

Mechanism of xanthine oxidase catalyzed biotransformation of HMX under anaerobic conditions

Bharat Bhushan,^a Louise Paquet,^a Annamaria Halasz,^a Jim C. Spain,^b and Jalal Hawari^{a,*}

^a Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Que., Canada H4P 2R2

^b US Air Force Research Laboratory, 139 Barnes Dr, Tyndall AFB, FL 32403, USA

Received 16 May 2003

Abstract

Enzyme catalyzed biotransformation of the energetic chemical octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) is not known. The present study describes a xanthine oxidase (XO) catalyzed biotransformation of HMX to provide insight into the biodegradation pathway of this energetic chemical. The rates of biotransformation under aerobic and anaerobic conditions were 1.6 ± 0.2 and 10.5 ± 0.9 nmol h⁻¹ mg protein⁻¹, respectively, indicating that anaerobic conditions favored the reaction. The biotransformation rate was about 6-fold higher using NADH as an electron-donor compared to xanthine. During the course of reaction, the products obtained were nitrite (NO₂⁻), methylenedinitramine (MDNA), 4-nitro-2,4-diazabutanal (NDAB), formaldehyde (HCHO), nitrous oxide (N₂O), formic acid (HCOOH), and ammonium (NH₄⁺). The product distribution gave carbon and nitrogen mass-balances of 91% and 88%, respectively. A comparative study with native-, deflavo-, and desulfo-XO and the site-specific inhibition studies showed that HMX biotransformation occurred at the FAD-site of XO. Nitrite stoichiometry revealed that an initial single N-denitration step was sufficient for the spontaneous decomposition of HMX. Crown Copyright © 2003 Published by Elsevier Science (USA). All rights reserved.

Keywords: Biotransformation; Cyclic nitramine; HMX; N-denitration; Xanthine oxidase

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) is a widely used energetic chemical for military and commercial purposes. The high scale manufacture, use, and disposal of this chemical have resulted in alarming levels of soil and groundwater contamination [1,2]. HMX is toxic to the biological systems and human health [3–7]. Hence there is a need for its safe removal from the contaminated sites. Several reports are available regarding biodegradation of HMX under aerobic [8] and anaerobic [9–11] conditions. However, no literature is available so far in regard to the enzymatic biotransformation of HMX. Such information is necessary to understand the mechanism(s) of biodegradation of RDX and HMX in anaerobic environments such as marine/estuarine water and sediments and thus to optimize the process for its mineralization. Unlike nitroaromatic compounds, cyclic nitramines lack aromatic electronic stability. Therefore, an initial enzymatic or chemical attack on one of its

N–NO₂ or –CH₂– groups should be sufficient for the ring cleavage and subsequent decomposition [12,13].

Xanthine oxidase (EC 1.1.3.22) is a metallo-flavo enzyme composed of two identical subunits, each subunit has a mol. wt. of 145 kDa and contains one each of molybdenum-site and FAD-site and two Fe–S centers [14]. Although XO has broad specificity for a wide variety of substrates, its physiological role is believed to be in purine catabolism where it catalyzes the oxidation of hypoxanthine to xanthine and then xanthine to uric acid with concomitant reduction of molecular oxygen [14]. In recent years, much attention has been given to XO catalyzed reduction of inorganic nitrate and nitrite in regard to pathogenesis of ischemia/reperfusion injury in mammals [15–18]. The present, *in vitro*, study could provide an understanding about how organisms that produce XO or similar enzyme(s) biotransform and thus counter the toxic effects of cyclic nitramine compounds such as HMX in the environment. To the best of our knowledge, no study has been reported so far in regard to the XO catalyzed biotransformation of a cyclic nitramine compound.

* Corresponding author. Fax: +514-496-6265.

E-mail address: jalal.hawari@nrc.ca (J. Hawari).

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE 2003		2. REPORT TYPE		3. DATES COVERED 00-00-2003 to 00-00-2003	
4. TITLE AND SUBTITLE Mechanism of Xanthine Oxidase Catalyzed Biotransformation of HMX Under Anaerobic Conditions				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) National Research Council of Canada, Biotechnology Research, Insitute, 6100 Royalmount Ave, Montreal, Quebec H4P 2R2, Canada,				8. PERFORMING ORGANIZATION 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					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

The presents study describes the initial reaction(s) involved in the biotransformation of HMX catalyzed by xanthine oxidase (XO). Several enzyme-modifications and site-specific inhibition studies were carried out in order to gain insights into the involvement of specific redox-center(s) of XO in the biotransformation of HMX. Carbon and nitrogen mass-balances, and product stoichiometry were also determined and a biotransformation mechanism is proposed.

Materials and methods

Chemicals. Commercial grade HMX (purity >99 %) was provided by the Defense Research and Development Canada (DRDC), Valcartier, Que., Canada. 4-Nitro-2,4-diazabutanal was obtained from SRI International, Menlo Park, CA, USA. Methylene-dinitramine (MDNA) was obtained from the rare chemical department of Aldrich, Oakville, Ont., Canada. Other chemicals, i.e., NADH, xanthine, uric acid, flavin adenine dinucleotide (FAD), allopurinol, diphenyleneiodonium chloride (DPI), and formaldehyde were purchased from Sigma Chemicals, Canada. Standard nitrous oxide (N_2O , 980 ppm by mole) was obtained from Scott specialty gases, Sarnia, Ont., Canada.

Enzyme preparation. Xanthine oxidase (EC 1.1.3.22) from buttermilk was obtained from Sigma chemicals, Canada. The enzyme suspension was mixed with five volumes of potassium phosphate buffer (50 mM, pH 7.0) and filtered through a Biomax-5K membrane (Sigma chemicals) twice before resuspending in the same buffer. The protein concentration was measured by bicinchoninic acid (BCA) kit (Sigma Chemicals) as per company instructions using bovine serum albumin as standard. The native xanthine oxidase (XO) activity was estimated using the standard method by measuring the increase in absorbance at 295 nm as a function of rate of oxidation of xanthine to uric acid.

Enzyme modifications and reconstitution. The deflavo- and reconstituted-XO were prepared as described by Komai et al. [19]. Reconstitution was carried out by incubating the deflavo-XO with 200 μM FAD in a potassium phosphate buffer (50 mM, pH 7.0) for 1 h at 30 °C. The unbound FAD was removed by washing the enzyme with the same buffer using Biomax-5K membrane centrifuge filter units. Preparation of desulfo-XO and its subsequent reactivation were done by the methods described by Massey and Edmondson [20]. The reactivation of desulfo-XO was performed by incubating it with a 10 mM solution of Na_2S at 30 °C in a potassium phosphate buffer (50 mM, pH 7.0) for 90 min. After incubation period, the free Na_2S was removed as described above.

Biotransformation assays. HMX biotransformation by the native-, desulfo-, and deflavo-forms of xanthine oxidase was performed under both aerobic (in the presence of air) and anaerobic conditions in 6 ml glass vials. Anaerobic conditions were created by purging all the solutions with argon three times (10 min each time at 10 min intervals) and replacing the headspace air with argon in a sealed vial. Each assay vial had 1 ml of an assay mixture containing HMX (40 μM), NADH (150 μM) or xanthine (150 μM), and xanthine oxidase (1 mg ml^{-1}) in a potassium phosphate buffer (50 mM), pH 7.0. Reactions were performed at 30 °C. Three different controls were prepared by omitting either enzyme, NADH or both from the assay mixture. Samples from the liquid and gas phase in the vials were withdrawn periodically to analyze for HMX and the biotransformed products as described in analytical procedures (discussed below). NADH was determined as described previously [12]. HMX biotransformation activity of the enzyme was expressed as $\text{nmol h}^{-1} \text{mg protein}^{-1}$ unless otherwise stated.

In order to detect and quantify the HMX metabolites which were produced in trace amounts, the energetic chemical was added to the reaction mixture at a final concentration of 11.8 mg L^{-1} (above

saturation level) from a 10,000 mg L^{-1} stock solution made in acetone. The aqueous solubility of HMX at 25 °C has been reported as 6.6 mg L^{-1} [21].

Biotransformation of the intermediates. In order to determine the fate of transient HMX intermediates, i.e., methylenedinitramine (40 μM), 4-nitro-2,4-diazabutanal (40 μM), and HCHO (100 μM), the standard intermediates were incubated separately with XO under the same experimental conditions as used for the biotransformation of HMX under anaerobic conditions. Samples from the liquid and gas phase in the vials were withdrawn periodically to analyze the residual compound and the biotransformed products as described in analytical procedures.

Enzyme inhibition studies. This study was performed by incubating XO with increasing concentrations of site-specific inhibitors, i.e., allopurinol or DPI for 30 min at room temperature. Thereafter, HMX biotransformation activity of the treated enzyme was determined by using two different electron-donors, i.e., NADH and xanthine. The nitrite mediated inhibition of HMX biotransformation was determined by assaying the XO against HMX in the presence of increasing NaNO_2 concentrations. The type of inhibition was inferred from the standard Lineweaver–Burk plots.

Analytical procedures. HMX and its nitroso-derivatives, i.e., octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine, octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine, octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine, octahydro-1,3,5-trinitroso-7-nitro-1,3,5,7-tetrazocine, and octahydro-1,3,5,7-tetranitroso-1,3,5,7-tetrazocine were analyzed by LC/MS as described previously [10,11].

Methylene-dinitramine and 4-nitro-2,4-diazabutanal were analyzed and quantified by a Waters HPLC system equipped with a photodiode array (PDA) detector (model 2996), a model 600 pump, a model 717 plus injector, and a temperature control module. A 50 μl of sample was injected. The separation was made on an interaction chromatography ION-310 column (15 cm \times 6.5 mm) supplied by Cobert Associates, St. Louis, MO, USA. The mobile phase was an acidified water (385 μl sulfuric acid in 4 L of deionized water, pH 2.0) at a flow rate of 0.6 ml min^{-1} and 35 °C. The detector was set to scan from 200 to 3250 nm wavelength and chromatograms were extracted at a wavelength of 225 nm.

Nitrite (NO_2^-), ammonium (NH_4^+), formaldehyde (HCHO), and nitrous oxide (N_2O) were analyzed by the previously reported methods [12,13,22]. Formic acid (HCOOH) was measured using an HPLC from Waters (pump model 600 and an autosampler model 717 plus) equipped with a conductivity detector (model 430). The separation was made on a DIONEX IonPac AS15 column (2 \times 250 mm). The mobile phase was 30 mM KOH at a flow rate of 0.4 ml min^{-1} and 40 °C. The detection of formic acid was enhanced by reducing the background with an autosuppressor from ALTECH (model DS-Plus) and the detection limit was 100 ppb (parts per billion).

Results and discussion

Biotransformation, mass-balance, and product stoichiometry of HMX

Xanthine oxidase biotransformed HMX, using NADH as electron-donor, under aerobic and anaerobic conditions at the rates of 1.6 ± 0.2 and 10.5 ± 0.9 $\text{nmol h}^{-1} \text{mg protein}^{-1}$, respectively, indicating that anaerobic conditions favored the reaction. Hence the subsequent study was carried out under anaerobic conditions.

In a time-course, HMX disappearance was accompanied by the formation of nitrite (NO_2^-), nitrous oxide

(N₂O), formaldehyde (HCHO), and formic acid (HCOOH) (Fig. 1). Methylenedinitramine (MDNA) and 4-nitro-2,4-diazabutanal (NDAB) were detected as transient metabolites and ammonium (NH₄⁺) was an end-product (Table 1).

XO also biotransformed HMX metabolites, i.e., NDAB and HCHO. For example, when standard compounds of NDAB and HCHO were incubated with XO under the same reaction conditions as those used for HMX, both compounds were biotransformed at the rates of 8.0 ± 0.6 and 16.5 ± 1.4 nmol h⁻¹ mg protein⁻¹, respectively. The NDAB produced N₂O, NH₄⁺, and HCOOH whereas HCHO was biotransformed to HCOOH quantitatively (data not shown). It is known that XO utilizes water (H₂O) as a source of oxygen

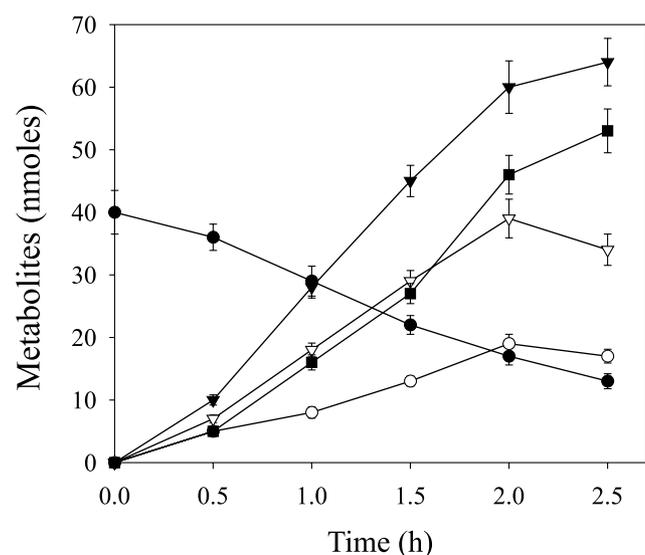


Fig. 1. Time-course of NADH-dependent biotransformation of HMX by xanthine oxidase under anaerobic conditions. HMX (●), nitrite (○), HCHO (▽), HCOOH (■), and nitrous oxide (▼). Data are means of duplicate experiments ($n = 2$), error bars indicate SD. Some error bars are not visible due to their smaller size.

during biotransformation of HCHO to HCOOH [14]. On the other hand, standard MDNA was readily decomposed abiotically at a rate of 9.2 ± 0.7 nmol h⁻¹ under similar reaction conditions. It has previously been reported that MDNA decomposes abiotically to produce N₂O and HCHO [23]. None of the nitroso-derivatives of HMX were observed during the course of reaction.

Some of the HMX products observed in the present study are consistent with those detected in our previous studies with HMX [11,24] and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) [12,13,22,25]. Biodegradation of HMX with anaerobic sludge produced an array of products including nitroso-derivatives of HMX, methylenedinitramine, formaldehyde, nitrous oxide, nitrogen, and carbon dioxide [11]. Recently, we observed nitrite, nitrous oxide, 4-nitro-2,4-diazabutanal, and formaldehyde during alkaline hydrolysis of HMX [24]. The 4-nitro-2,4-diazabutanal, besides being a HMX metabolite in the present study, was also a major product during biotransformation of RDX by cytochrome P450 [13] and *Rhodococcus* sp. DN22 [25]. On the other hand, methylenedinitramine was a major transient metabolite produced during RDX biotransformation by a diaphorase from *Clostridium kluyveri* [12] and nitrate reductase from *Aspergillus niger* [22].

The total carbon and nitrogen mass-balances in the present study were 91% and 88%, respectively (Table 1). Numerically, of the 4.0 carbon atoms per HMX molecule, 3.3 were recovered in forms of HCHO and HCOOH. On the other hand, of the 8 nitrogen atoms per HMX molecule, 6 were recovered in forms of NO₂⁻, N₂O, and NH₄⁺ (Table 1). The absence of nitroso-intermediates from HMX and the formation of 0.6 mol of NO₂⁻ (equivalent to 1 mol) per mole of reacted HMX indicated a single N-denitration of HMX. The remaining 0.4 mol may account for further bioconversion of NO₂⁻ to nitric oxide (NO) by XO under anaerobic conditions as reported previously [15,16].

Table 1

Stoichiometry and carbon and nitrogen mass-balances of metabolites produced during HMX biotransformation catalyzed by xanthine oxidase (1 mg ml⁻¹) at pH 7.0 and 30 °C for 2.5 h under anaerobic conditions

Metabolites	Amount (nmol)	Molar ratio (per mole of HMX)	% Carbon recovery ^a	% Nitrogen recovery ^a
1. Methylenedinitramine	3.5	0.1	3	6
2. 4-Nitro-2,4-diazabutanal	4.2	0.1	8	6
3. Formaldehyde (HCHO)	34.0	1.3	31	N.A.
4. Formic acid (HCOOH)	53.0	2.0	49	N.A.
5. Nitrous oxide (N ₂ O)	64.0	2.4	N.A.	59
6. Nitrite (NO ₂ ⁻)	17.0	0.6	N.A.	8
7. Ammonium (NH ₄ ⁺)	19.0	0.7	N.A.	9
Total % mass recovery			91	88

^a Calculated from the total carbon and nitrogen mass in 27 nmol of biotransformed HMX; N.A., not applicable. Data are means of duplicate experiments ($n = 2$). SDs were within 8% of the mean absolute values.

Involvement of FAD-site in HMX biotransformation

Xanthine oxidase has three redox-cofactors: the FAD-site, the molybdenum-site, and the two Fe–S centers (Fig. 2). The molybdenum-site interacts with a variety of substrates such as xanthine, inorganic nitrate, and nitrite [18], and aldehydes [14] whereas the FAD-site reduces oxygen [14] and organic nitrates [17]. Xanthine and NADH donate electrons at molybdenum-site and FAD-site, respectively (Fig. 2).

Chemically modified XO (deflavo- and desulfo-XO) were used in order to determine the reaction site for HMX biotransformation. In an experiment with deflavo-XO, both NADH and xanthine-dependent HMX biotransformations were inhibited (Table 2), indicating the direct involvement of FAD-site in HMX biotransformation. Furthermore, upon reconstitution of deflavo-XO with a 200 μ M of FAD, both NADH and xanthine-dependent HMX biotransformation activities were restored up to 78% (Table 2), providing an additional support for the involvement of FAD-site in HMX biotransformation. On the other hand, in desulfo-XO,

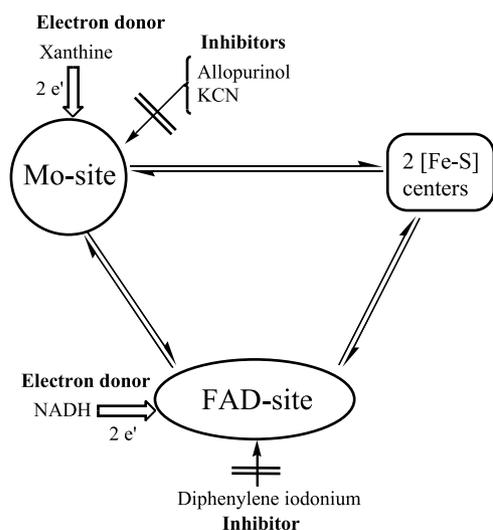


Fig. 2. A schematic representation of redox-cofactors present in xanthine oxidase (modified scheme from Komai et al. [19]) showing the interaction-sites for various electron-donors and site-specific inhibitors. Mo-site is molybdenum-site. Arrows indicate the direction of flow of electrons.

there was no significant change in HMX biotransformation activity when NADH was used as electron-donor, however, no activity was observed using xanthine as electron-donor (Table 2). This experiment indicated that HMX biotransformation did not occur at molybdenum-site of XO. Previously, Doel et al. [17] demonstrated that glyceryl trinitrate was biotransformed at FAD-site of XO with concomitant release of nitrite when the reaction was performed under anaerobic conditions using xanthine as an electron-donor.

The rate of HMX biotransformation by the native-XO was about 6-fold higher with NADH compared to xanthine as an electron-donor (Table 2). The most plausible reason may be the interaction of NADH and HMX at the same site (i.e., FAD-site) of XO for donating and accepting electrons, respectively, which led to a higher reaction rate. In contrast, the xanthine donates electrons at the molybdenum-site which subsequently transfers them to the FAD-site. The latter finally transfers the electron to HMX. This multi-step electron-transfer process probably decreases the resultant reaction rate.

Site-specific inhibition studies

DPI acts at FAD-site and prevents NADH from donating electrons at the site [19] whereas allopurinol binds at molybdenum-site and prevent its participation in electron transfer reactions [26]. In the present study, DPI inhibited the XO catalyzed biotransformation of HMX in a concentration-dependent manner (Fig. 3) whereas allopurinol did not inhibit the same reaction using NADH as electron-donor (Fig. 3). This experiment proved that HMX biotransformation occurred at the FAD-site and not the molybdenum-site. On the other hand, when xanthine was used as an electron-donor instead of NADH, both DPI and allopurinol inhibited the HMX biotransformation (data not shown) which additionally supported that FAD-site is the site of reaction.

Lineweaver–Burk plots showed that nitrite was a non-competitive inhibitor of HMX biotransformation (Fig. 4) which indicated that nitrite and HMX bind at different sites on XO, although they share a common electron source, NADH. Since nitrite binds at the molybdenum-site for its reduction [15,16,18] therefore

Table 2

Biotransformation of HMX by native and modified xanthine oxidase (XO) in the presence of NADH or xanthine as an electron-donor under anaerobic conditions at pH 7.0 and 30 °C

Electron-donor	HMX biotransformation activity (nmol h ⁻¹ mg protein ⁻¹) of:				
	Native-XO	Deflavo-XO	Reconstituted form of deflavo-XO	Desulfo-XO	Reactivated form of desulfo-XO
1. NADH	10.3 ± 0.8	1.9 ± 0.2	8.2 ± 0.4	9.6 ± 0.6	9.8 ± 0.8
2. Xanthine	1.8 ± 0.1	0.3 ± 0.04	1.4 ± 0.1	N.D.	1.3 ± 0.1

N.D., not detectable. Data are presented as means ± SD (n = 3).

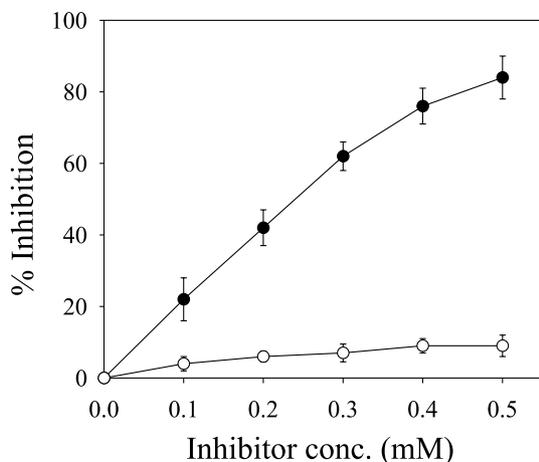


Fig. 3. Effect of site-specific inhibitors on NADH-dependent biotransformation of HMX by the xanthine oxidase. Allopurinol (○), diphenylene iodonium chloride (●). One hundred percent activity was equivalent to $10.5 \pm 0.9 \text{ nmol h}^{-1} \text{ mg protein}^{-1}$. Data are means of triplicate experiments ($n = 3$), error bars indicate SD.

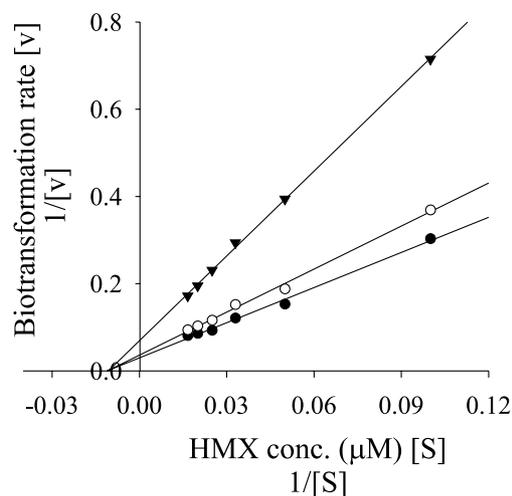


Fig. 4. Lineweaver-Burk plots showing nitrite as a non-competitive inhibitor of NADH-dependent biotransformation of HMX by the xanthine oxidase under anaerobic conditions. Nitrite concentrations, 0 mM (●), 0.5 mM (○), and 5.0 mM (▼). HMX biotransformation rate was expressed as $\text{nmol h}^{-1} \text{ mg protein}^{-1}$. Data are means of duplicate experiments.

HMX must bind at the FAD-site in order to accept electron.

Several lines of evidence have proved that biotransformation of HMX occurred at FAD-site. The present study is analogous with a previous report [17] which also showed the reduction of organic nitrates at FAD-site of xanthine oxidoreductase under anaerobic conditions.

Proposed biotransformation mechanism

Based on the above data, we propose that HMX undergoes a single N-denitration which requires an initial obligatory one electron transfer step. In the present

study, the electron transfer reaction occurred at the FAD-site which is known to catalyze one electron transfer to O_2 [14,19]. Therefore, under aerobic conditions, O_2 and HMX compete for the common binding-site (i.e., FAD-site) which accounts for the 6.5-fold lower HMX biotransformation rate in the presence of O_2 . On the other hand, under anaerobic conditions, XO could catalyze one electron transfer to HMX followed by a single N-denitration which eventually formed hypothetical structure I (Fig. 5). The structure I is analogous to the one we proposed during RDX biotransformation by cytochrome P450 [13]. The hypothetical structure I, being unstable, underwent hydrolytic decomposition to first produce structure II followed by ring cleavage to produce structure III (Fig. 5). The spontaneous hydrolysis of III would produce MDNA and NDAB (Fig. 5). As mentioned above, the NDAB was further biotransformed by XO to their respective products (i.e., N_2O , NH_4^+ , and HCOOH) whereas MDNA was abiotically decomposed to N_2O and HCHO . The latter being a substrate of XO was also partly biotransformed to HCOOH (mentioned above).

In a previous report, Chapman et al. [27] demonstrated a chemical N-denitration of HMX using a net

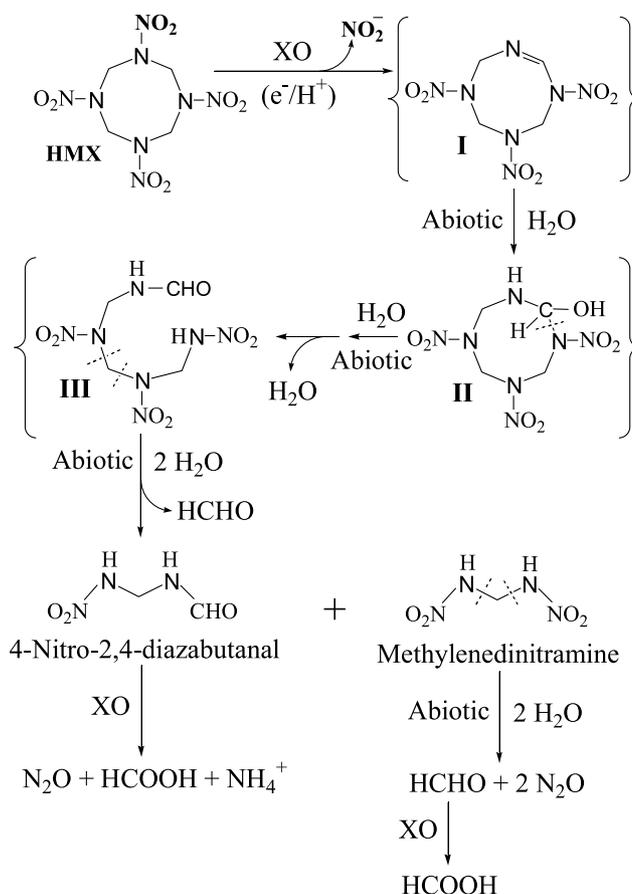


Fig. 5. Proposed pathway of xanthine oxidase (XO) catalyzed biotransformation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). Products shown inside brackets were not detected.

hydride transfer agent, 1-benzyl-1,4-dihydronicotinamide, in a photochemical reactor having a 200-W tungsten light bulb. They proposed that all the four N–NO₂ bond scissions occurred to produce a corresponding amine (octahydro-1,3,5,7-tetrazocine). The latter was unstable and underwent rearrangement to produce a more stable product hexamethylenetetramine [27]. In contrast, we observed a single N-denitration of HMX leading to its spontaneous decomposition (Table 1). Hence the present study apparently favors the XO catalyzed one electron transfer to HMX instead of a hydride transfer.

Several experimental evidences such as oxygen-sensitivity of the reaction, involvement of FAD-site in HMX biotransformation, and a stoichiometric single N-denitration of HMX have proved that XO catalyzed a one-electron transfer to HMX at FAD-site, necessary to cause a single N-denitration, leading to spontaneous decomposition.

In conclusion, we provided the first biochemical evidence of XO catalyzed biotransformation of HMX under anaerobic conditions. The mechanism described here is consistent with our previous results regarding enzymatic biotransformation of RDX [12,13] which also proved that one-electron transfer is necessary and sufficient to cause single N-denitration of RDX. The 4-nitro-2,4-diazabutanal, previously produced and accumulated during biodegradation of RDX with *Rhodococcus* sp. DN22 [25], was further biotransformed in the present study. This observation encourages one to use XO or similar enzyme(s) producing microorganisms for the complete degradation of RDX and HMX to innocuous products without accumulation of intermediates. The present study extends the fundamental knowledge to understand the biotransformation mechanism(s) of cyclic nitramine energetic compounds so as to design and optimize a well-controlled biochemical reaction for the complete mineralization of these compounds in soil and sediments.

Acknowledgments

We sincerely acknowledge the Natural Sciences and Engineering Research Council (NSERC) and the National Research Council (NRC) of Canada for a fellowship to Dr. B. Bhushan, and Strategic Environmental Research and Development Program (SERDP, CU 1213) and Office of Naval Research (N000140310269), USA, for funding. We also thank the Department of National Defense, Canada, for their support. Special thanks are due to Mr. D. Manno and Ms. T. Hooper for their assistance. We acknowledge the analytical and technical support of C. Beaulieu, C. Groom, A. Corriveau, and S. Deschamps.

References

- [1] R. Haas, E.v.L. Schreiber, G. Stork, Conception for the investigation of contaminated munitions plants. 2. Investigation of former RDX-plants and filling stations, *Fresenius J. Anal. Chem.* 338 (1990) 41–45.
- [2] C.A. Myler, W. Sisk, in: G.S. Sayler, R. Fox, J.W. Blackburn (Eds.), *Environmental Biotechnology for Waste Treatment: Bioremediation of Explosives Contaminated Soils (Scientific Questions/Engineering Realities)*, Plenum Press, New York, 1991, pp. 137–146.
- [3] J. Yinon, *Toxicity and Metabolism of Explosives*, CRC Press, Boca Raton, FL, 1990.
- [4] S.S. Talmage, D.M. Opreko, C.J. Maxwell, C.J.E. Welsh, F.M. Cretella, P.H. Reno, F.B. Daniel, Nitroaromatic munition compounds: environmental effects and screening values, *Rev. Environ. Contam. Toxicol.* 161 (1999) 1–156.
- [5] W. McLellan, W.R. Hartley, M. Brower, Health advisory for octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, Technical Report PB90-273533, Office of Drinking Water, US Environmental Protection Agency, Washington DC, 1988.
- [6] P.Y. Robidoux, J. Hawari, S. Thiboutot, G. Ampleman, G.I. Sunahara, Chronic toxicity of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in soil determined using the earthworm (*Eisenia andrei*) reproduction test, *Environ. Pollut.* 111 (2001) 283–292.
- [7] P. Gong, J. Hawari, S. Thiboutot, G. Ampleman, G.I. Sunahara, Toxicity of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) to soil microbes, *Bull. Environ. Contam. Toxicol.* 69 (2002) 97–103.
- [8] V.R. Harkins, T. Mollhagen, C. Heintz, K. Rainwater, Aerobic biodegradation of high explosives, phase I – HMX, *Bioremed. J.* 3 (1999) 285–290.
- [9] R. Boopathy, M. Gurgas, J. Ullian, J.F. Manning, Metabolism of explosive compounds by sulfate-reducing bacteria, *Curr. Microbiol.* 37 (1998) 127–131.
- [10] C.F. Shen, J. Hawari, G. Ampleman, S. Thiboutot, S.R. Guiot, Enhanced biodegradation and fate of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in anaerobic soil slurry bioprocess, *Bioremed. J.* 4 (2000) 27–39.
- [11] J. Hawari, A. Halasz, S. Beudet, L. Paquet, G. Ampleman, S. Thiboutot, Biotransformation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by municipal anaerobic sludge, *Environ. Sci. Technol.* 35 (2001) 70–75.
- [12] B. Bhushan, A. Halasz, J.C. Spain, J. Hawari, Diaphorase catalyzed biotransformation of RDX via N-denitration mechanism, *Biochem. Biophys. Res. Commun.* 296 (2002) 779–784.
- [13] B. Bhushan, S. Trott, J.C. Spain, A. Halasz, L. Paquet, J. Hawari, 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. DN22, *Appl. Environ. Microbiol.* 69 (2003) 1347–1351.
- [14] R. Hille, T. Nishino, Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase, *FASEB J.* 9 (1995) 995–1003.
- [15] B.L.J. Godber, J.J. Doel, G.P. Sapkota, D.R. Blake, C.R. Stevens, R. Eisenthal, R. Harrison, Reduction of nitrite to nitric oxide catalysed by xanthine oxidoreductase, *J. Biol. Chem.* 275 (2000) 7757–7763.
- [16] H. Li, A. Samouilov, X. Liu, J.L. Zweier, Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrate reduction: evaluation of its role in nitrite and nitric oxide generation in anoxic tissues, *J. Biol. Chem.* 276 (2001) 24482–24489.
- [17] J.J. Doel, B.L.J. Godber, R. Eisenthal, R. Harrison, Reduction of organic nitrates catalysed by xanthine oxidoreductase under anaerobic conditions, *Biochim. Biophys. Acta* 1527 (2001) 81–87.
- [18] H. Li, A. Samouilov, X. Liu, J.L. Zweier, Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrate reduction: evaluation of its role in nitrite and nitric oxide generation in anoxic tissues, *Biochemistry* 42 (2003) 1150–1159.

- [19] H. Komai, V. Massey, G. Palmer, The preparation and properties of deflavo xanthine oxidase, *J. Biol. Chem.* 244 (1969) 1692–1700.
- [20] V. Massey, D. Edmondson, On the mechanism of inactivation of xanthine oxidase by cyanide, *J. Biol. Chem.* 245 (1970) 6595–6598.
- [21] C.A. Groom, A. Halasz, L. Paquet, P. D'Cruz, J. Hawari, Cyclodextrin-assisted capillary electrophoresis for determination of the cyclic nitramine explosives RDX, HMX and CL-20: comparison with high-performance liquid chromatography, *J. Chromatogr. A* (2003) (published online 3rd April, 2003).
- [22] B. Bhushan, A. Halasz, J. Spain, S. Thiboutot, G. Ampleman, J. Hawari, Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) catalyzed by a NAD(P)H: nitrate oxidoreductase from *Aspergillus niger*, *Environ. Sci. Technol.* 36 (2002) 3104–3108.
- [23] A. Halasz, J.C. Spain, L. Paquet, C. Beaulieu, J. Hawari, Insights into the formation and degradation mechanisms of methylenedinitramine during the incubation of RDX with anaerobic sludge, *Environ. Sci. Technol.* 36 (2002) 633–638.
- [24] V.K. Balakrishnan, A. Halasz, J. Hawari, Alkaline hydrolysis of the cyclic nitramine explosives RDX, HMX and CL-20: new insights into degradation pathways obtained by the observation novel intermediates, *Environ. Sci. Technol.* 37 (2003) 1838–1843.
- [25] D. Fournier, A. Halasz, J.C. Spain, P. Fiurasek, J. Hawari, Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with *Rhodococcus* sp. strain DN22, *Appl. Environ. Microbiol.* 68 (2002) 166–172.
- [26] K. Ichimori, M. Fukahori, H. Nakazawa, K. Okamoto, T. Nishino, Inhibition of xanthine oxidase and xanthine dehydrogenase by nitric oxide, *J. Biol. Chem.* 274 (1999) 7763–7768.
- [27] R.D. Chapman, R.A. O'Brien, P.A. Kondracki, N-denitration of nitramines by dihydronicotinamides, *Tetrahedron* 52 (1996) 9655–9664.