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Bhushan, Bharat; Halasz, Annamaria; Hawari, Jalal

Publisher's version / Version de l'éditeur:
https://doi.org/10.1007/s00253-005-1992-4
Applied Microbiology and Biotechnology, 69, 4, pp. 448-455, 2005

Appl Microbiol Biotechnol (2005) 69: 448–455
DOI 10.1007/s00253-005-1992-4

Bharat Bhushan · Annamaria Halasz · Jalal Hawari

Biotransformation of CL-20 by a dehydrogenase enzyme from *Clostridium* sp. EDB2

Received: 7 March 2005 / Revised: 1 April 2005 / Accepted: 4 April 2005 / Published online: 20 April 2005

Abstract In a previous study, a marine isolate *Clostridium* sp. EDB2 degraded 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) under anaerobic conditions (Bhushan B, Halasz A, Thiboutot S, Ampleman G, Hawari J (2004c) Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX, HMX, and CL-20 by *Clostridium* sp. EDB2. Biochem Biophys Res Commun 316:816–821); however, the enzyme responsible for CL-20 degradation was not known. In the present study, we isolated and purified an enzyme, from strain EDB2, responsible for CL-20 degradation. The enzyme was membrane-associated and NADH-dependent and had a molecular weight of 56 kDa (with SDS-PAGE). N-terminal amino acid sequence of enzyme revealed that it belonged to dehydrogenase class of enzymes. The purified enzyme degraded CL-20 at a rate of 18.5 nmol/h mg protein under anaerobic conditions. Carbon and nitrogen mass balance of the products were 100 and 64%, respectively. In LC–MS–MS studies, we detected three different initial metabolites from CL-20, i.e., mono-nitroso derivative, denitrohydrogenated product, and double-denitrated isomers with molecular weight of 422, 393, and 346 Da, corresponding to presumed empirical formulas of $C_6H_6N_{12}O_{11}$, $C_6H_7N_{11}O_{10}$, and $C_6H_6N_{10}O_8$, respectively. Identity of all the three metabolites were confirmed by using ring-labeled [^{15}N]CL-20 and the nitro-group-labeled [$^{15}NO_2$]CL-20. Taken together, the above data suggested that the enzyme degraded CL-20 via three different routes: *Route A*, via two single electron transfers necessary to release two nitro-groups from CL-20 to produce two double-denitrated isomers; *Route B*, via a hydride transfer necessary to produce a denitrohydrogenated product; and *Route C*, via transfer of two redox equivalents to CL-20 necessary to produce a mono-nitroso derivative of CL-20. This is the first biochemical study which showed that CL-20 degradation can be initiated via more than one pathway.

B. Bhushan · A. Halasz · J. Hawari (✉)
Biotechnology Research Institute,
National Research Council of Canada,
6100 Royalmount Avenue,
Montreal, Quebec, H4P 2R2, Canada
e-mail: jalal.hawari@nrc.ca
Tel.: +1-514-4966267
Fax: +1-514-4966265

Introduction

2,4,6,8,10,12-Hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) is a newly synthesized future-generation energetic chemical (Nielsen et al. 1998), which is likely to replace the conventionally used explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and 2,4,6-trinitrotoluene (TNT) in the future. Initial reports on biological toxicity of CL-20 (Gong et al. 2004; Robidoux et al. 2004) have prompted the research in the area of determining its environmental fate and impacts. It is quite likely that extensive use of CL-20 in the near future (Giles 2004) may also raise similar environmental, biological, and health concerns as those previously experienced with structurally similar cyclic nitramines such as RDX and HMX (Etnier and Hartley 1990; Hoek 2004; McLellan et al. 1988; Talmage et al. 1999; Woody et al. 1986; Yinon 1990).

Several previous reports on microbial (*Pseudomonas* sp. FA1) (Bhushan et al. 2003), enzymatic (salicylate 1-monooxygenase and nitroreductase) (Bhushan et al. 2004a,b), and chemical (alkali hydrolysis) (Balakrishnan et al. 2003) degradation of CL-20 have shown that this compound can be degraded via an initial N-denitration route. Recently, it was found that photodegradation and Fe(0)-mediated degradation of CL-20 occurred via three different routes as shown in Fig. 1, i.e., N-denitration (route A), N-denitrohydrogenation (route B), and formation of mono-nitroso derivative of CL-20 (route C) (Balakrishnan et al. 2004; Hawari et al. 2004). However, no biological reaction has been reported so far, which showed that CL-20 biotransformation can proceed through a route other than initial N-denitration.

In our previous study, we showed that *Clostridium* sp. EDB2 degraded CL-20 under anaerobic conditions (Bhushan et al. 2004c); however, the enzyme responsible for CL-20

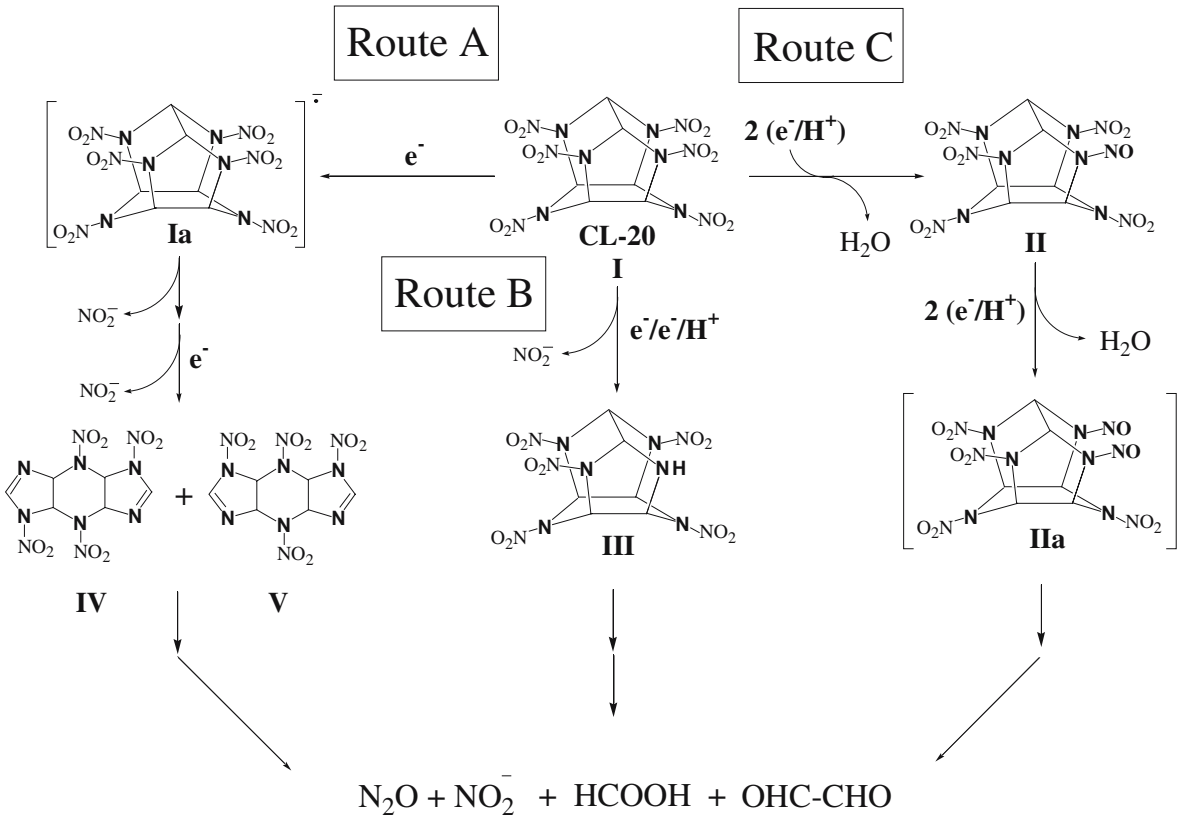

Fig. 1 Proposed pathway of biotransformation of CL-20 catalyzed by a dehydrogenase enzyme from *Clostridium* sp. EDB2. Intermediates shown inside bracket were not detected

degradation was not known. Therefore, the objective of the present study was to isolate and purify the enzyme(s), responsible for CL-20 degradation, from strain EDB2. LC–MS (ES^-) was used to determine the initial products and other intermediates produced during CL-20 degradation. We used uniformly ring-labeled [^{15}N]CL-20 and uniformly nitro-group-labeled [$^{15}NO_2$]CL-20 to identify the intermediate(s). Radiolabeled [^{14}C]CL-20 was used, due to the unavailability of authentic standards, to quantify the initial metabolites (in terms of radioactivity distribution) in order to supplement the product mass balance.

Materials and methods

Chemicals

2,4,6,8,10,12-Hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) in ε-form and 99.3% purity, uniformly ring-labeled [^{15}N]CL-20 (87.6% purity), uniformly nitro-group-labeled [$^{15}NO_2$]CL-20 (99.4% purity), and [UL-^{14}C]CL-20 (96.7% chemical purity, 95.7% radiochemical purity, and specific activity of 294.5 μCi/mmol) were provided by ATK Thiokol Propulsion (Brigham City, UT, USA). Nitrous oxide (N_2O) was purchased from Scott Specialty Gases (Sarnia, ON, Canada). All other chemicals were of the highest purity grade.

Enzyme purification and characterization

Cells of *Clostridium* sp. EDB2 were grown as described before (Bhushan et al. 2004c). The cell biomass was washed two times with normal saline and one time with suspension buffer (Tris–HCl 25 mM, sucrose 25 mM, PMSF 1 mM, EDTA 5 mM, DTT 0.5 mM). The washed cell biomass (0.2 g/ml) was subjected to disruption with a French press at 20,000 lb/in^2. The disrupted cell suspension was centrifuged at 9,000×*g* for 30 min at 4°C to remove cell debris and undisrupted cells. The supernatant thus obtained was ultracentrifuged at 165,000×*g* for 1 h at 4°C. The pellet (membrane protein fraction) and supernatant (soluble protein fraction) were separated. The membrane-associated protein fraction was dissociated from the membrane preparation by stirring with 0.2% Triton-X-100 for 1 h in an ice bath. The soluble and insoluble fractions of the membrane preparation were separated by ultracentrifugation, and the soluble membrane fraction, thus obtained, was retained. The low molecular weight proteins and peptides were removed from the soluble membrane fraction using 30 kDa cut off membrane, Centriprep YM30 (Amicon Bioseparations). The partially purified protein fraction was concentrated which showed activity against CL-20 in the presence of NADH under anaerobic conditions. Therefore, this protein fraction was subjected to further purification with ion-exchange chromatography with Q-sepharose column (HiTrap Q XL, Amersham Biosciences) using AKTA protein purification system

(Amersham Pharmacia). Column bed volume and flow rate were 5.0 ml and 1.5 ml/min, respectively. The proteins bound to the column were eluted with step-gradient method using a 20 mM Tris–HCl (pH 8.0) buffer supplemented with an increasing NaCl concentration (i.e., an increment of 50 mM of NaCl after every 7 min).

Enzyme purity and molecular weight were determined with standard SDS-PAGE procedure using 10% polyacrylamide gel (Laemmli 1970). The protein content was determined with a Bicinchoninic acid protein assay kit from Pierce Chemical Company (Rockford, IL). Enzyme specificity for electron donor was determined by assaying the enzyme against CL-20 in the presence of either NADH or NADPH. K_m and V_{max} values against CL-20 were determined with standard Lineweaver–Burk's plots.

N-terminal amino acid sequencing

The purified enzyme was blotted onto a polyvinylidine fluoride (PVDF) membrane (Problott membrane AB #400994) using the method as follows: protein sample (about 250 pmol) was subjected to electrophoresis using a mini gel (Mini Protean II, Electrophoresis Cell, Bio-Rad). Thereafter, the gel was soaked in a transfer buffer [10 mM of cyclohexylamino-1-propanesulfonic acid (CAPS) and 10% v/v methanol, pH 11.0] for 5 min. The gel was sandwiched between two sheets of PVDF and assembled into a blotting apparatus (Mini Protean II, Bio-Rad). Electroelution of proteins was carried out at room temperature for 15 min at 250 mA in transfer buffer. After blotting, the PVDF membranes were stained with Coomassie blue R-250. N-terminus sequence of the isolated protein, on PVDF membrane, was obtained by automated Edman degradation performed on a model Procise cLC 494 cLC protein sequencer from Applied Biosystems employing the general protocol of Hewick et al. (1981). About 1 pmol of the protein (on Problott membrane AB #400994) was loaded onto the sequencer. A standard program using liquid-phase TFA was employed for sequencing. The phenylthiohydantoin amino acid (PTH-aa) derivatives were determined by comparison with standards (PTH standards, AB) analyzed on-line on a capillary separation system (ABI 140D).

Biotransformation assays

Enzyme catalyzed biotransformation assays were performed under anaerobic conditions in 6-ml glass vials. Anaerobic conditions were created by purging the reaction mixture with argon gas for 20 min in sealed vials. Each assay vial contained, in 1 ml of assay mixture, CL-20 (25 μM or 11 mg l^{-1}), NADH (150 μM), enzyme preparation (250 μg), and potassium phosphate buffer (50 mM, pH 7.0). Higher CL-20 concentrations, as compared to its aqueous solubility of 3.6 mg l^{-1}, were used in order to allow detection and quantification of the intermediate(s). Reactions were performed at 30°C. Three different controls were prepared by omitting either enzyme, CL-20, or NADH from the assay mixture. Heat-inactivated enzyme was also used as a negative control. NADH oxidation was measured spectrophotometrically at 340 nm as described before (Bhushan et al. 2002). Samples from the liquid and gas phases in the vials were analyzed for residual CL-20 and biotransformed products.

To determine the residual CL-20 concentrations during biotransformation studies, at each time point, the total CL-20 content in one reaction vial was solubilized in 50% aqueous acetonitrile and analyzed by HPLC (see below). Enzyme activity against CL-20 was expressed as nanomoles per hour per milligrams of protein unless otherwise stated.

CL-20 and its intermediates, glyoxal (OHC–CHO), HCOOH, N_2O, and NO_2^-, were analyzed as described previously (Balakrishnan et al. 2004; Bhushan et al. 2003, 2004a,b,c; Hawari et al. 2004). Nitrous oxide detection method (gas chromatograph with electron capture detector) was much more sensitive (lowest detection limit 0.022 nmol/ml) compared to the nitrite detection method (HPLC ion conductivity detector) (lowest detection limit 5.434 nmol/ml).

Due to unavailability of the authentic reference samples of CL-20 intermediates, we quantified the initial metabolites in terms of dpm (radioactivity) counts using radiolabeled [^{14}C]CL-20. A reacted CL-20 sample was injected into HPLC-UV, the fractions corresponding to each metabolite were collected, and the radioactivity (in terms of dpm) associated with them were measured with a liquid scintillation counter (Packard, Tri-Carb 4530, model 2100 TR, Packard Instruments Company, Meriden, CT).

Attempted isolation and purification of metabolite IV, 1,4,5,8-tetranitro-1,3*a*,4,4*a*,5,7*a*,8,8*a*-octahydro-diimidazo[4,5-*b*:4′,5′-*e*]pyrazine ($C_6H_6N_{10}O_8$)

Previously, we hypothesized that metabolite, IV (and its isomer V) (Fig. 1), was unstable in water and decomposes spontaneously to eventually produce N_2O, glyoxal, and HCOOH (Balakrishnan et al. 2004; Bhushan et al. 2004a,b; Hawari et al. 2004). In order to determine the fate of metabolite IV in the degradation pathway, we produced and purified this compound by degrading CL-20 with resting cells of *Clostridium* sp. EDB2. The bacterial cells were grown in anaerobic batch culture containing 1 l of Luria–Bertani broth supplemented with 15 mg l^{-1} of CL-20. The cells were separated by centrifugation, washed three times with normal saline, and suspended in 100 ml of 50 mM phosphate buffer pH 7.0. CL-20 (10 mg) was added to the cell suspension from a stock solution made in acetone. Reaction was performed under anaerobic conditions (under argon atmosphere) for 1 h, and thereafter, cells were removed by centrifugation. The supernatant containing the metabolite(s) was passed through Sep-Pak RDX column (Supelco Co.). Metabolite IV and residual CL-20 were retained on the column. The loaded column was first washed with 10 ml of water followed by 10 ml of 10% aqueous acetonitrile solution. The bound metabolite fractions were

then eluted with 50% aqueous acetonitrile solution. One-milliliter-size fractions were collected and subjected to LC–MS (ES^-) to follow the elution profile of metabolite IV. The latter was eluted and collected in acetonitrile–water (1:1) mixture. Acetonitrile was evaporated under argon gas, and water phase, containing the metabolite, was lyophilized at −50°C. The dried material, thus obtained, was stored at −20°C. A part of purified metabolite IV was suspended in deionized water and its disappearance was followed over time using LC–MS (ES^-).

Results and discussion

Enzyme isolation and properties

Previously, we reported that resting cells of strain EDB2 biotransformed CL-20 under anaerobic conditions in the presence of NADH. We detected some end-products including NO_2^-, N_2O, and HCOOH; however, no other intermediates were detected (Bhushan et al. 2004c). In the present study, we isolated and purified an enzyme, responsible for CL-20 biotransformation, from strain EDB2. Various protein fractions were made from strain EDB2, and their activities against CL-20 were determined under both aerobic and anaerobic conditions. We found that enzyme activity towards CL-20 was largely associated with membrane protein fraction (Table 1). Subsequent ion-exchange chromatography using Q-sepharose gave a purified enzyme fraction at 300 mM NaCl step-elution gradient. The molecular weight of purified enzyme was found to be 56 kDa as determined with standard SDS-PAGE (Fig. 2). The N-terminal amino acid sequence (20 amino acids) of the enzyme was "AVKVAINGFGRIGRLAFRQM." A comparison with BLAST database revealed that N-terminal amino acid sequence of purified enzyme matched with a variety of dehydrogenases from several *Clostridium* spp., e.g., glyceraldehyde-3-phosphate dehydrogenases from *Clostridium pasteurianum* and *Clostridium acetobutylicum*, 20α-hydroxysteroid dehydrogenase from *Clostridium scindens*, *myo*-inositol 2-dehydrogenase from *Clostridium tetani*, and Fe–S oxidoreductase from *Clostridium thermocellum* with *E*-values of 8×10^{-9}, 3×10^{-8}, 2×10^{-5}, 0.018, and 0.19, respectively. Therefore, we classified the enzyme, isolated from strain EDB2, in dehydrogenase class.

Table 1 Purification of CL-20 degrading dehydrogenase enzyme from *Clostridium* sp. EDB2

Protein fraction	Total protein (mg)	Total enzyme activity[a]	Specific activity[b]	Yield (%)	Purification-fold
(1) Crude extract	280.0	1,568	5.6	100	1.0
(2) Soluble fraction	202.0	465	2.3	29.6	0.4
(3) Membrane-associated fraction	56.4	547	9.7	34.9	1.7
(4) Membrane fraction washed and concentrated with Centriprep YM30	23.2	283	12.2	18.0	2.1
(5) Ion exchange (Q-sepharose)	5.7	106	18.5	6.7	3.3

[a]Enzyme activity was determined against CL-20
[b]Specific activity was determined in terms of nanomoles of CL-20 degraded per hour per milligrams of protein

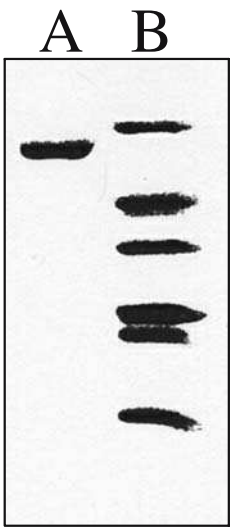

Fig. 2 SDS-PAGE picture showing band of purified dehydrogenase enzyme from *Clostridium* sp. EDB2 in *lane A*, and the standard molecular weight protein markers in *lane B* (from *top* to *bottom* 66, 45, 36, 29, 24, and 14.2 kDa)

The enzyme activity against CL-20 under anaerobic and aerobic conditions were 18.5 and 1.6 nmol/h mg protein, respectively, in the presence of NADH, suggesting that anaerobic conditions favored the reaction. As reported in our previous study (Bhushan et al. 2004b), molecular oxygen (O_2) quenches electron from the CL-20 free-radical anion, converting it back to the parent CL-20 molecule and thus enforcing a futile redox cycling. Analogously, in the present study, route A (Fig. 1), being the major pathway of CL-20 biotransformation that occurs via formation of CL-20 free-radical anion (discussed below), was inhibited under aerobic conditions. Therefore, subsequent experiments were carried out under anaerobic conditions. In enzyme-kinetic studies, we found that the purified enzyme degraded CL-20 at a rate of 18.5 and 2.7 nmol/h mg protein (Table 1) in the presence of NADH and NADPH, respectively, indicating that the enzyme was mainly NADH-dependent. The apparent K_m and V_{max} values against CL-20, determined with standard Lineweaver–Burks plots, were found to be 26.8 μM and 25 nmol/h mg protein, respectively.

Biotransformation of CL-20 with purified enzyme

In a time course study, the biotransformation of CL-20 was accompanied by gradual release of nitrite and nitrous oxide (Fig. 3). The concomitant formation of nitrite with the removal of CL-20 indicated that degradation of the energetic chemical proceeded via initial N-denitration route (Fig. 1a); however, we were not sure whether this was the only degradation route. Contrariwise, the release of nitrous

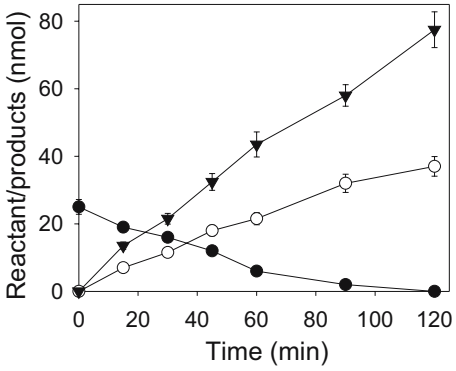

Fig. 3 Time course of biotransformation of CL-20 by the purified enzyme (1 mg) from *Clostridium* sp. EDB2 under anaerobic conditions. Residual CL-20 (●), nitrite (○), and nitrous oxide (▾). Data are means of results from triplicate experiments, and error bars indicate standard errors. Some error bars are not visible due to their small size

oxide from CL-20 was indicative of ring opening and decomposition of the molecule. This is to be noted that CL-20 is unstable in water if kept for longer hours (Monteil-Rivera et al. 2004); however, in the present study, we performed enzymatic reactions for short time period (up to 2 h) and found negligible CL-20 degradation in control experiments without enzyme.

Furthermore, during a time-course reaction using HPLC-UV, we detected three different initial metabolites, II, III, IV (and isomer V), that appeared simultaneously as early as 15 min of the reaction (Fig. 4a). The deprotonated molecular mass ion $[M-H]^-$ of the metabolites, II, IV, and V, as determined with LC–MS (ES^-) were 421, 345, and 345 Da, corresponding to presumed empirical formulas of $C_6H_6N_{12}O_{11}$, $C_6H_6N_{10}O_8$, and $C_6H_6N_{10}O_8$, respectively (Fig. 4c, e–f). Metabolites IV and V had identical masses and therefore were identified as isomers. However, IV and V were differed in their retention times of 8.65 and 8.10 min, respectively (Fig. 4 and Table 2), most probably due to difference in their polarities. On the other hand, metabolite III was detected as a mass of nitrate adduct $[M+NO_3]^-$ at 455 Da corresponding to a presumed empirical formula of $C_6H_7N_{11}O_{10}$ (Fig. 4d). The retention times and $[M-H]^-$ of all the detected metabolites were closely similar with those detected earlier during Fe(0)-mediated reduction and photodegradation of CL-20 (Balakrishnan et al. 2004; Hawari et al. 2004).

Further confirmation of the above metabolites was carried out by using uniformly ring-labeled $[^{15}N]$CL-20. The $[M-H]^-$ of products II, IV and V were observed at 427, 351, and 351 Da, respectively, whereas product III was observed as a nitrate adduct $[M+NO_3]^-$ at 461 Da, indicating an increase in mass by 6 Da in each case representing the incorporation of the six $[^{15}N]$-ring atoms in all the metabolites. Subsequently, when experiment was performed with nitro-group-labeled $[^{15}NO_2]$CL-20, the $[M-H]^-$ of products II, IV, and V were observed at 427, 349, and 349 Da, respectively. Contrariwise, product III was observed as a nitrate adduct $[M+NO_3]^-$ at 460 Da, indicating the incorporation of six $[^{15}N]$ atoms in II, five $[^{15}N]$ atoms in III, and only four $[^{15}N]$ atoms in IV and V. Experiments with uniformly ring-labeled $[^{15}N]$CL-20 and nitro-group-labeled $[^{15}NO_2]$CL-20 did confirm the identities of II, III, and IV and V as mono-nitroso derivative, denitrohydrogenated product, and double-denitrated product of CL-20, respectively (Fig. 1). All of the above metabolites were transient and disappeared with time to eventually produce nitrite, nitrous oxide, glyoxal, and formate (Figs. 1 and 3). The properties of the above metabolites are described in Table 2.

Due to unavailability of authentic standards of CL-20 metabolites, we quantified each metabolite by using ^{14}C-radiolabeled CL-20. Since each of II, III, IV, and V inherited six carbons from the parent CL-20 molecule, the radioactivity associated with each of them and their molar quantities should be comparable with that of CL-20. Therefore, relative quantification of radiolabeled metabolites was carried out by determining the amount of radioactivity (in terms of dpm) associated with each metabolite following their separation and fractionation with HPLC and by comparing them with that of CL-20. Radioactivity distribution associated with metabolites, in terms of percentage of CL-20, has been shown in detail in Table 3. We were able to recover almost 100% of the carbon mass at 15 and 45 min of the time course.

The nitrogen mass balance was determined from a parallel time-course experiment as shown in Fig. 3. At 2-h experiment, we recovered 1.5 mol NO_2^- and 3.1 mol N_2O per reacted mole of CL-20, which accounted for a total of 64% nitrogen. The remaining nitrogen would most probably be present in metabolites III, IV, V, and other unidentified polar products as shown in Table 3.

Insight into the secondary degradation pathway

In our previous studies, we hypothesized that metabolite IV should be unstable in aqueous medium due to the presence of two imine (–C=N–) bonds, which are very prone to attack by water molecules (March 1985). We also verified, with LC–MS–MS, the addition of two water molecules to IV using ^{18}O-labeled water ($H_2{}^{18}O$) and D_2O (Bhushan et al. 2004a; Hawari et al. 2004). In the present study, we isolated and purified IV using resting cells of strain EDB2 (see "Materials and methods"). A part of purified IV was suspended in deionized water and its disappearance was followed over time using LC–MS (Fig. 5a). We found that metabolite IV was unstable in water and was gradually converted to another metabolite IVa (with a $[M-H]^-$ at 381 Da) by the addition of two water molecules across –C=N– bonds. The apparent half-life of IV in deionized water at 10°C was determined as 150 min (Fig. 5a). Furthermore, IVa underwent rearrangement accompanied by ring cleavage to produce metabolite IVb, which also have a $[M-H]^-$ at 381 Da (Fig. 5b). The latter disappeared with time, suggesting that IVb was also unstable in water. Intermediates IV, IVa, and IVb were also detected during enzymatic and photodegradation of CL-20 in aqueous solution (Bhushan et al. 2004a; Hawari et al. 2004), suggesting that initial denitration whether chemical, physical, or biological leads

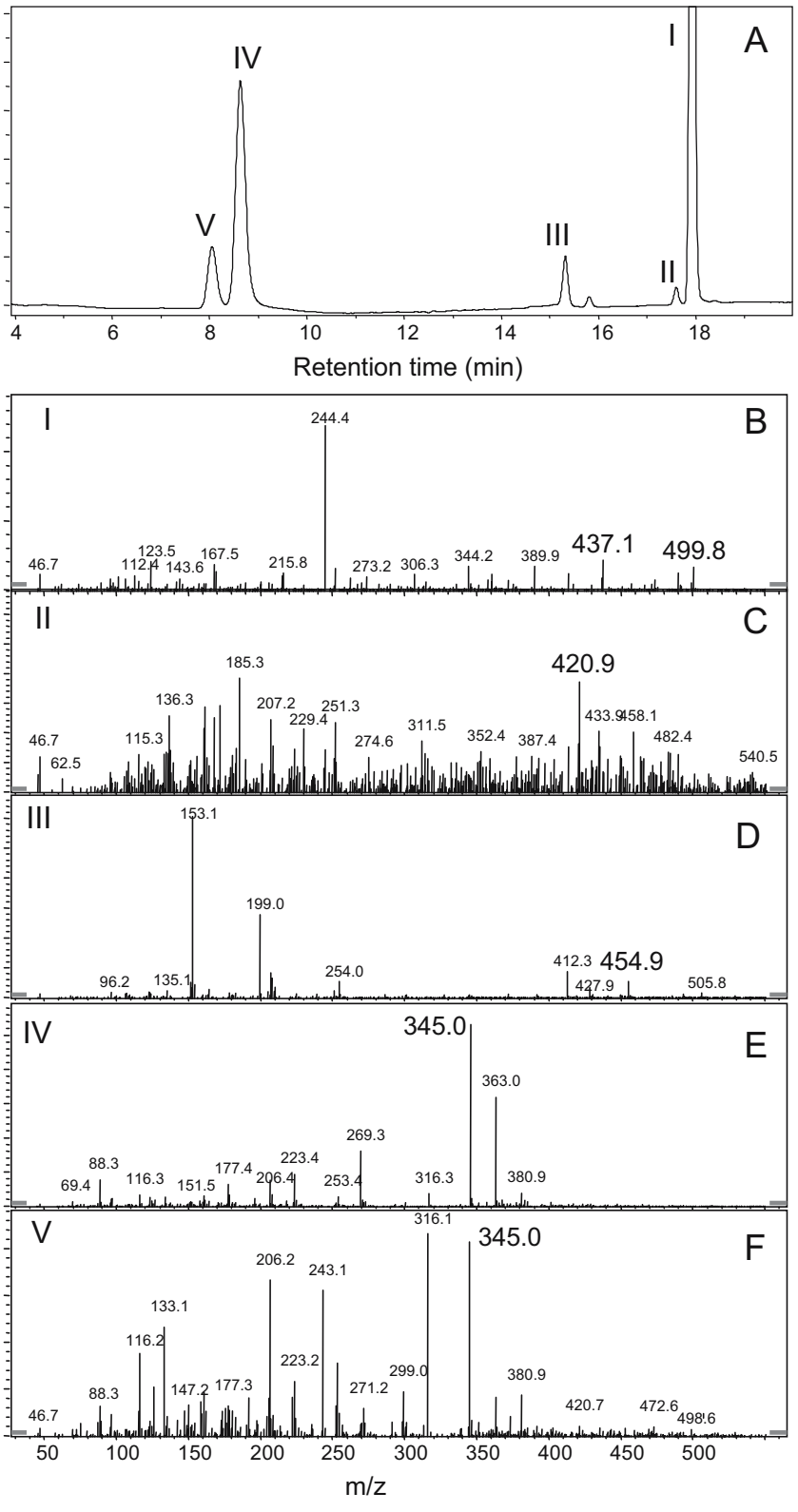

Fig. 4 (**a**) LC-UV chromatogram of CL-20 (I) and its metabolites (II–V) produced during the reaction of CL-20 with enzyme from *Clostridium* sp. EDB2. LC/MS (ES^-) spectra of the deprotonated mass ions $[M-H]^-$ of CL-20 (I) and its metabolites II, IV, and V were at 437 Da (**b**), 421 Da (**c**), 345 Da (**e**), and 345 Da (**f**), respectively, whereas metabolite III was detected as an adduct mass ion $[M+NO_3]^-$ at 455 Da (**d**)

to spontaneous decomposition of the molecule. However, intermediates II and III, as described above, were produced in small amounts and did not persist in the aqueous medium; hence, their fates were not determined.

Taken together, the above data suggested that the enzyme degraded CL-20 via three different routes (Fig. 1): *Route A*, via two single electron transfers necessary to release two nitro-groups from CL-20 to produce two double-denitrated isomers, IV and V; *Route B*, via a hydride transfer necessary

Table 2 Properties of CL-20 and the intermediates detected and identified by LC/MS (ES^-) during biotransformation of CL-20 catalyzed by dehydrogenase from *Clostridium* sp. EDB2

CL-20 and intermediates[a]	Retention time (min)	$[M-H]^-$ (Da)[b]	Nitrogen atoms from labeled ring [^{15}N]CL-20	Nitrogen atoms from labeled nitro-groups [$^{15}NO_2$]CL-20	Proposed empirical formula
(CL-20) I	17.90	437	6	6	$C_6H_6N_{12}O_{12}$
II	17.60	421	6	6	$C_6H_6N_{12}O_{11}$
III	15.25	392	6	5	$C_6H_7N_{11}O_{10}$
IV[c]	8.65	345	6	4	$C_6H_6N_{10}O_8$
V[c]	8.10	345	6	4	$C_6H_6N_{10}O_8$

[a]Tentative structures of these intermediates are shown in Fig. 1
[b]Deprotonated molecular mass ions are shown in Fig. 4
[c]Isomeric intermediates differ in their retention time

Table 3 Radioactivity distribution among [^{14}C]CL-20 and its degradation products recovered after 15- and 45-min incubation with dehydrogenase enzyme from strain EDB2

CL-20 and products	Percent of ^{14}C radioactivity counts	
	15 min	45 min
(1) Residual CL-20 (I)	58.2	28.4
(2) Intermediate II[a]	<1.0	Trace amount
(3) Intermediate III[a]	3.3	<1.0
(4) Intermediates (IV and V)[a]	7.0	10.1
(5) Other polar products[b]	33.0	63.2
(6) Total recovery of radioactivity	101.5	101.7

[a]These metabolites are shown in Figs. 1 and 4a
[b]Polar products contained glyoxal, formate, and unidentified product(s)

to produce a denitrohydrogenated product, III; and *Route C*, via transfer of two redox equivalents necessary to produce a mono-nitroso derivative (II) of CL-20.

In conclusion, we demonstrated for the first time that a dehydrogenase class of enzyme from anaerobic bacterial strain EDB2 biotransformed CL-20 via three different pathways which were previously not known in biological systems. The present data suggested that route A may be a major pathway in CL-20 biodegradation followed by routes B and C (Table 3 and Fig. 1). The present study has further strengthened our previous findings (Balakrishnan et al. 2004; Bhushan et al. 2003, 2004a,b; Hawari et al. 2004) that an initial reaction(s) either via denitration, denitrohydrogenation, and/or nitrosation is necessary and sufficient to destabilize CL-20 molecule leading to its ring opening and decomposition. The data presented here improved our basic

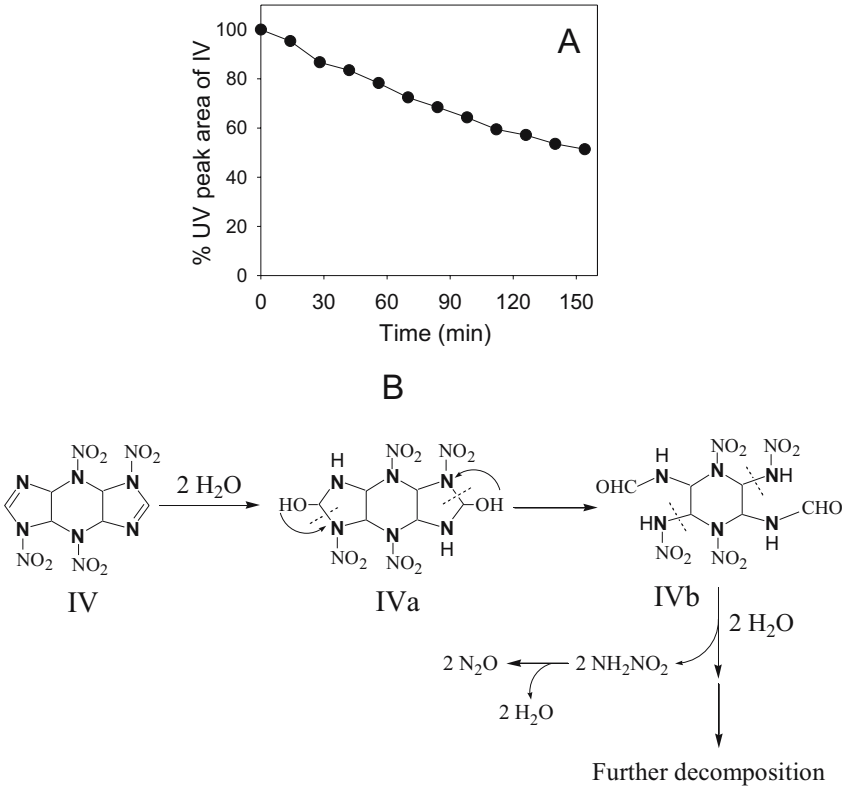

Fig. 5 **a** Abiotic decomposition of metabolite IV in deionized water at 10°C. **b** Proposed decomposition pathway of metabolite IV

understanding of microbial/enzyme-mediated degradation of CL-20 and revealed the possible fate of this energetic chemical in the environment.

Acknowledgements We sincerely acknowledge the DoD/DoE/EPA, Strategic Environmental Research and Development Program (SERDP), USA, for financial support (CP 1256). We also thank the Department of National Defense (DND), Val Bélair, Canada, for their support. We gratefully acknowledge Mr. A. Corriveau and Ms. C. Beaulieu for analytical and technical support. We thank two anonymous reviewers for their helpful suggestions.

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