Abstract

Introduction

Nitroaromatic compounds (NACs) resist microbial degradation due to their electron deficiency. Further substitution with electron-withdrawing groups, such as chloro group, increases the electron deficiency and recalcitrance. NACs and the closely related chloronitroaromatics (CNACs) are widely used in several industries, and are toxic and mutagenic, posing an ecological threat. Since the occurrence of natural NACs is limited, microbes have only recently been exposed to the anthropogenic burden of NACs. However, within this short exposure time, several microbes have evolved the capability to metabolise NACs. The enzymes initiating metabolism of NACs warrant special attention due to the thermodynamic and xenobiotic considerations involved. The current article reviews the state-of-the-art knowledge on some of the important enzymes that catalyse the initial step of NAC degradation.

Conclusion

Looking at the recent progress, dioxygenases hold a lot of promise for NAC degradation. In the near future, the structure–function relationship of the dioxygenases will be better understood. This will allow researchers to generate dioxygenases with desired specificity at will and, of course, at the mercy of the enzyme flexibility.

Introduction

Nitroaromatic compounds (NACs) contain at least one nitro group attached to an aromatic ring. The aromatic ring is relatively inert due to the delocalisation of π electrons. The nitro group, being a strong electrophile, further deactivated the ring towards electrophilic substitution through inductive effect and conjugation. Figure 1 shows the effect of various substituents on the reactivity of benzene ring. In general, increasing electronegativity of the substituents increases the degree of deactivation. Due to the electron deficiency over the aromatic ring, oxidative metabolism of NACs is very difficult. The presence of additional substituents also affects the reactivity of NACs. Electron-withdrawing groups increase the electron deficiency and consequently, deactivate the NACs, whereas electron-donating groups activate NACs towards oxidative metabolism (Figure 1). Thus, electron-withdrawing substituents like nitro and chloro groups make polynitroaromatics and chloronitroaromatics (CNACs) resistant to aerobic microbial degradation. It is, however, observed that the nitro group in NACs is easily reducible, and substitution with electron-withdrawing groups, like chloride or another nitro group, greatly increases the rate of reduction. For example, trinitrotoluene (TNT) readily undergoes reductive metabolism, which results in reduction of one nitro group to amino group. However, amino group, being electron-donating, decreases the electron deficiency, due to which the reduction of remaining nitro groups is less rapid and requires lower redox potential.

Majority of the NACs are produced and released into the environment by anthropogenic activities. They are widely used in the production of pesticides, dyes, polymers, pharmaceuticals, explosives, agrochemicals and speciality chemicals. CNACs are used as precursors for the synthesis of important chemicals, such as drugs, herbicides and dyes. Due to their recalcitrant nature, these compounds are discharged into the environment, where they accumulate and cause serious pollution. Several NACs and the closely related CNACs are listed as priority pollutants by the U.S. Environmental Protection Agency. Many of the NACs are toxic, mutagenic and established carcinogens. They show acute ecotoxicity towards algae and aquatic invertebrates, and are phytotoxic. Several NACs are associated with methaemoglobinemia, anaemia, hepatic toxicity, neurotoxicity, nephrotoxicity, and splenic, reproductive and respiratory disorders in

*Corresponding author
Email: abafana@rediffmail.com

1 Environmental Health Division, National Environmental Engineering Research Institute (NEERI), Council of Scientific and Industrial Research (CSIR), Nagpur-440020, India

Figure 1: Aromatic ring substituents in the order of their electron withdrawing and consequently, electrophilic substitution-deactivating effect.

For citation purposes: Bafana A. Who will attack the nitroaromatics first? The enzymatic diversity and conservation. OA Biotechnology 2013 May 01;2(2):18.
humans and other animals. NACs like nitrobenzene can induce tumour in multiple tissues like lung, thyroid gland, mammary gland, liver, and kidney in animals. The intermediates like nitroso and hydroxylamino derivatives, and reactive oxygen species generated during the metabolism of NACs are implicated in the toxicity and carcinogenicity of these compounds.

As discussed above, NACs resist aerobic microbial degradation and, hence, are generally discharged unchanged from the conventional aerobic wastewater treatment plants. Physico-chemical processes can effectively remove or degrade these compounds, but they are neither economically viable nor eco-friendly. There is great interest in developing biological methods or combinatorial methods to remove NACs. Hence, microbial metabolism of NACs has become an intense field of research. Several challenges like toxicity and bioavailability of NACs need to be addressed to make the biological treatment feasible. Furthermore, there are only very few naturally occurring NACs. Hence, bacteria were not exposed to them frequently, and consequently have limited ability to metabolise them. It is noteworthy that despite a short exposure period, bacteria quickly evolved pathways for the metabolism of anthropogenic NACs. Several pathways and enzymes involved in microbial NAC degradation have been delineated, although little is known about CNAC metabolism. Since the first step of NAC/CNAC degradation is thermodynamically unfavourable, it requires deployment of specialised enzymes. The current review describes the status of recent research on such enzymes.

Discussion
Pathways for NAC degradation
NACs are susceptible to fortuitous or cometabolic transformation by a number of microbes. They can be used as terminal electron acceptors, especially under anaerobic conditions. However, a number of aerobic microbes, capable of using NACs as a carbon and nitrogen source, have also been described. Metabolism of the nitro group from NACs can be accomplished by both oxidative and reductive mechanisms:
- In the oxidative pathway, the nitro group is removed in the form of nitrate through the action of mono-oxygenase and dioxygenase enzymes (Figure 2). This mechanism, however, is not applicable to NACs carrying multiple electron-withdrawing substituents, such as TNT. TNT is transformed reductively even in the presence of oxygen.
- The reductive pathway involves reduction of the nitro group to

![Diagram of NAC degradation](image)

**Figure 2:** NAC degradation though representative dioxygenases. Refer to Table 1 for details of the enzymes.
The current review deals with the enzymes involved in the initial attack on NACs in the first two pathways, i.e. dioxygenases and nitroreductases. These enzymes have evolved relatively quickly with broad substrate specificity in response to the anthropogenic release of wide variety of NACs. Additionally, the pathways initiated by these enzymes represent the major routes of microbial NAC metabolism.

Dioxygenases
Oxygenases can overcome the electron-withdrawing nature of the nitro group and catalyse electrophilic substitution of NACs by oxygen. Oxygenases can be grouped into monooxygenases which add a single oxygen atom, and dioxygenases that incorporate both atoms of the oxygen molecule into the substrate. Both types of reactions result in the elimination of nitro group. There are two major types of dioxygenases involved in NAC metabolism. Ring-hydroxylating dioxygenases use NAD(P)H and add hydroxyl group to the aromatic ring. On the other hand, ring-cleaving dioxygenases do not require NAD(P)H and oxygenolytically cleave the ring. It is the ring-hydroxylating dioxygenases that catalyse the first step of attack on NACs, and are the subject of this review (Figure 2). Generally, these enzymes are iron–sulphur proteins made up of α & β subunits, and require specific electron transport proteins for transfer of reducing equivalent from NAD(P)H. Some dioxygenases contain only the α subunit. For example, 3-nitrobenzoate oxygenase from Co.mamonas sp. JS46 contains a single catalytic subunit.

It is proposed that dioxygenases evolved rapidly in response to anthropogenic pollution, and have broad substrate specificity due to the varied chemical structures of the pollutants (Table 1). The substrate specificity is determined mainly by the α subunit, although β subunit can...
interact with the α subunit and alter its specificity in some cases. Several researchers attempted to utilise this concept by combining α & β subunits from different sources and generating a hybrid enzyme with new catalytic capabilities, though with limited success. When the α subunit of 2-nitrotoluene dioxygenase (2NTDO) from Acidovorax sp. JS42 was combined with the β subunit of 2,4-dinitrotoluene dioxygenase (24DNTDO), the hybrid enzyme had threefold lower activity towards 2-nitrotoluene (2NT), and no activity towards 2,4-dinitrotoluene (24DNT).13

Crystal structures of nitrobenzene dioxygenase (NBDO) and the structurally related naphthalene dioxygenase (NDO) are available in the literature, based on which active-site residues responsible for substrate specificity can be predicted. Figure 5 shows alignment of the protein sequences of representative NAC-metabolising dioxygenases with different substrate specificities. The alignment clearly shows significant variation in enzymes from different sources. Some of these amino acid substitutions may be responsible for the evolution of substrate specificity of the dioxygenase family. Several researchers have experimentally verified the catalytically important amino acid residues by site-directed mutagenesis studies. NDO from Ralstonia sp. U2 cannot oxidise NACs. However, Phe350Thr and Gly407Ser mutations in the α subunit (NagAc) enabled it to degrade 2,6-dinitrotoluene (26DNT) and 2,3-dinitrotoluene (23DNT). These mutations converted NDO residues to corresponding residues of 24DNTDO from Burkholderia sp. DNT, signifying the importance of these positions in substrate specificity14. The residue at the 350 position has also been shown to be important in other cases, as described below. Interestingly, wild-type 24DNTDO itself cannot act on 23DNT (see below), which indicates that other residues also play a role15. Keenan et al.14 also reported that Leu225Arg mutant of NDO showed faster degradation of 23DNT, although Leu is conserved in DNTDO. Gly50Ser and Ala269Thr mutations reduced the NDO activity when combined with any of the above mutations, indicating that these sites are also important for structure or activity.

Crystal structure of NBDO from Comamonas sp. JS765 gave great insight into the catalytic residues involved in

Table 1: Representative NAC-metabolising dioxygenases and their substrate profile

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Accession number</th>
<th>Enzyme and source</th>
<th>NAC substrate range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAD12610</td>
<td>Naphthalene dioxygenase (NDO) from Ralstonia sp. U2</td>
<td>Nil</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>AAL76202</td>
<td>Nitrobenzene dioxygenase (NBDO) from Comamonas sp. JS765</td>
<td>NB, 3NT, 13DNB, 2NT, 4NT, 26DNT, 2CNB, 3CNB, 4CNB</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>ADQ90222</td>
<td>2-Chloronitrobenzene dioxygenase (CnbAc), from Pseudomonas stutzeri ZWLR2-1</td>
<td>2CNB</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>AAB40383</td>
<td>2-Nitrotoluene dioxygenase (2NTDO) from Acidovorax sp. JS42</td>
<td>2NT, 2CNB, 3CNB, 4CNB, 3NT, 4NT</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>AAL50021</td>
<td>2,4-Dinitrotoluene dioxygenase (24DNTDO) from Burkholderia cepacia R34</td>
<td>24DNT, 26DNT, 4NT</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>AAB09766</td>
<td>2,4-Dinitrotoluene dioxygenase (24DNTDO) from Burkholderia sp. DNT</td>
<td>4NT, 3NT, 24DNT, 26DNT</td>
<td>15</td>
</tr>
</tbody>
</table>

*NB: nitrobenzene; DNB: dinitrobenzene; NT: nitrotoluene; DNT: dinitrotoluene; CNB: chloronitrobenzene. Numbers show the positions of the substituents.
Table 2: Representative NAC-metabolising nitroreductases and their substrate profile

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Accession number</th>
<th>Enzyme and source</th>
<th>NAC substrate range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAC43450</td>
<td>Nitroreductase (NfsA) from E. coli</td>
<td>Nitrofurazone</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>ABA55818</td>
<td>p-Chloronitrobenzene nitroreductase (PcnBC) from Pseudomonas putida 2NL73</td>
<td>NB, 3NP, 3CNB, 24DNT</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>BAF56676</td>
<td>2-Nitrobenzoate nitroreductase (NbaA) from Pseudomonas fluorescens KU-7</td>
<td>2NBA, 24DNBA</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>AAG01540</td>
<td>p-Nitrobenzoate nitroreductases (PnBA) from Pseudomonas putida strain TW3</td>
<td>4NBA</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>AAM95986</td>
<td>Nitroreductase (PnrA) from Pseudomonas putida JMP134</td>
<td>246TNT, 24DNT, 35DNT, 3NT, 3NBA, 4NBA</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>YP_293138</td>
<td>m-Nitrophenol nitroreductase (MnpA) from Cupriavidus necator JMP134</td>
<td>NB, 3NBA, 4NBA, 3CNB, 4CNB, 2CNB, 4NT, 3NT, 2NP, 4NP</td>
<td>28</td>
</tr>
</tbody>
</table>

*NP: nitrophenol; NBA: nitrobenzoate; DNBA: dinitrobenzoate; TNT: trinitrotoluene. Rest of the conventions are same as those in Table 1.

NBDO is highly specific towards the nitro-substituted position and produces chlorocatechols from CNBs. Substitutions at positions 258 and 350 increased oxidation at the chloro-substituted carbon. Although Asn258 is proposed to be critical for coordinating with nitro group, the Asn258Val substitution did not significantly affect CNB degradation.

2NTDO oxidises the nitro-substituted carbon and produces methylcatechols from nitrotoluenes. However, Ile350Phe substitution, which makes it similar to NDO at this position, resulted in preferential oxidation of the methyl group, producing nitrobenzyl alcohol. Similarly, Asn258Val substitution in 2NTDO significantly reduced oxidation of nitrotoluenes. This is unlike NBDO, indicating that dispensability of Asn258 depends on other residues.

Both *Burkholderia cepacia* R34 and *Burkholderia* sp. DNT can use 24DNT as the carbon source by 24DNTDO (R34-24DNTDO and DNT-24DNTDO respectively). Although both of them share significant sequence similarity, they exhibit overlapping but distinct substrate profile (Figure 5, Table 1). Mutagenesis experiments were carried out in these enzymes to identify the residues responsible for the substrate specificity. Mutagenesis of the α subunit of DNT-24DNTDO resulted in new metabolic capabilities. Ile204Leu and Ile204Tyr mutants could degrade 23DNT and 2,5-dinitrotoluene (25DNT). The mutants also showed enhanced activity on the wild-type substrates. Similar mutagenesis experiments were carried out in R34-24DNTDO by Keenan et al. They showed that mutations at Val350 significantly increased activity towards o-nitrophenol and m-nitrophenol, but they abolished the wild-type activity towards 24DNT.

To summarise, several residues have been predicted or shown to be important in dioxygenase activity on NACs. Substitution mutations in these residues show different effects.

Review

NAC metabolism. NBDO contains a Rieske Fe-S centre and an Fe atom at the active site. The β subunit of NBDO does not interact with the active-site iron atom, and may play only a structural role. The Rieske cluster is coordinated by Cys79, His81, Cys99 and His102, while the active-site Fe is coordinated by Asp360, His206 and His211. A hydrogen-bonding network connects the Rieske centre of one α subunit to active-site Fe in the neighbouring α subunit. In the Rieske centre, His102 is hydrogen bonded to Asp203, which, in turn, is hydrogen bonded to His206, an active-site Fe ligand. All the above residues involved in Rieske centre and active-site Fe coordination, and hydrogen bond networking are totally conserved in all the sequences in Figure 5. Thus, all these residues are catalytically essential, but cannot be involved in substrate specificity. Seventeen residues line the active site of NBDO, among which 5 are different from those in closely related NDO, and might be responsible for changing the substrate specificity towards NACs. These residues are Gly204, Phe251, Asn258, Phe293 and Ile350. All the residues show significant variation in different dioxygenases as expected (Figure 5). Asn258, which forms a hydrogen bond to the nitro group of the nitro-substituted carbon of the substrates, is a key residue for activity on NACs. However, it can be replaced with other residues in other dioxygenases as seen in Figure 5.

The importance of some of the above residues in determining the specificity of NBDO towards chloronitrobenzenes (CNBs) was studied by targeted mutagenesis experiments. To summarise, several residues have been predicted or shown to be important in dioxygenase activity on NACs. Substitution mutations in these residues show different effects.
Figure 5: Alignment of the α subunits form the NAC-metabolising dioxygenases. Conserved residues are shown against grey background. Residues involved in binding of Rieske centre, active-site Fe and substrate are in bold. More residues are predicted to be involved, but it remains to be demonstrated experimentally. Refer to Table 1 for the details of dioxygenases compared in this alignment.
in different enzymes, indicating that they are context-dependent. Hence, studies involving multiple mutagenesis at different sites are required to understand the mutual interaction of these positions.

**Nitroreductases**

Nitroreductases are flavoproteins that use NAD(P)H for the reduction of nitro group. It results in the formation of nitroso, hydroxylamino and amino groups, which are electron-donating substituents and activate the aromatic nucleus for further electrophilic attack by a dioxygenase (Figure 3). Nitroreductases are widely distributed among various organisms. Based on the sensitivity to oxygen and electron transfer pattern, nitroreductases may be grouped into type I or oxygen-insensitive, and type II or oxygen-sensitive enzymes. Type I nitroreductases catalyse the reduction of nitro group by the addition of electron pairs from NAD(P)H to produce hydroxylamino and amino derivatives via intermediate nitroso derivatives. Type II enzymes catalyse single-electron transfer to produce a nitro anion radical, which can reduce nitro group to a nitroso group. Under aerobic conditions, however, the nitro anion radical reacts with oxygen and produces superoxide anion in a futile cycle.

Crystal structure of the *Escherichia coli* nitroreductase (NfsA) has been solved, based on which catalytically important amino acid residues have been predicted. Several mutagenesis and structural modelling studies have also identified key residues in the nitroreductases. From the crystal structure, Kobori et al. predicted the residues Arg15, Ser39, Glu67, Tyr128, Gly130, Gly131 and Lys167 to be involved in the binding of FMN cofactor, while Arg203 was proposed to bind NADPH. In agreement with this, an Arg203Ala mutant showed significantly lower affinity for NADPH. However, these residues do not seem to be well conserved across different nitroreductases (Figure 6).

This indicates that nitroreductases are a heterogeneous class of enzymes...

---

**Figure 6:** Alignment of the NAC-metabolising nitroreductases. The sequence conservation is very low across different nitroreductases. Refer to Table 2 for the details of nitroreductases compared in this alignment.
with structural differences. Further, the residues lining the active site (Arg15, Ser39, Ser40, Gln67, Arg133, Lys167 and Arg225, Tyr199, Tyr200, Thr219, Lys222 and Ser224) were proposed to cause the differences in substrate profile.

Based on the structural modelling, Iwaki et al.\textsuperscript{23} proposed that a divalent metal ion, Ni\textsuperscript{2+}, mediated the interaction between FMN and 2-Nitrobenzoate Nitroreductase (NbaA) from \textit{Pseudomonas fluorescens} \textit{KU}-7. Mutations in any of the three predicted metal ion binding-site residues (Asn40Ala, Asp76Ala, and Glu133Ala) resulted in complete loss of the enzyme activity. Similarly, His69Ala replacement resulted in reduction of the nitroreductase activity, which is in agreement with the predicted NAD(P)H-binding role of His69. Kim et al.\textsuperscript{23} carried out a series of mutagenesis experiments to identify key residues in NbaA. The truncation mutations Δ65–74 and Δ193–216 markedly affected the enzyme activity. Site-directed mutagenesis indicated that the cysteines at positions 39, 103, 141, and 194 were important in determining the enzyme specificity.

Various studies have identified different residues to be involved in nitroreductase activity on NACs. Unfortunately, no clear consensus has emerged so far. This is probably because nitroreductases are heterogeneous groups, clubbed together by their function and not by their structure.

Conclusion

Both nitroreductases and dioxygenases evolved rapidly in response to anthropogenic pollutants such as NACs. A lot of factors might have played a role in this phenomenon. The broad substrate specificity of these enzymes and dependence of the substrate specificity on relatively few amino acid residues are notable in this context. Further, these enzymes may not have been essential for cell survival for such rapid evolution to occur. Thus, the physiological function of these enzymes may be dispensable, redundant or at least less essential. Mutagenesis studies in dioxygenases have identified the catalytically important residues. This information has been utilised by many researchers to generate enzymes with novel NAC substrate profile. Nitroreductases seem to be relatively heterogeneous group, and different residues are catalytically important in different enzymes. Crystal structures of a few more nitroreductases need to be solved before generalised inferences about NAC specificity can be drawn. The same is true for monooxygenases involved in NAC metabolism. Looking at the recent progress, dioxygenases hold a lot of promise for NAC degradation. In the near future, the structure–function relationship of the dioxygenases will be better understood. This will allow researchers to generate dioxygenases with desired specificity at will and, of course, at the mercy of the enzyme flexibility.

References

15. Leungsakul T, Keenan BG, Yin H, Smets BF, Wood TK. Saturation mutagenesis of...