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ARTICLE *in* BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS · JANUARY 2006

Impact Factor: 2.3 · DOI: 10.1016/j.bbrc.2005.09.166 · Source: PubMed

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Stereo-specificity for pro-(*R*) hydrogen of NAD(P)H during enzyme-catalyzed hydride transfer to CL-20

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Received 20 September 2005
Available online 5 October 2005

Abstract

A dehydrogenase from *Clostridium* sp. EDB2 and a diaphorase from *Clostridium kluyveri* were reacted with CL-20 to gain insights into the enzyme-catalyzed hydride transfer to CL-20, and the enzyme's stereo-specificity for either pro-*R* or pro-*S* hydrogens of NAD(P)H. Both enzymes biotransformed CL-20 at rates of 18.5 and 24 nmol/h/mg protein, using NADH and NADPH as hydride-source, respectively, to produce a N-denitrohydrogenated product with a molecular weight of 393 Da. In enzyme kinetics studies using reduced deuterated pyridine nucleotides, we found a kinetic deuterium isotopic effect of 2-fold on CL-20 biotransformation rate using dehydrogenase enzyme against (*R*)NADD as a hydride-source compared to either (*S*)NADD or NADH. Whereas, in case of diaphorase, the kinetic deuterium isotopic effect of about 1.5-fold was observed on CL-20 biotransformation rate using (*R*)NADPD as hydride-source. In a comparative study with LC–MS, using deuterated and non-deuterated NAD(P)H, we found a positive mass-shift of 1 Da in the N-denitrohydrogenated product suggesting the involvement of a deuteride (D^-) transfer from NAD(P)D. The present study thus revealed that both dehydrogenase and diaphorase enzymes from the two *Clostridium* species catalyzed a hydride transfer to CL-20 and showed stereo-specificity for pro-*R* hydrogen of NAD(P)H.

Keywords: CL-20; *Clostridium* sp.; Dehydrogenase; Diaphorase; Hydride transfer

CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) is a newly synthesized future-generation energetic chemical [1] which may 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) [2]. It is likely that extensive use of CL-20 in the near future may also raise similar environmental, biological, and health concerns as those previously experienced with other cyclic nitramines such as RDX and HMX [3–5]. Recent toxicological studies have shown the adverse effects of CL-20 in various biological receptors [6–8].

Several previous studies have shown that CL-20 can be degraded by microorganisms such as *Pseudomonas* sp. FA1, *Clostridium* sp. EDB2, *Agrobacterium* sp. strain JS71, and white-rot fungi [9–12], by enzymes e.g., monooxygenase, nitroreductase, and dehydrogenase [10,13,14], and by indigenous degraders present in soils and sediments [15,16]. However, none of these reports has emphasized on the mechanism of hydride-transfer to CL-20.

Previously, a DT-diaphorase from rat liver catalyzed a hydride transfer from NADPH to a nitramine compound, 2,4,6-trinitrophenyl-*N*-methylnitramine (Tetryl), to produce a corresponding N-denitrohydrogenated product [17]. In another report, a diaphorase from *Clostridium kluyveri* catalyzed a hydride transfer to a cyclic nitramine compound, RDX, followed by N-denitration [18]. Based on the stoichiometry of NADH consumed per reacted RDX molecule, Bhushan et al. [18] proposed the formation of a corresponding N-denitrohydrogenated product. The latter, however, could not be detected probably due to its very short half-life. More recently, a dehydrogenase enzyme from *Clostridium* sp. EDB2 degraded CL-20 via the formation of

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doi:10.1016/j.bbrc.2005.09.166

the N-denitrohydrogenated product [10]. None of the above studies elaborated on the enzyme's stereo-specificity for either pro-*R* or pro-*S* hydrogens of NADH.

In the present study, we employed two enzymes, a dehydrogenase from *Clostridium* sp. EDB2, and a diaphorase from *C. kluyveri*, to study the enzyme-catalyzed hydride transfer to CL-20, and to gain insights into the enzyme's stereo-specificity for either pro-*R* or pro-*S* hydrogens of NAD(P)H. We used LC–MS (ES-) to detect a mass-shift in the N-denitrohydrogenated product following CL-20 reactions with NAD(P)H and NAD(P)D as a hydride-source. Hydrogen–deuterium exchange between the ND-group of N-denitrohydrogenated product and the water was also examined during the reaction.

Materials and methods

Chemicals. CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) in ε-form and 99.3% purity was provided by ATK Thiokol Propulsion, Brigham City, UT, USA. NAD^+, $NADP^+$, ATP, 2-propanol-d_8, ethanol-d_5, alcohol dehydrogenase, D-glucose-d_1, glucose-6-phosphate dehydrogenase, and hexokinase were purchased from Sigma–Aldrich chemicals, Oakville, Ont., Canada. All other chemicals were of the highest purity grade.

Enzymes preparation. A dehydrogenase enzyme was isolated and purified from *Clostridium* sp. EDB2 as described before [10]. A diaphorase (EC 1.8.1.4) from *C. kluyveri* was obtained as a lyophilized powder from Sigma chemicals, Oakville, Ont., Canada. The enzyme was suspended in 50 mM potassium phosphate buffer (pH 7.0) and filtered through a Biomax-5K membrane filter (Sigma chemicals) before resuspending into the same buffer. The native enzyme activity of diaphorase was estimated spectrophotometrically at 340 nm (as per company guidelines) as the rate of oxidation of NADH using 2,6-dichlorophenol-indophenol as an electron acceptor.

Synthesis of deuterated and non-deuterated pyridine nucleotides. (*R*)NADD was synthesized by the method described by Ganzhorn and Plapp [19] with some modifications. A total of 25 mM NAD^+, 200 mM ethanol-d_5, and 75 U alcohol dehydrogenase were dissolved in 5 mL of 50 mM Tris-buffer, pH 9.0. The reaction was allowed to proceed at 37 °C, and the formation of (*R*)NADD was monitored by following the increase in absorbance at 340 nm. When no further increase in the OD_{340} was observed, the enzyme was separated from the reaction mixture using 10 kDa molecular weight cutoff filter (Centriprep YM10, Amicon Bioseparations, Bedford, MA).

(*S*)NADD was synthesized by the modified method described by Sucharitakul et al. [20]. The reaction mixture was composed of: 25 mM NAD^+, 35 mM D-glucose-d_1, 80 mM ATP, and 75 U each of hexokinase and glucose-6-phosphate dehydrogenase in 4 mL of 100 mM phosphate-buffer at pH 8.0. The reaction was allowed to proceed at room temperature until a maximum absorbance was achieved at 340 nm. Enzymes were removed with 5 kDa molecular weight cutoff filter. For proper controls, non-deuterated (*S*)NADH and (*R*)NADH were also synthesized by utilizing the non-deuterated components and by using the identical procedures that were used for (*S*)NADD and (*R*)NADD, respectively.

Furthermore, (*R*)NADPD and (*S*)NADPD were synthesized by the methods described by Pollock and Barber [21]. Non-deuterated (*S*)NADPH and (*R*)NADPH were also synthesized by utilizing the non-deuterated components and by using the identical procedures that were used for (*S*)NADPD and (*R*)NADPD, respectively.

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 assay mixture, CL-20 (25 μM or 11 mg/L), NADH(D) or NADPH(D) (200 μM), enzyme preparation (250 μg), and potassium phosphate buffer (50 mM, pH 7.0). Reactions were performed at 30 °C. Three different controls were prepared by omitting either enzyme, CL-20 or NAD(P)H(D) from the assay mixture. Heat-inactivated enzyme was also used as a negative control. NAD(P)H(D) oxidation was measured spectrophotometrically at 340 nm as described before [18]. Samples from the liquid phase in the reaction vials were analyzed periodically for the residual CL-20, and the N-denitrohydrogenated product.

For enzyme kinetics, enzyme and CL-20 reactions were performed at increasing CL-20 concentrations in the presence of NADH(D) in case of dehydrogenase and NADPH(D) in case of diaphorase. The data thus obtained were used to generate standard Lineweaver–Burk plots (double reciprocal plots).

Analytical techniques. CL-20 was analyzed with a LC–MS using a negative electro-spray ionization mode (ES-) to produce deprotonated molecular mass ion as described previously [10,22,23]. Whereas, N-denitrohydrogenated product was detected, with a LC–MS, as an $[M+TFA]^-$ adduct at *m*/*z* 506 Da following addition of trifluoroacetic acid (TFA) in the mobile phase. Protein concentrations were estimated by bicinchoninic acid (BCA) kit (Pierce Chemicals, Rockford, IL) using bovine serum albumin as standard.

Results and discussion

Two purified enzymes, a dehydrogenase from *Clostridium* sp. EDB2 and a diaphorase from *C. kluyveri*, biotransformed CL-20 (I) at rates of 18.5 and 24 nmol/h/mg protein, using NADH and NADPH as a hydride-source, respectively, to produce a denitrohydrogenated product (II) (Fig. 1). The latter had a HPLC-retention time and a molecular mass of 11.8 min and 393 Da (detected as

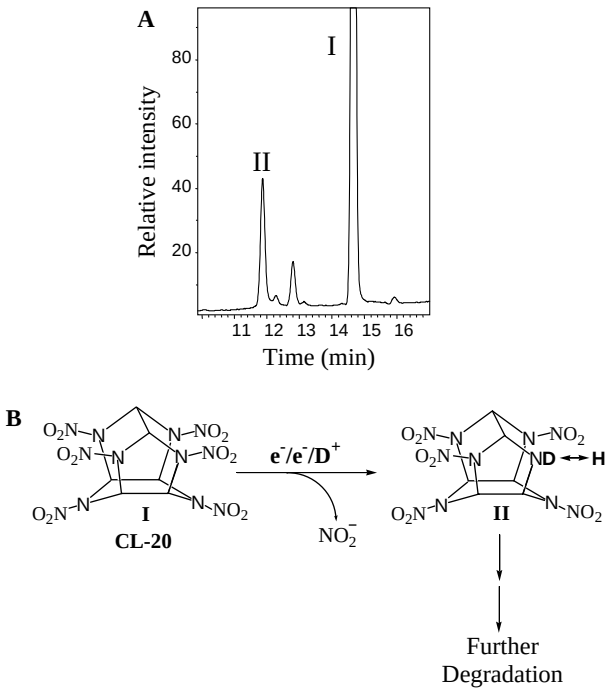

Fig. 1. (A) HPLC-UV chromatogram of CL-20 (I) and N-denitrohydrogenated product (II) obtained during CL-20 reaction with diaphorase from *C. kluyveri* at pH 7.0 and 30 °C. HPLC-UV chromatogram of N-denitrohydrogenated product obtained with dehydrogenase was published elsewhere [10]. (B) Proposed hydride transfer reaction of CL-20 and possible hydrogen–deuterium exchange between ND-group of product II and water.

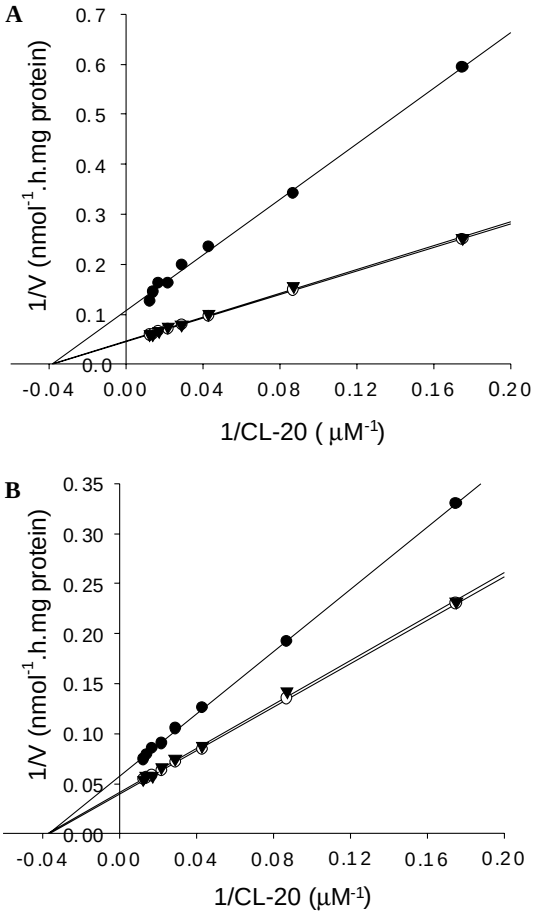

Fig. 2. Standard Lineweaver–Burk plots of CL-20 concentration versus its enzymatic biotransformation rate. (A) Plots using dehydrogenase enzyme from *Clostridium* sp. EDB2 in the presence of either NADH (○), (*S*)NADD (▼), or (*R*)NADD (●). (B) Plots using diaphorase enzyme from *C. kluyveri* in the presence of either NADPH (○), (*S*)NADPD (▼) or (*R*)NADPD (●). Data are means of the triplicate experiments, and the standard deviations were within 8% of the mean values.

$[M+TFA]^-$ adduct mass at *m*/*z* 506 Da), respectively. Product II was produced as a result of an obligate transfer of a hydride ion at the N-NO_2 group of CL-20 with the concomitant release of a nitro-group (Fig. 1). The product II, as reported in our previous study, was unstable in water, and therefore readily decomposed to finally produce NO_2^-, N_2O, and HCOOH [10]. Product II was previously detected during photolysis and Fe(0)-mediated degradation of CL-20; however in these reactions, NAD(P)H was not used as reducing agent [22,23]. On the contrary, in enzyme catalyzed reduction reactions, the reduced pyridine nucleotides such as NADH or NADPH mainly serve as the source of hydride [19–21,24].

To understand the stereo-specific effect of pro-*R* and pro-*S* hydrogens on hydride transfer reactions, both enzymes, dehydrogenase and diaphorase, were reacted with CL-20 in the presence of either deuterated or non-deuterated reduced pyridine nucleotides. In case of dehydrogenase enzyme, when Lineweaver–Burk plots of (*S*)NADD and (*R*)NADD were compared with that of NADH, we found a 2-fold deuterium isotopic effect with (*R*)NADD (i.e., V_{max} was 2-fold less with (*R*)NADD) compared to NADH, whereas results with (*S*)NADD were similar to those of NADH (Fig. 2A). This study revealed that dehydrogenase stereo-specifically utilized pro-(*R*)-hydride of NADH for CL-20 reduction and that the isotope effect is due to the cleavage of (4*R*)-deuterium-carbon bond in NADH.

Furthermore, in case of diaphorase, when Lineweaver–Burk plots of (*S*)NADPD and (*R*)NADPD were compared with that of NADPH, we found a 1.5-fold deuterium isotopic effect with (*R*)NADPD compared to NADPH, whereas results with (*S*)NADPD were closely identical to those obtained with NADPH (Fig. 2B). This study showed that diaphorase stereo-specifically transferred a pro-(*R*)-hydride of NADPH to CL-20 in order to produce denitrohydrogenated product II, and that the isotope effect is due to the cleavage of (4*R*)-deuterium–carbon bond in NADPH.

In order to confirm the presence of hydride, from NAD(P)H, in product II (MW 393 Da), we performed enzymatic reactions with CL-20 in the presence of either NADD or NADPD containing deuterated pro-*R* or deuterated pro-*S* hydrogens. In a comparative study with LC–MS, using dehydrogenase, we found a positive mass-shift of 1 Da in product II using (*R*)NADD compared to either NADH or (*S*)NADD, suggesting the involvement of a deuteride (D^-) transfer from (*R*)NADD. Surprisingly, the mass signal, corresponding to mono-deuterated product II with MW 394 Da (detected as $[M+TFA]^-$ adduct mass at *m*/*z* 507 Da), rapidly decreased with time with a concomitant increase in the non-deuterated mass signal corresponding to MW 393 Da (detected as $[M+TFA]^-$ adduct mass at *m*/*z* 506 Da) (Table 1). Similar results were obtained when diaphorase was reacted with CL-20 in the presence of (*R*)NADPD (data not shown). The above experimental evidence suggested that the deuterium at ND-group of the product II might be labile, and therefore we detected a rapid exchange of $D \leftrightarrow H$ between ND-group and water (Fig. 1B) as marked by the rapidly decreased mass signal of mono-deuterated product II during the course of reaction (Table 1).

Table 1
Time-course of $H \leftrightarrow D$ exchange between ND-group of N-denitrohydrogenated product and water as followed with a LC–MS during dehydrogenase catalyzed hydride transfer from (*R*)NADD to CL-20

Reaction time (min)	Mass signal intensity of N-denitrohydrogenated product	
	Non-deuterated $[M_{h7}+TFA^a]^-$ *m*/*z* 506 Da	Deuterated $[M_{h6,d_1}+TFA]^-$ *m*/*z* 507 Da
Control[b]	216,211	0
0	0	0
5	74,759	2440
10	104,799	578
20	120,673	342

[a] Molecular mass of N-denitrohydrogenated product of CL-20 as an adduct with TFA (trifluoroacetic acid).
[b] CL-20 was reacted with dehydrogenase in the presence of NADH for 20 min.

Several enzymes have previously been reported to be stereo-specific towards either (*R*)- or (*S*)-hydrogens of NAD(P)H for a hydride transfer to a variety of substrates [19–21,24]. However, the present study is the first one that showed stereo-specificity of two enzymes, dehydrogenase and diaphorase from *Clostridium* species, toward (*R*)-hydrogens of NAD(P)H for a hydride transfer to an environmentally significant cyclic nitramine compound, CL-20. Taken together, the data presented here extend our fundamental knowledge about the role of enzyme-catalyzed hydride transfer reactions in CL-20 biotransformation.

Acknowledgments

We sincerely acknowledge the DoD/DoE/EPA, Strategic Environmental Research and Development Program (SERDP), USA, for financial support (CP 1256). We thank the Department of National Defense (DND), Val Bélair, Canada, for their support. We gratefully acknowledge Mr. Alain Corriveau for analytical and technical support.

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