Journal of Molecular Catalysis B: Enzymatic 76 (2012) 1–8

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Dehydrogenation, oxidative denitration and ring contraction of *N*,*N*-dimethyl-5-nitrouracil by a *Bacillus* nitroreductase Nfr-A1

Sylvie Cortial[a], Philippe Chaignon[a], Didier Sergent[a], Sophie Dezard[b], Jamal Ouazzani[a,*]

[a] *Centre de Recherche de Gif. Institut de Chimie des Substances Naturelles, C.N.R.S, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France*
[b] *Commissariat à l'Energie Atomique, DSV/iBiTec-S/SCBM, 91191 Gif-sur-Yvette, France*

ARTICLE INFO

Article history:
Received 9 August 2011
Received in revised form
21 November 2011
Accepted 21 November 2011
Available online 17 December 2011

Keywords:
Nitroreductase
NADH-oxidase
Nitrouracil
Denitration
Ring contraction

ABSTRACT

Nfr-A1, a *Bacillus subtilis* nitroreductase, catalyzes the nitroreduction of a large panel of aromatic and heterocyclic nitro compounds, except those belonging to nitrouracil class of molecules. Besides nitroreduction, Nfr-A1 exhibits a strong NADH oxidase activity in the presence of oxygen, leading to high concentration of H_2O_2 (up to 200 μM). In the presence of (*N*,*N*)-dimethyl-5-nitrouracil **1** (dim-NU), Nfr-A1 achieves the reduction of dim-NU double bond to compounds **2** and **3** and in parallel the oxidation of dim-NU to the denitrated five membered derivatives **4** and **5**. The reduction is catalyzed by the reduced flavin Fl-$_{Red}$ and resembles those catalyzed by dihydropyrimidine dehydrogenases (DPD), during the catabolism of pyrimidines. The oxidative denitration is catalyzed in part by hydrogen peroxide generated through the NADH-oxidase activity, and certainly by the peroxyflavin intermediate Fl-$_{OOH}$ for the other part. The mechanisms of reaction were proposed according to experimental data and literature. These findings together with our previous results on the potential biological role of Nfr-A1, confirm the large spectrum of catalysis supported by this enzyme. The oxidative denitration is sporadically reported in literature and represents a safe and green alternative for the remediation of nitro-compounds.

1. Introduction

Nitroreductases are members of NAD(P)H/FMN oxidoreductases family. This appellation refers to their xenobiotic nitro substrates rather than their biological role witch is mostly unknown. The basis for their interest as pharmaceutical targets lies in the cell self-suicide, caused by the production of hydroxylamine intermediates that can be further metabolized to form cytotoxic DNA cross-linking agents. This property was exploited to design nitrofuran and nitroimidazole antibiotics [1]. Nitroreductases were also developed for enzyme/prodrug activation. The successful example is CB1954 that find applications in cancer prodrug therapy including antibody or gene-directed enzyme prodrug therapy [2] and Clostridia-directed enzyme prodrug therapy (CDEPT) [3]. Nitroreductases are involved in the activation of hypoxic anticancer nitro drugs in solid tumors [4]. The second application of nitroreductases is in the field of environment. These enzymes are involved in bioremediation of nitro-aromatic and nitro-heterocyclic pollutants, and were used for enzyme-based biosensor for nitro-sensitive ammunition [5,6].

Various known reductases are able to catalyze nitroreduction according to their sensitivity to oxygen. Type I or oxygen-insensitive nitroreductases catalyze the two-electron reduction of nitroaromatics. They include mammalian DT-diaphorase [7], xanthine dehydrogenase [8] and quinine reductase [9]. Type II or oxygen-sensitive nitroreductases includes various cytochrome reductases [10], ferredoxin and thioredoxin [11] and ubiquinone reductases [12]. These nitroreductases catalyze in the presence of oxygen the single-electron reduction of the nitro group to the nitro anion radical. This intermediates reacts with oxygen to generate superoxide, hydrogen peroxide and hydroxyl radicals. Some enzymes, initially classified as nitroreductases, catalyze the reduction of Cr(VI) to the less toxic Cr(III) compounds [13] and the reduction of various azo dyes in the presence of additional external FMN [14].

We have recently shown that *Bacillus subtilis* nitroreductase Nfr-A1 exhibits a strong NADH oxidase activity, catalyzes the degradation of the nicotinamide cofactor NAD^+ and scavenges high concentrations of H_2O_2 [15].

In order to further explore the catalytic potential of Nfr-A1, a large screening of diverse nitro substrates revealed that the nitro group of nitrouracil derivatives is not enzymatically reduced (results not shown). However, (*N*,*N*)-dimethyl-5-nitrouracil undergo a variety of unexpected reactions catalyzed directly or indirectly by Nfr-A1 and consisting of dihydrogenation, oxidative denitration and ring contraction.

* Corresponding author. Tel.: +33 1 69 82 30 01.
E-mail address: jamal.ouazzani@icsn.cnrs-gif.fr (J. Ouazzani).

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doi:10.1016/j.molcatb.2011.11.014

2. Experimental

2.1. Reagents

All chemicals were of the highest grade commercially available and purchased from Sigma–Aldrich, Fluka, or Merck. Parabanic acid is from TCI-Europe N.V., Belgium. Analytical grade solvents were from SDS (Peypin, France). Formic acid is HPLC-grade suprapur (Merck). (^{14}C)-methyl iodide was purchased from Amersham-Pharmacia-Biotech (Saclay, France). Superoxide dismutase (SOD, 4400 U/mg solid) and catalase (15,300 U/mg) were from Sigma–Aldrich.

2.2. N-methylation general procedure

This reaction was applied to uracil, 5-nitrouracil, 5-fluorouracil, parabanic acid and dialuric acid [24]. The desired quantity of the starting compound is solubilized in dimethylformamide DMF, and the solution stirred in ice under nitrogen atmosphere. 6 equiv. of NaH were added dropwise and allowed to react for 4 h. 2.5 equiv. of methyl iodide were then added and temperature rised to room temperature under stirring. The reaction is monitored by TLC or HPLC and stopped after total consumption of the starting material (3 h to overnight). DMF is evaporated under vacuum and the residue purified by flash chromatography on silica gel. Yields range from 65 to 90%.

N-N-dimethyl-5-nitrouracil ***1***: ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 8.72 (s, 1H), 3.59 (s, 3H), 3.38 (s, 3H). ^{13}C-NMR (62.5 MHz, $CDCl_3$): δ = 154.5, 150.0, 147.2, 125.2, 38.7, 29.0, MS (ES): m/z = 185.

N-N-dimethyl-parabanic acid: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.13 (s, 6H). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 160.4, 157.5, 28.43. MS (ES): m/z = 142.

2.3. Synthesis of 5-nitro-N,N-(^{14}C)-dimethyluracil

[^{14}C] methyl iodide (15 mCi, 55 mCi/mmole, from Amersham) was gauged into a vacuum ramp to afford 0.22 mmol of radioactive methyl iodide, i.e. 12.1 mCi. Non-labeled (0.56 mmol) methyl iodide in the gaseous form was mixed into the vacuum ramp with the radioactive methyl iodide to obtain an isotopic dilution to 15.4 mCi/mmol (0.78 mmol–12.1 mCi).

The [^{14}C] methyl iodide (12.1 mCi–15.4 mCi/mmol–0.78 mmol) was transferred under vacuum on a mixture of 53 mg (80% purity – 1.8 mmol) of sodium hydride, 45 mg (0.28 mmol) of nitrouracil in 5 mL of anhydrous dimethylformamide. The vacuum was broken under dry gaseous nitrogen and the mixture was allowed to react under stirring at room temperature during 5 h. The solution was evaporated and the residue purified.

Chromatography on silica gel offered 4.7 mCi of pure [^{14}C] dimethyl nitrouracil (radioactive yield: 39%). Chemical (98.7%) and radiochemical (96.1%) purities were determined by HPLC. Structure was assigned by NMR and mass spectrometry.

Specific activity was 27 mCi/mmol as measured by mass spectrometry and UV spectrophotometry. Stock solutions were prepared at 0.1 mCi/mL methanol (MeOH). Radiochemical purity was determined by HPLC. The original procedure is available in the lab book CEA/DSV/390 and the original spectra in the analysis report of CMM-2150.

2.4. Nitro-reduction of dim-NU

55 mg (0.3 mmol) of dim-NU were dissolved in 10 mL of MeOH then 15 mg of palladium on carbon catalyst (Pd/C) were added in a vessel equipped with a septum. Hydrogen was allowed to bubble into the mixture via a needle 2 h at atmospheric pressure and room temperature. The mixture is then filtrated and the solvent removed under reduced pressure. The product was purified by preparative thin layer silica gel chromatography to offer 26 mg (62% yield). ^{1}H NMR (300 MHz, CD_3OD): δ = 6.61 (s, 1H), 3.34 (s, 3H), 3.30 (s, 3H). ^{13}C NMR (62.5 MHz, CD_3OD): δ = 161.2, 150.4, 145.2, 121.5, 36.8, 28.4. m/z = 155 [MH^+].

2.5. Reduction of dim-NU double bond

500 mg (2.7 mmol) of dim-NU were dissolved in 50 mL of methylene chloride and 1 mL MeOH. 450 mg of $NaBH_4$ were added dropwise in ice and the reaction monitored by TLC. The reaction is stopped by the addition of water, and the compound extracted in dichloromethane. 370 mg of the ketone **2** were obtained from flash chromatography (75% yield). **2**: ^{1}H NMR (300 MHz, CD_3OD): δ = 5·24 (t, J = 4.7 Hz, 1H), 3.95 (q, J = 13.9 Hz, 1H), 3.94 (q, J = 13.9 Hz, 1H), 3.22 (s, 3H), 3.05 (s, 3H). ^{13}C NMR (62.5 MHz, CD_3OD): δ = 160.1, 152.1, 81.3, 46.9, 36.2, 28.7. MS (ES): m/z = 187.

Ketone **2** was stirred in distilled water overnight. 12 mg of the enol **3** were obtained by preparative HPLC. **3**: ^{1}H NMR (300 MHz, CD_3OD): δ = 4.15 (q, J = 14.4 Hz, 1H), 3.20 (s, 3H), 3.08 (s, 3H). ^{13}C NMR (62.5 MHz, CD_3OD): δ = 164.8, 156.0, 83.0, 48.4, 38.1, 30.8. MS (ES): m/z = 187.

2.6. Synthesis of 5-hydroxy-N,N-dimethyluracil

Methylation of 5-hydroxyuracil according to the procedure reported above lead to the trimethylated derivative 5-methoxy-*N*,*N*-dimethyluracil. The methoxy group was selectively demethylated as follows: 132 mg of 5-methoxy-*N*,*N*-dimethyluracil were dissolved in 3 mL of methylene chloride and mixed with 15.5 mL of 1 M solution of BBr_3 in methylene chloride. The mixture was allowed to react at 4 °C until total disappearance of the starting material in TLC, then the pH is adjusted to 9 with NH_4OH and the solvent evaporated. The mixture is recovered in 100 mL water and extracted with 6 × 50 mL of methylene chloride. 5-hydroxy-*N*,*N*-dimethyluracil was purified by flash chromatography in 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.85 (s, 1H), 3.39 (s, 3H), 3.36 (s, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 161.4, 150.5, 131.8, 121.5, 37.2, 28.7. MS (ES): m/z = 185.

2.7. Reduction of N,N-dimethyl-parabanic acid to N,N-dimethyl-hydroxyhydantoin

140 mg (1 mmol) of *N*,*N*-dimethyl-parabanic acid were dissolved in 20 mL of methanol and the solution cooled in ice. 40 mg of $NaBH_4$ (1 equiv.) were added dropwise and the reaction closely monitored by HPLC to avoid any formation of the diol. The reaction is stopped by the addition of water, and the mixture evaporated. 52 mg of pure *N*,*N*-dimethyl-hydroxyhydantoin were obtained through flash chromatography.

N-N-dimethyl-hydroxyhydantoïn ***4***: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.25 (s, 1H), 3.02 (s, 3H), 2.99 (s, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 173.3, 157.2, 79.6, 26.4, 24.5. MS (ES): m/z = 144.

2.8. Synthesis of N,N-dimethylhydantoin

N,*N*-dimethylhydantoin was synthesized according to the literature [16]. 176 mg (2 mmol) of dimethyl-urea and 200 mg of glyoxal (2 mmol) were mixed in pH 4 aqueous solution and held to react at 50 °C for 4 h. The solution is evaporated by vacuo, recovered in 20% $NaHCO_3$ and heated to 60 °C for 4 h. The mixture was evaporated by vacuo and extracted by ether. Ether was removed and the residue purified by flash chromatography to offer 115 mg of *N*,*N*-dimethyl-hydantoin.