The flavoprotein-catalyzed reduction of aliphatic nitro-compounds represents a biocatalytic equivalent to the Nef-reaction†

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The bioreduction of aliphatic *sec*-nitro compounds catalyzed by purified flavoproteins from the old-yellow-enzyme family unexpectedly furnished the corresponding carbonyl compounds instead of the expected amines and thus represents a biocatalytic equivalent to the Nef-reaction. The pathway was shown to proceed *via* initial reduction of the nitro-group to yield the nitroso-derivative, which spontaneously tautomerized to yield the more stable oxime, which was enzymatically reduced in a second step to furnish a hydrolytically unstable imine-species, which spontaneously hydrolyzed to finally give a carbonyl compound and ammonia.

Introduction

Nitroaromatic compounds, such as dyes, pesticides and (mostly toxic and mutagenic) explosives, are released into the biosphere almost exclusively from anthropogenic sources. Due to the resistance of the electron-deficient aromatic core towards biological oxidation, the biodegradation of nitroaromatics predominantly proceeds through the three-step reduction of the nitro-group *via* the nitroso- and hydroxylamino-stage to furnish the corresponding aromatic amine.[1] This metabolic activity is widespread among bacteria,[2] fungi, and plants[3] and was even detected in baker's yeast.[4] The majority of enzymes responsible for this reductive biotransformation are flavoproteins from the 'old-yellow-enzyme' family, generally denoted as 'nitroreductases',[5] whereas Mo-dependent enzymes, such as xanthine oxidase,[6] or alcohol dehydrogenases[7] play a minor role. The main three-hydride pathway,[1,8] is often accompanied by spontaneous (non-enzymatic) self-condensation of reactive nitroso- with hydroxylamino-intermediates to yield azoxybenzene derivatives; in addition, diaryl-hydroxylamines or -amines may be formed.[9] As an alternative pathway, flavoproteins may deliver the hydride directly onto the electron-deficient aromatic system thereby forming the so-called 'Meisenheimer-complex', which is stabilized *via* elimination of nitrite.[10] As a consequence of these diverse metabolic pathways, product mixtures are common and isolated yields of aromatic amines are modest at best. Overall, this biotransformation does not seem to be an advantageous alternative to existing methodology.[11]

On the other hand, the bioreduction of *aliphatic* nitro compounds would be an elegant method for the generation of amines in nonracemic form, bearing in mind that dynamic kinetic resolution processes based on the facile (spontaneous) racemization of *sec*-nitro groups may be envisaged. However, the data on the bioreduction of nitroaliphatics are very scarce, and this activity appears to be exclusively associated to (facultative or obligately) anaerobic organisms. Early studies by H. Simon *et al.*[12] revealed that 2-nitroethanol was reduced to 2-aminoethanol by crude extracts of *Clostridium* spp. using H_2, CO or formate as reductant. The relative rate was about one order of magnitude lower than that of *p*-nitrobenzoate and the pathway was shown to proceed *via* the corresponding oxime[13] and hydroxylamine.[14] However, partially purified NADH-dependent 'nitroaryl reductases' were shown to be inactive on 2-nitroethanol.[15] In a related fashion, 2-nitro-1-phenylpropane was reduced to traces of amphetamine ($\leq$1 %) by anaerobic intestinal bacteria.[16] More recently, G. Stephens *et al.* observed that the reduction of 2-nitro-1-phenylpropene by *Peptostreptococcus productus* DSM 3507 did not stop as expected at the nitroalkane-stage,[17] but proceeded further to yield 1-phenyl-2-propylamine with an e.e. of up to 19%, depending on the substrate concentration.[18] This observation prompted us to investigate the possibility to employ oxygen-stable flavin-reductases from the old-yellow-enzyme family for the reduction of aliphatic nitro-compounds to the corresponding amines.

Results and discussion

In order to cover a diverse set of substrates, cyclic, short- and long-chain *prim*/*sec*-nitroaliphatic compounds **1a-5a** were chosen and tested by employing the following flavin-dependent oxidoreductases (Scheme 1): NADH:FMN-Dependent oxidoreductases Lot6P, YcnD, and YhdA,[19] 12-oxophytodienoic acid reductase isoenzymes 1 and 3 (OPR1, OPR3),[20] the old-yellow-enzyme homolog YqjM,[21] nicotinamide-dependent cyclohexenone reductase (NCR-Red),[22] old-yellow-enzymes 1–3 from yeast (OYE1-3),[23] pentaerythritol tetranitrate reductase (PETN-Red), morphinone reductase (Mor-Red), *N*-ethylmaleimide reductase (NEM-Red),[5] glycerol trinitrate reductase (NerA),[24] and xenobiotic reductases (XenA, XenB).[9b,25]

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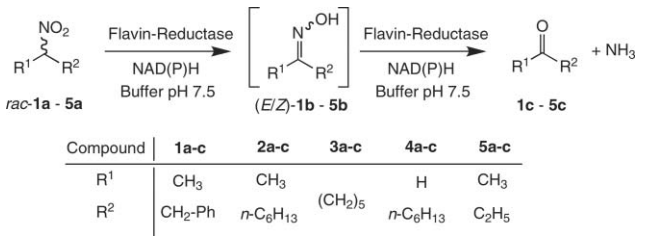


Compound	1a-c	2a-c	3a-c	4a-c	5a-c
R^1	CH_3	CH_3	$(CH_2)_5$	H	CH_3
R^2	CH_2-Ph	n-C_6H_{13}		n-C_6H_{13}	C_2H_5

Scheme 1 Enzymatic reduction of aliphatic nitro-compounds **1a-5a**.

Much to our surprise, during the bioreduction of substrates **1a-3a** using XenA, PETN-Red, Mor-Red and OPR3 in presence of NADH or NADPH (2.0 equiv.), the *N*-functionality was lost and the corresponding carbonyl compounds **1c-3c** were formed instead of the expected amines (Table 1). Overall, the difference in activities between NADH or NADPH (2.0 equiv.) as cofactors was modest (entries 1/2 and 4/5, resp.). In combination with an appropriate recycling system (NADH/glucose dehydrogenase/glucose or NADPH/glucose-6-phosphate dehydrogenase/glucose-6-phosphate, resp.) the conversion remained approximately the same (data not shown) or was markedly increased (entries 3, 6 and 13). The fact that this transformation was enzyme-catalyzed was proven by blank-experiments using heat-denaturated protein (XenA) or in the absence of enzyme (entry 17). The remainder of enzymes proved to be inactive, and the *prim*-nitroalkane **4a** and short-chain-*sec*-analog **5a** were not converted by any of the enzymes (entry 18).

Among the possible intermediates of this bioreduction, the nitroso-derivatives are too unstable to be synthesized or isolated. In contrast, oximes **1b-3b** are stable compounds, which were converted to ketones **1c-3c**, albeit in low yields (entries 19-25). More data on key intermediates of this pathway could be obtained from the bioreduction of nitroalkene (*E*)-**6a** (Scheme 2).

Careful monitoring of the sequential bioreduction of nitroalkene (*E*)-**6a** allowed the identification of the corresponding nitroalkane **6b** and oxime **6c** as intermediates en route to 2-phenylpropanal **6d** as the final product of the cascade (Table 2). The relative proportion of products strongly depended on the enzyme: While NerA and XenB predominantly acted as 'enoate reductase' by selectively reducing the activated C=C-bond to give nitroalkane (*S*)-**6b** in 54% and 21% e.e., resp. (entries 1,2), OPR3 furnished substantial amounts of oxime **6c** in up to 15% (entries 3,4). This trend was even more pronounced with XenA and morphinone reductase, where oxime formation was dominant (entries 5-8). The best enzyme was clearly PETN-reductase, which gave (*R*)-**6b** (42% conversion, 57% e.e.) and **6c** in 40% yield, respectively.[26] Aldehyde **6d** was formed in up to 4% yield when the substrate concentration was reduced to 5 mM

Table 1 Bioreduction of nitro-compounds *rac*-**1a-5a** and oximes (*E*/*Z*)-**1b-3b**

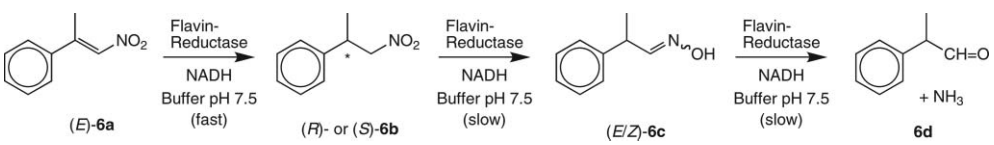
Entry	Substrate	Enzyme	Conditions[a]	Product (%)
1	**1a**	XenA	NADH	**1c** (12)
2	**1a**	XenA	NADPH	**1c** (10)
3	**1a**	XenA	$NADP^+$/G6PDH	**1c** (17)
4	**1a**	PETN-Red	NADH	**1c** (9)
5	**1a**	PETN-Red	NADPH	**1c** (5)
6	**1a**	PETN-Red	$NADP^+$/GDH/Glu	**1c** (10)
7	**1a**	Mor-Red	NADH	**1c** (8)
8	**1a**	OPR3	NADH	**1c** (8)
9	**2a**	PETN-Red	NADH	**2c** (10)
10	**2a**	Mor-Red	NADH	**2c** (12)
11	**2a**	OPR3	NADH	**2c** (7)
12	**3a**	XenA	NADPH	**3c** (8)
13	**3a**	XenA	$NADP^+$/G6PDH	**3c** (16)
14	**3a**	PETN-Red	NADH	**3c** (5)
15	**3a**	Mor-Red	NADH	**3c** (5)
16	**3a**	OPR3	NADPH	**3c** (5)
17	**1a-3a**	none	NADH	**1c-3c** ($0 \leq 3$)
18	**4a**,**5a**	all enzymes	NADH	**4c**,**5c** (0)
19	**1b**	OPR3	NADH	**1c** (2)
20	**2b**	OPR3	NADH	**2c** (2)
21	**2b**	PETN-Red	NADH	**2c** (2)
22	**2b**	Mor-Red	NADH	**2c** (1)
23	**3b**	PETN-Red	NADH	**3c** (3)
24	**3b**	Mor-Red	NADH	**3c** (3)
25	**3b**	OPR3	NADH	**3c** (3)

[a] Substrate **1a-5a** (10 mM), Tris-buffer (50 mM, pH 7.5) NADH or NADPH (2.0 eq); alternatively, $NAD(P)^+$ (100 μM)/glucose dehydrogenase (10 U)/glucose (20 mM) or $NADP^+$ (100 μM)/glucose-6-phosphate-dehydrogenase (10 U)/glucose-6-phosphate (20 mM), respectively; reaction time 24 h.

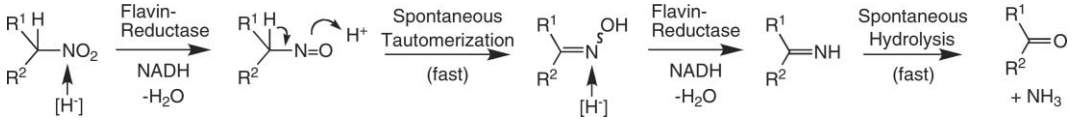
Table 2 Enzymatic reduction of nitroalkene **6a** *via* nitroalkane **6b** and oxime **6c** to aldehyde **6d**

Entry	Substrate	Enzyme	Conditions[a]	Product (%)[b] **6b**	(*E*/*Z*)-**6c**	**6d**
1	**6a**	NerA	NADH	(29)[c]	(8)	(2)
2	**6a**	XenB	NADH	(66)[d]	(8)	(1)
3	**6a**	OPR3	NADH	(19)	(1)	(1)
4	**6a**	OPR3	NAD^+/GDH/Glu	(17)	(15)	(2)
5	**6a**	XenA	NADH	(2)	(5)	(2)
6	**6a**	XenA	NAD^+/GDH/Glu	(2)	(16)	(1)
7	**6a**	Mor-Red	NADH	(6)	(1)	(1)
8	**6a**	Mor-Red	NAD^+/GDH/Glu	(9)	(23)	(2)
9	**6a**	PETN-Red	NADH	(38)	(15)	(1)
10	**6a**	PETN-Red	NAD^+/GDH/Glu	(42)[e]	(40)	(4)[f]
11	**6a-6c**	none	NADH	($\leq$1)	(0)	($\leq$3)

[a] Substrate **6a-c** (10 mM), Tris-buffer (50 mM, pH 7.5), NADH (2.0 eq) or NAD^+ (100 μM)/glucose dehydrogenase (10 U)/glucose (20 mM), reaction time 24 h; [b] traces of acetophenone were formed; [c] e.e. 54%, (*S*); [d] e.e. 21%, (*S*); [e] e.e. 57%, (*R*);[26] [f] substrate concentration 5 mM.



Scheme 2 Sequential bioreduction of nitroalkene **6a** *via* nitroalkane **6b** and oxime **6c** to furnish aldehyde **6d**.



Scheme 3 Proposed mechanism of the reductive transformation of nitroalkanes to carbonyl compounds.

(entry 10). No trace of the corresponding hydroxylamine could be detected.

Conclusion

Based on these data, the mechanism of the sequential reductive biotransformation of nitroalkanes to carbonyl compounds is delineated (Scheme 3). In the first step, the nitro-moiety is reduced by a hydride delivered *via* the flavin cofactor to furnish the nitroso-compound. The latter species are very unstable intermediates and are known to dimerize (if tertiary) or tautomerize (if secondary) thereby forming the more stable oximes.[27] This tendency is well documented and was found to occur under a remarkable variety of reaction conditions.[28] In a subsequent step, the oxime is reduced *via* hydride-delivery at nitrogen to furnish an imine.[7] In contrast to oximes, which are remarkably stable towards hydrolysis,[29] imines rapidly hydrolyse in aqueous solution to finally yield a carbonyl compound and ammonia. While our data nicely confirm the reductive biotransformation of a structurally related nitroalkene [(*E*)-2-nitro-1-phenyl-1-propene] to the corresponding carbonyl compound (1-phenyl-2-propanone) *via* the corresponding oxime using whole cells of *Peptostreptococcus productus*, some of the intermediate stages of the proposed pathway should to be reconsidered.[18,30] Overall, the reductive biotransformation of *sec*-nitro compounds furnishes carbonyl compounds and hence represents a biocatalytic equivalent to the Nef-reaction.[31] The latter is the hydrolysis of a *prim*- or *sec*-aliphatic nitro compound to yield the corresponding carbonyl product, *i.e.* an aldehyde or ketone, respectively and nitrous oxide. Whereas the chemical route is catalyzed by strong acids (*e.g.* H_2SO_4), the biological equivalent proceeds at ambient reaction conditions and produces NH_3 (rather than N_2O) as co-product. An analogous oxidative biological pathway—*i.e.* the O_2-dependent enzymatic oxidation of nitroalkanes to furnish the corresponding carbonyl compounds, nitrite and H_2O_2 has been observed with nitroalkane oxidases[32] and horseradish peroxidase.[33] Detailed studies will show whether the bio-Nef-reaction represents a serious alternative for preparative-scale reactions and/or as a method of choice for effluent treatment of nitro-compounds.

Experimental

Cloning, expression and purification of XenA, XenB and NerA

Xenobiotic reductase A from *Pseudomonas putida* (XenA, 8.39 mg/ml), xenobiotic reductase B from *Pseudomonas putida* JLR11 (XenB, 1.78 mg/ml), and glycerol trinitrate reductase from *Agrobacterium radiobacter* (NerA, 7.34 mg/ml) were cloned, expressed and purified (Ni^{2+}-NTA) as follows: The DNA sequences were identified using a search of the NCBI Genebank (accession files Q9R9V9 for XenA, Q9RPM1 for XenB and O31246 for NerA). The corresponding genes were synthesized at Geneart AG Regensburg (C-terminal hexahistidine tagged protein). After restriction with the adequate restriction enzymes (*NdeI*/*XhoI*), the insert was ligated into pET21a and transformed into competent *E-coli* TOP10 cells. The sequence was determined at Geneart AG Regensburg. Heterologous expression of the genes in *E-coli* BL21 (DE3) was performed as follows: 100 ml starter LB_{Amp} cultures were inoculated with aliquots from a frozen stock culture and grown overnight at 37 °C. Each starter culture was used to inoculate a 700 ml culture, which was vigorously aerated until the OD_{600} reached 0.6-0.8. The cultures were incubated with 0.2 mM IPTG (final concentration) and protein expression was performed for 4 h at 37 °C. Pellets were frozen at -80 °C or used directly for the protein purification. The cell pellets were resuspended in lysis buffer (5 ml/g wet weight; 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole) and sonicated on ice (t = 5 min, pulse 1 sec, pause 2 sec, amplitude 40%) with addition of FMN (*ca.* 3 mg). After centrifugation (30 min, 18000 rpm, 4 °C), the supernatant was subjected to protein purification on Ni^{2+}-NTA according to the manufacturer's protocol.[34,35]

General procedure for the bioreduction of substrates 1a-6a, 1b-3b, 6b and 6c

An aliquot of enzyme [OPR1, OPR3, two isoenzymes of 12-oxophytodienoate reductase from *Lycopersicon esculentum*, YqjM from *Bacillus subtilus*, three OYEs from yeast OYE1 (*Saccharomyces carlsbergensis*), OYE2, OYE3 (both from *Saccharomyces cerevisiae*), NADH-dependent cyclohexenone reductase from *Zymomonas mobilis* (NCR-Red), three flavin-oxidases (Ycnd, Lot6p, YhdA), PETN-reductase from *E. cloacae* PB2 (PETN-Red), N-ethylmaleimide (NEM-Red) and morphinone reductase (Mor-Red), xenobiotic reductases (XenA, XenB) and glycerol trinitrate reductase (NerA)] corresponding to a protein content of 100 μg/ml was added to a Tris-HCl buffer solution (0.8 ml, 50 mM, pH 7.5) containing the substrate (10 mM) and the cofactor NADH or NADPH (20 mM). Alternatively, the oxidized form of the cofactor NAD^+ or $NAD(P)^+$ (100 μM) was used in combination with a recycling enzyme (glucose dehydrogenase or glucose-6-phosphate dehydrogenase, respectively, 10 U) and the cosubstrate (glucose or glucose-6-phosphate, respectively, 20 mM). The mixture was shaken at 30 °C and 120 rpm. After 24 h products were extracted with EtOAc (2 × 0.8 ml); GC-analysis showed that product recovery from the aqueous phase was >95% in all cases. The combined organic phases were dried (Na_2SO_4) and the resulting samples were analyzed on achiral GC after TLC-control. Products were identified by comparison with authentic reference materials (which were synthesized as described in ESI †) *via* co-injection on GC-MS and achiral GC.

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