Biotransformation of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) by a Rabbit Liver Cytochrome P450: Insight into the Mechanism of RDX Biodegradation by Rhodococcus sp. Strain DN22

Bharat Bhushan, Sandra Trott, Jim C. Spain, Annamaria Halasz, Louise Paquet, and Jalal Hawari

Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec H4P 2R2, Canada, and U.S. Air Force Research Laboratory, Tyndall Air Force Base, Florida 32403

Received 18 September 2002/Accepted 12 December 2002

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a cyclic nitramine explosive commonly used for military and commercial purposes worldwide. The extensive manufacturing, use, and disposal of RDX have resulted in severe environmental contamination (7, 14). RDX is toxic, mutagenic, and carcinogenic for humans and other biological systems (9, 19); hence, there is an urgent need for safe removal of this compound from the environment. In several reports workers have described biodegradation of cyclic nitramine explosives, such as RDX, under both aerobic (2, 3, 5, 18) and anaerobic (8, 10, 12, 13, 15, 20) conditions, but little information is available on the initial reactions that led to its synthesis were not known. In the present study, we produced and purified the unknown metabolite by biotransformation of RDX with Rhodococcus sp. strain DN22 and identified the molecule as 4-nitro-2,4-diazabutanal using nuclear magnetic resonance and elemental analyses. Furthermore, we tested the hypothesis that a cytochrome P450 enzyme was responsible for RDX biotransformation by strain DN22. A cytochrome P450 2B4 from rabbit liver catalyzed a very similar biotransformation of RDX to 4-nitro-2,4-diazabutanal. Both the cytochrome P450 2B4 and intact cells of Rhodococcus sp. strain DN22 catalyzed the release of two nitrite ions from each reacted RDX molecule. A comparative study of cytochrome P450 2B4 and Rhodococcus sp. strain DN22 revealed substantial similarities in the product distribution and inhibition by cytochrome P450 inhibitors. The experimental evidence led us to propose that cytochrome P450 2B4 can catalyze two single electron transfers to RDX, thereby causing double denitrification, which leads to spontaneous hydrolytic ring cleavage and decomposition to produce 4-nitro-2,4-diazabutanal. Our results provide strong evidence that a cytochrome P450 enzyme is the key enzyme responsible for RDX biotransformation by Rhodococcus sp. strain DN22.

A unique metabolite with a molecular mass of 119 Da (C₂H₅N₃O₃) accumulated during biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by Rhodococcus sp. strain DN22 (D. Fournier, A. Halasz, J. C. Spain, P. Fiurasek, and J. Hawari, Appl. Environ. Microbiol. 68:166-172, 2002). The structure of the molecule and the reactions that led to its synthesis were not known. In the present study, we produced and purified the unknown metabolite by biotransformation of RDX with Rhodococcus sp. strain DN22 and identified the molecule as 4-nitro-2,4-diazabutanal using nuclear magnetic resonance and elemental analyses. Furthermore, we tested the hypothesis that a cytochrome P450 enzyme was responsible for RDX biotransformation by strain DN22. A cytochrome P450 2B4 from rabbit liver catalyzed a very similar biotransformation of RDX to 4-nitro-2,4-diazabutanal. Both the cytochrome P450 2B4 and intact cells of Rhodococcus sp. strain DN22 catalyzed the release of two nitrite ions from each reacted RDX molecule. A comparative study of cytochrome P450 2B4 and Rhodococcus sp. strain DN22 revealed substantial similarities in the product distribution and inhibition by cytochrome P450 inhibitors. The experimental evidence led us to propose that cytochrome P450 2B4 can catalyze two single electron transfers to RDX, thereby causing double denitrification, which leads to spontaneous hydrolytic ring cleavage and decomposition to produce 4-nitro-2,4-diazabutanal. Our results provide strong evidence that a cytochrome P450 enzyme is the key enzyme responsible for RDX biotransformation by Rhodococcus sp. strain DN22.

In the present study, we evaluated the role of a cytochrome P450 enzyme in RDX biotransformation by using cytochrome P450 2B4 (EC 1.14.14.1) from rabbit liver. We carried out this study with a model system in order to identify the metabolites produced and to understand the initial reaction mechanism(s) involved in the degradation process. We also purified and characterized the dead end metabolite C₂H₅N₃O₃ produced during RDX degradation by Rhodococcus sp. strain DN22. We compared cytochrome P450 2B4-catalyzed RDX biotransformation with RDX biotransformation by Rhodococcus sp. strain DN22 to obtain insight into the RDX biotransformation pathways of the two systems.

MATERIALS AND METHODS

Chemicals. Commercial grade RDX (purity, >99%) and [³⁵N]RDX (purity, >98%) were provided by Defense Research and Development Canada, Valcartier, Quebec, Canada. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) was obtained from SRI International, Menlo Park, Calif. Pentoxysorulfin, cytochrome c, NADPH, 1-aminobenzoazolizole, and ellipticine were purchased from Sigma Chemicals, Canada. Methyleneendinitramine, 2-methyl-1,2-di-3-pyrild-1-propanone (metyrapone), and phenylhydrazine were purchased from Aldrich, Canada. ¹⁸O-labeled water (enrichment, 95 atom%) and ¹⁸O-labeled mo-
**Title:** Biotransformation of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) by a Rabbit Liver Cytochrome P450: Insight into the Mechanism of RDX Biodegradation by Rhodococcus sp. Strain DN22

**Abstract:**

1. **REPORT DATE**
   2002

2. **REPORT TYPE**

3. **DATES COVERED**
   00-00-2002 to 00-00-2002

4. **TITLES AND SUBTITLE**

5. **AUTHOR(S)**

6. **PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
   U.S. Air Force Research Laboratory, Tyndall Air Force Base, FL, 32403

7. **SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

8. **REPORT NUMBER**

9. **SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

10. **SPONSOR/MONITOR’S ACRONYM(S)**

11. **SPONSOR/MONITOR’S REPORT NUMBER(S)**

12. **DISTRIBUTION/AVAILABILITY STATEMENT**
    Approved for public release; distribution unlimited

13. **SUPPLEMENTARY NOTES**
    APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2003, p. 1347-1351, vol 69, No. 3

14. **ABSTRACT**

15. **SUBJECT TERMS**

16. **SECURITY CLASSIFICATION OF:**
    a. **REPORT** unclassified
    b. **ABSTRACT** unclassified
    c. **THIS PAGE** unclassified

17. **LIMITATION OF ABSTRACT**
    Same as Report (SAR)

18. **NUMBER OF PAGES**
    6

19a. **NAME OF RESPONSIBLE PERSON**

---

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18
TABLE 1. Elemental analysis of 4-nitro-2,4-diazabutanal

<table>
<thead>
<tr>
<th>Value</th>
<th>% Nitrogen (SD)</th>
<th>% Carbon (SD)</th>
<th>% Hydrogen (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured</td>
<td>34.25 (0.028)</td>
<td>20.27 (0.021)</td>
<td>4.19 (0.005)</td>
</tr>
<tr>
<td>Calculated</td>
<td>35.29</td>
<td>20.17</td>
<td>4.23</td>
</tr>
</tbody>
</table>

The suggested molecular formula is C₆H₁₂N₃O₃ (molecular weight, 119.08).

TABLE 2. Molecular structure of 4-nitro-2,4-diazabutanal as elucidated by NMR analysis in d₆-dimethyl sulfoxide

<table>
<thead>
<tr>
<th>¹H signals</th>
<th>¹³C signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>H²: δ 8.06 (d, 1H, CHO)</td>
<td>C₆: δ 165.05 (CHO)</td>
</tr>
<tr>
<td>H³: δ 4.70 (d, 2H, CH₂)</td>
<td>C⁷: δ 47.73 (CH₂)</td>
</tr>
<tr>
<td>H⁴: δ 8.93 (s, broad, 1H, NH)</td>
<td></td>
</tr>
</tbody>
</table>
water, thus indicating that this proton was not coupled with other protons. Carbon spectra had two signals at 47.73 and 165.05 ppm, which were assigned to CH₂ and CHO groups, respectively, as confirmed by a DEPT 135 experiment. All of the results described above and especially the two distinct NH rates of 9.0 and 2.8 nmol min⁻¹ release of nitrite and HCHO from RDX at the expense of the prosthetic heme group or binds at a nearby site (e.g., heme ligand position 6) and undergoes single-electron reduction by oxidizing the Fe²⁺. In addition to the substrate, the Fe²⁺ of the prosthetic heme is also a binding site for O₂. Therefore, O₂ competes with the substrate for binding at the same site, and this provides a probable explanation for the inhibition of RDX biotransformation by cytochrome P450 2B4 under aerobic conditions. In the case of Rhodococcus sp. strain DN22, although aerobic conditions are needed for bacterial growth, RDX transformation does not involve the incorporation of molecular oxygen. When RDX was incubated with strain DN22 in the presence of ¹⁸O₂, we detected the metabolite, 4-nitro-2,4-diazabutanal, without ¹⁸O (5). In the presence of H₂¹⁸O we detected 4-nitro-2,4-diazabutanal (molecular weight, 121; [M + 2]⁺ with one ¹⁸O atom. Similarly, in the case of cytochrome P450 2B4, we observed that ¹⁸O was incorporated into 4-nitro-2,4-diazabutanal from H₂¹⁸O but not from ¹⁸O₂.

Inhibition study. A comparative inhibition study was carried out with cytochrome P450 2B4 and Rhodococcus sp. strain DN22 by using various cytochrome P450 inhibitors. At a concentration of 200 uM, all of the inhibitors tested inhibited the RDX biotransformation activity of cytochrome P450 2B4, as well as the activity of Rhodococcus sp. strain DN22 (Table 4).

These inhibitors act on the exposed prosthetic heme group of cytochrome P450 (16). The inhibition study provided additional support for the hypothesis that the enzyme responsible for the RDX biotransformation activity of Rhodococcus sp. strain DN22 is cytochrome P450. Coleman et al. (4) previously reported that metyrapone and menadione strongly inhibited the RDX biotransformation activity of Rhodococcus sp. strain DN22, which indicated that the cytochrome P450 enzyme was probably involved in RDX biotransformation by Rhodococcus sp. strain DN22. Seth-Smith et al. (18) also described metyrap-

---

**TABLE 3. Carbon and nitrogen mass balance and stoichiometry of reactants consumed and metabolites produced during RDX biotransformation catalyzed by rabbit liver cytochrome P450 2B4 (100 μg/ml) at pH 7.2 and 37°C for 2 h under anaerobic conditions**

<table>
<thead>
<tr>
<th>Reactants or metabolite</th>
<th>Amt (nmol)</th>
<th>% Carbon recovery</th>
<th>% Nitrogen recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDX</td>
<td>84</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NADPH</td>
<td>108</td>
<td>NA b</td>
<td>NA b</td>
</tr>
<tr>
<td>Metabolites produced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite (NO₂⁻)</td>
<td>162</td>
<td>NA</td>
<td>32.0</td>
</tr>
<tr>
<td>4-Nitro-2,4-diazabutanal</td>
<td>68</td>
<td>54</td>
<td>40</td>
</tr>
<tr>
<td>Formaldehyde (HCHO)</td>
<td>69</td>
<td>27</td>
<td>NA</td>
</tr>
<tr>
<td>Ammonium (NH₄⁺)</td>
<td>65</td>
<td>NA</td>
<td>13</td>
</tr>
</tbody>
</table>

a Recovery values were calculated from the total carbon and nitrogen masses in the biotransformed RDX (84 nmol). The initial amounts of RDX and NADPH were 100 and 150 nmol, respectively. The data are means for duplicate experiments, and the standard deviations were within 6% of the mean absolute values (n = 2). The levels of total mass recovery were 81% for carbon and 85% for nitrogen.

b NA, not applicable.

---

**FIG. 1.** Time course study of biotransformation of RDX catalyzed by rabbit liver cytochrome P450 2B4. The data are means of duplicate experiments, and the standard deviations were within 6% of the mean absolute values (n = 2). Symbols: ●, RDX; ○, NADPH; ▽, NO₂⁻; ◇, HCHO.

---

A comparative inhibition study was carried out with cytochrome P450 2B4 and Rhodococcus sp. strain DN22 by using various cytochrome P450 inhibitors. At a concentration of 200 μM, all of the inhibitors tested inhibited the RDX biotransformation activity of cytochrome P450 2B4, as well as the activity of Rhodococcus sp. strain DN22 (Table 4).

These inhibitors act on the exposed prosthetic heme group of cytochrome P450 (16). The inhibition study provided additional support for the hypothesis that the enzyme responsible for the RDX biotransformation activity of Rhodococcus sp. strain DN22 is cytochrome P450. Coleman et al. (4) previously reported that metyrapone and menadione strongly inhibited the RDX biotransformation activity of Rhodococcus sp. strain DN22, which indicated that the cytochrome P450 enzyme was probably involved in RDX biotransformation by Rhodococcus sp. strain DN22. Seth-Smith et al. (18) also described metyrap-
TABLE 4. Effects of cytochrome P450 inhibitors on RDX biotransformation activities of rabbit liver cytochrome P450 2B4 and Rhodococcus sp. strain DN22

<table>
<thead>
<tr>
<th>Inhibitor (200 µM)</th>
<th>Cytochrome P450 2B4 (100 µg/ml)</th>
<th>Rhodococcus sp. strain DN22 (5 mg (wet wt)/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no inhibitor)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>75 ± 4</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>66 ± 8</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>70 ± 7</td>
<td>77 ± 5</td>
</tr>
<tr>
<td>1-Aminobenzotriazole</td>
<td>55 ± 7</td>
<td>43 ± 7</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>82 ± 6</td>
<td>48 ± 5</td>
</tr>
</tbody>
</table>

a The RDX transformation activity without inhibitor was considered 0% inhibition; the 100% activities for cytochrome P450 2B4 and Rhodococcus sp. strain DN22 were 9.0 nmol min⁻¹ mg⁻¹ of protein⁻¹ and 0.12 nmol min⁻¹ mg⁻¹ of biomass⁻¹, respectively.

b A washed and centrifuged cell pellet was used as wet biomass.

c Carbon monoxide was bubbled through the aqueous phase and headspace for 60 s in sealed vials.

The values are means ± standard deviations (n = 3).

one-mediated inhibition of RDX biotransformation by Rhodococcus rhodochrous strain 11Y expressing a cytochrome P450 gene.

Stoichiometry and mass balance of RDX biotransformation by cytochrome P450 2B4. We found that 84 nmol of RDX was transformed at the expense of 108 nmol of NADPH, suggesting that the stoichiometry is 1:1. The remaining 24 nmol of NADPH was presumably consumed by cytochrome P450 reductase present in the cytochrome P450 2B4 preparation (see above), and/or a small fraction of NADPH could also have reacted directly with RDX or its metabolites. The total recovered carbon mass balance was 81% and was distributed as 4-nitro-2,4-diazabutanal (54%) and HCHO (27%) (Table 3), and the total nitrogen mass recovery was 85% and was distributed as nitrite (32%), 4-nitro-2,4-diazabutanal (40%), and ammonium (13%) (Table 3).

Based on the product distribution and mass balance of RDX transformation by cytochrome P450 2B4, we concluded that of the six nitrogen atoms and three carbon atoms present in one RDX molecule, two nitrogen atoms were recovered as nitrite ions, whereas three nitrogen atoms and two carbon atoms were recovered in a dead end metabolite, 4-nitro-2,4-diazabutanal. The remaining one nitrogen atom and one carbon atom were present in NH₄⁺ and HCHO (Table 3).

Taken together, the results described above provided several lines of evidence which support the hypothesis that a cytochrome P450 type of enzyme is responsible for RDX transformation by Rhodococcus sp. strain DN22. A comparison of the biotransformation of RDX catalyzed by rabbit liver cytochrome P450 2B4 and the biotransformation of RDX by Rhodococcus sp. strain DN22 (5) revealed that the product distributions and stoichiometries were strikingly similar. In a previous study, cytochrome P450s from R. rhodochrous participated in the degradation of 2-ethoxyphenol and 4-methoxybenzoate (11). Our enzyme inhibition studies are consistent with those of Coleman et al. (4) regarding involvement of cytochrome P450 in RDX degradation by Rhodococcus sp. strain DN22. Finally, Seth-Smith et al. (18) recently provided strong molecular evidence that the constitutively expressed cytochrome P450-like gene xplA from R. rhodochrous strain 11Y is responsible for RDX degradation.

Proposed mechanism. Based on stoichiometry and mass balance studies and the key observation that ¹⁸O was incorporated into 4-nitro-2,4-diazabutanal from H₂¹⁸O but not from ¹⁸O₂, we suggest a plausible mechanism for the initial biotransformation of RDX by cytochrome P450 2B4. According to this mechanism, cytochrome P450 2B4 catalyzes sequential transfer of two single electrons to RDX; the first electron causes denitration to form compound I (Fig. 2), and the second electron causes a second denitration to produce compound II. The latter product is unstable in water and should be hydrolyzed by incorporation of two ¹⁸OH groups from two H₂¹⁸O molecules to give hypothetical compound III. The spontaneous decomposition of compound III produces 4-nitro-2,4-diazabutanal (Fig. 2).

In conclusion, we provide direct biochemical evidence that a rabbit liver cytochrome P450 catalyzed the biotransformation of RDX. We propose a plausible mechanism for the initial enzymatic attack on RDX by cytochrome P450, which eventually produced the same products as the products produced by Rhodococcus sp. strain DN22. This mechanism is consistent with our observation that two electrons (approximately one NADPH molecule) were consumed and two nitrite ions were produced per reacted RDX molecule. Several lines of evidence in the present study and the results of previous studies (4, 5, 18) support the conclusion that an enzyme(s) belonging to the cytochrome P450 family is responsible for RDX biotransformation by Rhodococcus sp. strain DN22.

ACKNOWLEDGMENTS

We thank the Natural Sciences and Engineering Research Council (NSERC) and the National Research Council (NRC) of Canada for...
 awarding a visiting fellowship to B. Bhushan and the U.S. Strategic Environmental Research and Development Program (SERDP) for funding this project (grant CU 1213). We also thank the Department of National Defense, Canada, for its support.

We especially thank S. Bilodeau, P. V. M. Tan, H. Dinel, and F. Bélanger-Gariépy of the University of Montreal and Fanny Montreuil of BRI, Montreal, Quebec, Canada, for conducting NMR studies and elemental analysis of 4-nitro-2,4-diazabutanal. We sincerely acknowledge the analytical and technical support of C. Beaulieu, C. Groom, A. Corriveau, and S. Deschamps. Special thanks also go to S. Nishino for her technical assistance. We thank Neil Bruce, University of York, York, United Kingdom, for sharing unpublished results concerning RDX biodegradation by R. rhodochrous. Finally, we thank N. Coleman for providing Rhodococcus sp. strain DN22.

REFERENCES