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Nitroreductase catalyzed biotransformation of CL-20

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Abstract

Previously, we reported that a salicylate 1-monooxygenase from *Pseudomonas* sp. ATCC 29352 biotransformed CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaaza-isowurtzitane) ($C_6H_6N_{12}O_{12}$) and produced a key metabolite with mol. wt. 346 Da corresponding to an empirical formula of $C_6H_6N_{10}O_8$ which spontaneously decomposed in aqueous medium to produce N_2O, NH_4^+, and HCOOH [Appl. Environ. Microbiol. (2004)]. In the present study, we found that nitroreductase from *Escherichia coli* catalyzed a one-electron transfer to CL-20 to form a radical anion ($CL\text{-}20^{\bullet-}$) which upon initial N-denitration also produced metabolite $C_6H_6N_{10}O_8$. The latter was tentatively identified as 1,4,5,8-tetranitro-1,3a,4,4a,5,7a,8,8a-octahydro-diimidazo[4,5-*b*:4′,5′-*e*]pyrazine [IUPAC] which decomposed spontaneously in water to produce glyoxal (OHC—CHO) and formic acid (HCOOH). The rates of CL-20 biotransformation under anaerobic and aerobic conditions were 3.4 ± 0.2 and 0.25 ± 0.01 nmol min^{-1} mg of $protein^{-1}$, respectively. The product stoichiometry showed that each reacted CL-20 molecule produced about 1.8 nitrite ions, 3.3 molecules of nitrous oxide, 1.6 molecules of formic acid, 1.0 molecule of glyoxal, and 1.3 ammonium ions. Carbon and nitrogen products gave mass-balances of 60% and 81%, respectively. A comparative study between native-, deflavo-, and reconstituted-nitroreductase showed that FMN-site was possibly involved in the biotransformation of CL-20.

Keywords: Biotransformation; CL-20; Nitroreductase; *Escherichia coli*; Glyoxal

Anticipated military and commercial use of the newly synthesized energetic chemical, CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane), in the near future [1] has opened the scope of research to study its environmental fate and impact. Biological and health impacts of CL-20 and its metabolic products are not known. There is one recent report which suggested an acute and chronic toxicity of CL-20 to earthworms exposed to amended natural soils [2]. It is likely that CL-20 may also raise similar environmental concerns as those experienced with RDX and HMX [3–6].

Several previous reports showed that biodegradation [7,8], photodegradation [9], and alkali-hydrolysis [10] of CL-20 produced HCOOH as a carbon-product in addition to several nitrogen-containing products such as nitrite, nitrous oxide, and ammonium. For instance, a membrane-associated flavoenzyme(s) from *Pseudomonas* sp. FA1 produced 2.3 nitrite ions, 1.5 molecules of nitrous oxide, and 1.7 molecules of formic acid from each reacted CL-20 molecule [7]. Whereas, salicylate 1-monooxygenase from *Pseudomonas* sp. ATCC 29352 produced 1.7 nitrite ions, 3.2 molecules of nitrous oxide, 0.6 ammonium ion, and 1.5 molecules of formic acid from each reacted CL-20 molecule [8]. In both previous studies [7,8], only formic acid (2 mol equivalents per mole of CL-20) was confirmed as a carbon product of CL-20. However, carbon mass-balance could not be determined due to the missing four mole equivalents of carbon in CL-20.

More recently, a new carbon-containing product, glyoxal, was detected and quantified (2 mol glyoxal/mol

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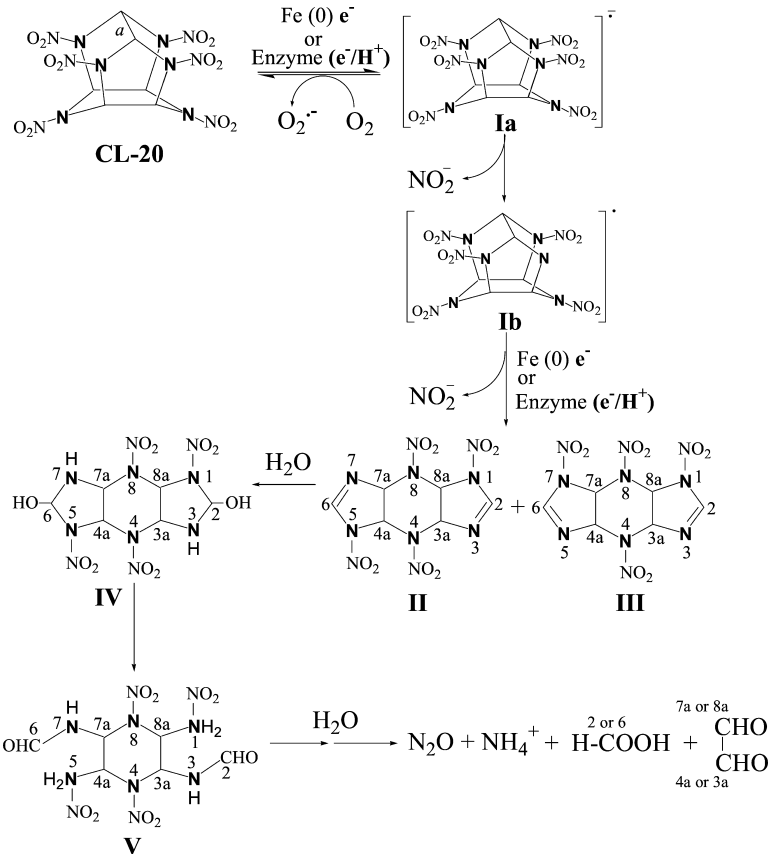

Fig. 1. Proposed pathway of CL-20 transformation (modified from [11]) by a nitroreductase from *Escherichia coli*. Intermediate shown inside bracket was not detected. Numbering on carbon products designates the source of carbon atoms from the CL-20 molecule.

CL-20) during CL-20 reaction with Fe(0) under anaerobic conditions and a pathway for glyoxal formation was proposed (Fig. 1) [11]. Balakrishnan et al. [11] proposed that CL-20$^{\bullet-}$ radical-anion was produced via a one-electron transfer from Fe(0) to CL-20. The resulting CL-20$^{\bullet-}$ upon denitration (loss of $2NO_2^-$) produced unstable intermediate II ($C_6H_6N_{10}O_8$) and its isomer III ($C_6H_6N_{10}O_8$). Both II and III undergo spontaneous hydrolysis in water to produce IV ($C_6H_{10}N_{10}O_{10}$) and its corresponding isomer (not shown in Fig. 1). The IV, being an α-hydroxyalkyl nitramine, was unstable in water [12] and therefore decomposed spontaneously in water to produce glyoxal and formic acid (Fig. 1).

In the present study, we hypothesized that an oxygen-sensitive nitroreductase from *Escherichia coli* should also biotransform CL-20 via an initial denitration to eventually give a similar product distribution as obtained with Fe(0). Nitroreductase catalyzed biotransformation of CL-20 allowed us to detect and quantify glyoxal in order to support the carbon mass-balance of the reaction. Superoxide dismutase (SOD)-sensitive cytochrome *c* reduction experiment was performed to determine the potential formation of an anion radical CL-20$^{\bullet-}$ prior to N-denitration. Additionally, we determined the involvement of flavin-site of nitroreductase in CL-20 biotransformation.

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, and uniformly ring-labeled [^{15}N]CL-20 (ε-form and 90.0% purity) were provided by ATK Thiokol Propulsion, Brigham City, UT, USA.

NADH, flavin mononucleotide (FMN), glyoxal (40% solution), superoxide dismutase (SOD, EC 1.15.1.1, from *E. coli*), and cytochrome *c* (from horse heart, MW 12,384 Da, purity 90%) were purchased from Sigma chemicals, Oakville, Ont., Canada. Nitrous oxide (N_2O) was purchased from Scott specialty gases, Sarnia, Ont., Canada. All other chemicals were of the highest purity grade.

Enzyme preparation and modification. Nitroreductase (purity of 90% by SDS–PAGE), from *E. coli*, was purchased from Sigma chemicals, Oakville, Ont., Canada. The enzyme was washed with phosphate buffer (pH 7.0) at 4 °C using Biomax-5K membrane centrifuge filter units (Sigma chemicals, Oakville, Ont.) to remove preservatives and then re-suspended in the same buffer. Native enzyme activity was determined as per company guidelines. The protein content was determined by Pierce BCA (bicinchoninic acid) protein assay kit from Pierce chemicals company, Rockford, IL, USA.

Apoenzyme (deflavo-form) was prepared by removing FMN from the holoenzyme using a previously reported method [13]. Reconstitution was carried out by incubating the apoenzyme with 100 μM FMN

in a potassium phosphate buffer (50 mM, pH 7.0) for 1 h at 4 °C. The unbound FMN was removed by washing the enzyme with the same buffer using Biomax-5K membrane centrifuge filter units.

Biotransformation assays. Enzyme catalyzed biotransformation assays were performed under aerobic as well as anaerobic conditions in 6 ml glass vials. Anaerobic conditions were created by purging the reaction mixture with argon gas for 20 min and by replacing the headspace air with argon in sealed vials. Each assay vial contained, in 1 ml of assay mixture, CL-20 or uniformly ring-labeled [^{15}N]CL-20 (25 μM or 11 mg L^{-1}), NADH (100 μM), enzyme preparation (50 μg), and potassium phosphate buffer (50 mM, pH 7.0). Higher CL-20 concentrations, than its aqueous solubility of 3.6 mg L^{-1} [14], 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 (90 °C for 30 min) was also used as a negative control. NADH oxidation was measured spectrophotometrically at 340 nm as described before [8]. Samples from the liquid and gas phase in the vials were analyzed for residual CL-20 and biotransformed products.

To determine the residual CL-20 concentrations during biotransformation studies, the reaction was performed in multiple identical vials. 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). CL-20 biotransformation activity of nitroreductase was expressed as nmol min^{-1} mg of $protein^{-1}$ unless otherwise stated.

To demonstrate the effect of enzyme concentration on CL-20 biotransformation, a progress curve was made under anaerobic conditions by incubating nitroreductase at an increasing concentration (25–150 μg/ml) with CL-20 (45 μM) and NADH (200 μM). The reaction conditions were the same as described above.

The effect of molecular oxygen (O_2) on CL-20 biotransformation activity of nitroreductase was determined by performing the assays under aerobic conditions at pH 7.0 and 30 °C. Formation of anion-radical CL-20$^{\bullet-}$ was determined by incubating CL-20 with nitroreductase in the presence of NADH, cytochrome *c* (75 μM), and SOD (150 μg ml^{-1}) as described previously [15,16]. Inhibition of cytochrome *c* reduction in the presence of SOD was monitored at 550 nm.

Analytical procedures. CL-20 and its intermediates, N_2O ($^{14}N^{14}NO$ and $^{15}N^{14}NO$), HCOOH, NO_2^-, and NH_4^+, were analyzed as described previously [7,8].

Glyoxal was analyzed by a derivatization method as described by Bao et al. [17] with some modifications. A 20 μl solution (15 mg ml^{-1}) of *O*-2,3,4,5,6-pentafluorobenzyl-hydroxylamine hydrochloride (PFBHA) was added to 0.5 ml of an aqueous sample containing CL-20 products and 0.5 ml acetonitrile. pH was adjusted to 3.0 with 5% (v/v) of HCl. The reaction mixture was stirred in dark for 2 h at room temperature. The derivatized samples were analyzed with a LC/UV-MS (Platform LC, Micromass, Manchester, UK) at a wavelength of 250 nm by using positive electrospray (ES^+) ionization mode. Separation was performed on a Supelcosil C8 column (25 cm × 4.6 mm ID, 5 μm) at 35 °C using acetonitrile:water gradient (acetonitrile from 60% to 90% in 10 min, 90% to 60% in 2 min, and then 60% for 6 min) at a flow rate of 1 ml min^{-1}.

Results and discussion

Nitroreductase catalyzed biotransformation of CL-20

A purified nitroreductase, from *E. coli*, biotransformed CL-20 in a NADH-dependent manner at pH 7.0 and 30 °C under anaerobic conditions. A progress curve demonstrated a linear increase in CL-20 biotransformation as a function of enzyme concentration (data not shown). The rates of CL-20 biotransformation were 3.4 ± 0.2 and 0.25 ± 0.01 nmol min^{-1} mg of $protein^{-1}$ (mean ± SD; $n = 3$) under anaerobic and aerobic conditions, respectively, indicating the involvement of an oxygen-sensitive process. In all controls (see Materials and methods), we found a negligible abiotic removal of CL-20 during one hour of reaction time.

Oxygen-sensitivity of the reaction suggested that nitroreductase catalyzed a one-electron transfer to CL-20 to first produce an anion radical (CL-20$^{\bullet-}$) before N-denitration as previously reported during biotransformation of CL-20 with salicylate monooxygenase [8] or its reduction with Fe(0) [11]. Subsequently, we found that under aerobic conditions, superoxide dismutase (SOD) inhibited 30% of reduction of cytochrome *c* which suggested the formation of oxygen free-radical ($O^{\bullet-}$) during the reaction. Hence, molecular oxygen (O_2) inhibited CL-20 biotransformation by quenching an electron from the CL-20 anion-radical and converting it back to the parent molecule (CL-20), and thus enforcing a futile redox-cycling as shown in Fig. 1. Control experiments without nitroreductase showed that CL-20 was neither auto-oxidized nor it directly reduced cytochrome *c*. Analogously, O_2-mediated inhibition of RDX anion-radical formation was previously reported during biotransformation of RDX with diaphorase [15]. Furthermore, the phenomenon of regeneration of parent nitro-compound during reaction of nitro anion-radical with molecular oxygen (O_2) in aerobic systems is well established [16,18–20]. Due to the inhibitory effect of oxygen, the subsequent experiments were carried out under anaerobic conditions.

Time-course of formation of initial metabolite II ($C_6H_6N_{10}O_8$)

In LC/MS (ES-) studies, we found two isomeric key intermediates II and III (Fig. 1) which appeared simultaneously as early as 5 min of the reaction with retention times (R_t) of 8.2 and 7.5 min, respectively (Fig. 2A). A time-course, considering the relative HPLC-UV areas, showed that both intermediates reached their maximum level after 30 min and then gradually declined during the course of reaction (Figs. 2A and B). As reported before [8,9], II and its isomer III were detected by LC/MS with similar deprotonated molecular mass ion $[M–H]^-$ at 345 Da corresponding to an empirical formula of $C_6H_6N_{10}O_8$ and were confirmed by using uniformly ring-labeled [^{15}N]CL-20. The product II was tentatively identified as 1,4,5,8-tetranitro-1,3a,4,4a,5,7a,8,8a-octahydro-diimidazo[4,5-*b*:4′,5′-*e*]pyrazine which is a double-denitrated product of CL-20 (Fig. 1). Both II and III contain imine bonds (—C═N—) which are known to be unstable in water [21] and therefore decompose rapidly to produce ring-cleavage products (described below).

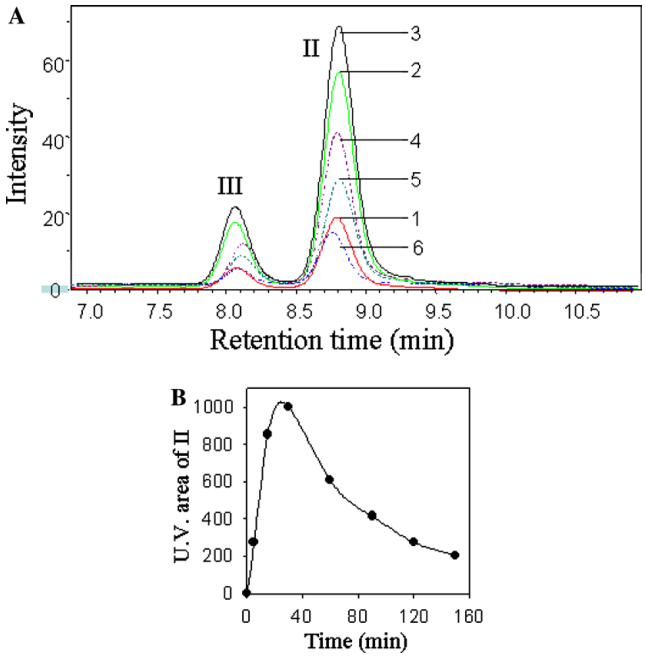

Fig. 2. (A) Time course of formation and disappearance of key metabolites II and III during biotransformation of CL-20 by nitroreductase. Chromatograms corresponding to numbers 1–3 indicate increasing formation of metabolite II at times 5, 15, and 30 min, respectively, whereas chromatograms 4–6 indicate disappearance of II at times 60, 90, and 150 min, respectively. Proposed molecular structures of II and III are shown in Fig. 1; (B) Formation and disappearance of II in terms of HPLC-UV-area as a function of time.

Detection and quantification of end-products including glyoxal (OHC—CHO)

Time-course studies showed a gradual disappearance of CL-20 at the expense of the electron-donor NADH with concomitant release of nitrite (NO_2^-), nitrous oxide (N_2O), and formate (Fig. 3). After 3 h of reaction, each reacted CL-20 molecule consumed about 1.3 NADH molecules and produced 1.8 nitrite ions, 3.3 molecules of nitrous oxide, 1.6 molecules of formic acid, 1.3 ammonium ions, and 1.0 molecule of glyoxal (Table 1). With regard to glyoxal stoichiometry, we expected 2.0 mol of glyoxal per reacted mol of CL-20 as found previously during CL-20 reaction with Fe(0) [11]. However, in the present study, we could recover only 1.0 mol equivalent of glyoxal due to the reason that glyoxal binds to the proteins via a non-enzymatic process called glycation [22] and therefore goes undetected. For instance, when we incubated glyoxal (35 μM) with enzyme (50 μg nitroreductase) in one ml buffer for 2 days, about 44% of glyoxal disappeared and could not be recovered either by heating (80 °C for 1 h) or by denaturing the enzyme with acetonitrile.

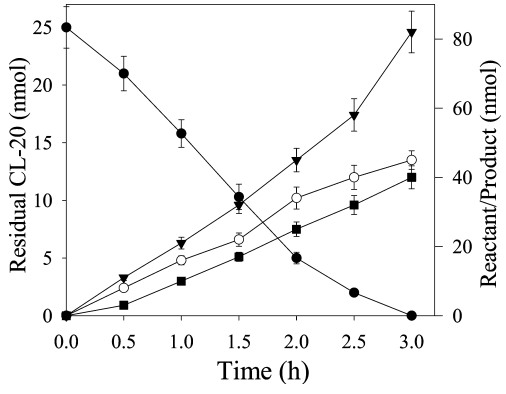

Fig. 3. Time-course study of NADH-dependent biotransformation of CL-20 by nitroreductase under anaerobic conditions. CL-20 (●), nitrite (○), nitrous oxide (▼), and formate (■). Data are means ± SE ($n = 2$). Some error bars are not visible due to their small size.

On the other hand, stoichiometry of NADH versus nitrite suggested that two single-electron transfer steps on CL-20 molecule released two nitrite ions. Of the total 12 nitrogen atoms (N) and 6 carbons (C) per reacted CL-20 molecule, we recovered approximately 10 N (as NO_2^-, N_2O, and NH_4^+) and 4 C (as HCOOH and glyoxal), respectively. The product distribution gave carbon and nitrogen mass-balance of 60% and 81%, respectively (Table 1). The product distribution in the present study was very similar, though with different relative yields, to those observed previously during photolysis of CL-20 [9] and the CL-20 reaction with Fe(0) [11]. A close similarity in product distributions among the above-investigated biotic and abiotic reactions suggested that once CL-20 undergoes initial N-denitration, the subsequent spontaneous reactions in water are basically similar.

We found that N_2O, even though produced at later steps of CL-20 biotransformation (described below), appeared before NO_2^- in the assay medium as shown in Fig. 3. This could be explained by two facts, first, there was a large difference in stoichiometries of nitrite (1.8) and nitrous oxide (3.3); second, nitrous oxide detection method (GC-electron capture detector) was much more sensitive (lowest detection limit of 0.022 nmol) than the nitrite detection method (HPLC-conductivity detector) (lowest detection limit of 5.434 nmol ml^{-1}). A comparative study between ^{14}N-CL-20 and uniformly-ring-labeled-[^{15}N]CL-20 using GC-MS analysis showed that N-NO_2 groups in CL-20 were the source of N_2O, detected as $^{14}N^{14}NO$ (44 Da) and $^{15}N^{14}NO$ (45 Da), respectively, which is in accordance with our previous study [8].

On the other hand, we observed that formate appeared earlier in the assay medium compared to glyoxal (data not shown) which indicated that formate might have been originated as the ring-cleavage product as a result of initial denitration followed by rapid cleavage of the weakest C—C bond designated as *a* in CL-20 structure [23] (Fig. 1). Whereas, the source of glyoxal was most probably the other two C—C bonds (C_{4a}—C_{7a} or C_{3a}—C_{8a}) in CL-20 (Fig. 1).

Formation of glyoxal during CL-20 degradation appears to be thermodynamically favorable since it is used

Table 1
Stoichiometry and mass-balance of reactants and products after 3 h of reaction between CL-20 and nitroreductase under anaerobic conditions at pH 7.0 and 30 °C

Reactant/product	Amount (nmol)	Molar ratio per mole of reacted CL-20	% Carbon recovery	% Nitrogen recovery
Reactants				
CL-20	25 ± 1.8	1.0 ± 0.07	100	100
NADH	34 ± 2.3	1.3 ± 0.08	NA	NA
Products				
Nitrite	45 ± 2.7	1.8 ± 0.10	NA	15.0
Nitrous oxide	82 ± 6.4	3.3 ± 0.25	NA	55.0
Ammonium	33 ± 2.5	1.3 ± 0.05	NA	10.8
Formic acid	40 ± 3.3	1.6 ± 0.13	26.6	NA
Glyoxal	25 ± 1.3	1.0 ± 0.05	33.3	NA
Total mass-balance			59.9	80.8

Data are means ± SE ($n = 2$).

to synthesize CL-20 [24]. Under alkaline conditions or in the presence of Fe(0), glyoxal further converts to glycolic acid [11,25]. However, in the present study at neutral conditions (pH 7.0), glyoxal was accumulated as an end-product. Under biological conditions, further conversion of either glyoxal (OHC—CHO) or glycolic acid (HOH_2C—COOH) to glyoxylic acid (HOOC—CHO) requires involvement of glyoxal oxidase or glycolate oxidase, respectively [25,26]. The formation of glyoxal as a CL-20 product is environmentally significant since glyoxal is a reactive α-oxoaldehyde and its biological toxicity is well known [22]. Glyoxal is also produced in biological systems as a result of glucose auto-oxidation, DNA oxidation by oxygen free-radicals, and lipid oxidation [22,27]. It reacts with proteins and nucleic acids by forming covalent bonds via a non-enzymatic process called glycation thus leading to a variety of clinical manifestations [22]. It is a known mutagen [22] and an important allergen [28]. Thus, glyoxal produced from CL-20 may significantly contribute to the biological toxicity of CL-20.

Involvement of flavin-moiety in CL-20 biotransformation

Nitroreductase, from *E. coli*, is a monomeric protein with a mol. wt. of 24 kDa and contains one molecule of flavin-moiety (FMN) per enzyme monomer [29]. The involvement of FMN in CL-20 biotransformation was determined by assaying deflavo- and reconstituted-form of nitroreductase against CL-20. The specific activities of the native-, deflavo-, and reconstituted-form of nitroreductase against CL-20 were 3.4 ± 0.2, 0.4 ± 0.03, and 2.75 ± 0.2 nmol min^{-1} mg of protein^{-1}, respectively, revealing that deflavo-enzyme lost about 88% of its activity compared to the native-enzyme (Fig. 4). The remaining 12% activity observed in deflavo-enzyme was due to incomplete removal of FMN (data not shown). Whereas, the reconstituted enzyme, prepared by reconstitution of deflavo-enzyme with FMN, restored the CL-20 biotransformation activity up to 81%

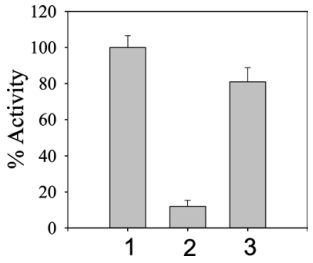

Fig. 4. Biotransformation of CL-20 by the native- (1), deflavo- (2), and reconstituted-nitroreductase (3). One hundred percent CL-20 biotransformation activity was equivalent to 3.4 ± 0.2 nmol min^{-1} mg of protein^{-1}. Data are mean percentages of CL-20 biotransformation activity ± SE ($n = 3$).

(Fig. 4). The above results suggested the involvement of FMN in biotransformation of CL-20. Furthermore, the free FMN (100 μM) also transformed CL-20 in the presence of NADH (200 μM) at a rate of 0.17 ± 0.02 nmol min^{-1}, however, the biotransformation rate was only 5% of the enzyme-bound-FMN present in native nitroreductase. This finding additionally supported the involvement of FMN in CL-20 biotransformation and also suggested that flavin-moiety functions more efficiently in enzyme-bound form.

In conclusion, the present study provided the first biochemical evidence for the quantitative formation of glyoxal and HCOOH during enzymatic biotransformation of CL-20. This study supported the previous finding of glyoxal production during chemical degradation of CL-20 with Fe(0). In the latter case, however, glyoxal was further converted to glycolic acid [11]. Detection and quantification of glyoxal supported the carbon mass-balance. Literature reported so far revealed that very few reports are available with regard to microbial and enzymatic degradation of CL-20. The present study thus provides a better understanding of the products and mass-balance of CL-20 reaction with nitroreductase(s)- or similar enzyme(s)-producing bacteria. Further work, however, is required to determine the fate

and impact of glyoxal, a known toxic compound, in the environment.

Acknowledgments

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