 24-2 mit Succinat als Kohlenstoffquelle und 2,4-DNP als Stickstoffquelle wurde die Entstehung von 4,6-DNH als Metabolit beobachtet (Lenke et al. 1992).

Beim anaeroben Umsatz des Strukturanalogons, 2-Chlor-4,6-dinitrophenol, durch Zellen des Stammes HL 24-1, die mit 2,4-DNP angezogen worden waren, war 4,6-DNH im Überstand in nahezu stöchiometrischen Mengen nachzuweisen. 4,6-DNH-analoge Diastereomere, *R*- und *S*-4,6-Dinitro-2-methylhexanoat, wurden entsprechend aus dem Überstand eines DNOC Umsatzes isoliert und identifiziert (Lenke und Knackmuss 1996).

Der sich aus diesen Experimenten ergebende und vorgeschlagene Abbauweg von 2,4-DNP beinhaltet eine hydrogenolytische Ringspaltung unter Bildung von 4,6-DNH. Aus Kulturüberständen von *Rhodococcus* sp. Stamm RB1, mit Acetat als Kohlenstoffquelle und 2,4-DNP als Stickstoffquelle, wurde 3-Nitroadipat isoliert und identifiziert (Blasco et al. 1999). Eine oxidative Ringspaltung unter gleichzeitiger Nitritelimination, mit 3-Nitroadipat als Produkt, wurde als Teil des produktiven Abbauweges postuliert.

Behrend und Heesche-Wagner isolierten einen Bakterienstamm CB22-2, der zu den *Nocardioiden* gehört. Das Isolat kann entweder mit Pikrinsäure als Kohlenstoff- und Energiequelle oder als Stickstoffquelle wachsen. Bei der Umsetzung von 2,4-DNP mit Zellextrakten entstand ein Hydrid-σ-Komplex. Der postulierte Abbauweg beinhaltet im Weiteren eine NADPH-abhängige Monooxygenierung in *para*-Stellung, welche unter Elimination von salpetriger Säure und Protonierung zu 2-Nitrohydrochinon führt.

Durch die Art des initialen Angriffs unterscheiden sich Pikrinsäure und 2,4-DNP in ihrem Abbau wesentlich von anderen Nitroaromaten. Bakterien, welche diese xenobiotischen Stoffe mineralisieren, sind *Rhodococcus erythropolis* HL PM-1 (Lenke und Knackmuss 1992), HL 24-1, HL 24-2 (Lenke et al. 1992), *Nocardioides simplex* FJ2-1A (Rajan et al. 1996) und *Nocardioides* sp. Stamm CB 22-2 (Behrend und Heesche-Wagner 1999). Alle untersuchten Stämme sind gram-positiv, obwohl in der Literatur auch gram-negative *Pseudomonaden* beschrieben sind, die 2,4-DNP und

Pikrinsäure unter Freisetzung stöchiometrischer Mengen an Nitrit umsetzen (Jensen und Lautrup-Larsen 1967).

Bei der aeroben Umsetzung von TNT kann grundsätzlich ein ähnlicher initialer Angriff erfolgen. Der Pikrinsäure abbauende *Rhodococcus-erythropolis*-Stamm, HL PM-1, der 4-Nitrotoluol-Abbauer *Mycobacterium* sp., Stamm HL 4-NT-1 (Vorbeck et al. 1998) und der Penta-erythritol-tetranitrat und Glycerol-trinitrat abbauende *Enterobacter cloacae* PB2 (Williams und Bruce 2000) reduzieren im ersten Schritt das aromatische System des TNT. Es entsteht zunächst der Hydrid-σ-Komplex von TNT (H^--TNT) als Intermediat. Dieser wird zum Dihydrid-σ-Komplex des TNT ($2H^-$-TNT) umgesetzt. Er gilt als Dead-end-Metabolit bei der Umsetzung durch HL 4-NT-1 und HL PM-1. Bei *E. cloacae* wurde ein langsames Wachstum mit TNT als Stickstoffquelle beobachtet.

Neben dem voranstehend beschriebenen, reduktiven initialen Angriff wird auch eine direkte oxygenolytische Umsetzung des 2,4-DNP diskutiert. Der Bakterienstamm *Sphingomonas* sp UG 30 setzt 2,4-DNP und andere *para*-substitutierte Nitrophenole oxygenolytisch um. Dabei wurde die Entstehung polarer Metaboliten beobachtet. Gaschromatographische Untersuchungen, gekoppelt mit Massenspektrometrie, deuten hier auf die Substitution einer Nitrogruppe durch eine Hydroxylgruppe hin. Beim Wachstum auf 2,4-DNP wurde 4,6-DNH nicht nachgewiesen. Das aus *Sphingomonas* sp. UG 30 stammende pcpB Genprodukt (PCP-4-Monooxygenase) wurde in *Escherichia coli* überexprimiert. Dieser transgene *E.-coli*-Stamm setzte 2,4-DNP unter Elimination von Nitrit um. Metabolit dieser monooxygenolytischen Reaktion ist 2-Nitrohydrochinon.

Enzyme, die einen reduktiven Angriff am aromatischen Ring katalysieren, sowie deren Gene waren zu Beginn der hier beschriebenen experimentellen Arbeiten nicht bekannt.

Die Frage nach dem Mechanismus eines initialen reduktiven Angriffs am aromatischen System und nach der Herkunft von Reduktionsäquivalenten machten im Rahmen der vorliegenden Arbeit eine Untersuchung der bisher unbekannten Enzyme unverzichtbar. Aus diesem Grunde wurden eine Enzymreinigung und Identifizierung der benötigten Kofaktoren durchgeführt. Die ermittelten N-terminalen Sequenzen der Proteine ermöglichten es, die Gene der an der initialen Reduktion beteiligten Enzyme zu lokalisieren und zu klonieren. Zur Aufklärung des oberen

Abbauweges wurden die Produkte der enzymatisch katalysierten Reaktion identifiziert, sich anschließende enzymatische Reaktionen detektiert und deren Produkte aufgeklärt.

2. Der Abbau von Pikrinsäure

Pikrinsäure und 2,4-DNP abbauende Bakterienstämme

Pikrinsäure und 2,4-DNP sind sehr persistente Stoffe, die in der Natur von natürlichen Populationen nicht abgebaut werden. Dennoch sind Mikroorganismen isoliert worden, die in der Lage sind diese Stoffe zu mineralisieren.

Die biologische Abbaubarkeit von Pikrinsäure, durch Belebtschlamm und den Bakteriestamm *Nocardioides simplex* FJ2-1A, wurde in einem Standardtestverfahren der OECD (DOC Die-Away, Test Nr. 301A) verglichen. *N. simplex* war in der Lage, Pikrinsäure unter Freisetzung von Nitrit innerhalb eines vorgegebenen Zeitfensters (28 Tage) zu mineralisieren. Der Gehalt des gelösten organischen Kohlenstoffes (DOC) wurde über diesen Zeitraum hinweg aufgezeichnet. Nach vier Tagen war der anfängliche DOC-Wert um fast 100% gesunken. Die Nitritfreisetzung betrug 78% des erwarteten Wertes. Der Organismus verwertete vermutlich einen Teil des Nitrits als Stickstoffquelle. Der Stamm *N. simplex* baut Pikrinsäure vollständig ab. Belebtschlamm setzte Pikrinsäure nicht um. Dies zeigt, dass Pikrinsäure von selektiv gewonnenen Mikroorganismen als Stickstoff-, Kohlenstoff- und Energiequelle genutzt wird, aber kein vollständiger Abbau in der Kläranlage stattfindet.

Enzyme und Kofaktoren des initialen Abbauschrittes von Pikrinsäure und 2,4-DNP

Für die enzymatischen und biochemischen Untersuchungen wurde der im OECD-Test vergleichend untersuchte Bakterienstamm FJ2-1A verwendet.

Es stellte sich heraus, dass die Pikrinsäure und 2,4-DNP umsetzende Aktivität von NADPH abhängig ist. Dieser Kofaktor dient als Quelle von Reduktionsäquivalenten. Nur sehr frischer, zellfreier Extrakt war in der Lage, 2,4-DNP, Pikrinsäure oder den Meisenheimer-Komplex der Pikrinsäure ohne Zugabe von NADPH umzusetzen. Routinemäßig diente die Absorptionsabnahme bei 340 nm zur Kontrolle der Aktivität. Da sich aber die Absorptionen der untersuchten Nitroaromaten, der Produkte und des Kofaktors überlagern, konnte mit einem photometrischen Test die Aktivität nicht quantifiziert werden. Für genaue Betrachtungen der Reaktionen wurden deshalb Proben, die zu verschiedenen Zeitpunkten einem Enzymtest entnommen wurden, mit

konzentrierter Phosphorsäure abgestoppt. Die Substratkonzentrationen wurden mittels HPLC (high-performance liquid chromatography) bestimmt.

Für eine Enzymreinigung mit FPLC (fast-protein liquid chromatography) wurde der cytosolische Extrakt mit einer Anionenaustauschersäule (Q Sepharose) fraktioniert. Die Aktivität in den Einzelfraktionen ging komplett verloren. Kombinationsexperimente mit den Fraktionen deckten drei für die Aktivität nötige Komponenten auf. Eine dieser Fraktionen wurde mittels HPLC/PDA-Untersuchungen als Kofaktor F_{420} identifiziert. Koenzym F_{420} aus *Methanobacterium thermoautotrophicum* diente als Standard. Eine Substitution der analysierten Komponente im Enzymtest durch den Standard von Koenzym F_{420} ergab eine gleiche bis höhere Aktivität. Dies war sehr überraschend, da es sich hier um einen eher im Bereich der methanogenen Archaea verbreiteten Kofaktor handelt, der an der Methanogenese beteiligt ist.

Die beiden verbleibenden enzymatischen Fraktionen wurden mittels FPLC weiter bis zur elektrophoretischen Homogenität aufgereinigt. Eine Proteinfraktion zeigte ein apparentes Molekulargewicht von ca. 30 kDa. Die N-terminale Sequenz enthielt das charakteristische Muster einer Nukleotidbindungsstelle.

Der N-Terminus dieses Proteins war, nach Vergleichen mit Datenbanken (BLAST), den F_{420}-abhängigen NADP-Oxidoreduktasen aus *Methanobacterium thermoautotrophicum, Methanogenium organophilum* und *Methanococcus jannaschii*, und aus dem nicht-methanogenen *Archaeoglobus fulgidus* sehr ähnlich. Tatsächlich katalysierte dieses Enzym die Reduktion des Kofaktors F_{420} unter Verbrauch von NADPH. Diese Homologie gab erneut einen Bezug zu Archaea und der Methanogenese. Zwar wurden eine F_{420}-abhängige NADP-Oxidoreduktase in *Streptomyces griseus* gefunden, aber diesem Enzym konnte keine physiologische Funktion zugeschrieben werden (Eker et al. 1989).

Die letzte Komponente, welche zur Hydrid transferierenden Aktivität des Enzymtests notwendig war, zeigte ein apparentes Molekulargewicht von 38 kDa. Datenbankenvergleiche der N-terminalen Sequenz ergaben keine Ähnlichkeit mit anderen Proteinsequenzen. Aufgrund der Funktion dieses Enzyms wurde es als Hydridtransferase bezeichnet.

NADH konnte NADPH als Hydriddonor nicht ersetzen. Dagegen hatten vergleichende Untersuchungen mit zellfreiem Extrakt des *Rhodococcen*-Stamms

RB1 nur Aktivität mit NADH nicht jedoch mit NADPH als Quelle von Reduktionsäquivalenten ergeben (Lara Sáez-Meléro, mündliche Mitteilung).

Die Identifikation der nötigen Kofaktoren und der Enzyme, die am initialen reduktiven Angriff des nitroaromatischen Systems beteiligt sind, deckte ein faszinierendes Enzymsystem auf, das den ersten Schritt des Abbaus katalysiert (Abbildung 3).
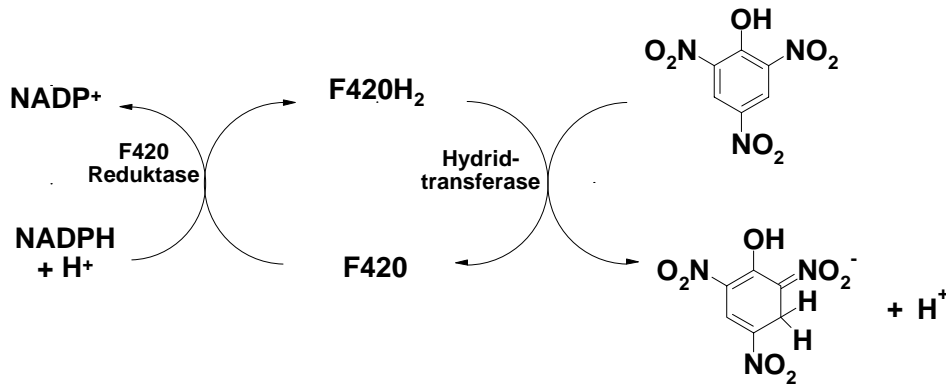
Abbildung 3. Hydrid transferierendes Enzymsystem am Beispiel von Pikrinsäure aus *Nocardioides simplex* FJ2-1A.

Hydrid wird von NADPH mittels Koenzym F_{420}, einem Zweielektronencarrier, der als Mediator fungiert, auf das elektronenarme aromatische System des Nitroaromaten übertragen. Zwei Enzyme sind an der Katalyse dieses Prozesses beteiligt.

Vergleichbare Enzymsysteme sind bei methanogenen Organismen zu finden (Abbildung 4).

Abbildung 4. Oxidation von Ethanol in methanogenen Archaea.

Dabei mediieren NADPH und $F_{420}H_2$ die Übertragung der Reduktionsäquivalente, die durch Oxidation von Ethanol zu Acetaldehyd gebildet werden, auf N^5,N^{10}-Methenyltetrahydromethanopterin, das zu N^5,N^{10}-Methyltetrahydrometha-nopterin reduziert wird. Drei Enzyme sind in diese Übertragung involviert, wobei eines davon eine F_{420}-abhängige NADP Reduktase ist (Berk und Thauer 1997).

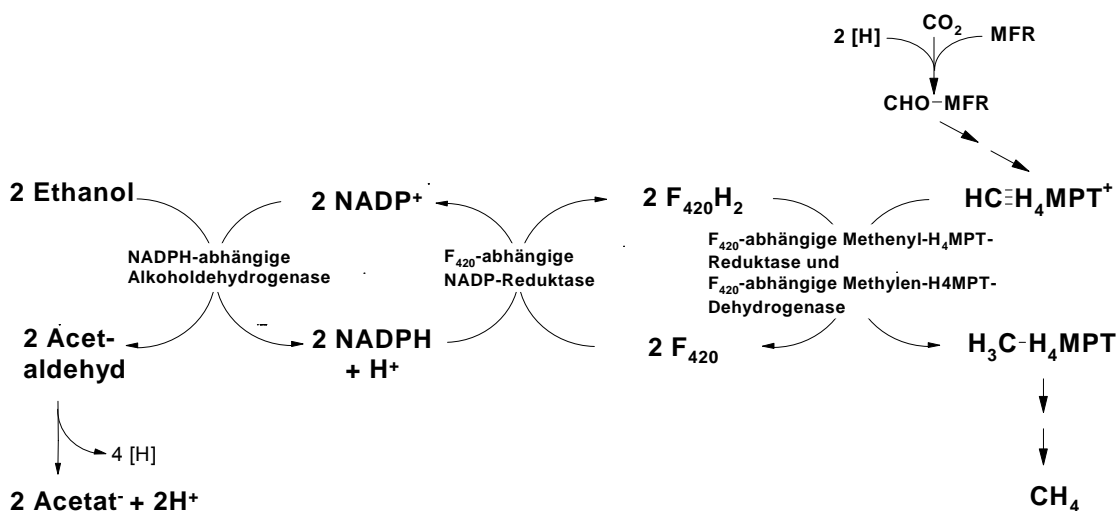Obwohl Kofaktor F_{420} lange Zeit als ein typisches Koenzym der Methanogenese angenommen wurde, sind in der Literatur aerobe Bakterien beschrieben, die Kofaktor F_{420} enthalten. Einige Mikroorganismen der Gattungen *Mycobacterium* und *Nocardia* besitzen eine F_{420}-abhängige Glucose-6-phophat-Dehydrogenase (Purwantini und Daniels 1996; Purwantini et al. 1997), welche aber bei Bakterien der Gattungen *Streptomyces* und *Corynebacterium* (Purwantini et al. 1997) und dem Stamm *N. simplex* FJ2-1A nicht nachgewiesen werden konnte.

Der Kofaktor ist an der biologischen Reduktion von 5a,11a-Dehydrochlortetrazyklin zu Chlortetrazyklin, einem Antibiotikum, in *Streptomyces griseus* beteiligt (McComerick und Morton 1982). Die Synthese von Lincomycin, einem weiteren Antibiotikum in *Streptomyces lincolnensis,* läuft mit F_{420} als kosynthetischem Faktor (Coats et al. 1989).

Charakterisierung des Enzymsystems

Der Gehalt an F_{420} in den Zellen von *N. simplex* wurde untersucht und mit dem anderer Organismen verglichen. Es zeigte sich, dass dieser Stamm für aerobe Bakterien relativ hohe Mengen des Kofaktors enthält, aber weniger als anaerobe methanogene Stämme. Der methanogene Stamm *Methanobacterium thermo-autotrophicum* enthält ungefähr die 2,7-fache Menge des Kofaktors. Eine Induzierbarkeit der Kofaktorproduktion in Abhängigkeit von der Präsenz des Pikrats im Kulturmedium konnte nicht nachgewiesen werden.

Die Enzymkomponenten des Systems wurden näher untersucht. Der zweite Reaktionsschritt, die Hydridübertragung, konnte nicht vom ersten Schritt, der F_{420}-Reduktion, abgekoppelt werden. Eine chemische Reduktion des Kofaktors F_{420} war mit den aus der Erforschung der Methanogenese bekannten Methoden nicht möglich, da in Abwesenheit eines Reduktionsmittels, auch unter anaeroben Bedingungen, eine spontane Reoxidation erfolgte. Andererseits reduzierte ein anwesendes Reduktionsmittel Nitrosubstituenten am aromatischen Ring zu

Aminogruppen. Da hierdurch der Elektronenzug am aromatischen Ring herabgesetzt wird, d.h. die Elektronendichte im aromatischen π-System sich erhöht, unterbleibt die Hydrierung am aromatischen Ring. Die Vorschaltung der enzymatischen Reaktion war demnach unvermeidbar. Die Hydridübertragung wurde folglich mit Hilfe eines gekoppelten Tests mit einem Überschuß an NADPH- und NADPH-abhängiger F_{420}-Reduktase untersucht.

Zunächst wurden die pH-Optima bestimmt. Für die Reduktion von F_{420} ergab sich ein Wert von 5,5, welcher im Bereich anderer F_{420}-abhängiger NADP-Reduktasen liegt (Eker et al. 1989; Berk und Thauer 1997). Das pH-Optimum des Hydridtransfers liegt um 2 pH-Einheiten höher und war vergleichbar mit Werten, wie sie für homologe Enzyme aus Archaea beschrieben wurden (Schmitz et al. 1991). Der K_m-Wert für die Hydridtransferase mit Pikrat als Substrat lag bei 0,06 mM. Bei höheren Substratkonzentrationen kam es zu einer Hemmung des Hydridtransfers. Da das Produkt der Reaktion mit 2,4-DNP als Hydridakzeptor zum Teil wieder nicht-enzymatisch das Edukt liefert, konnte für diese Reaktion kein K_m-Wert bestimmt werden.

Substitutionsversuche des Mediators mit kommerziell verfügbaren Kofaktoren wie FMN oder FAD führten zum Verlust der Aktivität im Enzymtest. Aus diesem Grund wurde das nicht käufliche Koenzym F_{420} aus gesammelten F_{420}-haltigen Abfällen zurückgewonnen.

Die Aktivität der Hydridtransferase blieb während der nativen Gelelektropherese erhalten. Eine Mischung der nötigen Komponenten wurde auf das Gel aufgeträufelt. Es konnte eine deutliche Orangefärbung einer Bande beobachtet werden. Diese wurde aus einer nicht gefärbten Kontrollspur ausgeschnitten und auf ein SDS-Gel aufgetragen. Es wurde nur eine Bande gefunden, was auf die Existenz nur einer Untereinheit hinweist. Das apparente Molekulargewicht stimmt mit dem nativen überein. Dies deutet auf eine Enzymstruktur mit nur einer Peptidkette hin.

Interessant war die Analyse der Produkte der Hydridübertragung. Rieger et al. wies das Entstehen und die Struktur des orangefarbigen Hydrid-σ-Komlexes von Pikrat (H⁻-Pikrat) beim Umsatz von Pikrinsäure mit dem Stamm *Rhodococcus erythropolis* HL PM-1 nach (Rieger et al. 1999). Dieser Komplex trat auch während der hier durchgeführten Enzymreaktionen mit Pikrat intermediär auf. Die durch H⁻-Pikrat hervorgerufenen Orangefärbung ging in blassgelb über. Das Produkt dieser durch

das Enzymsystem katalysierten Reaktion wurde als Dihydrid-σ-Komplex von Pikrat (Dianion des 2,4,6-Trinitrocyclohexanons) identifiziert. Dadurch wurde das Auftreten eines zweifachen Hydridtransfers auf Pikrat nachgewiesen.

Im Fall von 2,4-DNP führt die enzymatische Hydridübertragung zum Hydrid-σ-Komplex dieser Substanz. Die chemische Synthese des authentischen Komplexes durch Reduktion von 2,4-DNP mit Natriumborhydrid in Lösung und das bekannte UV/Vis-Spektrum (Behrend und Heesche-Wagner 1999) ermöglichte eine Identifikation dieses Produktes anhand von HPLC gekoppelt mit einem Photodiodenarray-(PDA) Detektor. Dieser Hydrid-σ-Komplex ist im Vergleich zum σ-Komplex des Pikrats nicht sehr stabil. Er disproportioniert in 2,4-DNP und Aminonitrophenol (Behrend 1999).

Mit verschiedenen Nitroaromaten wurde ein Substratspektrum erstellt. Dabei stellte sich heraus, dass Mononitrophenole wie *p*- oder *o*-Nitrophenol von dem betrachteten Enzymsystem nicht reduktiv angegriffen werden. Der disubstituierte Aromat 2,6-Dinitrophenol wird ebenfalls nicht umgesetzt. Von diesen Nitroaromaten ist bekannt, dass sie oxidativ angegriffen werden (Munnecke und Hsieh 1974; Spain et al. 1979; Ecker et al. 1992; Hanne et al. 1993; Kadiyala und Spain 1998). Dies stünde aber keiner Kernhydrierung bei einem Umsatz durch andere Stämme entgegen.

Strukturanaloga zu Pikrinsäure wie 2-Chlor-4,6-dinitrophenol und DNOC werden beide durch das Hydrid transferierende Enzymsystem umgesetzt. Bei 2-Chlor-4,6-dinitrophenol ist diese Umsetzung schneller als bei DNOC. Dies ist auf den positiven induktiven Effekt der Methylgruppe bei DNOC zurückzuführen. Dadurch ist die Elektronendichte des aromatischen Systems verglichen mit 2,4-DNP größer. Chlor als Substituent hat einen negativen induktiven Effekt, was die Elektronendichte am aromatischen Ring herabsetzt und somit einen nukleophilen Angriff erleichtert. In beiden Fällen ist eine Zunahme der Absorption im Bereich oberhalb von 420 nm zu beobachten. Dies deutet auf die Entstehung eines Hydrid-σ-Komplexes hin. Eine nachfolgende Abnahme der Absorption, wie es bei einer weiteren Hydridübertragung der Fall sein müsste, war nicht zu beobachten. Eine zweite Hydrierung hätte eine sp^3-Hybridisierung der Kohlenstoffatome 3 und 5 des aromatischen Rings zu Folge. Damit könnten π-Elektronen über diese Positionen nicht delokalisiert werden. Die Mesomerie wäre somit eingeschränkt und die Absorption bei niedrigen Wellenlängen

müsste geringer werden. Aus den geschilderten Beobachtungen bei Enzymtests mit verschiedenen Nitroaromaten konnte eine Strukturvorhersage für mögliche Substrate gefolgert werden: Zwei Nitrogruppen in 2,4- oder 4,6-Position am aromatischen Ring sind notwendige Bedingung für einen durch das Enzymsystem katalysierten Hydridtransfer in Position 3 oder 5.

Detektion der Gene des initialen Abbauweges

Die N-terminalen Sequenzen der beiden Enzyme des Enzymsystems wurden verwendet, um Primer für eine PCR (Polymerase chain reaction) abzuleiten. Dabei wurde die Annahme getroffen, dass die NADPH-abhängige F_{420}-Reduktase vor der Hydridtransferase auf der DNA kodiert ist. Der Forward-Primer wurde deshalb aus dem N-Terminus der Reduktase nach der die Nukleotidbindungsstelle kodierenden Region abgeleitet. Der Reverse-Primer wurde an einer wenig gewobbelten Stelle

```
cgggacagcgagtccaggatggccgctctcggctaccgcaccaccgcctaccggctcggtgcgttcgtcctcagcggggtcctcgcgccgtcagcggtg  100

cgctgctgacctaccagcttcagttcgcgagcccggcgcagctcaacttcgtcgtgtcgatccaggctctgttgatcgtcgtcatcggagccggcacgct  200

ctacgggcccgcggtcgccggagtcggggtcatctggctccaggagctcctccagcagcacaccgaccactggctcctcgtgctcggctgcatctacatc  300

gcggtggcgctgggcaacccgccggcactggtgcagcggctgcggagtcggctgggcccggcgcgtccggggtcgaggatgtcggatcggacgacgcagg  400

aggtggccgcatgagcgcccgatccaccgcggcgacgagcgggcaggccgcgctcagcgcgacccgggatctgcgtccgtctcggcggcgtggagatcctt  500

cgggacctgtccatcgaggtggctccggggggagcgacgcgcgatcgtcggtccgaacggagccggcaagacgacgatgttcaacgtcctgaccggtgtgc  600

tgacgcccgagtcgggccacgtctccctcgggggtcgtgacgtcacccgctgggatgtgagtcgacgtgcctcgcagggcatggccaggaccttccagat  700

caccaacctgatgcacacgatgacggtggagcagaacgtggtgctagcgcttgcggccgatagacctcgggtccgtcgcgacctcttccgtcgcatcgag  800

cgagccggcctccacgagcaggcgggtgccctgatcgagcagtggggtctcggcgcgcgccggtccactgccgtgcacctgctctcctacggcgagcggc  900

gcgagctcgagatggtgctggcggtcgccggcgagccagacgtcctcctgctcgacgagccggctgcagggctgtcgcccgtcgagacagagcgcgtcgc  1000

gggactcgtggagcggttgccccggtccacgagcatcgtcctgatcgagcacgacctggacatggcgctggcgctctcggacacggtcacggtcatgtcc  1100

gagggctgcatccgcggcaccgggcgcgcagacgatcctgcgatctccgcgctcgtggagcgcgtctatgtcggcggcggccagga**ggagga**cgacg**atg**  1200
                                                                        ****** **orf**

ttgcaggttgaggcgattcgctccggctacggctcgatcgagatcgtgcgcggactcgatctcaccgtgggagcgggcgaggtggtgtcgctgatcggac  1300
**2->**

gcaacggcgccggcaagacgacactggtcaacaccctgatgggcttcaatcgcctcgggtccggcagtgtcaccctcgccgggagggacatctcgcgggc  1400

caagccgcacctgcgcaaccgcctgggcatggccgtggtcccgcaggggcgtcgcgtcttcggcagcctgacggtcgagcagtgcgtcgatctcgccgct  1500

cgcgaaggcgggtgggacctggaccgcatcgcgaccgcctttcctcgcctggccgagcgtcggggaacccgagcgcggagtctgtcgggcggggagcaga  1600

gcgcgctcgcgatggcgcggggcgctggcgacggggccgcagttgatgctcctcgacgagccctcggagggcatgtcgccgcacgttctgaggtcgctcgc  1700

gcacatcatctccgggctgagggagtccggtgccggcgtactgcttgtcgagcagaacctgccgttcgcggtcggtgtcgctgaccgggtcgtcgtgatg  1800

gagcgtggtcatgtcgcgctcgaggtcacgggcgacgaggcacgggcggaccgaccgttctctcccagtacttggtgatcaaccgctcggag**tga**gcag  1900
                                                                               -> **orf2**

atcaccgcatcaccagagtcatcttcgga**aaggaa**tccaaccaa**atg**cgcaagttctggcatgtcggcatcaacgtgaccgacatggacaagtcgatcga  2000
                             ****** **orf3->**

gttctaccgcaaggtgggcttcgacgtcgtgcaggacaaggaggtcgaggacgagaacctcgcccgtgccttcatggtcgacggcgtcagcaggctgcgg  2100

ttcgcccacctgcggatgaacgaatcgccgacgaggccctgctcgacctcatcgagtggaaggacgtggccccgagcggcccagccaacgccgatctcgc  2200

tccacccggtctgtgcaggttctcgatcctgaccgacgacatccagggtgagttcgaccggctcacggcggacgcgtggagttcctccacgagccgca  2300

gaccgtcatgggacccgacggcgtgaacggatggaagctgctcttcgcccgcgacccggacggaacgctcttccacttcgcagagctggtcggc**tga**gcg  2400
                                                                                -> **orf3**

ataccgtcgtcggtgcaggatcggagaggctggaaccagtgatcgggc**aggagg**gtcag**atg**ccggagagccgcgaggagtcgggcagtggtacgacggg  2500
                                                ****** **orf4 ->**

tgcccaggtggtgcccgcggtgagtcgggcgacccgtgtcctcgaggcgctggtccgcagtccgctcggcgccagcctcaccgagctcgccgaggcctgc  2600

gacctcgccaagagcagctcgtcgaacctcctgcgcaccatggtggccgagggactggtcagctatgacgcgggcacccgccgctacaacctcggcccga  2700
```

```
tgctcgtcgagttcggtgtcgcggccatcacccggaccaccgcggtgacggaggcacggcccttcatggaggagctggccgcgcgcaccgagctggcctg   2800

cctggcgatccagccgatgcccgacggacacttcacggcgatcgccaagatcgagagccgcaaggcgatcaaggtgaccatcgaggtgggcgcgcggttc   2900

cacaccacctcaccgctcctgagccggttgcaccgcgcctggcgactggagtcggagttcgaggacgggcccgcaacccccggcgacgcggcgctggcac   3000

tcgtgcgcgagcgtggttacggagcggtcttcggcgagtaccgcccggagctcaatgtcgccggcttcccggtcttcgaccgcgacgccctgccctgccg   3100

ctacgtcacgctgctcgggatgggtgaggacttcaccggaccccgggtggaggagatcggcgactacctcatcgaggctgcgcacgccatcacgacccgc   3200

agcgggggccacctccctgccgactacccgaaggccgacggctcgccgactctcggctgacctgatcgcaccatccatccgctgcgcgcgcgagcgcgcg   3300
                                                           -> orf4

                       M  Q  P  T  T  I  A  V  V  G  G  T  G  P  Q  G  R  G  L  A  R  W  F
gctctctttgccatcccttttttgaggagacctgatgcaacccaccaccatcgccgtcgtcggcgggaccggcccccagggggcgaggactcgcccgatggt   3400
                       *******       orf5->

  A  Q  A  G  H  T  V  I  L  G  S  R  S  A  E  R  A  S  A  V  A  D  D  V  T  A  R  S  G  A  T  V  T
tcgcccaggccgggcacaccgtgatcctcggctcgcgctccgccgagcgtgcctccgccgtggcagacgacgtcaccgcgcgcagcggggccacggtgac   3500

  G  A  S  N  A  D  A  V  A  S  A  D  I  V  L  I  V  V  P  W  D  G  H  E  E  L  V  A  D  L  A  P  R
cggcgcgagcaacgccgacgccgtcgcgagcgctgacatcgtgctgatcgtcgtcccctgggacggtcacgaggagctcgtcgccgatctggctccccgc   3600

L  A  G  K  L  V  V  S  C  V  N  P  L  G  F  D  K  R  G  A  Y  G  L  D  V  V  D  G  S  A  A  E  Q  T
ctggccggcaagctggtcgtcagctgcgtgaacccgctgggcttcgacaagcgcggcgcctacggactcgacgtcgtcgacggcagcgccgccgagcaga   3700

  Q  R  L  V  P  T  A  T  V  V  G  A  F  H  H  L  S  A  V  T  L  W  D  S  E  E  P  L  D  H  E  D  V
cccagcgcctggtgcccacggccacggtcgtcggcgccttccaccatctcagcgccgtgacgctgtgggacagcgaggagcctctcgaccacgaggacgt   3800

  L  V  C  G  D  D  A  D  A  R  A  R  V  L  E  L  A  T  A  V  T  G  R  A  G  I  E  V  G  P  L  R  L
gctcgtgtgcggtgacgatgccgacgcgagggcgagggtcctcgagctggcgacagccgttacgggccgggccgggatcgaggtcggcccgttgcggctg   3900

A  A  Q  L  E  P  L  T  A  V  L  I  G  V  N  R  L  Y  K  T  R  S  G  V  A  L  A  G  V  P  R  *
gcggcccagctcgagccgttgaccgccgtcctgatcggcgtcaaccgcctctacaagacgcgctccggagtcgccctggcaggagtgccgcgatgatcca   4000
                                                                            -> orf5
                                                                    *****       orf6 ->

cgagctgcgtgagtacgtcgccctcccggggcgggcagaggagctgcaccggcgattcgctgatcgcacgctcgagttgtggcgcgagatcggactcgac   4100

ctggtcggcttctggcacgtctccgacgagccccggcggatcgtctacctggtgcgcttcgcgaccgaggacgaggcgcggagtcactgggaggccttca   4200

cacgcgatcccaggtggctgcagatcaagggggagacggaggccgacgggcccctgatcgagtcgatctcgtcgacgtaccttgccacacctgcctactt   4300
                 PstI                            Stop of orf5??

                                                                                        M  I  K
ccgggcaccggatccgttgccctcggaacctgcggctctctaggttcctcgaaatgtcgacccccctgttcatcgcaatgaatcgagctctctcatgatca   4400
            BamHI                        -> orf 6                                *******       orf7 ->

  G  I  Q  L  H  A  W  A  G  G  P  E  M  V  E  V  A  E  I  A  A  Q  Q  F  E  T  V  W  V  T  D  Q  L
aaggaatccagctgcacgcatgggccggaggcccggagatggtcgaggtcgccgagatcgcggcccaacagttcgagacggtctgggtgaccgaccagct   4500

  Q  S  R  G  V  A  A  V  L  G  A  I  A  A  R  T  G  M  G  V  G  T  A  V  T  F  P  F  G  R  N  P  L
ccagtcacgcggcgtggccgcggtgctcggcgcgatcgcggcccgtactggcatgggtgtcggcactgccgtgaccttcccgttcggccgcaatccgctc   4600

E  Q  A  S  T  M  A  T  L  A  E  F  L  P  E  G  R  Q  V  S  M  G  I  G  T  G  G  G  L  V  S  A  L  M
gagcaggcctccacgatggcgaccctggccgagttcctccccggagggacggcaggtcagcatggggatcggcaccggtggtgggctggtgagcgccctca   4700

  D  R  H  Q  P  V  G  R  V  A  E  L  I  A  L  S  R  V  L  W  S  G  E  E  I  T  L  G  D  Y  P  L  T
tggaccgacaccagcccgtgggccgtgttgcggagctgatcgccctgtcgcgtgtgctgtggagcggcgaggagatcaccctgggcgactacccgctgac   4800

  C  S  S  L  G  L  R  P  E  A  K  A  S  F  K  W  S  K  D  P  A  V  R  V  V  V  A  G  A  G  P  R  I
gtgctcgagcctgggcctgcgccccgaggcaaaggcgtccttcaagtggtcgaaggacccggctgtcagggtcgtggtcgccggcgccgggcccggatc   4900

L  E  M  A  G  E  I  A  D  G  V  I  C  A  S  N  Y  P  A  H  S  L  A  A  F  R  S  G  Q  F  D  Q  V  S
ctggagatggcgggcgaaatcgcggacggggtgatctgcgcgagcaactaccccgcgcacagcctcgcagcgttccgcagcggccagttcgaccaggtca   5000

  N  L  D  A  L  D  R  G  R  L  R  S  E  R  P  D  F  S  R  I  Y  G  V  N  I  S  V  A  E  D  R  E  A
gcaacctcgacgcactcgaccgcggtcgcttgcgcagcgagcggcccgacttctcgcggatctacggcgtcaacatctccgtggccgaggatcgtgaggc   5100

  A  N  A  A  A  R  R  Q  A  T  L  I  V  S  Q  Q  P  H  E  N  L  E  R  V  G  F  D  R  G  D  Y  S  A
ggccaacgccgcggcccgacgtcaggcgacgctgatcgtcagccagcagccgcacgagaacctcgagcgcgtcggcttcgaccggggtgactacagcgcc   5200

T  R  E  A  V  H  A  G  A  G  V  E  R  A  A  E  L  L  P  Q  F  V  A  D  Q  L  I  V  S  G  T  P  E  D
acccgcgaggcggtgcacgcaggcgccggtgtcgagcgcgctgctgagctgctgccgcagttcgtggcggaccagctcatcgtgtcgggcaccccggagg   5300
```

```
  C  V  A  G  L  G  E  L  W  A  T  R  S  G  P  A  S  T  R  S  T  W  A  R  P  S  A  P  T  R  G  R  R
actgcgtcgccggcctgggtgagctctgggccacgcgagcgggccggcttcgacgaggtctacctgggcgcgcccatcggccccgacccgagggaggcg        5400


  C  K  L  I  T  S  R  V  L  P  A  L  S
gtgcaagctgatcaccagccgcgtcctcccggcactgtcgtgaccgagtcggccgggcgggcgttcatcgtcgacaggaacgacgcggtcgtcaccatca    5500
                                  -> orf7
cgctgaaccggcccgaggcacgcaacgcgctgaatctcgacctgctcgacgggctgctgtccacgctgcggtccacgcgggatgaaggcgcggagatcgt    5600

catcctgcggggggccgggcggggcttctgtgcgggagccgacacccgctccgacgacggcacggccatcggccgcccgcctgcgtcgcgagctgatc       5700

ggcgacctgatcgaggaggtccgacgctttcccgccagcatcgcggccgtccacggcgctgccgtcggcggcggttgggggctggccttcgccgccgacg    5800

tcgttctggccgcacccgacgcgctgttcaagtttcccgagctggccctggggatcctgccgccgaaaccgctggtggatcgattcgtcgaggtcgccgg    5900

ggcggcccgagcgatccggctgctcggctccggcgagcccttcgccgccgcttcaccagcgttggtgggtctggtcgagccggtccccgacggcggcctc    6000

gacgaggcgtgcgcacgtctcgtcggtcacttctcgcaggcacgccccggcctgctgcatgcactcaagcgagccctcgaccctgcggtcgcctgacgaa    6100

aggtagaacagccatgagcacacgtagtccgttggagaccgacgaccagagcgagctgcgcaagaccgcccgcgatctcttcgctgccgaggcgcccgag    6200

cgcgacgtccggatctgggacgaggacggcgtctaccggagcgtctctaccgccggatcgccgacctcggctggtacgacatcgcgaccgacgactcgg     6300

tcgaggagcgagcgagccttctgaccgctttgtgcgaggagatcgggcgcagcagctccgacctggtcgcgctcttcaacctgaacttcagcggcgtccg    6400

cgacctccagcgttggggcacgcccgcccagcaggagaagtacgcgcagccggtcctcgacggcacgcaccgattctcgatcgcagtcagcgaacccgac    6500

gtcgggtccgacgcggccagcgtggtgacccgggccgagtcggccggcgatggttggatcctcaacggtcagaagacctactgcgagggcgccgggatcg    6600

acgggacggtcatggaggtgctcgccaaggtggggggagggcgagcggaagcgcgaccggttggccgtcttcctcgtcccaaccgacttgccgggcatcga    6700

ggtgcggcgcatgcctgcgctcgggcgcaacatcagcggcatctacgaggtcttcctccgcgacgtcgagctccccgcggatgcgctgctcggtgaggtc    6800

gggcagggatggcagatactcaaggagcgactggtgctcgagcgcctgctgatcagctccggtttcgtcggcagctcggcggcggtgctcgacatgaccg    6900

tcggctatgccaacgagagggagcagttcggcaaggccatctcgtcgtaccaaggcgtctcgctgcccctggcggagatgcacatccggctcgatgctgc    7000

ccggtgcgccgtacgtcgatccgcagagctcttcgaccgcggcattccctgcgaggtggagagcaccatggccaagttcctctgcggccagctctatgcg    7100

gagtcgtcggcccttgcgatgcaggtgcagggggcctacggctacgtccgcgaccacgcgctgccgatgcaccactccgacgcgcatcatcgcccgcgtcg    7200

tggcgggaccgccggcgctgcag
```

Abbildung 5. Contig aus BamHI und PstI Fragment. * kennzeichnet potentielle ribosomale Bindungsstellen.

des N-Terminus der Hydridtransferase gewählt. Die getroffene Annahme bestätigte sich durch den Erhalt eines PCR-Produktes. Das Verdauen von DNA mit verschiedenen Restriktionsenzymen und die geschickte Wahl von Sonden führte mittels Southern-blot-Analyse zur Isolation eines BamHI- und eines PstI-Fragments, welche miteinander überlappten und die Gene der beiden Enzyme enthielten. Der so erhaltene Contig ist in Abbildung 5 dargestellt. Eine Analyse auf Open Reading Frames (ORF) ergab 7 ORFs. Eine Zusammenfassung über die Ähnlichkeiten der in Protein übersetzten ORFs mit Proteinen aus anderen Organismen ist in Tabelle 1 abgebildet. Vor jedem ORF befindet sich eine potentielle ribosomale Bindestelle (in Abbildung 5 durch Stern gekennzeichnet). ORF 5 (663 bp) enthält zu Beginn den N-Terminus der NADPH-abhängigen F_{420}-Reduktase und ORF 7 (1704 bp) am Start den der Hydridtransferase. Dazwischen liegt ein kleiner ORF (351 bp), dessen Funktion nicht bekannt ist. Das korrespondierende Enzym zu ORF 7 hat ein Molekulargewicht von 37,07 kDa. Dies entspricht dem durch SDS-Page bestimmten apparenten Molekulargewicht des Enzyms. Das Protein enthält keine

charakteristischen Sequenzmotive, die auf bestimmte Funktionen oder Bindestellen hinweisen. ORF 5 enthält außer der schon vom N-Terminus her bekannten Nukleotidbindestelle keine weiteren charakteristischen Sequenzen. Das von ORF 5 abgeleitete Protein hat ein Molekulargewicht von 22 kDa. Dadurch ergibt sich eine Differenz von 8 kDa zu dem Gewicht, welches mit SDS-Page bestimmt wurde. Die DNA Sequenz ist der Consensus einer Überlagerung mehrfach Strang und Gegenstrang sequenzierter kleinerer Fragmente. Deshalb ist ein Frameshift, verursacht durch einen Sequenzierfehler, eher unwahrscheinlich. Eine mögliche Glykosylierung des Proteins könnte diese Differenz erklären. Prokaryonten enthalten zwar weitaus weniger Glykoproteine als Eukaryonten, aber es sind dennoch Glykoproteine aus *Streptococcus* (Kawamura und Shockman 1983), *Mycobacterium* (Garbe et al. 1993) und *Streptomyces*-Arten (Kluepfel et al. 1990; Mihoc und Kluepfel 1990) bekannt. Die Art der Verknüpfung könnte N- oder O-glykosidisch sein. Bei Prokaryonten tauchen auch spezielle Verknüpfungsarten auf (Gerwig et al. 1992; Christian et al. 1993). Ong et al. (Ong et al. 1994) postulierten, dass eine O-Glykosylierung Proteine vor proteolytischem Zerfall schützt und die Struktur stabilisiert.

Eine weitere Ursache wäre eine Retardation des Proteins im SDS-Gel, bedingt durch die Bindung eines Kofaktors. Dies würde sich mit hoher Wahrscheinlichkeit im UV/Vis-Spektrum bemerkbar machen. Es konnten aber keine signifikanten Absorptionsbanden festgestellt werden.

Im Anschluss an das Stopcodon des ORF 5 folgt ein weiteres Stopcodon (4255-4258 bp). Das hieraus abgeleitete Protein hätte ein Molekulargewicht von 31 kDa. Dies liegt näher im Bereich des apparenten, mit SDS-Page bestimmten Molekulargewichts. Untersuchungen mittels MALDI-ToF-MS (Matrix-assisted laser desorption time of flight mass spectroscopy) könnten Hinweise über die reale Masse des Proteins sowie Aufschlüsse über eine eventuelle Glykosylierung oder gebundene Kofaktoren geben.

Des weiteren sind für *Rhodococcus erythropolis* HL PM-1, bei welchem die Enzyme des Abbaus von Pikrinsäure induzierbar sind, Gencluster durch die Methode des Differential Display aufgedeckt worden. Hierbei wird die gesamte mRNA des Organismus im induzierten und nicht induzierten Zustand verglichen. Die im induzierten Zustand vorkommende mRNA wird weiter analysiert. Zusammen mit den

in der vorliegenden Arbeit beschriebenen N-Termini der *N. simplex* Enzyme konnte ein Gencluster isoliert werden, welcher eine NADPH-abhängige F_{420}-Reduktase und eine Hydridtransferase codiert. Ein Alignment der Hydridtransferasen aus *N. simplex* und *R. erythropolis* wies sehr hohe Ähnlichkeiten auf. Es konnte eine Aktivität der in *E. coli* exprimierten Enzyme aus dem HL PM-1 Stamm nachgewiesen werden (Russ et al. 2000). Zwischen den Genen dieser Enzyme ist analog zu dem oben untersuchten Organismus ein kleiner Orf mit bisher unbekannter Funktion lokalisiert. Homologien der zu ORF 5 und ORF 7 korrespondierenden Proteine mit in Datenbanken (BLAST) vorhandenen Proteinsequenzen sind in Tabelle 2 und 3 zusammengefasst.

Weil Pikrinsäure und 2,4-DNP in der Natur auf einen anthropogenen Ursprung zurückzuführen sind, können diese Nitroaromaten nicht die natürlichen Substrate für das untersuchte Enzymsystem sein. Da nur sehr wenig natürliche aromatische Nitroverbindungen bekannt sind, ist ein analoger Nitroaromat, der in der Natur vorkommt, als Substrat des Enzymsystems sehr unwahrscheinlich. Deshalb haben Enzyme, die in diesen Abbauweg involviert sind, vermutlich eine andere physiologische Funktion und sind in den Organismen konserviert. Eine mögliche Konservierung der Gene, die F_{420}-abhängige $NADP^+$-Reduktasen kodieren, lässt auf die Entwicklung von einem gemeinsamen Vorfahren schließen. Diese Überlegung legt die Vermutung nahe, dass es sich um eine unter den Prokaryonten eventuell weiter verbreitete genetisch konservierte Eigenschaft handelt. Eine andere Möglichkeit wäre der horizontale Gentransfer bei näherzurückliegenden Vorfahren (Chistoserdova et al. 1998).

Tabelle 1. Ähnlichkeiten der von den Orfs der DNA-Sequenz abgeleiteten Proteine zu Proteinen in der Datenbank BLAST (Altschul, 1997).

Orf	Identifizierte Ähnlichkeit	Identität	Ähnlichkeit	Lücken	Score	E-Wert
1	vermutliches aliphatisch-verzweigte Aminosäuren transportierendes ATP-bindendes Protein [*Streptomyces coelicolor A3(2)*]	108/271 (39%)	150/271 (54%)	22/271 (8%)	163 bits (409)	1,00E-39
2	ABC-Transporter aliphatisch - verzweigter Aminosäuren, ATP-bindendes Protein [*Deinococcus radiodurans*]	103/230 (44%)	144/230 (61%)	5/230 (2%)	178 bits (448)	3,00E-44
3	Ähnlich zu Protein gb|Z74962 von Brassica oleracea welches ähnlich ist zu bakteriellen YRN1 und HEAHIO Proteinen. [*Arabidopsis thaliana*]	40/148 (27%)	64/148 (43%)	25/148 (16%)	49.2 bits (115)	2,00E-05
4	Transkriptionsregulator, IclR Familie [*Thermotoga maritima*]	59/245 (24%)	110/245 (44%)	19/245 (7%)	68.3 bits (164)	9,00E-11
5	Vermutliche Oxidoreduktase. [*Streptomyces coelicolor A3(2)*]	105/213 (49%)	136/213 (63%)	4/213 (1%)	194 bits (489)	5,00E-49
6	FrnE [*Streptomyces roseofulvus*]	18/55 (32%)	27/55 (48%)		32.5 bits (72)	1.6
7	N5,N10-Methylenetetrahydro-methanopterin-Reduktase (mer-1) [*Archaeoglobus fulgidus*]	85/331 (25%)	151/331 (44%)	36/331 (10%)	97.9 bits (240)	1,00E-19

Tabelle 2. Ähnlichkeiten zum von Orf 5 abgeleiteten Protein mit Proteinen der Datenbank BLAST (Altschul et al. 1997).

Ähnlichkeiten zu Orf 5	Ähnlichkeit	Positive	Lücken	Score	E-Wert
vermutliche Oxidoreduktase. [*Streptomyces coelicolor* A3(2)]	105/213 (49%)	136/213 (63%)	4/213 (1%)	194 bits (489)	5,00E-49
Vermutliche F420-abhängige NADP-Reduktase - konserviertes Protein [*Methanobacterium thermoautotrophicum*]	83/222 (37%)	122/222 (54%)	9/222 (4%)	123 bits (307)	1,00E-27
Vermutliche F420-abhängige NADP-Reduktase - konserviertes hypothetisches Protein [*Methanococcus jannaschii*]	75/212 (35%)	117/212 (54%)	9/212 (4%)	115 bits (284)	5,00E-25
Konserviertes hypothetisches Protein AF0892 [*Archaeoglobus fulgidus*]	70/202 (34%)	112/202 (54%)	6/202 (2%)	113 bits (281)	1,00E-24
F420-abhängige NADP-Reduktase (EC 1.6.8.-) [*Methanobacterium thermo-autotrophicum* (Stamm Marburg)]	76/222 (34%)	116/222 (52%)	9/222 (4%)	111 bits (276)	4,00E-24
konserviertes hypothetisches Protein [*Archaeoglobus fulgidus*]	65/205 (31%)	113/205 (54%)	3/205 (1%)	103 bits (255)	1,00E-21
vermutliche Oxidoreduktase [*Streptomyces coelicolor* A3(2)]	62/191 (32%)	99/191 (51%)	7/191 (3%)	92.1 bits (225)	4,00E-18

Tabelle 3. Ähnlichkeiten zum von Orf 5 abgeleiteten Protein mit Proteinen der Datenbank BLAST (Altschul et al. 1997).

Ähnlichkeiten zu Orf 7	Ähnlichkeit	Positive	Lücken	Score	E-Wert
N5,N10-Methylene-tetrahydromethanopterin-Reduktase (mer-1) [*Archaeoglobus fulgidus*]	85/331 (25%)	151/331 (44%)	36/331 (10%)	97.9 bits (240)	1,00E-19
vermutliche F420-dependent N5,N10-Methylene-tetrahydro-methanopterin-Reduktase [*Methanolobus tindarius*]	90/335 (26%)	147/335 (43%)	28/335 (8%)	97.9 bits (240)	1,00E-19
N5,N10-Methylenetetrahydro-methanopterin-Dehydrogenase (Koenzyme F420-abhängig) [*Methanococcus jannaschii*]	99/330 (30%)	148/330 (44%)	36/330 (10%)	94.8 bits (232)	1,00E-18
N5,N10-Methylene-tetrahydro-methanopterin-Reduktase (mer) [*Methanococcus jannaschii*]	99/330 (30%)	148/330 (44%)	36/330 (10%)	94.8 bits (232)	1,00E-18
Koenzyme F420-abhängige N5,N10-Methylene tetrahydro-methanopterin Reduktase [*Methanobacterium thermo-autotrophicum* (Stamm Delta H)]	87/335 (25%)	141/335 (41%)	47/335 (14%)	70.6 bits (170)	2,00E-11

Oberer Abbauweg von Pikrinsäure und 2,4-DNP

Die bislang postulierten Abbauwege basierten auf der Identifizierung von Metaboliten, die aus Kulturüberständen und Überständen von Ruhezellumsätzen isoliert wurden. Ein Beweis durch in-vitro-Untersuchungen stand zu Beginn der vorliegenden Arbeit noch aus. Die Entdeckung von $2H^-$-Pikrat als Produkt des initialen Hydridtransfers deckte neue Möglichkeiten für einen weiteren Abbau auf. Deshalb wurde der obere Teil der Abbausequenz vor allem auf enzymatischer Ebene eingehend untersucht. Die Aktivität mit verschiedenen Substraten des betrachteten Enzymsystems wurde mit den Ruhezellumsätzen verglichen. 2,6-Dinitrophenol wurde nicht umgesetzt. Es konnte ein Wachstum auf p-Nitrophenol beobachtet werden, obwohl keine Aktivität des Hydrid übertragenden Enzymsystems detektiert wurde. Da p-Nitrophenol in vielen Stämmen oxygenolytisch abgebaut wird, deutet dies darauf hin, dass der Organismus möglicherweise für dieses Substrat einen separaten, durch Oxygenasen initiierten, Abbauweg besitzt (Spain und Gibson 1991; Jain et al. 1994; Kadiyala und Spain 1998). DNOC wird bei einem Umsatz mit Ruhezellen nur in geringem Maße umgesetzt, was mit der beim Enzymtest gemachten Beobachtung einer geringen Aktivität korreliert.

Der $2H^-$-Pikrat-Komplex, welcher bei der vom Hydrid übertragenden Enzymsystem katalysierten Reaktion von Pikrat entsteht, wurde nach einer Vorschrift von Severin und Schmitz (Severin und Schmitz 1962) synthetisiert. Dazu wurde Pikrat mit Natriumborhydrid in wäßriger Lösung reduziert und das Produkt durch Zugabe von Methanol ausgefällt. Da in der Literatur bisher keine NMR-Spektren vorlagen, wurde zur Absicherung der Struktur des erhaltenen Produktes eine ^{1}H- und ^{13}C-NMR Analyse durchgeführt. Die Struktur konnte dadurch bestätigt werden. Auch die Absorptionsbande bei 390 nm, in alkalischer Lösung, im UV/Vis–Spektrum stimmte mit dem in der Literatur beschriebenen überein.

Bisher blieb die Frage offen, ob aus H^--Pikrat oder aus dem Produkt des Enzymsystems, $2H^-$-Pikrat, eine Nitritgruppe als Nitrit eliminiert wird. Eine Nitritfreisetzung aus H^--Pikrat wurde in früheren Untersuchungen an *Rhodococcen* postuliert (Lenke und Knackmuss 1992; Behrend und Heesche-Wagner 1999; Ebert et al. 1999; Rieger et al. 1999). Desweiteren war bisher unklar, ob es sich dabei um eine enzymatisch katalysierte Reaktion handelt. Eine Freisetzung von Nitrit aus H^--Pikrat hätte eine Rearomatisierung unter Bildung von 2,4-DNP zufolge. Würde

Nitrit aus 2H⁻-Pikrat frei gesetzt, entstünde der Hydrid σ-Komplex von 2,4-DNP (H⁻-2,4-DNP) als Produkt.

Tatsächlich wurde bei Umsatzversuchen von Pikrat mit *R. erythropolis* HL PM-1 unter anaeroben Bedingungen 2,4-DNP beobachtet (Lenke und Knackmuss 1992). Rieger et al. beschrieben die Umsetzung von H⁻-Pikrat mit zellfreiem Extrakt und wiesen 2,4-DNP durch HPLC analytische Untersuchungen nach. Da der H⁻-Pikrat Komplex bei pH ≤ 7,4 zu 5% protoniert vorliegt und eine Eliminierung von Nitrit aus H⁻-Pikrat die Wanderung eines Protons von C-3 nach C-2 erfordert, schloss man auf eine energetisch günstigere Abspaltung aus der protonierten Form des Hydrid-σ-Komplexes (Rieger et al. 1999).
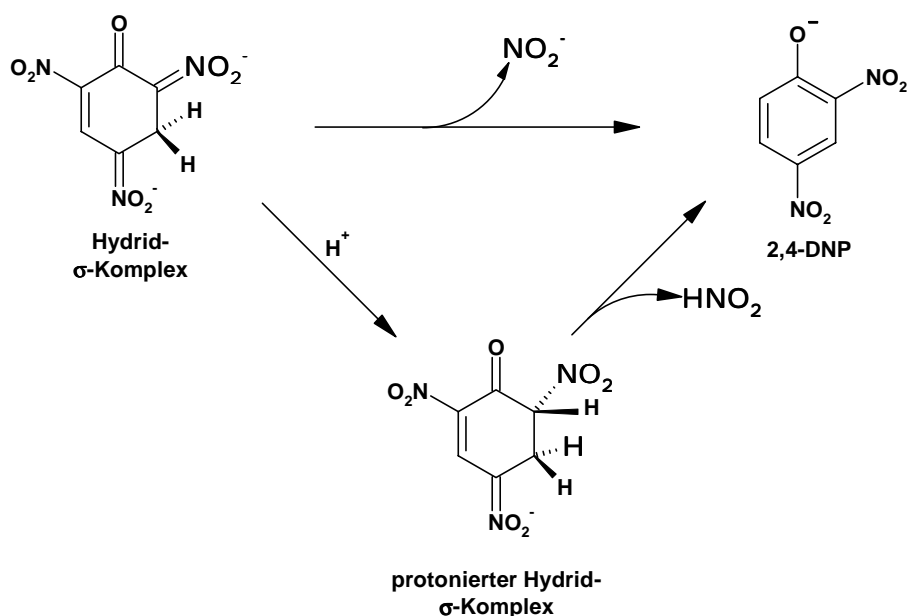
Abbildung 6. Mechanismus der Nitriteliminierung bei *R. erythropolis* nach Rieger et al. (1999).

Bei eigenen Untersuchungen konnte, nach einem ersten Reinigungsschritt des zellfreien Extrakts von *N. simplex*, in keiner Fraktion eine Aktivität für H⁻-Pikrat festgestellt werden. Lediglich eine Kombination der einzelnen Komponenten des Enzymsystems zeigte eine Aktivität mit H⁻-Pikrat. Bei dieser Umsetzung wurde jedoch kein Nitrit detektiert, aber 2H⁻-Pikrat als Produkt identifiziert. Das bedeutet, dass H⁻-Pikrat einer weiteren Hydrierung durch das Enzymsystem unterliegt.

Der chemisch synthetisierte Dihydrid-Komplex wurde deshalb in weiteren Untersuchungen als potentieller Metabolit des oberen Abbauweges in Betracht gezogen. Der zellfreie Extrakt zeigte eindeutig Aktivität mit 2H⁻-Pikrat. Nitrit konnte in der Testlösung nachgewiesen werden. Die Auftrennung in Cytosol- und

Membranfraktion ergab, dass die Aktivität klar in der cytosolischen Fraktion lokalisiert ist. Eine denitrierende Aktivität ließ sich durch FPLC mit einem Anionenaustauscher (Q Sepharose) anreichern. Weitere Schritte mit einer Hydroxyapathit und einer Mono-Q-Säule führten allerdings zum völligen Verlust der Aktivität, welche auch durch Zugabe von verschiedenen Metallsalzen (Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, K^+, oder Na^+) nicht wieder hergestellt werden konnte. Die Anreicherung im ersten Schritt bewirkte die Abtrennung des initialen Hydrid übertragenden Enzymsystems. Nitrit in stöchiometrischen Mengen und H^--2,4-DNP wurden als Denitrierungsprodukte nachgewiesen.

Wie bei der Identifizierung von H^--2,4-DNP als Produkt der enzymkatalysierten Hydrierung schon erwähnt, ist dieser σ-Komplex sehr instabil und disproportioniert, was die frühere Beobachtung von 2,4-DNP als scheinbarem Metabolit von Pikrat erklären würde. Folgende Überlegungen müssen daher berücksichtigt werden: Entweder der Abbauweg von Pikrinsäure in *R. erythropolis* unterscheidet sich in der Nitriteliminierung von dem in *N. simplex* oder das gebildete H^--2,4-DNP zerfällt derart schnell, dass die Entstehung von 2,4-DNP als Metabolit von Pikrat suggeriert wird.

Aus den mit *N. simplex* gemachten Untersuchungen ergibt sich ein konvergenter Abbauweg für Pikrat und 2,4-DNP mit H^--2,4-DNP als gemeinsamem Metaboliten.

Die weitere Umsetzung des H^--2,4-DNP scheint NADPH-abhängig zu sein (Behrend 1999) (Abbildung 7). Auf chemischem Wege durch die Reduktion von 2,4-DNP mit
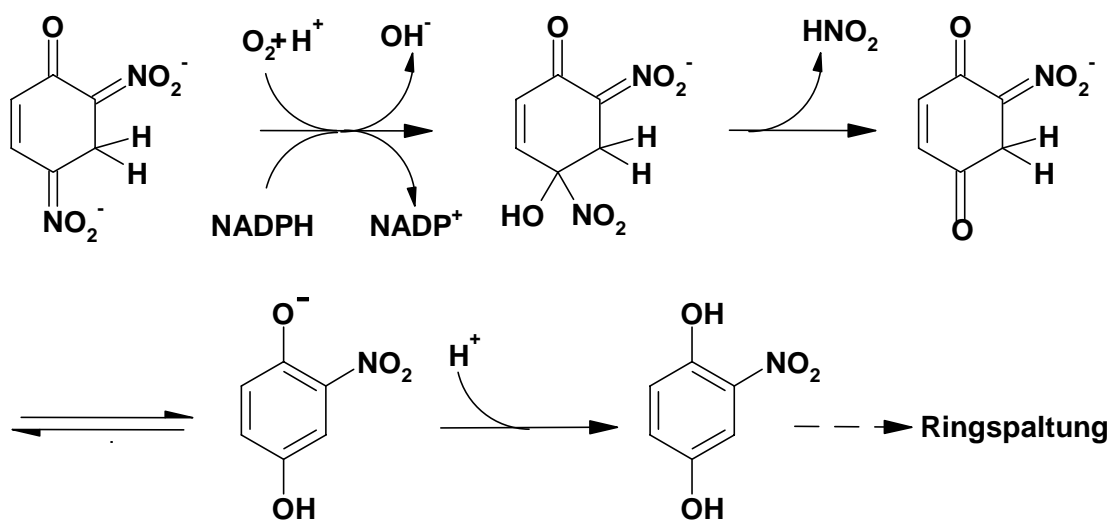

Abbildung 7. Postulierter Mechanismus der Umsetzung von H^--2,4-DNP in Nocardioides sp. Stamm CB 22-2 nach Behrend und Heesche-Wagner (1999).

Natriumborhydrid synthetisiertes H$^-$-2,4-DNP muss für in-vitro-Untersuchungen gereinigt werden, da offenbar Nebenprodukte die enzymatische Umsetzung von H$^-$-2,4-DNP hemmen (Behrend 1999). Die Instabilität von H$^-$-2,4-DNP dürfte die Reindarstellung des Komplexes äußerst schwierig gestalten.

Behrend und Heesche-Wagner (Behrend und Heesche-Wagner 1999) nahmen für die weitere Reaktion den Angriff einer Monooxygenase in Abhängigkeit von NADPH an. Der Vergleich mit Monooxygenierungen bei Phenol in *ortho*-Position (Neujahr und Gaal 1973; Detmer und Massey 1985), 3-Hydroxybenzoat in *para*-Position (Jones und Cooper 1990) und *p*-Nitrophenol unter Nitritfreisetzung (Spain et al. 1979; Spain und Gibson 1991) ließ die Autoren den weiteren Abbau postulieren: Die *para*-Position des Aromaten würde hydroxyliert und die Abspaltung von salpetriger Säure würde zur Bildung von 2-Nitro-*p*-hydrochinon führen. Dieses könnte unter Ringspaltung weiter abgebaut werden. Da im Rohextrakt keine Aktivität für 4-Nitrobrenzcatechin, dem Produkt einer möglichen *ortho*-Hydroxylierung, gemessen wurde, wurde die Hydroxylierung in *para*-Stellung postuliert (Behrend und Heesche-Wagner 1999).

Zablotowicz et al. (Zablotowicz et al. 1999) untersuchten den Abbau von 2,4-DNP an dem *Sphingomonas*-Stamm UG30. 2,4-DNP wird von diesem Stamm in Anwesenheit von Glucose als Kohlenstoffquelle und Glutamat als Stickstoffquelle unter Freisetzung von Nitrit umgesetzt. Die PCP-4-Monooxygenase (Pentachlorphenol-4-Monooxygenase) aus diesem Stamm wurde in *E. coli* überexprimiert. Dieser *E. coli* Stamm setzte bei der Transformation von *p*-Nitrophenol, 4-Nitrobrenzcatechin, 2,4-DNP und DNOC Nitrit frei. GC-MS Analysen von Ethylacetat-Extrakten des Kulturüberstandes identifizierten 2-Nitrohydrochinon als Produkte der Umsetzung. Die so gewonnenen Hinweise ließen die Autoren (Zablotowicz et al. 1999) einen monooxygenolytischen initialen Angriff von 2,4-DNP mit einer zentralen Rolle der PCP-4-Monooxygenase postulieren.

Für einen anderen unteren Abbauweg (Abbildung 8) sind sowohl 4,6-Dinitrohexanoat wie 3-Nitroadipat als mögliche Metabolite diskutiert worden. 4,6-DNH wurde aus Überständen bei Umsatzversuchen mit 2,4-DNP isoliert und 3-Nitroadipat aus Kulturüberständen während des Wachstums. 4,6-DNH wurde als Produkt einer hydrogenolytischen Ringspaltung angenommen, 3-Nitroadipat als das einer oxygenolytischen (Lenke et al. 1992; Blasco et al. 1999).
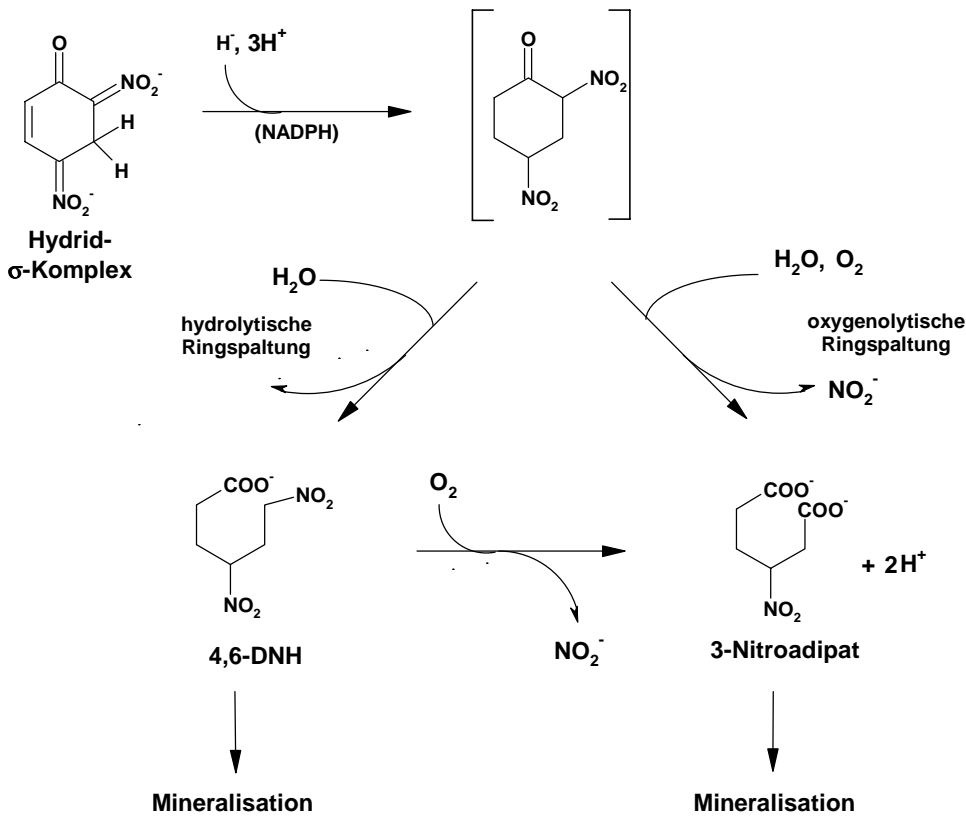
Abbildung 8. Zusammenfassung der bisher bekannten Reaktionen und Metaboliten in einem unteren Abbauweg mit hypothetischem Hydrid.

Beide möglichen Produkte der Ringspaltung würden 2-Nitrohydrochinon nicht als Vorgänger einer Ringspaltung im Abbauweg zulassen. Beim Umsatz des Strukturanalogons von 2,4-DNP, DNOC, wurden 4,6-DNH-analoge Diastereomere R- und S-4,6-Dinitro-2-methylhexanoat isoliert (Lenke und Knackmuss 1996). Diese Beobachtung würde für 4,6-DNH als Metaboliten und eine hydrogenolytische Ringspaltung im Abbauweg von Pikrinsäure und 2,4-DNP sprechen.

HPLC-Untersuchungen von 2,4-DNP-Umsätzen mit zellfreiem Extrakt oder Ruhezellen von *N. simplex* zeigten das Entstehen von geringen Mengen an 4,6-DNH. Andere Metaboliten, wie 2-Nitrohydrochinon oder 3-Nitroadipat, konnten jedoch nicht identifiziert werden. Ruhezellen von *N. simplex* setzten DNOC nur in geringem Maße um. Dies machte einen Nachweis der bei *R. erythropolis* beobachteten Diastereomere R- und S-4,6-Dinitro-2-methylhexanoat unmöglich.

Die Detektion und Analyse des Enzymsystems, welches die initiale Hydridübertragung katalysiert, die detektierten und klonierten Gene, die Untersuchung des Mechanismus der Hydridübertragung und die Identifikaiton der Produkte belegen die Konvergenz der Abbauwege von 2,4-DNP und Pikrinsäure.

Desweiteren war eine Analyse der Gene in *R. erythropolis* HL PM-1 durch Differential Display möglich.

Die hier gezeigten Ergebnisse bieten neue Perspektiven für einen aeroben Abbau von TNT. Wie Vorbeck et al. (Vorbeck et al. 1998) und Williams und Bruce (Williams und Bruce 2000) beobachteten, sind aerobe Stämme bekannt, die TNT am aromatischen Ring zum Dihydrid-σ-Komplex des TNT reduzieren. Wäre eine Nitriteliminierung aus dieser Substanz unter Bildung des Hydrid-σ-Komplexes von 2,4-Dinitrotoluol möglich, so könnte eine aerobe mikrobielle Mineralisierung des TNT erwogen werden. Spangford et al. (Spangford et al. 1991) beschrieben den vollständigen aeroben Abbau von 2,4-Dinitrotoluol durch einen *Pseudomonas*-Stamm. Basierend auf diesen Ergebnissen wäre die Konstruktion eines rekombinanten Organismus vorstellbar, der TNT aerob mineralisiert. Prinzipiell liese sich damit ein produktiver Abbau von TNT für die Sanierung von Altlasten bewerkstelligen. Ein solcher Prozess wäre jedoch nur auf reine TNT-Verunreinigungen anwendbar. Die als Kontamination häufig beobachteten Aminonitroverbindungen und deren Folgeprodukte wären jedoch dem initialen Hydridtransfer nicht zugänglich.

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Anhang 1:   Function of Coenzyme F$_{420}$ in Aerobic Catabolism of 2,4,6-Trinitrophenol and 2,4-Dinitrophenol by *Nocardioides simplex* FJ2-1A

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ABSTRACT

2,4,6-Trinitrophenol (picric acid) and 2,4-dinitrophenol were readily biodegraded by the strain *Nocardioides simplex* FJ2-1A. Aerobic bacterial degradation of these π-electron deficient aromatic compounds is initiated by hydrogenation at the aromatic ring. A two-component enzyme system was identified which catalyzes hydride transfer to picric acid and 2,4-dinitrophenol. Enzymatic activity was dependent on NADPH and coenzyme F_{420}. The latter could be replaced by an authentic preparation of coenzyme F_{420} from *Methanobacterium thermoautotrophicum*. One of the protein components functions as a NADPH-dependent F_{420} reductase. A second component is a hydride transferase which transfers hydride from reduced coenzyme F_{420} to the aromatic system of the nitrophenols. The N-terminal sequence of the F_{420} reductase showed high homology with a F_{420}-dependent NADP reductase as described in archaea. In contrast, no N-terminal similarity to any known protein was found for the hydride transferring enzyme.

Abbreviations

2,4-DNP, 2,4-dinitrophenol; F_{420}, coenzyme F_{420}; TNT, 2,4,6-trinitrotoluene; 4,6-DNH, 4,6-dinitrohexanoate; DOC, dissolved organic carbon; HPLC, high pressure liquid chromatography; FPLC, fast-protein liquid chromatography.

INTRODUCTION

The majority of nitroaromatic compounds in the environment are due to anthropogenic activities. Since nitrogroups can readily be converted into other functional groups nitroaromatic compounds are important starting materials for the production of aromatic amines, hydrazo- and azo-compounds, isocyanates, benzidin derivatives and haloaromatic structures. Hence, some of them occur as contaminants in waste water. Trinitroaromatics are used as explosives and thus were found as contaminants in ground water at certain military sites and former production facilities (28).

Due to the presence of the electron withdrawing nitro groups as substituents di- and particular trinitroaromatic compounds like 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenol (picric acid) are readily susceptible to initial reductive rather than oxidative attack (23, 27). Consequently, initial oxidations by microbial mono- or dioxygenases of aerobic microorganisms are unknown for this class of xenobiotic compounds. Besides specific and unspecific reductions of the nitro groups (26) unusual hydrogenations of the aromatic ring system have been observed for picrate and TNT. Thus, hydride and dihydride complexes have been identified as initial metabolites (23, 27). In addition, the identification of 2,4-dinitrophenol (2,4-DNP) and 4,6-dinitrohexanoate (4,6-DNH) (13, 14, 23, 22) as metabolites of picrate indicates that extensive hydrogenation of the aromatic system (6 H per mol picrate and 4 H per mol 2,4-DNP) give rise to a non-oxygenolytic ring cleavage. As outlined in Fig.1, transformation of picrate *via* a hydride σ-complex to 2,4-DNP and 4,6-DNH is considered as part of a productive catabolic sequence in *Rhodococcus erythropolis* HL PM-1, whereas the dihydride complexes of trinitroaromatics are dead end products (14, 27).

However, information on the origin and transfer of the hydride ion was still missing. The present paper describes an enzyme system from *Nocardioides simplex* FJ2-1A that catalyzes hydride transfer from NADPH to picrate and 2,4-DNP. These enzymes of strain FJ2-1A are readily accessible when compared to the formerly described *Rhodococcus erythropolis* HL PM-1 (14), which resists to common cell disrupting techniques.
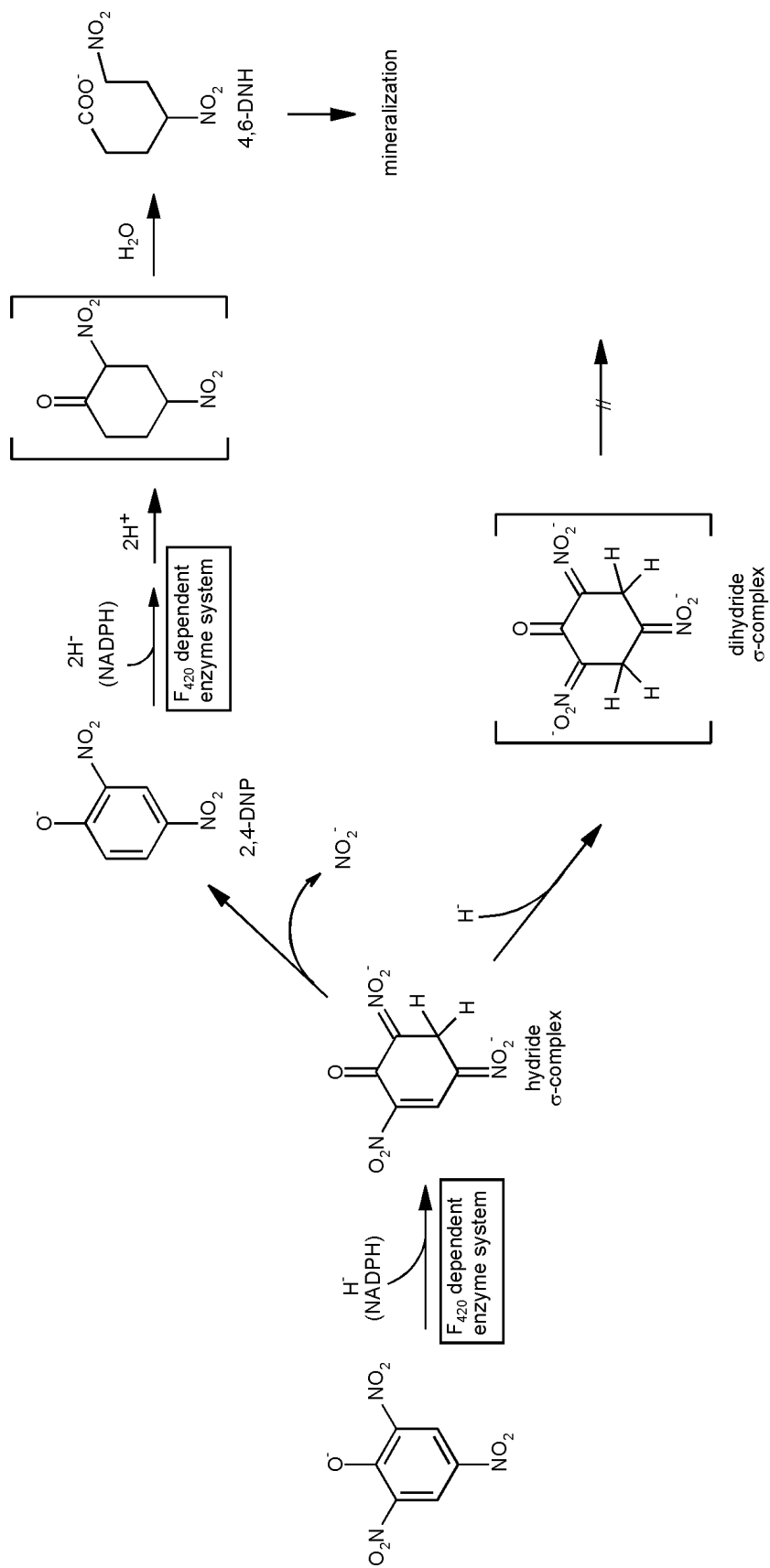
Figure 1. Proposed steps in initial degradation of nitroaromatic compounds.

MATERIALS AND METHODS

Bacterial strain and growth condition. Nocardioides simplex FJ2-1A was previously isolated from picric acid containing waste water and identified by (16S) rRNA analysis by Rajan et. al (21). The strain was grown in batch cultures in a 10 l fermenter (BIO-MAG; Fa. BCC, Göttingen) at 30°C, 550 rpm and 1.2 l air min^{-1} with 50 mM phosphate buffer (pH = 7.1), 0.7 mM picrate, 20 mM acetate, 0.5 g l^{-1} yeast extract, 0.5 g l^{-1} proteose peptone, 0.5 g l^{-1} casamino acids and mineral salts. Mineral salts without nitrogen contained 20 mg l^{-1} Fe(III)-citrate, 1 g l^{-1} MgSO$_4$ · 7H$_2$O, 50 mg l^{-1} CaCl$_2$ · 2H$_2$O and 1 ml trace element solution (19). After consumption of 0.7 mM picrate we added 0.35 mM picrate to maintain induction. The cells were then harvested by centrifugation immediately after decolorization of the medium. They were frozen in liquid nitrogen and stored at -30°C.

DOC Die-Away test. The test was performed as described in the OECD guideline for testing chemicals 301A (18). Pre-cultures were grown in a medium containing only mineral salts as described above and 0.7 mM picrate as sole source of nitrogen, carbon and energy. Cells were harvested by centrifugation and washed twice. The test medium was inoculated to a final concentration of 30 mg suspended solids l^{-1} with an initial concentration of 146 mg l^{-1} (0.64 mM) picrate corresponding to 46 mg l^{-1} dissolved organic carbon. Cells were incubated at a temperature of 20°C. The DOC concentration was followed over a time period of 28 days. For comparison the same test was performed with unadapted activated sludge from a municipal sewage treatment plant. Benzoate at a concentration of 72 mg l^{-1} (0.6 mM) corresponding to 50 mg l^{-1} dissolved organic carbon served as reference substance.

Enzyme assay. The activity of the system towards picrate or 2,4-DNP as substrates was routinely assayed under aerobic conditions by photometric determination of the decrease of absorption at 340 nm (NADPH) or repeated recording of UV visible spectra between 200 nm and 600 nm at 20°C. The test was conducted in 50 mM TRIS/HCl (pH = 7.5) containing 0.2 mM NADPH, 0.05 mM 2,4-DNP or 0.07 mM picrate, F$_{420}$ and component A and B. Tests followed by HPLC analysis contained 50 mM TRIS/HCl (pH = 7.5), 1.6 mM NADPH, 0.4 mM 2,4-DNP or 0.56 mM picrate, 0.04 mg protein ml^{-1} of component A, 0.05 mg protein ml^{-1} of component B containing Q Sepharose fractions and nearly 5 nM coenzyme F$_{420}$. Reactions were started by addition of substrate (2,4-DNP or picrate). Reactions were

stopped by addition of phosphoric acid (85%), and the samples were frozen in liquid nitrogen, and stored at -30°C until being analyzed by HPLC.

The assay for testing the function of component A contained citric acid - phosphate buffer (pH = 5.5) (9), 0.3 mM NADPH, 0.06 mM coenzyme F_{420} and the partially purified component A from the Q Sepharose step at a protein concentration of 5 μg ml^{-1}. The test was conducted under anaerobic conditions in rubber-stoppered cuvettes with nitrogen as the gas phase and started by addition of substrate.

Preparation of cell-free extract. Frozen cells were resuspended in 50 mM TRIS/HCl (pH = 7.5) and disrupted by multiple French press treatment at 137 MPa. Cell debris were removed by centrifugation at 100,000 x g and 4°C for 45 min.

Purification of coenzyme F_{420}, the F_{420}-dependent NADPH oxidoreductase and the hydride transferase. The cell extract (105 mg protein) was passed through a Q Sepharose column (1.6 x 10 cm) pre-equilibrated with basic buffer (50 mM TRIS/HCl (pH = 7.5)). Three of the different fractions were eluted from the column with a linear gradient (300 ml) from 0 to 1 M NaCl in basic buffer at NaCl concentrations of approximately 0.34, 0.42 and 0.5 M, respectively. They were designated component A, B, and C.

The fraction containing component C was heated to 100°C for 15 min. Precipitate was removed by centrifugation. The supernatant was diluted with the basic buffer at a ratio of 1:1 and added to a Mono Q column (0.5 x 5 cm) pre-equilibrated with the basic buffer. Component C eluted at a NaCl-concentration of 0.42 M.

Ammonium sulfate was added to the fraction containing component A to a final concentration of 1.25 M. The solution was applied to a Phenyl Superose HR column (1 x 10 cm) pre-equilibrated with 1.25 M ammonium sulfate in basic buffer. The protein was eluted with a linear gradient (100 ml) from 1.25 to 0 M ammonium sulfate in basic buffer at a concentration of 0.81 M $(NH_4)_2SO_4$. Fractions containing component A were pooled. The sample was diluted with basic buffer and concentrated with a microconcentrator (10K; Filtron, Northborough, USA) by centrifugation. This step was repeated twice to desalt the sample. Then it was passed through a Mono Q column (0.5 x 5 cm) pre-equilibrated with basic buffer. Component A was eluted with a linear gradient (70 ml) from 0 to 1 M NaCl in basic buffer at a concentration of 0.26 M NaCl.

The fraction containing component B was treated the same way as described above for component A. Component B eluted with 0.69 M (NH$_4$)$_2$SO$_4$ from the Phenyl Superose column and with 0.37 M NaCl from the Mono Q column. Protein concentration was estimated according to Bradford (5) or Scopes (25) with bovine serum albumin as the standard. The molecular mass of protein subunits was determined using a 10% SDS polyacrylamide gel stained with silver. The standard proteins were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The concentration of F$_{420}$ was calculated from the UV visible spectrum with an extinction coefficient at 420 nm of 41,4 mM^{-1} cm^{-1} (pH = 7.5) (20).

Analysis of protein sequences. Purified proteins were blotted onto a polyvinylidene difluoride membrane (ProSorb, Applied Biosystems, Weiterstadt, Germany)and were subjected to automatic sequencing (491 protein sequencer, Applied Biosystems, Weiterstadt, Germany). Data base searches were performed with BLAST (2).

Chemical reduction of F$_{420}$. To obtain reduced coenzyme F$_{420}$ we used a anaerobic solution of F$_{420}$ in water, 50 mM TRIS/HCl (pH = 7.5), or 50 mM phosphate buffer (pH = 7.1). Reductants were applied in portions of crystals or as solution. Sodium borohydride, sodium dithionite, zinc dust and also NADPH in presence of component A served as reductants. Progress in reduction was followed by UV visible spectroscopy. The loss of absorption at 420 nm indicated the reduction of coenzyme F$_{420}$.

Analytical methods. For quantification of substrates and identification of coenzyme F$_{420}$ a high pressure liquid chromatography (HPLC) system with a Gromsil 120 Oc4 column (125 x 4 mm, particle size 5 µm) was used. The mobile phase consisted of 80% water, 20% acetonitrile and 0.26% H$_3$PO$_4$. Concentrations were determined at 210 nm. Identification via UV visible spectrum was performed with a photodiode array detector (PDA) (UVD 340S) from Gynkotek (Germering, Germany). Dissolved organic carbon (DOC) was analyzed with a Beckmann Industrial 915B total organic carbon (TOC) analyzer. Nitrite was determined by HPLC as described previously (23). Fluorescence of compound C was detected with a common UV lamp at 360 nm.

Materials. All chemicals used were of the highest available purity and were purchased from Aldrich (Steinheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), and Sigma (Deisenhofen, Germany).

RESULTS

Picrate belongs to those xenobiotic compounds that are generally not degraded in natural populations although single isolates were described to degrade this compound. Therefore biodegradeability of picrate by *Nocardioides simplex* FJ2-1A was investigated.

Biodegradability of picrate. Complete biodegradation of picrate by *N. simplex* and unadapted activated sludge was investigated by a standard OECD method (test on ready biodegradability). As shown in Fig. 2 biodegradation was followed over a time period of 28 days. To avoid an accumulation of storage products the inoculum of *N. simplex* was pregrown with picrate as sole source of carbon, nitrogen and energy over three generations. The test was inoculated to a dry mass concentration of 30 mg l^{-1}. After four days nearly 100% of the initial carbon concentration was removed. Nitrite elimination amounted to 78% of the theoretically expected value. Supposedly the organism assimilated some of the nitrite. In control experiments using activated sludge (30 mg suspended solids l^{-1}) or heated inactivated cells of *N. simplex* picrate was not degraded under the condition of the OECD test. This underlines the ability of *N. simplex* to use the carbon backbone of picrate as sole source of carbon.
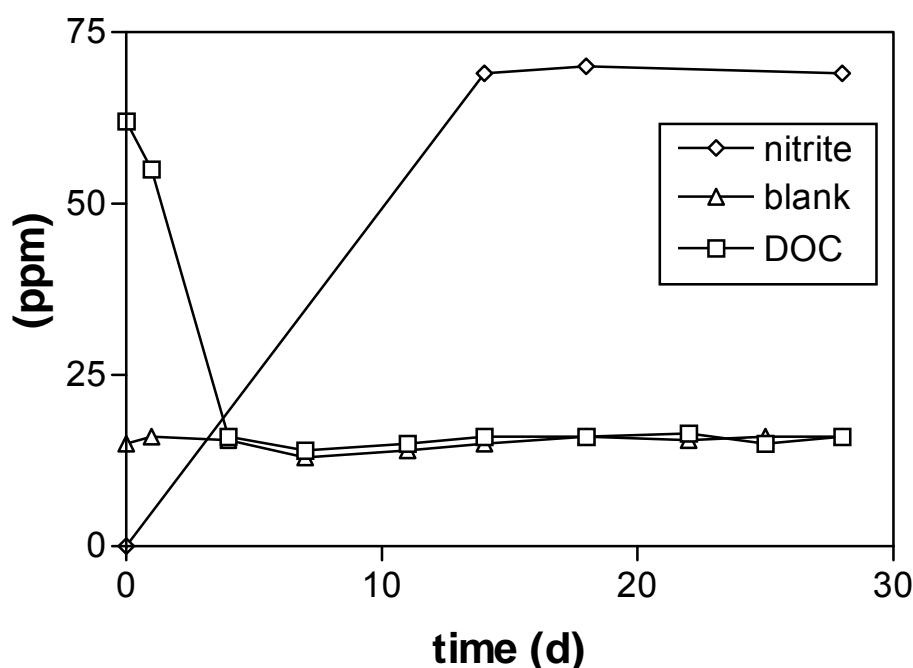

Figure 2. Decrease of DOC and release of nitrite by N. simplex in an OECD biodegradation test. The initial concentration of picrate was 146 mg l-1 or 0.64 mM picrate. The test was inoculated to a final cell density of 30 mg suspended solids/l.

Purification of a coenzyme F_{420}-dependent NADPH oxidoreductase and a hydride transferase. To identify the enzyme responsible for the initial hydrogenation step of picrate and 2,4-DNP cell-free extract was fractionated by an anion exchanger column. This resulted in a complete loss of activity. Combination of distinct fractions restored activity of picrate and 2,4-DNP turnover. These fractions were designated component A, B, and C. Component C showed a yellowish green color and a bright green fluorescence at an excitation wavelength of 360 nm. In contrast, UV visible spectra of components A and B displayed no characteristic absorbance.

The component C containing fractions were heated to 100°C for 15 min in order to denature protein if present. This procedure did not affect the activity of this component in the enzyme assay. Further investigations were performed with HPLC/PDA analysis and revealed a sharp band eluting at 1.9 min, which displayed a characteristic UV visible spectrum (Fig. 3). Comparison of the retention time and UV visible spectrum with an authentic preparation from *Methanobacterium thermoautotrophicum* identified this component as coenzyme F_{420}. Enzyme tests, in which component C was substituted by the authentic coenzyme F_{420}, revealed the same or even greater activity as in the reconstituted mix of components A, B, and C.
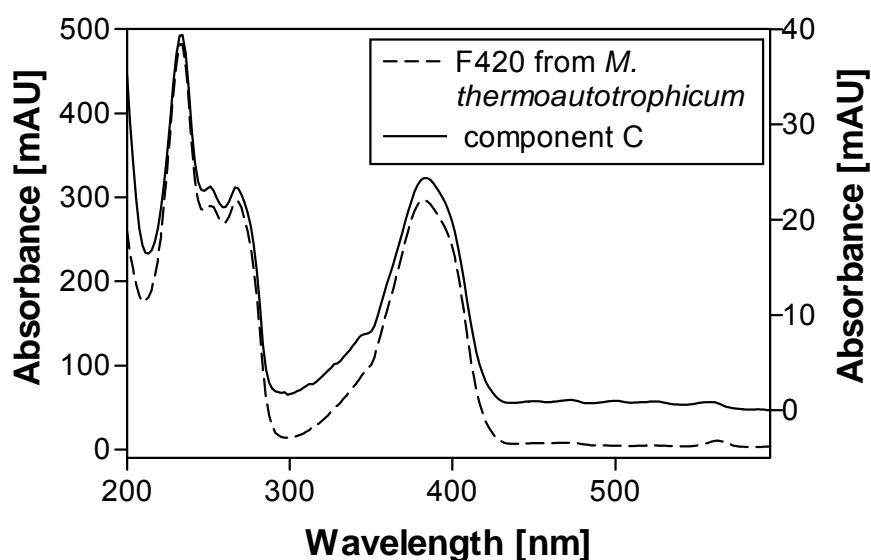

Figure 3. UV visible spectra recorded during HPLC run of coenzyme F420 of Methanobacterium thermoautotrophicum and component C under acidic conditions.

Heating of component C containing fractions or the authentic coenzyme F_{420} at 100°C for 10 min in 2 M HCl resulted in a total loss of activity. This indicates, that hydrolysis products of coenzyme F_{420} cannot function as a cofactor. In contrast, a

hydrolysis product from *Streptomyces aureofaciens* is acting as a catalyst in the reduction of 5a,11a-dehydrochlortetracycline to chlortetracycline (17) or is involved in an enzymatic step leading to the synthesis of propyl proline in lincomycin production of *Streptomyces lincolnensis* (8).

Practically no major activity was observed if one of the three components were missing in the enzymatic test. Obviously, all three components are necessary for activity. Further purification of component A and B yielded small amounts of homogenous proteins. Component A was judged to be homogenous on the basis of SDS-PAGE (Fig. 4). A 10% SDS-PAGE revealed a single protein band. The apparent molecular mass was calculated to be 30 kDa. N-terminal amino acid sequencing of the purified component A by Edman degradation revealed the sequence MQPTTFAVVGGTGPQGRGLAARFAQQG (Fig. 5). It is important to mention that the characteristic pattern for a nucleotide binding site is found within the 27 amino acid residues. Comparison with BLAST (2) showed very high similarity of nearly 66% to a F$_{420}$-dependent NADP reductase of *Methanobacterium thermoautothrophicum.* Component A from *N. simplex* catalyzes the reduction of coenzyme F$_{420}$ (Fig. 6). This was also demonstrated for the reference sample F$_{420}$ from *M. thermoautothrophicum* (not shown). NADPH is required as hydride donor and could not be substituted by NADH. The assay was carried out at pH = 5.5, which was the pH optimum for F$_{420}$ reduction by the oxidoreductase of *Methanogenium organophilum* (4).
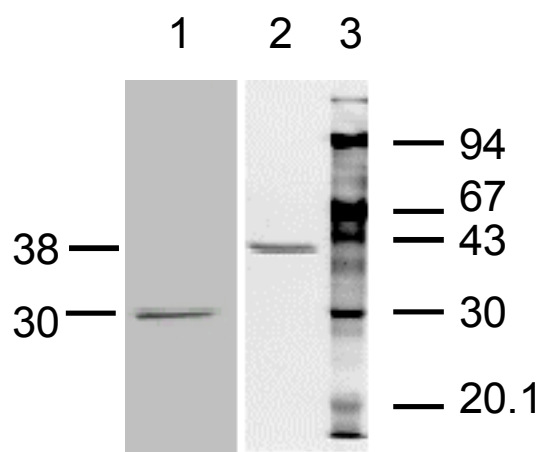

Figure 4. SDS-PAGE of component A and B. A 10% polyacrylamide gel was used and stained with silver. The molecular mass markers in lane 3 were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Lane 1 represents the pure enzyme A and lane 2 the pure enzyme B.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
nucleotide-binding site	-	-	-			X	V	X	V	X	G	X	G	X	X	G	X	X	X	A	X	X	A	X	X	X	X	X	X	X	V	X	P	X	A	X
N-terminal sequence of component A	M	Q	P	T	T	F	A	V	V	G	G	T	G	P	Q	G	R	G	L	A	A	R	F	F	A	Q	Q	G								
N-terminal sequence of reductase in *M. thermoautotrophicum*				M	K	I	A	V	L	G	G	T	G	D	Q	G	L	G	L	A	L	R	L	A	L	A	G	E								

Positions for hydrophobic residues
Positions for invariant glycine residues
Invariant aspartat or glutamate

Figure 5. N-terminal sequence of component A compared to the homologous region of the F420-dependent NADP reductase of M. thermoautotrophicum and the nucleotide-binding motif.
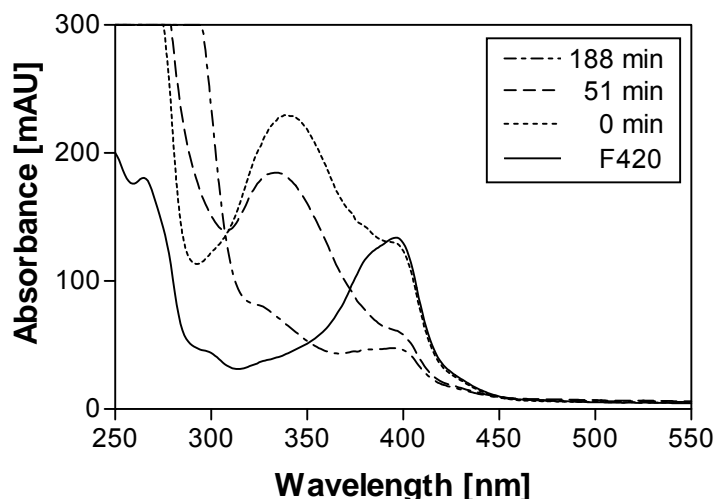
Figure 6. UV visible spectrum of coenzyme F420 from Nocardioides simplex FJ2-1A in citric acid /phosphate buffer at pH = 5.5 and repeated UV visible spectra during the reduction of coenzyme F420 with NADPH-dependent F420 reductase from N. simplex after 0, 55, a nd 188 minutes in citric acid /phosphate buffer at pH = 5.5.

For component B an apparent molecular mass of 38 kDa was determined by a 10 % SDS-PAGE shown in Fig. 4. N-terminal amino acid sequencing revealed the sequence MIKGIQLHAWAGGPEMVEFAEIAAQEF. Comparing it with BLAST (2) gave no similarity to known protein sequences. The function of component B was investigated by repeated recording of the UV visible spectrum (Fig. 7) during an aerobic enzymatic assay, which contained of 2,4-DNP, the partially purified components A, B, and C plus NADPH as described in the methods section. A

decrease of absorption in the spectra represents disappearance of both NADPH and 2,4-DNP. The final spectrum corressponds to excess NADPH.
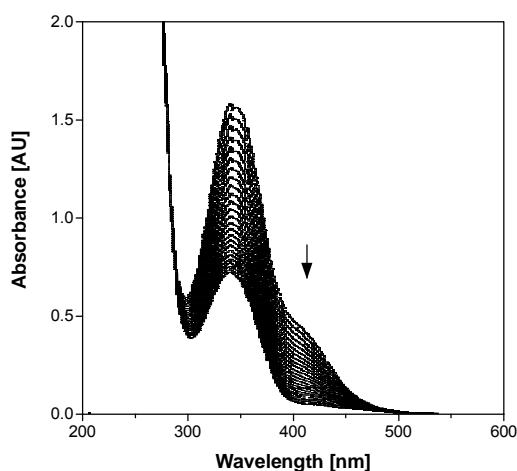

Figure 7. Repeated recording of the UV visible spectrum during an enzymatic assay containing 0.05 mM 2,4-DNP, the partially purified component A and component B (0.244 mg protein, respectively), and component C in a concentration of 8.3 µM F$_{420}$ plus 0.2 mM NADPH. The spectra were recorded at intervalls of 3 min. The arrow indicates the disappearance of the characteristic absorption shoulder of 2,4-DNP. This was confirmed by HPLC.

Complete disappearance of 2,4-DNP was confirmed by HPLC. As described above, component A reduced coenzyme F$_{420}$ under consumption of NADPH. Addition of component B enabled the system to transfer hydride from coenzyme F$_{420}$ to the aromatic ring of 2,4-DNP. Reduction of the aromatic ring is indicated by the following observations: First the loss of UV visible absorption characteristic for the nitrophenolic chromophore, second detection of 4,6-dinitrohexanoate by HPLC (non-stoichiometric amounts), and third the absence of an amino aromatic structure, which can not be detected by HPLC and might have been generated *via* nitro group reduction. Stoichiometry of 4,6-DNH formation can not be expected because of its instability at pH = 7.5. No hydride transfer from coenzyme F$_{420}$ to 2,4-DNP was observed without component B. Hence, component B is assigned as a hydride transferase.

To investigate the enzymatic turnover by HPLC higher concentrations of substrate, cofactor, and partially purified protein were applied. Finally, the reaction was stopped with phosphoric acid. Furthermore picrate was tested as a substrate for the hydride transferase, because in picrate metabolism the first step is the formation of a hydride σ-complex (21) which subsequently leads to 2,4-DNP (14, 23). As shown in Fig. 8 the

initial activities for both substrates were similar. For picrate it was 39 U (mg protein)$^{-1}$ and for 2,4-DNP 51 U (mg protein)$^{-1}$. This suggests that the hydride transferase is responsible for the initial reduction of the ring of 2,4-DNP and picrate.
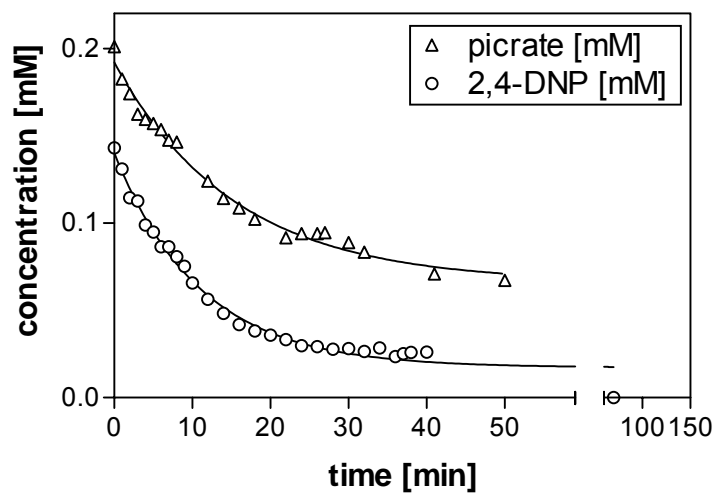

Figure 8. Conversion of picrate and 2,4-DNP by partially purified enzymes of *N. simplex*. Concentrations were determined by HPLC. The test contained 0.04 mg ml^{-1} of component A, 0.05 mg ml^{-1} of component B containing Q Sepharose fractions, and nearly 5 nM coenzyme F_{420}.

DISCUSSION

During the present *in vitro* studies on biodegradation of picrate and 2,4-DNP surprisingly coenzyme F$_{420}$ and its reductase, which are typical for methanogenic archaea, were isolated from the aerobic bacterium *Nocardioides simplex*. These and a novel hydride transferase were identified as parts of a redox enzyme system which appears to have a key function in catabolism of picrate and 2,4-DNP.

Picrate is readily biodegraded by *N. simplex* as shown by the DOC Die-Away test. Remarkably, fast and complete carbon removal within four days strongly indicates that picrate is completely mineralized and utilized as sole source of carbon and energy. In contrast, under the same standardized conditions picrate is not degraded neither by activated sludge nor by inactivated cells of *N. simplex*. Hence, the highly efficient catabolic system of *N. simplex* and the formerly described *Rhodococcus erythropolis* HL PM-1 (14) is unique and obviously such organisms are not or only marginally present in unadapted activated sludge.

With partially purified enzymes the system showed activity with 2,4-DNP and picrate as substrates. Activity was dependent on NADPH. From transformation of picrate transiently 2,4-DNP is generated and 4,6-dinitrohexanoate is identified as a metabolite from both nitrophenols. So it is evident that hydride ions were transferred to the π-electron deficient system of these nitroaromatic compounds. The initial activity towards picrate and 2,4-DNP was very high but soon leveled off in the course of the reaction. This may be due to a low substrate affinity of the initial hydride transferase or, more likely, to decomposition of coenzyme F$_{420}$ upon exposure to oxygen and light during the test. Such decomposition of coenzyme F$_{420}$ from *M. thermoautotrophicum* by oxygen has been described by Schönheit *et.al.* (24). Also photodecomposition of coenzyme F$_{420}$ has been reported in cell-free extract from *Methanobacterium* strain M.o.H. (6).

In order to resolve the hydride transfer from NADPH to picrate and 2,4-DNP into single reaction steps we reduced coenzyme F$_{420}$ separately. Under anoxic conditions reduction of coenzyme F$_{420}$ could be achieved with reductants such as sodium borohydride, sodium dithionite, zinc dust, or NADPH in presence of component A. In the absence of reductant fast reoxidation of coenzyme F$_{420}$ occurred. Excess of inorganic reductant, however, inevitably gave rise to chemical reduction of the nitrophenols, most likely by attacking the nitrogroups. Thus, enzymatic F$_{420}$ reduction

could not be detached from the hydride transferring reaction and an isolated system containing only component B, reduced coenzyme F_{420}, and substrate could not yet be established. In addition, UV visible spectroscopic measurements were hampered by the overlapping absorption of 2,4-DNP, picrate and NADPH in a rather narrow wavelength region, so that the stoichiometry for the hydride transfer to the aromatic system of 2,4-DNP could not yet be fully defined. UV visible spectroscopic estimation of NADPH consumption in enzymatic turnover of 2,4-DNP indicates that reduction of one mol 2,4-DNP required 2 mol NADPH.

Coenzyme F_{420} appears in archaea (4, 6, 10, 12, 15, 20) and also in some actinomycetes (11). It functions as a two electron carrier for several redox reactions. It transfers for example hydride to methenyl- and methylene-tetrahydromethanopterin during reduction of CO_2 by methanogenic archaea. The reduced cofactor is generated by a F_{420}-reducing hydrogenase or by two enzymes that together *in vitro* can catalyze the reduction with H_2 (1) or by a F_{420}-dependent NADP reductase (4). Up to now in aerobic bacteria only a hydrolysis product of F_{420} was detected in *Streptomyces aureofaciens* (17) and *Streptomyces lincolnensis* (8).

Component A from *N. simplex* catalyzed the reduction of coenzyme F_{420} in a NADPH dependent reaction and is therefore assigned to a NADPH-dependent F_{420} reductase. The N-terminal amino acid sequence of component A if compared with BLAST (2) showed high similarity to a F_{420}-dependent NADP reductase occurring in methanogenic archaea such as *Methanobacterium thermoautotrophicum* (4)*, Methanogenium organophilum*, and *Methanococcus jannaschii* and also non-methanogenic archaea such as *Archaeoglobus fulgidus*. Despite the large evolutionary distance between archaea and proteobacteria the oxidoreductase of the *Nocardioides simplex* strain has the same function as the F_{420}-dependent NADP reductases in archaea. According to L. Chistoserdova, *et. al.* (7), this indicates that either the gene encoding this enzyme has been conserved, because these organisms evolved from a common ancestor, or it has been transferred horizontally between more recent ancestors. It implies that the same enzymes in archaea and in proteobacteria are involved in the reductive metabolism. Other authors reported on a F_{420}-dependent NADP reductase that was purified and characterized from *Streptomyces griseus* (11). However, a metabolic function could not be assigned to the oxidoreductase except of a ground-state electron carrier and a photosensitizer (11).

For the hydride transferase (component B) no N-terminal similarity to any known protein was found. It seems to be a novel enzyme, which is responsible for the hydride transfer to the nitroaromatic ring. Due to the electron withdrawing effect of the nitro substituents π-electron deficiency of the aromatic system favors nucleophilic hydride additions. Such reactions are known for chemical hydride donors like sodium borohydride generating hydride or even dihydride σ-complexes of nitroaromatic compounds (23). More recently, hydride complexes were observed in our lab as microbial metabolites of picrate and TNT. Whereas the hydride σ-complex of picrate is an intermediate of a productive catabolic pathway (s. Fig. 1) (14) the hydride σ-complex of TNT (2,4,6-trinitrotoluene) is further reduced to a dihydride σ-complex which proved to be a stable dead end product (27).

The hydride transferase (component B) of strain FJ2-1A obviously transfers hydride not only to picrate but also to 2,4-DNP. Thus, it appears to have a multiple function in the degradation of picrate *via* 2,4-DNP. Since 4,6-dinitrohexanoate (4,6-DNH) is formed from 2,4-DNP with the partially purified enzyme two hydride ions must be transferred. This is supported by estimation of NADPH consumption. In the first step a hydride σ-complex of 2,4-DNP may be generated. Thereafter, a second hydride ion may give rise to a dihydride-complex which can form 2,4-dinitrocyclohexanone. As described in the literature for 2-nitrocyclohexanone (3, 16) such an activated α-nitroketo group may hydrolyze easily yielding 4,6-DNH (see Fig. 1). As shown by the OECD test for ready biodegradeability it results in mineralization. Current analytical work with larger amounts of pure enzyme must provide quantitative data with respect to stoichiometry of hydride transfer to picrate or 2,4-DNP and formation of 4,6-DNH.

This report describes a novel enzyme system which is responsible for the transfer of hydride ions from NADPH to 2,4-DNP and picrate (s. Fig. 9). It consists of three components, the NADPH-dependent F_{420} reductase, coenzyme F_{420} as a mediator, and a new hydride transferase. Such a F_{420}-dependent enzyme system seems to be of general importance in picrate and 2,4-DNP metabolism: Picrate or 2,4-DNP degrading strains from different habitats were all gram positive and exhibited the characteristic blue green fluorescence as strain FJ2-1A when examined under the microscope.
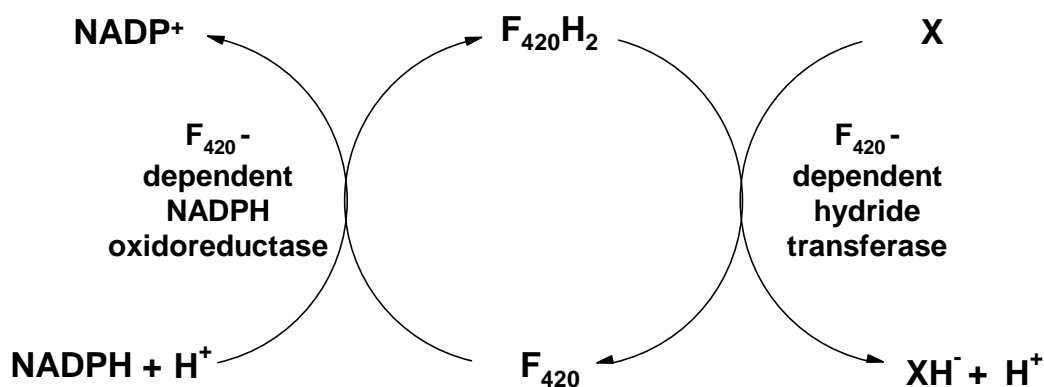
NADP⁺ $F_{420}H_2$ X

F_{420}-dependent NADPH oxidoreductase

F_{420}-dependent hydride transferase

NADPH + H⁺ F_{420} XH⁻ + H⁺

Figure 9. Tentative reaction scheme of the enzyme system transferring hydride from NADPH to 2,4-DNP or picrate (X).

ACKNOWLEDGEMENTS

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Anhang 2:   Enzymes, Genes and Products of Initial Degradation of 2,4,6-Trinitrophenol (Picric Acid) and 2,4-Dinitrophenol by *Nocardioides simplex* FJ2-1A

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ABSTRACT

Both cytosolic proteins of the formerly described enzyme system catalyzing the initial F_{420}-dependent hydrogenation of 2,4,6-trinitrophenol (picric acid) and 2,4-dinitrophenol degradation show high homology to known enzymes of archaea. In *N. simplex* the coenzyme F_{420} content is significantly lower than in archaea but higher than in other aerobic bacteria. Hydride and dihydride σ-complexes of picrate and 2,4-dinitrophenol were identified as reaction products in vitro. The enzyme system showed activity against several substituted 2,4-dinitrophenols but not with mono-nitrophenols. A K_m-value of 0.06 mM of the hydride transfer for picrate as substrate was found. The pH optima of the oxidoreductase and for the hydride transferase were 5.5 and 7.5 respectively. The genes encoding these enzymes were identified and sequenced. They are homologous to genes of enzymes of archaea and streptomycetes. The hydride transferase gene is located upstream the gene coding for the oxidoreductase.

Abbreviations

2,4-DNP, 2,4-dinitrophenol; DNOC, 4,6-dinitro-*ortho*-cresol; F_{420}, coenzyme F_{420}; TNT, 2,4,6-trinitrotoluene; 1,3,5-TNP, 1,3,5-trinitropentane; 2H⁻-picrate, dihydride σ-complex of picrate; H⁻-2,4-DNP, hydride σ-complex of 2,4-DNP; orf, open reading frame; HPLC, high pressure liquid chromatography; FPLC, fast-protein liquid chromatography.

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 hence in surface and ground waters (10). 2,4,6-Trinitrophenol (picric acid) and its ammonium or potassium salts (picrates) were used as explosive 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 (30). The main source of dinitrophenols and picric acid in industrial wastewater is the production of nitrobenzene from benzene (SRI International, Menlo Park, C.A.).

Because of the presence of 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 (31) and *p*-nitrophenol (11, 13, 17, 26). Consequently, initial biological reduction of the aromatic system is more likely than an oxidative attack (20, 29). Characteristically, gratuitous reduction of picric acid generates picramic acid as a dead end product (28). Unitil recently aerobic bacterial degradation of 2,4,6-trinitrophenol, 2,4,6-trinitrotoluene (TNT), or 2,4-dinitrophenol (2,4-DNP) has been through initial mono- or dioxygenation. An exception is 2,6-dinitrophenol (2,6-DNP) which is subject to denitration by an initial dioxygenase (8). Reduction occurs preferably at the nitrogroups revealing unproductive dead end metabolites as products. For picric acid and 2,4-DNP an unusual initial hydrogenation of the aromatic ring system as part of a complete catabolism is observed. For these compounds hydride σ-complexes were identified as the initial metabolites (14, 21, 29). Despite the inability of activated sludge to degrade picrate and 2,4-DNP (7) these compounds are mineralized aerobically by *Nocardioides simplex* FJ2-1A (7) and several *Rhodococcus* strains (6, 14). It is suggested that extensive ring hydrogenation results in a non-oxidative ring cleavage yielding 4,6-dinitrohexanoate. This metabolite is found in the supernatant of resting cell experiments with *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2 (15). 3-Nitroadipate was identified as a metabolite in the degradation of 2,4-DNP by *Rhodococcus* sp. strain RB1 which is proposed to be a product of oxidative ring cleavage and the elimination of nitrite (6).

In our earlier work we described the hydride transfer to the aromatic ring system by a two-component enzyme system containing coenzyme F_{420} as mediator for the

hydride transfer and NADPH as the source of reduction equivalents (7). A NADPH-dependent F_{420} reductase and a hydride transferase were identified. The N-terminal sequences of these enzymes are homologous to enzymes of archaea.

In the present paper we describe the characterization of the hydride-transferring enzyme system, its substrate specifity, the identification of hydrogenation products, and the detection of genes coding for enzymes involved in initial hydride transfer.

MATERIALS AND METHODS

Bacterial strain and growth condition. *N. simplex* FJ2-1A was isolated from picric acid containing wastewater and identified by 16S rRNA analysis (19). For enzyme purification the strain was grown and harvested as described previously (7). Cells were frozen in liquid nitrogen and stored at -30°C. For isolation and quantification of coenzyme F_{420} the strain was grown in batch culture in a 100 l fermenter (Bioengineering, Wald, Switzerland) at 30°C and 550 rpm with 50 mM phosphate buffer, 0.7 mM picrate, 20 mM acetate, 0.5 g/l yeast extract, 0.5 g/l protease peptone, 0.5 g/l casamino acids and mineral salts. Mineral salts without nitrogen contained 20 mg/l Fe(III)-citrate, 1 g/l $MgSO_4 \cdot 7H_2O$, 50 mg/l $CaCl_2 \cdot 2H_2O$ and 1 ml trace element solution (7, 18)). The cells were fed once more with 0.35 mM picrate and harvested by centrifugation immediately after decolorization of the medium. They were frozen in liquid nitrogen and stored at -30°C.

E.coli DH5α was grown on LB agar plates or liquid media containing 100 µg·ml^{-1} ampicillin at 37°C under aerobic conditions.

Preparation of cell-free extract. Frozen cells were resuspended in 50 mM TRIS-HCl (pH = 7.5) and disrupted by multiple French press treatment at 137 MPa. Cell debris were removed by centrifugation at 100,000 x g and 4°C for 45 min.

Purification of coenzyme F_{420}, the F_{420}-dependent NADPH oxidoreductase and the hydride transferase. The enzymes were purified to electrophoretic homogeneity as described previously(7). Native polyacrylamide gel electrophoresis was performed with partially purified and concentrated enzymes. Lanes were cut in three parts. One part was silver stained, the second part was utilized for activity staining using a reaction mixture consisting of F_{420}-dependent NADPH oxidoreductase, coenzyme F_{420}, NADPH and picrate. The third part was used for subsequent SDS-gel electrophoresis.

For isolation and quantification of coenzyme F_{420} 500 g wet cells of *N. simplex* were resuspended in 70 % ethanol. After stirring for a few minutes the cells were precipitated by centrifugation. The extraction procedure was repeated twice. The supernatants containing cofactor F_{420} were pooled and the cells were discarded. To avoid overloading the extract was divided in five equal volumes and each was applied on a Q Sepharose column (1.6 x 10 cm) equilibrated with basic buffer

(50 mM TRIS-HCl (pH = 8.5)). After washing the column with 100 ml of 0.2 mM NaCl the coenzyme was eluted with a linear gradient of 0.2 to 0.6 mM NaCl in basic buffer (250 ml) at a NaCl concentration of 0.4 to 0.5 M. Coenzyme F_{420} containing fractions were pooled diluted fourfold with glass distilled water and divided into three parts. Each was passed through a Q Sepharose HP 16/10 column equilibrated with distilled water. The loaded column was washed with 2.5 mM HCl (20 ml) and coenzyme F_{420} eluted with 50 mM HCl. Fractions containing cofactor F_{420} were pooled and the solution adjusted to pH = 1 by 2 M HCl. It was applied to a XAD 4 HP 26/40 column equilibrated with 0.1 M HCl and washed with 1 M HCl. The coenzyme was eluted with a linear water/methanol gradient (600 ml). F_{420} containing fractions were pooled and methanol was removed completely by distillation in vacuum. The residual aqueous solution of coenzyme F_{420} was lyophilized. The concentration of F_{420} was calculated on the basis of an extinction coefficient at 420 nm of 41,4 $mM^{-1} \cdot cm^{-1}$ (pH = 7.5) (7).

For recycling of the cofactor coenzyme F_{420} containing waste fractions were heated to 100°C for 15 min. The precipitate was removed by centrifugation. The supernatant was diluted 1:1 with the basic buffer and applied to a Resource Q column (6 ml) equilibrated with 50 mM TRIS-HCl (pH = 8.5). The cofactor was eluted with 0.42 M NaCl.

Enzyme assay. The activity of the hydride-transferring system towards picrate or 2,4-DNP as substrates was routinely assayed photometrically by following the decrease of absorption at 340 nm (NADPH) or repeated recording of UV-visible spectra between 200 nm and 600 nm at 25°C. The test was carried out in 50 mM TRIS-HCl (pH = 7.5) containing 0.5 mM NADPH, 0.05 mM 2,4-DNP or picrate, 10 to 26 µM F_{420}, F_{420}-dependent NADPH oxidoreductase and hydride transferase. Tests which were followed by HPLC analysis contained 50 mM TRIS-HCl (pH = 7.5), 1 mM NADPH, 0.05 to 0.15 mM 2,4-DNP or picrate, 0.15 µg protein $\cdot$ ml^{-1} oxidoreductase, 0.09 to 0.16 µg protein / ml hydride transferase and 10 to 30 nM coenzyme F_{420}. Reactions were stopped by addition of phosphoric acid (85%). The samples were frozen in liquid nitrogen and stored at -30°C until they were analyzed by HPLC. All reactions were started by addition of substrate (2,4-DNP or picrate) or for repeated recording of UV-visible spectra with the hydride transferase.

For determining the pH optimum of the F_{420}-dependent NADPH oxidoreductase the assay mixture contained citric acid - phosphate buffer / pH = 3 to 7 or 50 mM TRIS-HCl pH = 7 to 8.5, 0.5 mM NADPH, 0.044 mM coenzyme F_{420} and 0.3 µg · ml^{-1} F_{420}-dependent NADPH oxidoreductase. The test was carried out anaerobically in microtiterplates with a microtiterplate reader (Bio-tek Instruments, Vermont, USA) under nitrogen at 30°C and started by addition of substrate.

The buffers used were 100 mM Succinat · NaOH buffer, pH = 3.5 to 5, 100 mM potassium phosphate buffer, pH = 5 to 7.5, 100 mM TRIS-HCl, pH = 7.5 to 10, 100 mM glycin · NaOH, pH = 10 to 12 containing 1 mM NADPH, 3.5 µM coenzyme F_{420}, 0.1 mM 2,4-DNP, 0.1 µg · ml^{-1} oxidoreductase and 78 ng · ml^{-1} hydride transferase.

Analytical methods. For quantification of substrates in stopped tests a high performance liquid chromatography (HPLC) system with a Gromsil 120 Oc4 column (125 x 4 mm, particle size 5 µm) was used. The mobile phase consisted of 80% water, 20% acetonitrile and 0.26% H_3PO_4 (acidic conditions). For determination of reduced nitroaromatics a Gromsil TSK-Gel Q-5PW column (125 x 4 mm, particle size 20 µm) was used as a solid phase. The mobile phase consisted of 25 mM TRIS-HCl (pH = 8) (basic conditions). A linear gradient from 0.35 to 0.6 M NaCl in basic buffer was applied. 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 (PDA) (UVD 340S) from Gynkotek (Germering, Germany).

Cloning of the NADPH-dependent F_{420} reductase gene (*fr*) and the hydride transferase gene (*ht*). The isolation of DNA from *N. simplex*, restriction, DNA ligation and other standard techniques were performed as described elsewhere (2). Plasmid DNA for cloning and sequencing was prepared with the Flexi Prep Kit (Pharmacia, Freiburg, Germany).

A homologous probe for *fr* was generated using genomic DNA from *N. simplex* as template. The oligonucleotides (ATGCARCCNACNTTYGCIGT and CCATGCG TGTAGYTGDATNCC) were derived from N-terminal protein sequences of the oxidoreductase and hydride transferase (7). PCR mixtures (50 µl) for amplification of genomic DNA contained 50 pmol of each primer, 30 ng of chromosomal template DNA, 0.1 mM of each desoxynucleotide triphosphate, Goldstar DNA polymerase (Eurogentech, Seraing, Belgium) reaction buffer, 1.5 mM $MgCl_2$, and

8% dimethylsulfoxide. The PCR-program started with initial denaturating (3 min, 95°C). The addition of 0.7 U of Eurogentech Goldstar polymerase was followed by 30 cycles of polymerization. Conditions for the first cycle were 1 min at 55°C, 1.5 min at 72°C and 0.5 min, 95°C. In the following 19 cycles a touch down of 0.5°C for the annealing temperature was used. In the last 10 cycles (1min, 45°C; 1.5 min, 72°C; 0.5 min, 95°C) the annealing temperature was kept constant. A final step with a prolonged elongation period (1 min, 45°C; 5 min, 72°C) was set at the end. A 1.0 kb fragment was amplified and cloned into a T-tailed vector (16) prepared from pBluescript II SK (+/-) (Strategene, Heidelberg, Germany). The resulting plasmid named *pFJ* was partially sequenced. The identity of the fragment was confirmed by comparison of the deduced amino acid sequence with available N-terminal protein sequences.

Genomic DNA was digested with several restriction endonucleases. The DNA fragments generated were separated by agarose gel electrophoresis, transferred to a nylon membrane using the capillary transfer method (25) and hybridized at 68°C with the 1 kb PCR product labeled with digoxigenin using the DIG DNA Labeling and Detection Kit Nonradioactive as indicated by the supplier (Boehringer, Mannheim, Germany). Genomic BamHI fragments were isolated from agarose gels by the Biozyme Easy Pure kit (Biozym, Oldendorf, Germany), ligated into pBluescript SK (+/-) and transformed into *E.coli* DH5α cells (12). Positive clones were identified by Southern hybridization with the DIG-labeled 1 kb PCR product using the DIG DNA Detection Kit Nonradioactive. Two clones named pI43B and pI055B containing a 4.3 kb BamHI and a 0.55 kb BamHI fragment were used for further analyses. Again chromosomal DNA was digested with several restriction endonucleases. Resulting DNA fragments were separated and transferred to a nylon membrane as described above. They were hybridized at 68°C with the 0.55 kb BamHI fragment labeled with digoxigenin using the DIG DNA Labeling and Detection Kit Nonradioactive. Isolation, ligation, transformation and identification of positive clones were performed as described above. This revealed a clone named pI3P containing a 3 kb PstI fragment.

Sequencing and analyses. The 4.3 kb BamHI or 3.0 kb PstI fragment was prepared for sequencing with the GPS-1 Genome Priming system kit (New England Biolabs, INC., Beverly, Massachusetts, USA). Obtained plasmid templates were purified and sequenced as described in the manual.

Materials. All chemicals used were of the highest available purity and were purchased from Aldrich (Steinheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), and Sigma (Deisenhofen, Germany).

RESULTS

Purification and content of coenzyme F_{420}. To quantify the amount of coenzyme F_{420} involved in the hydride transfer by the enzyme system, wet cells of *Nocardioides simplex* FJ2-1A were extracted with 70% ethanol and the supernatant purified by FPLC. This revealed 139 µmol per kg of wet cells of cofactor F_{420}. Obviously the content is lower than in *Methanobacterium thermoautotrophicum* (376 µmol per kg) but nearly 16-fold higher than in *Streptomyces griseus* (8 µmol per kg), *Streptomyces coelicolor* (9 µmol per kg), or *Halobacterium cutirubrum* (9 µmol · (1 kg wet cells)$^{-1}$) (9).

Enzyme characterization. The NADPH-dependent F_{420}-reductase and the hydride transferase are cytosolic enzymes. No activity was found in membrane fractions. Activity of hydride transfer was maximal at pH 7.5. The oxidoreductase had a maximum activity at pH 5.5. The enzyme system could be stimulated by ammonium sulfate. 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. Chemical reductants such as sodium borohydride or sodium dithionite were inappropriate because of inhibiting effects on the enzyme system or reduction of nitrogroups of substrates (7). Additionally, NADH could not substitute for NADPH (7) and FAD or FMN could not substitue for coenzyme F420. Hence, the hydride transfer was investigated in a coupled test with an excess of the F_{420}-reductase and NADPH.

Native gel electrophoresis was performed with a concentrated FPLC fraction of the hydride transferase. To detect protein bands one part of the gel was silver stained. Another part was used for activity staining. A color change from yellow to orange could be observed when the reaction mixture was dropped on the slab. From the third part the activity band was cut out and SDS gel electrophoresis was performed revealing a single band for the hydride transferase.

The affinity of the hydride transferase for picrate was determined by enzyme tests stopped with phosphoric acid and subsequent quantification of substrate turnover by HPLC. The apparent Km 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. The hydride σ-complex of 2,4-DNP was very unstable and spontaneously regenerated 2,4-DNP.

Substrates. Several nitroaromatic compounds were tested as substrates for the enzyme system. Reactions were followed by repeated recording of UV-visible spectra and HPLC measurements. Mono-nitrophenols such as *o*-nitrophenol and *p*-nitrophenol were not transformed by the enzyme system. 2,6-DNP was also not reduced. 2-Methyl-4,6-dinitrophenol (4,6-dinitro-*ortho*-cresol, DNOC) as a substrate showed only very low activity for the enzyme system (≤ 1 % of activity with picrate). But increasing absorption during repeated recording of UV-visible spectra between 400 and 550 nm indicates formation of a hydride σ-complex. This is even more pronounced with 2-chloro-4,6-dinitrophenol as substrate.

Identification of products of hydride transfer. Repeated scans of UV-visible spectra during picrate transformation 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 the reaction assay mix turned quickly to orange and then slowly to pale yellow. Therefore the orange red hydride σ-complex of picrate, as described from Rieger et. al. (21), cannot be the final product of the enzymatic reaction and further transformation must occur.
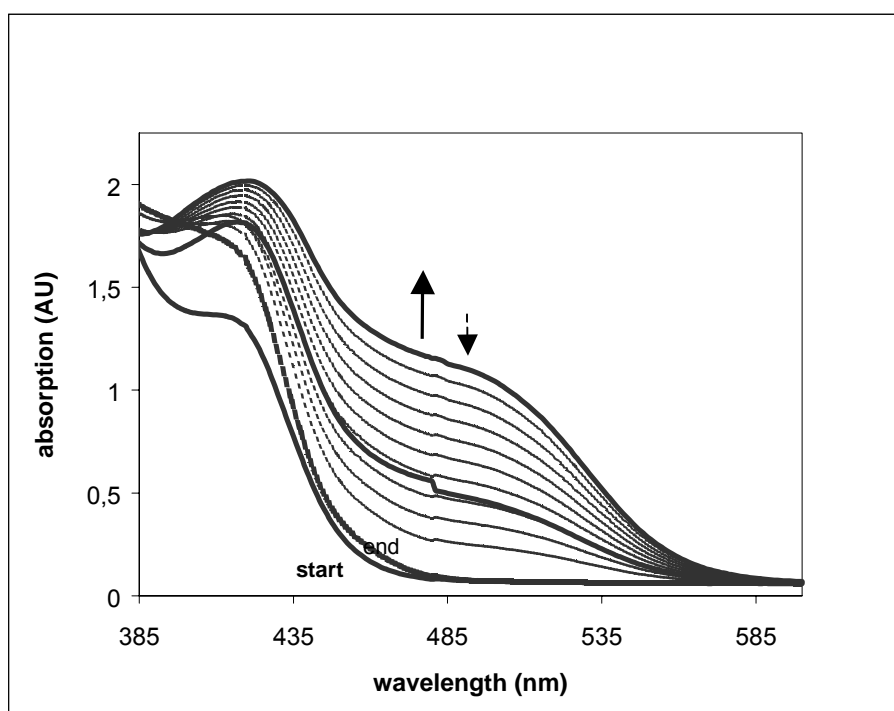

Figure 10. Repeated recording of UV-visible spectrum at intervals of 35 seconds during an enzymatic transformation containing 0.1 mM picrate, NADPH-dependent F420 reductase and hydride transferase (11.5 µg/ml and 0.04 µg/ml protein, respectively), coenzyme F_{420} (11 µM), NADPH (1 mM), and TRIS-HCl (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 (broken lines) corresponding to the formation of 2H$^-$-picrate (105 – 455 sec).

Samples taken from an enzymatic reaction were stopped with phosphoric acid and analyzed with HPLC under acidic conditions. A sharp band at a retention time of 7.6 min and a maximum absorption of 203 nm corresponds to the data measured for a standard of 1,3,5-trinitropentane (1,3,5-TNP). As described from Severin et. al. (24) 1,3,5-TNP is generated by decarboxylation of a dihydride σ-complex of picrate (2H⁻-picrate) under acidic conditions. This suggests that two hydride ions were transferred by the enzyme system and 2H⁻-picrate was the product (figure 3).

During transformation of 2,4-DNP an increasing absorption between 400 and 550 nm and an isosbestic point at 405 nm was observed (figure 2). Obviously, the hydride σ-complex of 2,4-DNP (H⁻-2,4-DNP) as described by Behrend (4) was formed.
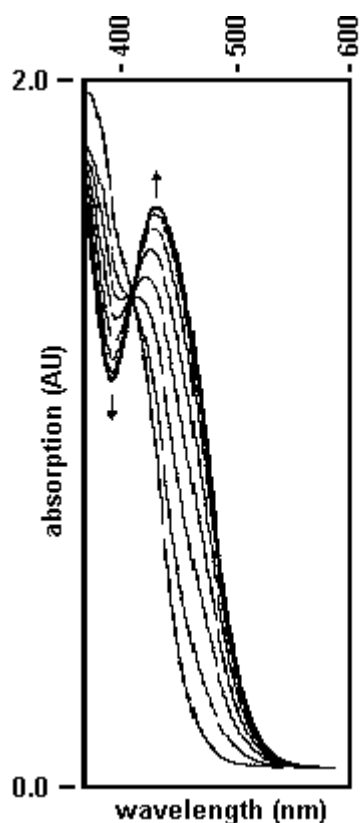
Figure 2. Recording of UV-visible spectrum during an enzymatic transformation of 2,4-DNP at intervals of 5 min. The reaction mixture contained 0.1 mM 2,4-DNP, NADPH-dependent F420 reductase and hydride transferase (11.5 µg/ml and 1.53 µg/ml protein, respectively), coenzyme F_{420} (7.1 µM), NADPH (0.4 mM), and TRIS-HCl (50 mM, pH = 7.5).

An authentic standard was prepared by adding sodium borohydride to a 2,4-DNP solution. 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 was rather unstable and could only be used for up to 2 hours even if stored on ice. The

UV-visible spectrum showed the characteristic absorption spectrum of the hydride σ-complex of 2,4-DNP. The reduction was not complete as analyzed in HPLC measurements. Minor byproducts and 2,4-DNP were identified in the chromatogram. The byproducts of this reduction as described by Behrend et al. (3) could not be identified so that quantification of H⁻-2,4-DNP as the reaction product was not possible. HPLC analysis using an anion exchanger column and UV-visible spectra recorded in situ proved the identity of the chemically synthesized standard and the enzymatic product (figure 3).
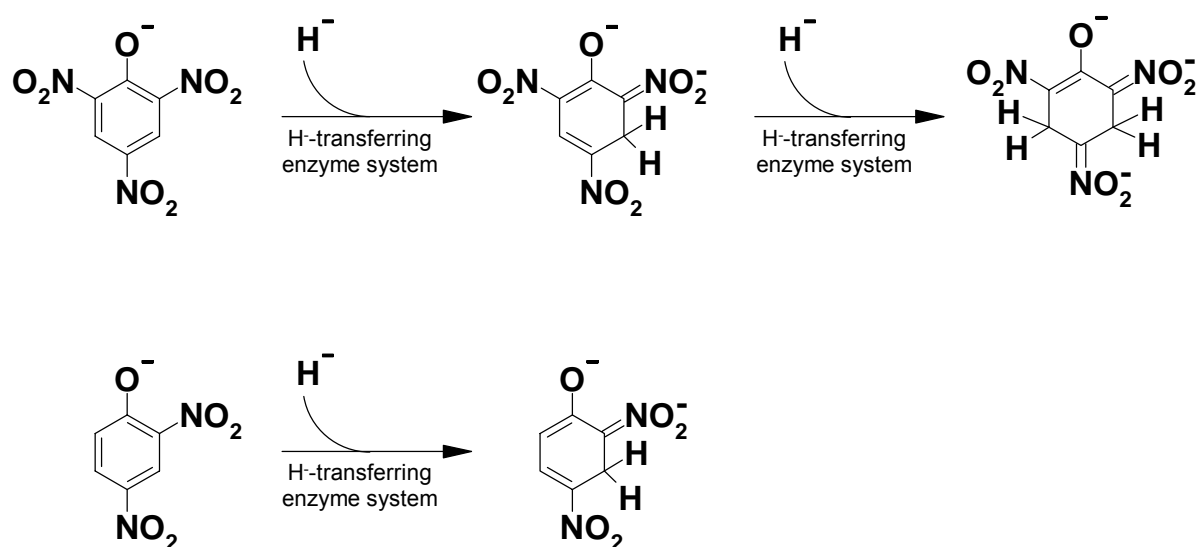

Figure 3. Reaction scheme of hydride transfer to picrate or 2,4-DNP catalyzed by the hydride-transferring enzyme system from *N. simplex*.

Cloning and sequencing of the genes of the NADPH-dependent F_{420}-reductase and the hydride transferase (*fr* and *ht*). Both enzymes of *N. simplex* FJ2-1A were purified from the cytoplasmatic fraction of picrate grown cells as described earlier (7). The N-termini were used to deduce two degenerated oligonucleotides, a forward primer of the N-terminal sequence of the F_{420}-reductase and a reverse primer of the N-terminal sequence of the hydride transferase. A 1 kb fragment was amplified by PCR using genomic DNA of *N. simplex.* The PCR product was cloned into pBluescript SK II (+). The resulting plasmid named pFJ was partially sequenced. The N-terminal sequences of the enzymes were found in the deduced amino acid sequence of the 1kb fragment and PCR product contained the *fr*

(F_{420}-reductase gene). A gene probe labeled with digoxigenin was prepared with the PCR product as template.

Genomic DNA of *N. simplex* was digested with several restriction endonucleases and the fragments were separated by agarose gel electrophoresis. Southern-blot analysis from the genomic DNA fragments using the labeled gene probe identified two BamHI fragments of about 4.3 kb and 0.55 kb containing at least parts of the two genes, *fr* and *ht*. Genomic BamHI fragments of 4.0-4.6 kb and 0.5-0.6 kb were isolated from an agarose gel, ligated into the pBluescript vector, and transformed into *E.coli* DH5α. Five out of 600 colonies for each fragment hybridized with the gene probe. The plasmid of one of the positive clones prepared from the 4-6 kb BamH1 insert library was called pI43B and contained a insert of about 4.3 kb. The plasmid of one of the positive clones from the 0.5-0.6 kb BamH1 insert library (pI055B) contained a insert of about 0.55 kb. Both inserts were partially sequenced and analyzed for possible restriction sites. It turned out that the insert of pI055B was located directly beyond the 4.3 kb BamHI fragment. It contained the start of the hydride transferase gene. Therefore the insert of pI055B was isolated and labeled with digoxigenin. After a further restriction analysis, it was determined that there was no Pst1 site in the gene. Again digestion of genomic DNA of *N. simplex* with several restriction endonucleases, separation of fragments with agarose gel electrophoresis and southern blot analysis from the genomic DNA fragments using the labeled gene probe of the 0.55 kb fragment were performed. A PstI fragment of about 3 kb containing at least the *ht* (hydride transferase gene) was identified. Genomic PstI fragments of 2.7-3.5 kb were isolated, ligated and transformed as described above. Four out of 600 colonies hybridized with the 0.55 kb gene probe. The plasmid of one of the positive clones contained a PstI insert of about 3 kb. It was named pI3P. The plasmids pI43B and pI3P were used for double strand sequencing (figure 4).

Sequence analysis of the F_{420}-reductase gene and the hydride transferase gene. Open reading frames (orfs) coding for the F_{420}-reductase and the hydride transferase were found. They are located downstream of an orf identified as a transcription regulator. In both orfs the derived protein contained the N-terminal amino acid sequence of the corresponding enzyme. Ribosomal binding sites were found before each orf.

A 220-amino acid protein was derived from the gene containing the N-terminal sequence of the F_{420}-reductase. At the beginning the N-terminal sequence including the nucleotide binding motif as described in (7) was detected.
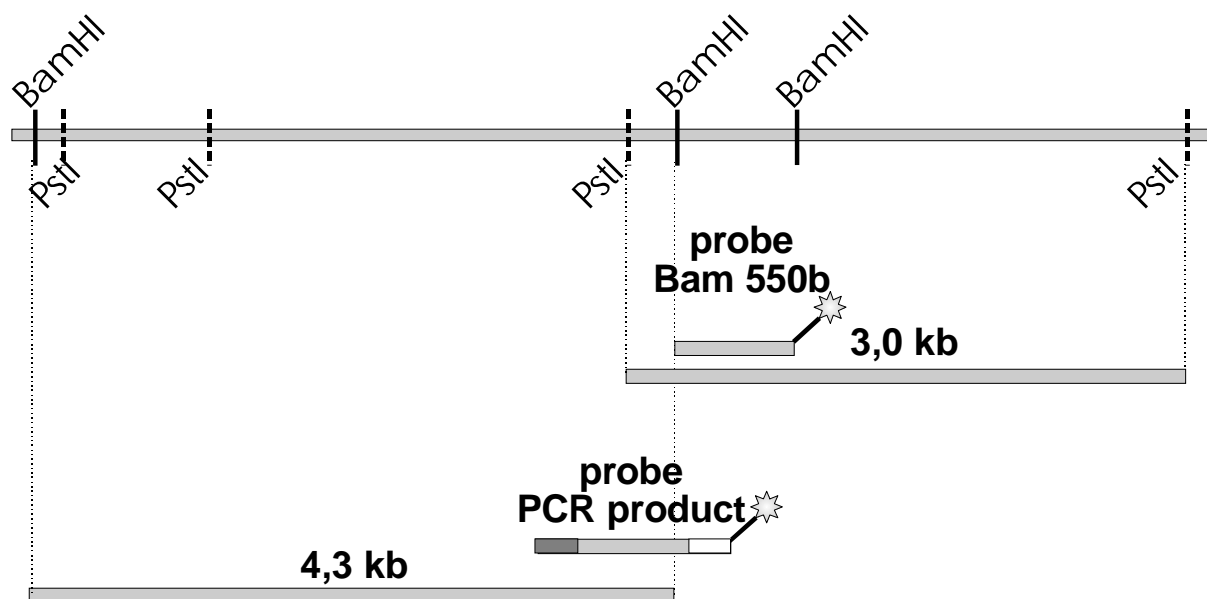

Figure 4. Strategy of gene detection. BamHI restriction sites (▬) and PstI restriction sites (▪▪▪▪) on DNA of _N. simplex_ in the region of the PCR product.

An enzyme of _Rhodococcus erythropolis_ HL PM-1 showed a homologous N-terminal sequence (22). Homologs are putative F_{420}-dependent NADP reductases of _Streptomyces coelicolor_, _Methanobacterium thermoautotrophicum_, _Methanococcus jannaschii_, and to an F_{420}-dependent NADP reductase of _Methanobacterium thermoautotrophicum_ (strain Marburg) and other putative enzymes of _Streptomycetes_ as found when comparing to BLAST (1).

The molecular mass of a 349-amino acid protein was calculated to be 37.07 kDa and corresponded to the mass determined for the hydride transferase by SDS Page (7). In searches through several data bases as BLAST (1), these proteins did not show any characteristic patterns or binding motives for cofactors etc. with any other prokaryotic proteins. High homologies to N5,N10-methylene-tetrahydromethanopterin reductases of archaea as _Archaeoglobus fulgidus_, _Methanococcus jannaschii_, and _Methanopyrus kandleri_ were found when comparing with BLAST (1). The derived protein sequence showed a similarity of 70 % to an F_{420}-dependent dehydrogenase #2 of _Rhodococcus erythropolis_ HL PM-1 when compared with ClustalW (27)

(figure 5). An alignment of the derived protein sequences including the archaeal sequences is shown in figure 5.

```
mk   MAEVSFGELLPDDKPTKIAHLIKVAEDNGFEYAWICDHYNNYSYMGVTTLAAVITSKIKLGPGTTNPYTRHFLITASNIATLDWIS--GRAIIGMGPGDKATFDKMGLPFPCKIPIWN 118
mj   ---MKFGIEFVPNEPIQKLCYYVKIAEDNGFEYCWITDHYNNRNVYMALTAIAMNTNKIKLGPGVTNPYVRSPAITASAIALDELS--GGRAVLGIGPGDKATFDALGIEW------- 107
af   ---MKFGIEFVPDMKYYELEYYVKIAEDSGFDYTWITDHYNNRNVYSMITILAKTRTIKLGPGVTNPYHISPALTASAIGTINEIS--GGRAVLGIGAGDKVTFERIGITW------- 107
ht   ---MIKGIQLHAWAGGPEMVEVAEIAAQQ--FETVWVTDQLQSRGVAAVLGAIAARTG-MGVGTAVTFPFGRNPLEQASTMATLAEFLPEGRQVSMGICTGGGLVSALMDRHQ----- 107
dh2  ---MIKGIQLHGWADGPQMVEVAEIAAGS--FETVWLSDQLQSRGVAVLLGAIAARTG-VGVGTAVTFPFGRNPLEMASSMATLAEFMPEGRRVTMGIGTGGGLVSALMPLQN----- 107
          :  *   :::       *     *  : .   *      *  :    *   : :* : ::**  *   *     *  :        *:   *   . :: . :

mk   PEAEDEVGCPATAIREVMEVIYQYLEGGPVEYEYG-------KYVKTGTADVKARSIQGSDIPFYMGAQGFIMLKTAGEIANGVLVNASNPKD--------FEVAVPKIEEGAKEAGRSLDE 223
mj   ------VKFVTTLKESTEVIRKLLAGERVSYEG--------KVVKIAGAALAVKPIQKA-VPVYMGAQGFKMLETAGMIADGVLINASNPKD--------FEAAIPLIKKGARAAGRSMDE 205
af   ------EKFLKRMRFAVEIIRQLTEGKAVKYDG-------EIFKFNGAKLGFK--PGS-IPIYIGAQGFKMLQLAAELGDGVLINASHPKD--------FEVAKENIDAGLAKAGKSRDA 203
ht   ------PGVRVAEFIALSRVLWSGEEITIGDYPLTCSSLGLRFPEAKASFKWSKDPAVRVVVAGEFRILNAGEIADGVICASNYPAHSLAAFRSGQFDQVSNLDALDRGRLRSERPD 219
dh2  ------EIDRVAEFIAMCRLLWQGEAIRMGDYPQICTALGLREDARASFSWTSKPDVRVVVAGAGEHKVLEWAGELADGVICASNFPAHSLAAFRSGQFDAVSNLDALDRGKRSRRGE 219
           .   *    . :    :     *         :  :      . .:*    ::**** :*  ::**.: .**:: **. ::    *   . .. ::  *:

mk   IDVAAYTCFSIDKDEDKAIEATKIVVAFTVMGSPDVVLERHGIDTEKAEQIAEAIG----KGDFGTAIGLVDEDMIEAFSIAGDPDTVVDKIEETLKAG----VTQVVVGSPIGPDKEKA 335
mj   IDVAAYACMSVDKNADKAKQCAAVPVVAFIAAGSPPVVLERHGIDMEKVEAIRNALK----SGNFPEAFKNVDDTMLEAFSIYGTPEDVVEKCKKLAEMG----VTQIVAGSPIGPNKETA 317
af   FDTVAYASMSVDKDRDKARNAAARIVVAFIVAGSPPTVLERHGLSEDAVNAVREALNNAFTKGDWGGVAKSVTDEMIDIFSISGTPDDVIERINFLSKAG----VTQVVAGSPIGPDKKKS 319
ht   FSRIYGVNISVAEDREANAAARRQATLIVSQQFHENLERVGFDRGDYSATREAVHAG---AGVERAAELLPQFVADQLIVSGTPEDCVAGLGFLWATRSGPASTRSTWARPSAPTRGRR 336
dh2  FTRIYGVNILSVSADRESACAAARRQATLIVSQQFPENIHRVGFEPSDYAATRAALKAG---DGVDAAADLLPQEVADQLVVSGTPEDCIEALAEFILGYAEDAGFTEAYIGAEVGEDPREA 336
       .   .   . :  . .     : :  *      *  . *   .        *      .         . .:: :*    *   :          *   *

mk   IELVGQEVIPHFKE 349
mj   IKLIGKKVIPALKE 331
af   IQLIGKEIIPKLK- 332
ht   CKLITSRVLPALS- 349
dh2  VELLTSQVLPELA- 349
       :*: ..::*  :

*  identical amino acids
:  similar amino acids
.  less similar amino acids
```
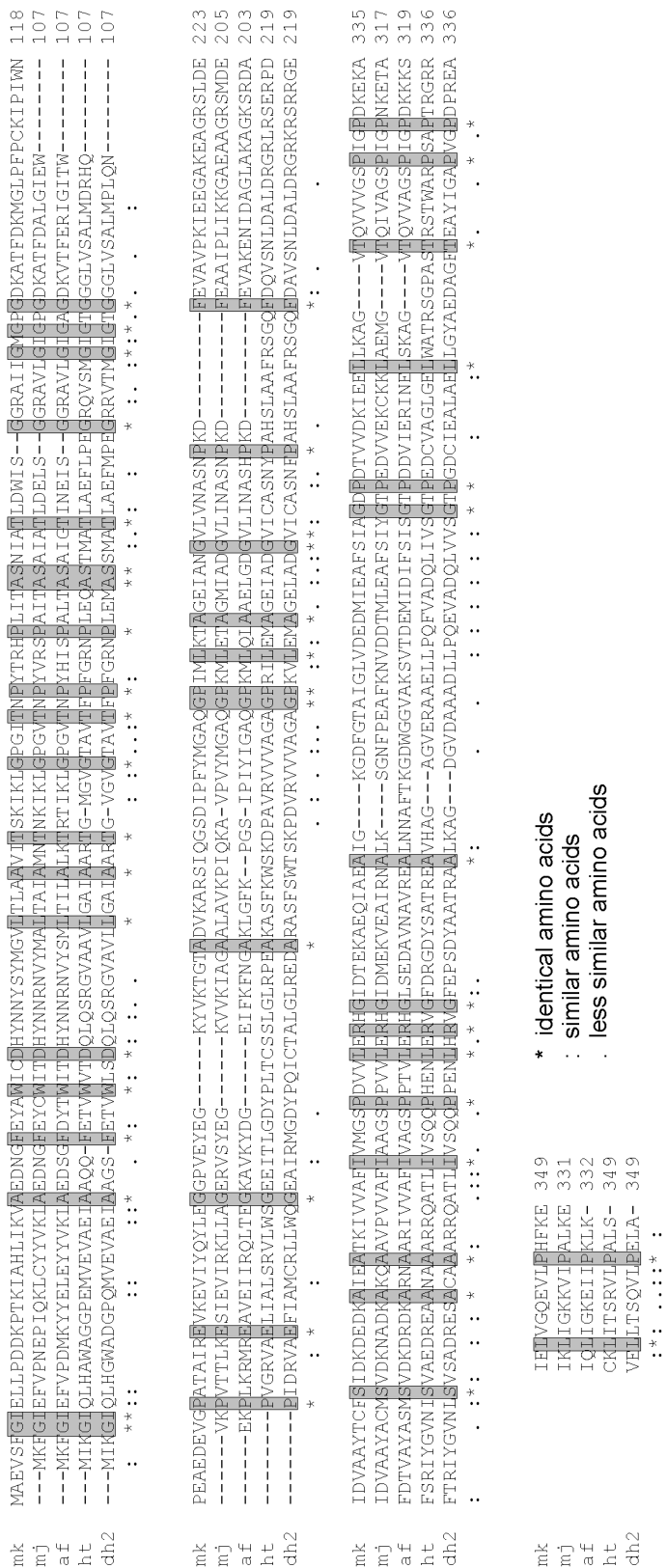
Figure 5. Sequence alignments of dehydrogenase #2 (dh2) of *Rhodococcus erythropolis* HL PM-1 with the hydride transferase of *N. simplex* (ht), the hypothetical protein (*Archaeoglobus fulgidus*) (af), the F_{420}-dependent N^5,N^{10}-methylene-terahydromethanopterin reductase (*Methanococcus jannaschii*) (mj), and the F_{420}-dependent N^5,N^{10}-methylene-tetrahydromethanopterin reductase (*Methanopyrus kandleri*) (mk).

DISCUSSION

During the present *in vitro* studies on the initial hydrogenation of picrate by an enzyme system described earlier (7) a second hydrogenation step generating a dihydride σ-complex of picrate ($2H^-$-picrate) was discovered. By UV visible spectroscopy two subsequent enzymatic hydride transfer steps were identified. The $2H^-$-complex, originally described by Severin et. al. (24), can also be generated chemically from picrate by reduction with sodium borohydride in an aqueous solution. The complex spontanously decarboxylates if the solution is acidified and 1,3,5-TNP is generated. This allows for indirect identification and quantification by HPLC under acidic conditions (14), if enzymatic transformation of picrate as substrate was stopped with phosphoric acid. 1,3,5-TNP as a product of the decarboxylation occurred as a peak in chromatograms and increased with the ongoing reaction. The delocalisation of the π-electron system in the nitroaromatic compound involving all three nitrogroups, the phenolate oxygen and the aromatic ring is abolished step by step through hydride transfer to the ring system. Position 3 and 5 of the carbon backbone are sp^3 hybridized and snapped in contrast to the planar aromatic system of picrate. Therefore a color change from the bright yellow of picrate to the orange red of the hydride σ-complex and finally to a pale yellow of the $2H^-$ -complex is observed during the reaction. The enzymatic activity of the hydride-transferring enzyme system could not be quantified by a photometric test because of interferring absorption of substrates, products, and cofactors. Therefore, in standard measurements activities were determined only qualitatively by the decreasing absorbance at 340 nm.

The NADPH-dependent F_{420}-reductase and the hydride transferase in the FJ2-1A strain were constitutively expressed which is in contrast to *Rhodococcus erythropolis* HL PM-1, another picrate degrading strain (22). Correspondingly, the concentration of cofactor F_{420} in *N. simplex* is constant irrespective of the presence of picrate in culture medium. Remarkably, the concentration of the coenzyme was higher than in other aerobic bacteria such as *Streptomycetes* or halophilic archaea but 2.7 fold less than in *Methanobacterium thermoautotrophicum* (9)*.*

Native gel electrophoresis with subsequent SDS electrophoresis of the hydride transferase band revealed a single band. Thus, it can be concluded that the hydride transferase of *N. simplex* consists of a single subunit.

The pH optimum of the F_{420}-reductase at pH 5.5 corresponds to the pH optima of other enzymes in *Methanogenium organophilum* (5), in *Streptomyces griseus*, in *Methanococcus vannielii* and in *Halobacterium cutirubrum* (9) with the same function. Hydride was transferred to picrate or 2,4-DNP by the hydride transferase with the highest activity at pH 7.5. The derived enzyme of the corresponding gene is homologous to N5,N10-methylene-tetrahydromethanopterin reductases of several archaea. Similar pH optima for such archaea were found with enzymes of *Archaeoglobus fulgidus, Methanosarcina bakeri, Methanopyrus kandleri,* and *Methanobacterium thermoautotrophicum*. The reductases of *Methanopyrus kandleri* and *Methanobacterium thermoautotrophicum* are stimulated by high salt concentrations, an effect also observed for the hydride transferase of *N. simplex* with ammonia sulfate. The K_m values for methylene-tetrahydromethanopterin reductases with methylene-tetrahydromethanopterin as a substrate are 2-10 fold lower than the K_m of 0.06 mM for picrate measured for the hydride transferase (23).

Hydride transfer to picrate, the hydride σ-complex of picrate, and 2,4-DNP takes place at position 3 of the aromatic ring. Hydride is transferred only to nitrophenols with nitro groups in 2 and 4 position. Obviously, the two nitro groups, which are located in the direct neighborhood to the position of hydrogenation, are necessary for the activity of the hydride transferase. Transforming 2,4-DNP with the enzyme system of *N. simplex* FJ2-1A revealed a hydride σ-complex of 2,4-DNP as described by Behrend et al. (4). It was identified with a chemically synthesized reference by in situ UV-visible spectra during HPLC analyses and repeated recording of UV visible spectra during enzymatic turnover of 2,4-DNP.

The genes coding for the NADPH-dependent F_{420}-reductase and the hydride transferase from *N. simplex* FJ2-1A were cloned and sequenced. Ribosomal binding sites were found for all open reading frames. A schematic organization is shown in figure 6. Orf 4 was identified to be a transcription regulator; orf 5 contained the corresponding N-terminal sequence of the NADPH-dependent F_{420} reductase and corresponds to *fr*. A small orf (orf 6) which was also detected in the *Rhodococcus* strain follows but no specific function has been identified. Orf 7 contained the corresponding N-terminal sequence of the hydride transferase as described in (7) and corresponds to *ht*. It is remarkable that in spite of the large evolutionary distance between archaea and actinomycetes the two enzymes in *N. simplex* are closely related to proteins of archaea.

Orf 1-3   **ABC transporter subunits**

Orf 4   **transcription regulator**

Orf 5   **NADPH-dependent F$_{420}$ reductase (*fr*)**

Orf 6   **small orf**

Orf 7   **hydride transferase (*ht*)**

Figure 6. Assembly of sequences obtained with transposon reaction in contigs and organization of the gene cluster tentatively involved in hydrogenation of the aromatic ring system of picric acid and 2,4-DNP.

For the NADPH-dependent F$_{420}$ reductase high homologies to enzymes with the same function of *Streptomycetes* and archaea were found. Comparing the amino acid sequence of the hydride transferase to BLAST (1) revealed homologs to N5,N10-methylene-tetrahydromethanopterin reductases of several archaea. This shows that not only cofactor F$_{420}$ is referring to methanogenic archaea but also the genes of the involved enzymes.

The N-terminal sequence of a F$_{420}$–dependent NADP reductase of *Rhodococcus erythropolis* HL PM-1 is almost identical to the earlier purified enzyme of *N. simplex*. The reductase of *R. erythropolis* had been overexpressed in *E. coli* and purified. It reduced coenzyme F$_{420}$ with NADPH as hydride donor (22). The hydride transferase of *N. simplex* was compared with the protein sequence of the F420-dependent dehydrogenase #2 of *Rhodococcus erythropolis* HL PM-1. Dehydrogenase #2 revealed high similarity to the hydride transferase of *N. simplex* FJ2-1A (figure 5). Furthermore enzymatic tests with crude extract of *E. coli* overexpressing the dehydrogenase #2 and 2,4-DNP as substrate revealed the same spectra as described by Behrend et al. (4, 22). Both enzymes could transfer hydride to 2,4-DNP and picrate (7, 22). Obviously they have the same physiological function in degradation of picric acid and are highly homologous although the enzymes of the hydride-transferring enzyme system are inducible in the *Rhodococcus* strain and constitutively expressed in the *Nocardioides* strain.

ACKNOWLEDGEMENTS

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Anhang 3:   Converging Catabolism of 2,4,6-Trinitro-phenol (Picric Acid) and 2,4-Dinitrophenol by Nocardioides simplex FJ2-1A

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ABSTRACT

An enzymatic activity has been identified which catalyzes the release of stoichometric amounts of nitrite from the dihydride σ-complex of picrate (2H⁻-picrate), formerly addressed as a dead end metabolite. The complex was synthesized independently by chemical reduction of picrate and characterized by ^{1}H and ^{13}C NMR spectroscopy. The hydride σ-complex of 2,4-dinitrophenol was identified as the denitration product. The nitrite-eliminating activity was concentrated and separated from the hydride-transferring enzyme system by fast-protein liquid chromatography (FPLC). The activity is not stimulated by any cofactors or metal ions. Since 2,4-dinitrophenol has now been disproven as a metabolite in the degradation pathway of picrate a convergent catabolic pathway for picrate and 2,4-dinitrophenol must exist with the hydride σ-complex of 2,4-dinitrophenol as the common key intermediate.

Abbreviations

2,4-DNP, 2,4-dinitrophenol; 2-Cl-4,6-DNP, 2-chloro-4,6-dinitrophenol; DNOC, 2-methyl-4,6-dinitrophenol; F_{420}, coenzyme F_{420}; TNT, 2,4,6-trinitrotoluene; 4,6-DNH, 4,6-dinitrohexanoate; 1,3,5-TNP, 1,3,5-trinitropentane; H⁻-picrate, hydride σ-complex of picrate; 2H⁻-picrate, dihydride complex of picrate; HTES, hydride-transferring enzyme system; HPLC, high pressure liquid chromatography; FPLC, fast-protein liquid chromatography.

INTRODUCTION

Several pathways have in the past been postulated for the degradation of picric acid and 2,4-dinitrophenol (2,4-DNP) (3, 5, 7, 9, 13). A hydride σ-complex of picrate, suggested as a metabolite by Lenke et al.(9), has been proven as the initial product of the pathway by Rieger et al. (13). 4,6-Dinitrohexanoate (4,6-DNH) (10) as well as 3-nitroadipate (3) were isolated from culture media and supposed to be formed via hydrogenolytic (10) or oxidative (3) ring cleavage of a fully hydrogenated six-membered ring carbon backbone. Both are considered metabolites in the lower pathway of picrate and 2,4-DNP degradation. An enzyme system, catalyzing the initial hydride transfer, was purified (7); the products were characterized (8). The corresponding genes were detected and cloned, and the content of the hydride mediating cofactor F_{420} in cells of *Nocardioides simplex* FJ2-1A was determined (8). Enzyme systems with the same function were detected in the 2,4-DNP- and the picrate-degrading *Rhodococcus erythropolis* strain HL PM-1 (14), and also in the *Nocardioides* sp. strain CB 22-2 (1).

The first step in picrate and 2,4-DNP degradation is formation of the corresponding hydride σ-complex catalyzed by the hydride transferring enzyme system (HTES)(7). Nitrite elimination from the hydride σ-complex of picrate and rearomatisation were proposed as the follow-up catabolic steps, with 2,4-DNP being formed as the denitration product (7, 9, 13). The dihydride σ-complex of picrate (2H⁻-picrate) has been identified as the product of the initial hydride transfer reaction with picrate as substrate (8). In the present paper, we describe the identification of a denitrating activity for 2H⁻-picrate, formerly assumed to be a dead end product, by employing authentic 2H⁻-picrate. These findings contradict the prior assumption that nitrite is eliminated from the σ-complex of picrate (H⁻-picrate).

MATERIALS AND METHODS

Bacterial strain and growth condition. *N. simplex* FJ2-1A was isolated from picric acid containing wastewater and identified by 16S rRNA analysis (12). The strain was grown and harvested as previously described (7). Cells were frozen in liquid nitrogen and stored at -30°C until they were used for protein purification. Cell density was monitored photometrically by measuring the turbidity at 546 nm.

Turnover of nitrophenols by cells of Nocardioides simplex FJ2-1A. As described earlier (7), cells were grown in a medium with mineral salts without nitrogen, yeast extract, peptone, casamino acids, picrate as nitrogen source and acetate as carbon source. Cells were 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.

Preparation of cell-free extract. Frozen cells were resuspended in 50 mM TRIS-HCl (pH = 7.5) and disrupted by multiple French pressure treatment at 137 MPa. Cell debris were removed by centrifugation at 100,000 x g and 4°C for 45 min.

Purification of coenzyme F_{420}, the F_{420}-dependent NADPH oxidoreductase and the hydride transferase. The enzymes were purified to electrophoretic homogeneity as described formerly (7). Nitrite eliminating activity was concentrated by FPLC (fast-protein liquid chromatography). Cell extract (74.1 mg of protein) was passed through a Q Sepharose column (1.6 × 10 cm) equilibrated with basic buffer (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 basic buffer at a NaCl concentration of 0.28 M, respectively.

Protein concentrations were estimated by the method of Bradford (4). Coenzyme F_{420} was purified and recycled by FPLC as described earlier (7). The concentration of coenzyme F_{420} was calculated from its absorption at 420 nm using the extinction coefficient of 41,4 $mM^{-1} \cdot cm^{-1}$ for coenzyme F_{420} at pH = 7.5 (7).

Enzyme assay. The activity of the two component enzyme system towards picrate or 2,4-DNP as substrates was assayed as described earlier (8). Transformation of $2H^-$-picrate was routinely monitored spectrometrically by following the decrease of absorption at 390 nm or by repeated recording of 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. Nitrite was estimated photometrically by the method described by Montgomery and Dymock (11).

For quantification of substrates, a high-pressure liquid chromatography (HPLC) system with a Gromsil 120 Oc4 column (125 x 4 mm, particle size 5 µm) was used. The mobile phase consisted of 80% water, 20% acetonitrile and 0.26% H_3PO_4 (acid eluent conditions). Purity control of the dihydride complex of picrate was examined by ion pair chromatography on the Gomsil column with an isocratic eluent consisting of 20 % methanol-water, 5mM tetrabutylammonium hydrogen sulfate, NaOH (pH = 12, alkaline conditions). For determination of σ-complexes a HPLC system with a Gromsil TSK-Gel Q-5PW column (125 x 4 mm) was used. The mobile phase consisted of 25 mM Tris · Cl / pH = 8. A linear gradient from 0.35 to 0.6 M NaCl in buffer was used for elution. Chromatograms were monitored at 210 nm, 240 nm, 260 nm and 300 nm. Identification via UV-visible spectrum was performed with a photodiode array detector (PDA) (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 (16). All other chemicals used were of the highest available purity and were purchased from Aldrich (Steinheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), or Sigma (Deisenhofen, Germany).

RESULTS

Growth of _Nocardioides simplex_ FJ2-1A and turnover of substrates by whole cells. _Nocardioides simplex_ FJ2-1A is able to grow with picrate as a nitrogen, carbon, and energy source. Growth with the nitroarene substrate in mineral medium is very slow, though; acetate, yeast extract, peptone, and casamino acids were therefore supplied to grow the strain within 26 h to an optical density of 3.5 at 546 nm. Picrate consumption was monitored by HPLC under acid eluent conditions. No formation of 1,3,5-trinitropentane (1,3,5-TNP) which would indicate 2,4,6-trinitrocyclohexanone as a metabolite, was observed. During growth of _Rhodococccus erythropolis_ HL PM-1 (9) some 1,3,5-TNP was generated by hydrogenolytic ring cleavage and decarboxylation of the dianion of 2,4,6-trinitrocyclohexanone under acid eluent conditions (15).

Several nitroaromatic compounds were tested for their substrate potential with whole cells grown on picrate. 2,6-DNP was not transformed. 2-Methyl-4,6-dinitro-phenol (4,6-dinitro-_o_-cresol, DNOC) was converted by the _Nocardioides_ strain only to a small amount ($0.1 \ nM \cdot min^{-1} \cdot OD^{-1}$). Picrate was transformed at a rate of $1.8 \ nM \cdot min^{-1} \cdot OD^{-1}$ with whole cells and small amounts of 1,3,5-TNP were detected when samples were analyzed by HPLC under acidic conditions (Fig. 1). 2,4-DNP was converted at a rate of $5.74 \ nM \cdot min^{-1} \cdot OD^{-1}$ (Fig. 2). 4,6-DNH was detected by HPLC and accumulated in the medium in non-stoichiometric amounts. None of these substrates were transformed by heat-inactivated cells (as control).
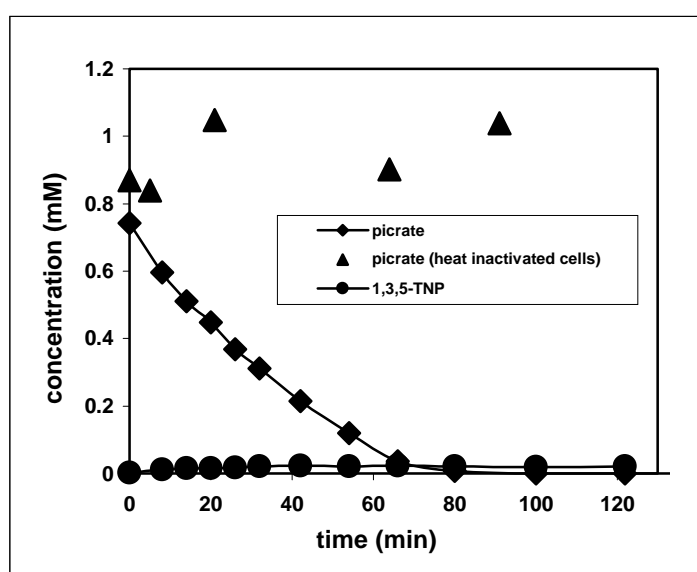

Figure 1. Conversion of picrate with a suspension of whole cells of _N. simplex_ ($OD_{546 \ nm} = 4.15$) and by heat inactivated cells as control.
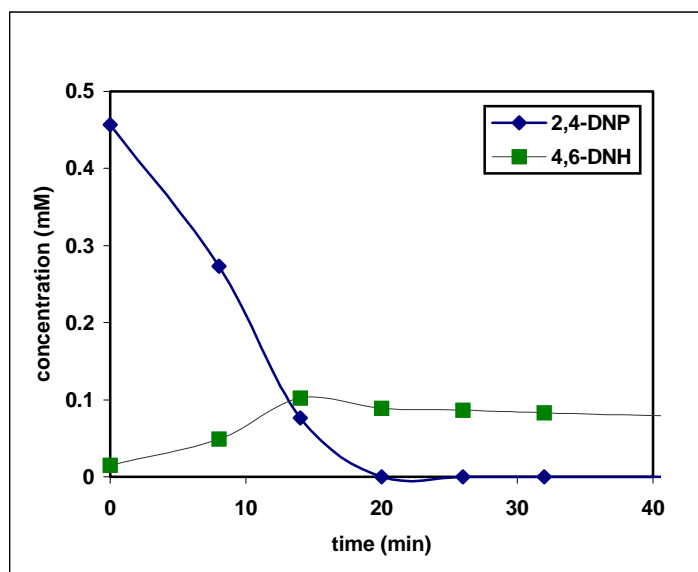
Figure 2. Conversion of 2,4-DNP by suspension of whole cells of *N. simplex* (OD$_{546 \text{ nm}}$ = 4.15).

Synthesis of the authentic dihydride complex of picrate (2H⁻-picrate) and spectroscopic characterization. The dihydride complex of picrate was synthesized by reducing of picrate in alkaline solution with sodium borohydride (16) (Fig. 3) and precipitated by addition of methanol. HPLC Analysis under alkaline conditions gave a single peak (retention time 6.2 min). The UV-visible spectrum in alkaline solution (pH = 13) showed a maximum at 390 nm as described by Severin and Schmitz (16) which changes characteristically both as to position and intensity with decreasing pH (Fig. 4).
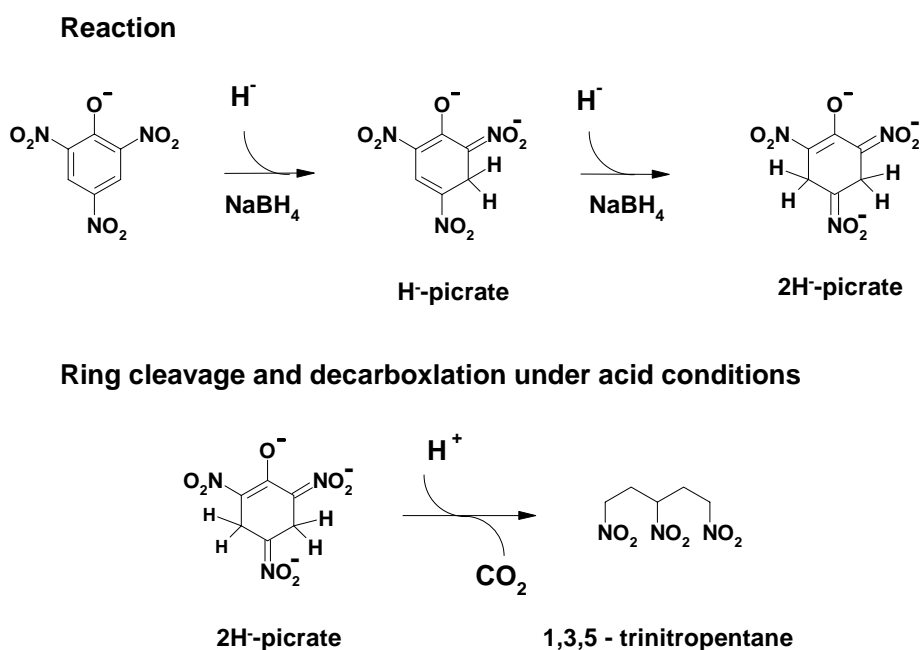
Reaction

Ring cleavage and decarboxlation under acid conditions

Figure 3. Chemical reactions relevant for identification of metabolites.
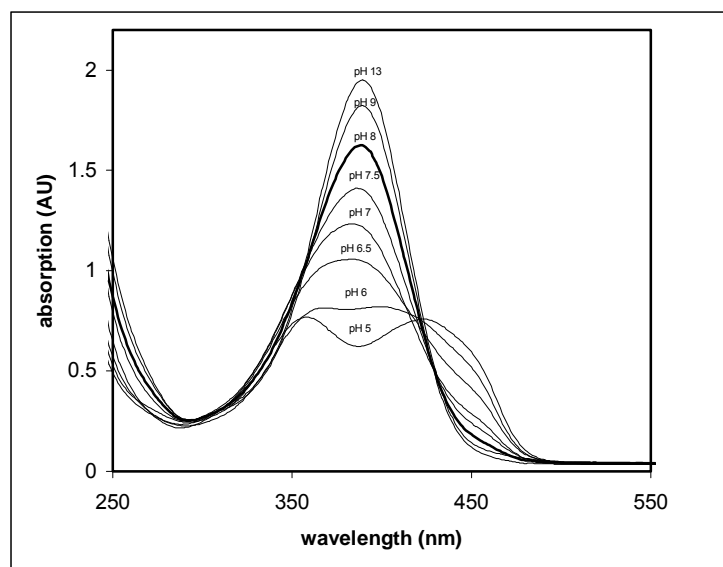
Figure 4. pH-Dependence of the UV-visible spectrum of 2H⁻-picrate (0.1 mM).

The ^{1}H and ^{13}C NMR spectra (D$_2$O, 300K) unequivocally confirm the dihydride σ-complex structure of 2H⁻-picrate. Apart from the water peak, the ^{1}H spectrum shows one singlet only, at δ = 4.04 ppm, for the four chemically equivalent protons in positions 3,5 of the cyclohexanoid ring system. (Another small singlet at δ = 3.36 ppm is due to residual methanol from the precipitation step.) 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 shows 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 is 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, is 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,^{1}H COSY (C,H correlation). A ^{1}H test spectrum, taken after the time-consuming ^{13}C and correlation NMR experiments, clearly demonstrates 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; both 2H⁻-picrates had identical HPLC retention times and UV-visible spectra. The nitrite-eliminating activity was investigated using the authentic chemical reduction 2H⁻-picrate.

Hydride transfer and subsequent nitrite elimination. Enzymatic activity of the HTES and the denitrating activity were tested with relevant nitroaromatic compounds. Picrate was transformed by fresh cell-free extract. The first hydride transfer is faster than the second one so that the hydride σ-complex of picrate (H⁻-picrate) could be identified as an only intermediate by its UV-visible spectrum (8). Nitrite was detected in the reaction mixture.

The freshly prepared cell extract also transformed chemically synthesized H⁻-picrate. Successive UV-visible spectral traces, taken at 10 min intervals, (Fig. 5) revealed two well-defined isosbestic points which indicate the transformation of H⁻-picrate to 2H⁻-picrate.

Transformation of 2H⁻-picrate with concomitant nitrite elimination was also catalyzed by crude extract as demonstrated by successive UV-visible spectral traces in Fig. 6 and the detection of nitrite in the reaction mix.
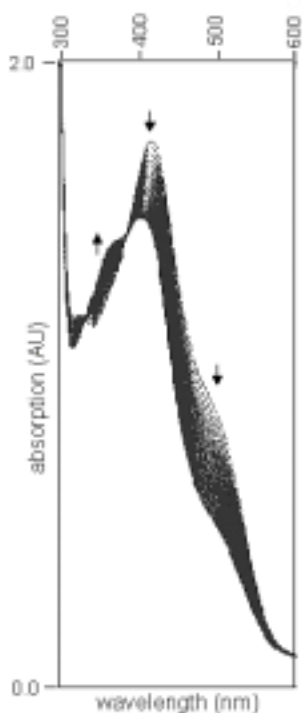

Figure 5. Successive UV-visible spectral traces (10 min intervals) during the turnover of H⁻-picrate by a fresh extract of picrate-grown cells of *N. simplex* (0.153 mg protein in 50 mM TRIS-HCl (pH = 7.5)).
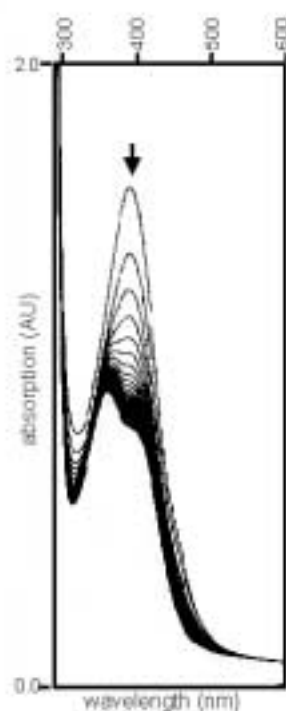
Figure 6. Successive UV-visible spectral traces (5 min intervals) during turnover of 2H⁻-picrate by fresh crude extract of *N. simplex* (0.17 mg protein in 50 mM TRIS-HCl (pH = 7.5)).

All fractions obtained by FPLC separation of the crude extract were tested for activity to clarify whether nitrite is eliminated from H$^-$-picrate as assumed earlier or from 2H$^-$-picrate which is the final product of the reaction catalyzed by the HTES. 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. 2H$^-$-picrate was found as product of the reaction catalyzed by the enzyme system (8). Both H$^-$- and 2H$^-$-picrate are stable. No nitrite was released either spontaneously or unspecifically by bovine serum albumin or denatured enzyme. In contrast, nitrite-eliminating activity was observed in one protein fraction after the first enrichment step with 2H$^-$-picrate as substrate. Hence, the nitrite eliminating step must follow the two hydrogenation steps of picrate and is catalyzed enzymatically. Release of nitrite from H$^-$-picrate can be excluded.

Quantification of nitrite and the product of nitrite elimination. 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. Activity eluted at a ionic strength of 0.28 M sodium chloride. This purification step allowed a separation from the components of the hydride transferring enzyme system which eluted at higher ionic strength (7). All further purification efforts to get an electrophoretically homogenous fraction of the nitrite-eliminating enzyme failed because of loss of activity. Several tested buffers such as Tris $\cdot$ Cl/ 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^{2+}, Cu^{2+}, Co^{2+}, Ni^{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 (Fig. 7). 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 a hydride σ-complex of 2,4-DNP. Nitrite was released in stoichiometric amount. The product of the reaction was identified as the hydride σ-complex of 2,4-DNP (H$^-$-2,4-DNP) by HPLC using a freshly synthesized authentic preparation of H$^-$-2,4-DNP as reference. At 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. The characteristic UV-visible spectrum measured in situ using a photodiode array detector was identical with that of the reference H$^-$-2,4-DNP.
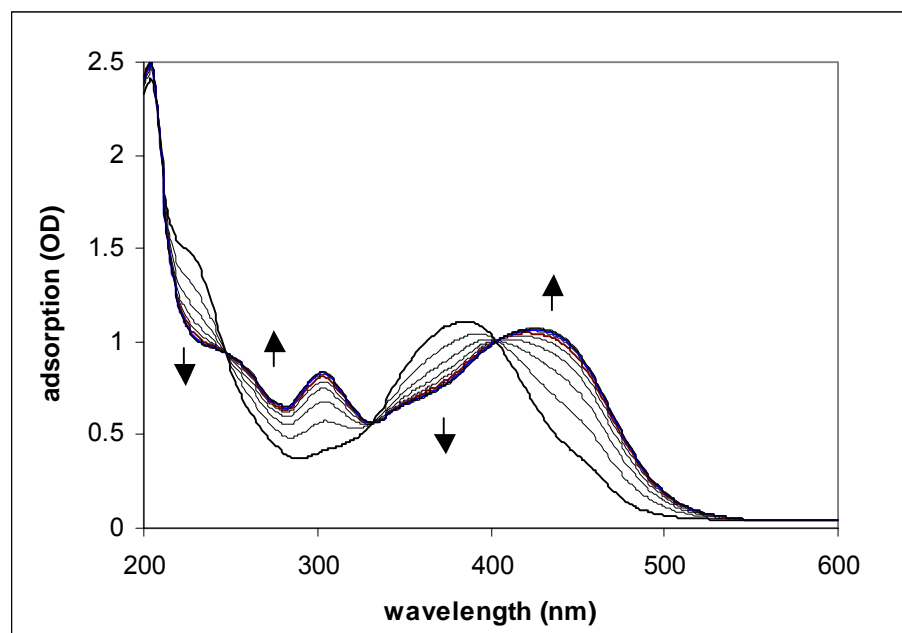

Figure 7. Successive UV-visible spectral traces (5 min intervals) during turnover of 2H$^-$-picrate by enriched nitrite eliminating activity (0.024 mg protein in 50 mM TRIS-HCl (pH = 7.5)).

A combination of the HTES and the nitrite-eliminating activity transformed both picrate and the mono-hydride σ-complex of picrate to H$^-$-2,4-DNP and nitrite as the only products.

Nitrite release from picrate, hydride σ-complex of picrate, or 2H$^-$-picrate as substrate was compared and quantified colorimetrically. Only with 2H$^-$-picrate as substrate the nitrite eliminating protein was sufficient to release stoichiometric amounts of nitrite. Amounts of nitrite eliminated from different substrates are compared in Table 1.

Table 1. Release of nitrite from nitroaromatic compounds by crude extract and enriched nitrite-eliminating activity.

substrate	concentration (mM)	nitrite (mM)	conversion with
picrate	0.1	0.099	crude extract
H$^-$-picrate	n.a.	0.04	crude extract
2H$^-$-picrate	0.1	0.08	enriched nitrite eliminating activity
2,4-DNP	0.1	0.01	crude extract

No nitrite elimination was observed when DNOC or 2-chloro-4,6-dinitrophenol (2-Cl-4,6-DNP) was transformed by the combination of the HTES and the denitrating activity. All substrates tested and the products characterized from the respective transformations are summarized schematically in Table 2.

Table 2. Nitrophenols and their products.

substrate	extent of conversion in culture	conversion by	product
picrate	++	HTES[a]	$2H^-$-picrate
H^--picrate	+	HTES	$2H^-$-picrate
$2H^-$-picrate	n.a.	enriched nitrite-eliminating activity	H^--2,4-DNP
2,4-DNP	++	HTES[a]	H^--2,4-DNP
H^--2,4-DNP	n.a.	crude extract[b]	n.a.
2-Cl-4,6-DNP	+	HTES[a]	[H^--2-Cl-4,6-DNP]
DNOC	(+)	HTES[a]	[H^--DNOC]

[a] Ebert et. al. (8)
[b] Behrend Dissertation (1)

DISCUSSION

$2H^-$-Picrate has been identified as a new metabolite in the upper degradation pathway of picrate. It is generated in a two-step hydrogenation process catalyzed by the two-component enzyme system of *N. simplex* (7), with NADPH as the hydride donor and cofactor F_{420} as mediator for the hydride transfer. The enzymes involved are a NADPH-dependent F_{420} reductase and a hydride transferase. In the first step of the catabolic sequence, the orange H^--picrate is formed. The second hydrogenation step, reducing 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. (18) as one of the catabolic reactions that prevent mineralization 2,4,6-trinitrophenol (TNT). Formation of hydride and dihydride σ-complexes of TNT has also been observed with the picric acid-utilizing *Rhodococcus erythropolis* strain HL PM-1, the 4-nitrotoluene-utilizing *Mycobacterium sp.* strain HL 4-NT-1 (18), and the pentaerythritol tetranitrate and glycerol trinitrate-utilizing *Enterobacter cloacae* PB2 (19). All these hydrogenation reactions with TNT are gratuitous reactions that give rise to dead end hydride complexes, although slow growth of the *E. cloacae* strain on TNT as a nitrogen source was described (19).

Enzymatic release of nitrite from H^--picrate and concomitant rearomatisation to 2,4-DNP were up to now considered as the initial key steps for productive breakdown of picrate. In contrast to this hypothesis, the results presented here clearly show the cell free extract of *N. simplex* to eliminate nitrite from authentic $2H^-$-picrate synthesized chemically. This activity is lost completely in the course of an attempted purification, although it is not affected by concentration at an anion exchanger column, the first step of the purifying protocol. H^--2,4-DNP and stoichiometric amounts of nitrite were found in enzyme tests containing the enriched nitrite-eliminating activity and $2H^-$-picrate as substrate. No such activity was found with H^--picrate as substrate.

Hence, 2,4-DNP is not a metabolite in the degradation of picric acid by *N. simplex* as assumed for the degradation of picrate by *Rhodococcus erythropolis* HL PM-1 (2, 7, 9, 13). Rather, 2,4-DNP and picrate are degraded via a convergent pathway, with the hydride σ-complex of 2,4-DNP constituting a common key metabolite.

Picrate, 2,4-DNP, and other structurally related dinitrophenols, e.g. DNOC, 2-Cl-4,6-DNP, and 2,6-DNP, were tested both in vivo and as substrates for the HTES

and the denitrating activity (Table 2). DNOC is transformed only very slowly both by whole cells and the HTES. Hydrogenation seems to stop at the level of a hydride σ-complex of DNOC as indicated by characteristic changes of the UV-visible spectra (8). No nitrite was eliminated when enriched nitrite-eliminating activity was added to the reaction mixture. Under the same conditions, fast transformation of 2-Cl-4,6-DNP by the HTES was observed. UV-visible spectroscopy indicates hydride σ-complex formation (8). However, nitrite was not eliminated when denitrating activity was added.

2,4-DNP was converted faster than picrate by whole cells. Within 20 min the concentration of 2,4-DNP decreased to zero. 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. Because of the chemical instability of 4,6-DNH under physiological conditions ($t_{1/2}$ approximately 55.6 min at pH =7.2 (7)) its occurrence in culture fluid does not prove its role as a metabolite in the degradation pathway. The first step in 2,4-DNP degradation is hydrogenation to the hydride σ-complex which is less stable than the corresponding H^--picrate. Nevertheless, Behrend and Heesche-Wagner (2) had synthesized the hydride σ-complex of 2,4-DNP. It could be identified by immediate recording of NMR spectra. The complex readily disproportionates to amino-nitro-phenols and 2,4-DNP and unknown byproducts (1). The hydride σ-complex of 2,4-DNP is converted by crude extract and NADPH stimulates the reaction (1). A NADPH-dependent monooxygenation in *para*-position is suggested for the degradation of H^--2,4-DNP by the authors (2), but also further hydrogenation may occur. Zablotowicz et al. (20) proposed an initial attack at 2,4-DNP by a PCP-4 monooxygenase (pentachlorophenol-4 monooxygenase) under release of nitrite and a concomitant hydroxylation of the *para* carbon of 2,4-DNP by a 2,4-DNP degrading *Sphingomonas* sp UG 30 strain.

Our observations with different substituted nitrophenols clearly identify two structural elements that are necessary for hydrogenation and subsequent nitrite elimination. (i) Two nitro groups in 2- and 4-position prerequisite for hydride transfer (8). (ii) Nitrite elimination requires a twofold hydrogenation of the 2,4,6-trinitroaromatic ring. By these conversion experiments and enzyme tests 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 and degradation by bacteria able to mineralize picrate.

Both enzymes of the HTES, the NADPH-dependent F_{420} reductase and the hydride transferase, are homologous to proteins and genes of methanogenic archaea. Up to now no anaerobic organism is known to mineralize picrate or 2,4-DNP, though several investigations under anaerobic conditions were made and hydride σ-complex formation should be favored. Instead, Donolon et al. (6) described the transformation of 2,4-DNP to the corresponding aminoarenes in upflow anaerobic sludge blanket reactors. Reduction of 2,4-DNP to 2-amino-4-nitrophenol under anaerobic conditions is mentioned by Uberoi and Bhattacharya (17).

Further investigation will be necessary to elucidate the lower pathway of picric acid and 2,4-DNP degradation. Fig. 8 gives an overview of the upper pathway of picrate and 2,4-DNP catabolism.
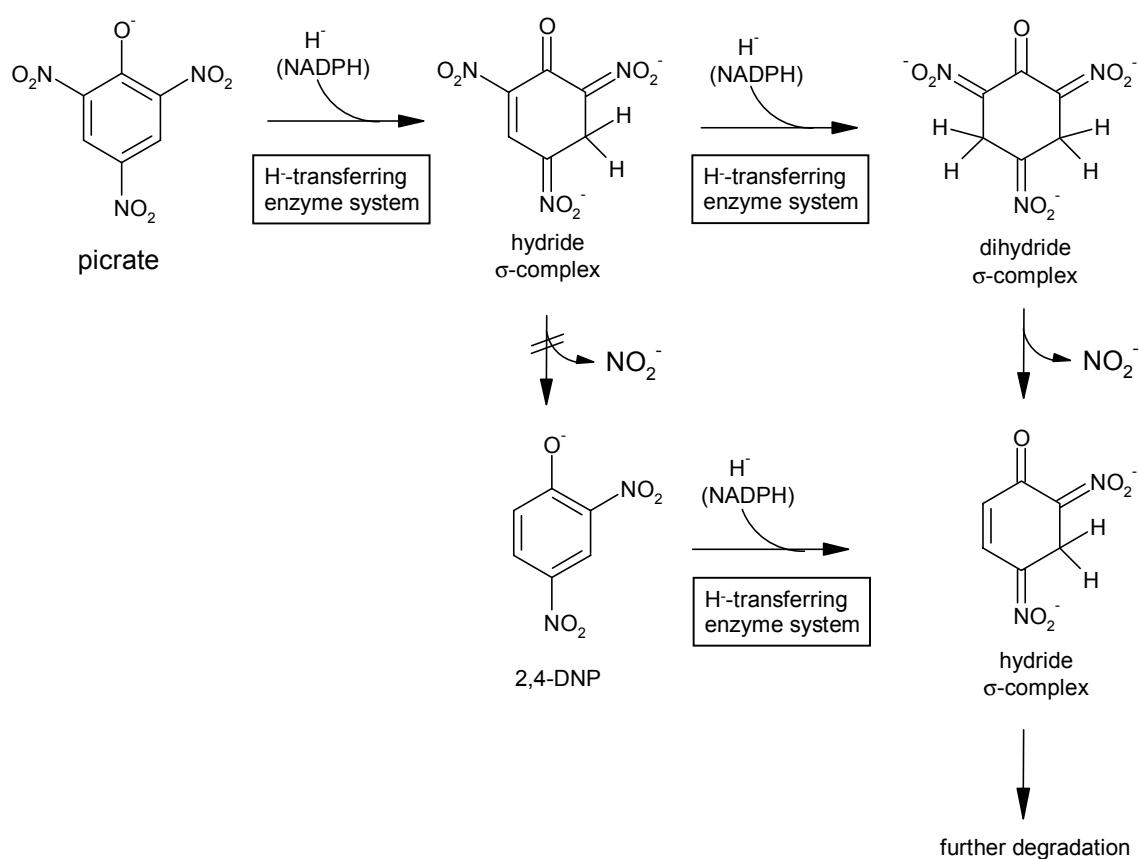

Figure 8. Revised upper pathway for the convergent degradation of picrate and 2,4-DNP by *N. simplex*.

The present conversion experiments and enzyme tests furnish unequivocal evidence of 2H$^-$-picrate as a key metabolite. Nitrite is eliminated from this complex. Therefore, 2,4-DNP is not a metabolite of picrate degradation. Both nitrophenols are degraded in a convergent pathway.

ACKNOWLEDGEMENTS

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Danksagung

Herrn Professor Knackmuss danke ich für die Überlassung des Themas und anregende Diskussionen. Ich bedanke mich bei ihm für die Möglichkeit zur Knüpfung internationaler Kontakte durch die Teilnahme an internationalen Kongressen.

Herrn Dr. Paul-Gerhard Rieger danke ich für inspirierende Diskussionen, Tips in Theorie und Praxis und eine gute Zusammenarbeit.

Bei René Handrick bedanke ich mich für hilfreiche proteinchemische und genetische Diskussionen und das Korrekturlesen der Arbeit.

Herrn PD Dr. Andreas Stolz danke ich für die Übernahme des Korreferates.

Herrn Dr. Rafael Blasco danke ich für einen angenehmen und lehrreichen Laboraufenthalt in Córdoba (Spanien).

Den „Mibis" sei herzlicher Dank für die angenehme Laboratmosphäre, die gute Zusammenarbeit und für wissenschaftliche Diskussionen bei einer Tasse Kaffee. Bei den „Männern" aus Büro 4, Olaf Dickel, Wolfgang Haug und Dr. Paul-Gerhard Rieger, bedanke ich mich für technische sowie seelische und moralische Unterstützung und die nette Schreibraumatmosphäre.

Bei meinen Eltern, Gerlinde und Josef Ebert, bedanke ich mich für ihre Unterstützung.

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