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Isotopic effects in mechanistic studies of biotransformations of fluorine derivatives of L-alanine catalysed by L-alanine dehydrogenase

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ABSTRACT

Synthesis of 3-fluoro-[2-^{2}H]-L-alanine (3-F-[^{2}H]-L-Ala) in reductive amination of 3-fluoropyruvic acid catalysed by L-alanine dehydrogenase (AlaDH) was described. Fluorine derivative was used to study oxidative deamination catalysed by AlaDH applied kinetic (for 3-F-L-Ala in H_2O - KIE's on V_{max}: 1.1; on V_{max}/K_M: 1.2; for 3-F-L-Ala in $^{2}H_2O$ – on V_{max}: 1.4; on V_{max}/K_M: 2.1) and solvent isotope effect methods (for 3-F-L-Ala - SIE's on V_{max}: 1.0; on V_{max}/K_M: 0.87; for 3-F-[2-^{2}H]-L-Ala – on V_{max}: 1.4; on V_{max}/K_M: 1.5). Studies explain some details of reaction mechanism.

1. Introduction

Dehydrogenases are large group of enzymes belonging to the class of oxidoreductases which catalyse biochemical reactions of great significance. Enzyme L-alanine dehydrogenase (EC 1.4.1.1, AlaDH), a NAD^{+}- dependent amino acid dehydrogenase, was found in various bacteria species i.a. *Bacillus* (Kim et al., 2000), *Archaeoglobus, Streptomyces,* and their catalytic along with kinetic properties and the structure have been well identified (Ohshima and Soda, 1990). AlaDH was also found and described in pathogenic *Mycobacterium tuberculosis*, what makes the enzyme a potential target for pathogen control by antibacterial compounds (Hutter and Singh, 1999). AlaDH, characterized by narrow substrate specificity, catalyses the reversible transformation of L-alanine into pyruvic acid. According to the direction of reaction, it acts towards pyruvate, 2-oxobutyrate, 2-oxovalerate and 3-hydroxypyruvate in reductive amination, though in terms of the opposite reaction - oxidative deamination - the enzyme predominantly decomposes L-alanine (Ohshima and Soda, 1979) and some of its halogen derivatives (Gonçalves et al., 2003). In case of the stereochemistry of hydrogen transfer, AlaDH isolated from *Bacillus subtilis* shows *pro-R* stereospecificity, which indicates that hydrogen is transferred from the *pro-R* position at C-4 of the NADH nicotinamide ring to C-2 of pyruvate to form L-alanine (Alizade et al., 1975).

Halogenated amino acids, including fluorinated, don't occur naturally in living organisms, but they can be introduced like natural amino acids due to the similar van der Waals radius of fluorine (1.35 Å) and hydrogen (1.2 Å). High electronegativity of fluorine causes abnormalities in metabolism and functions of proteins containing unnatural fluorinated amino acids. A few years ago it was discovered that compounds such as 3-fluoro-L-alanine (3-F-L-Ala) and 3-fluoro-D-alanine (3-F-D-Ala) exhibit antibacterial and antivirus properties (Kollonitsch and Barash, 1976) and they are broad spectrum antibiotics due to irreversible inactivation of alanine racemase - enzyme involved in biosynthesis of the bacterial cell wall (Ohshima et al., 1989; Esaki and Walsh, 1986). 3-F-L-Ala - an unnatural amino acid containing fluorine substituent, is the product of enzymatic conversion of 3-fluoropyruvic acid (3-F-PA) catalysed by AlaDH (Fig. 1).

Enzymatic methods of syntheses are characterized with rapid, facile and specific course of action; therefore they are subjects of great interest in development of syntheses involving short-lived isotopes. Unnatural amino acids labelled with β^{+}-emitting fluorine-18 can be applied as radioactive traces in Positron Emission Tomography (PET) for the diagnosis of cancer and neurodegenerative diseases (Laverman et al., 2002). ^{18}F-Fluorinated amino acids such as 3-^{18}F-L-Ala can be additional tools in cancer imaging by tracing of the protein biosynthesis, mainly by measuring amino acids transport rate to cancer cells via different mechanisms, providing fundamental components for tumour growth (Wang et al., 2012).

In addition, fluorinated α-hydroxyacids and α-amino acids can be potentially highly versatile chiral building blocks for the asymmetric synthesis of compounds of pharmacological interest and they can be potential precursors of fluoroamine compounds (Gonçalves et al., 2000). Amino acids containing fluorine can be used in bioorganic applications such as biological traces, mechanistic probes (Qui et al., 2004) and as potential irreversible inhibitors of PLP-dependent en-

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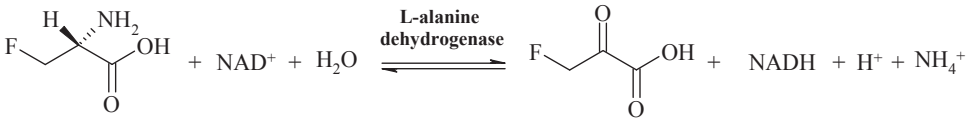

3-F-L-Ala **3-F-PA**

Fig. 1. Reversible reaction of enzymatic biotransformation of 3-F-L-Ala catalysed by AlaDH from *Bacillus subtilis*.

zymes like decarboxylases, transaminases and racemases (Seo et al., 2011).

Despite the literature reports concerning the reactions catalysed by AlaDH, their mechanisms are still under investigation. In previous article (Szymańska and Kańska, 2013) we reported deuterium isotope effects for reductive amination of 3-F-PA catalysed by AlaDH. In this report we have studied some intrinsic details of enzymatic reaction catalysed by AlaDH involved in oxidative deamination of 3-F-L-Ala (AlaDH) (Fig. 1) using solvent (SIE) or kinetic (KIE) isotope effect methods. The numerical values of deuterium effects were determined using a non-competitive spectrophotometric method. This technique involves determining the ratios of rate constants using protium and deuterium. By labelling of the molecule at different specific positions and determining primary and secondary isotope effects it is possible to designate the rate determining step and characterize many details of the mechanism crucial for kinetics such as bond breaking/forming and the structure of active complex (Cook, 1991).

2. Experimental

2.1. Materials

The enzyme L-alanine dehydrogenase (EC 1.4.1.1) from *Bacillus subtilis*, cofactors NAD^+, NADH and other chemicals required for enzymatic synthesis and trail experiments, such as sodium 3-fluoropyruvate were from Sigma. Deuteriated water (99,9% ^2H) and 30% $KO^2H/^2H_2O$ were from POLATOM (Poland). Thin layer chromatography (TLC) plates with UV indicator (DC-Plastikfolien Keiselgel 60 F_{254}) and Amberlite IR-120 (Na^+) were from Merck and Aldrich respectively. Selectively deuteriated cofactor [(*4R*)-^{2}H]-NADH (100% ^2H incorporation) was obtained using enzymatic method described in the recent article (Szymańska and Kańska, 2014).

2.2. Methods

The proton nuclear magnetic resonance (^1H NMR) spectra were recorded in ^2H_2O using tetramethylsilane (TMS) as an internal standard on Varian 500 MHz Unity-plus spectrometer. The extent of deuterium incorporation was determined from ^1H NMR spectrum. The kinetic assays were performed using Shimadzu-UV-1800 spectrophotometer. The reaction progress was checked by TLC (thin layer chromatography) using silica gel plates and ethanol/ammonia, 4:1; *v/v* as a developing solvent. 0,1% ninhydrin in ethanol and UV lamp were the methods of visualization of chromatograms.

2.3. Synthesis of 3-F-[2-^{2}H]-L-Ala

To the 10 mL vial containing 9.2 mg (0.072 mmol) of sodium 3-fluoropyruvate and 52 mg (0.074 mmol) of [(*4R*)-^{2}H]-NADH (enriched in near 100% in the pro-R-position), 7 U of enzyme AlaDH was added and solubilized in 4 mL of 0.3 M deuteriated ammonium buffer ($NH_4Cl/NH_3{\cdot}H_2O$) adjusted to pD 9.2 with 30% $KO^2H/^2H_2O$. The reaction mixture was incubated at 30 °C for 24 h and its progress was monitored by TLC. The reaction was quenched by acidification with concentrated hydrochloric acid to pH~2 and the denaturized enzyme was removed by centrifugation. Then, the remaining 3-F-PA was extracted with 8×1 mL portions of diethyl ether and the mixture was loaded onto an Amberlite IR 120H^+ column (100×10 mm). Afterwards the column was washed with 500 mL of water in order to remove the salts and deuterium from labile positions of the product. 3-F-[2-^{2}H]-L-Ala was eluted with 1 M NH_3(aq), and collected as 7 mL fractions. The presence of the product in fractions was confirmed by TLC as mentioned before. The proper fractions were combined, concentrated under reduced pressure at 50 °C and lyophilized. As a result 5.7 mg (0.053 mmol) of 3-F-[2-^{2}H]-L-Ala was obtained with 74% chem. yield. The extent of deuterium enrichment into α position of the product was determined by the ^1H NMR spectrum (TMS, D_2O, 500 MHz): δ [ppm] 4.1 (1H, H-α, 72% ^2H), δ 4.9 (2H, H-β). The deficit of signal of proton at the α-carbon atom indicates 72% enrichment of deuterium at this position.

2.4. Kinetic assay

2.4.1. Determination of deuterium SIE's and KIE's for oxidative deamination of 3-F-L-Ala in reaction catalysed by AlaDH

The kinetic experiments for studying SIE's and KIE's of oxidative deamination of 3-F-L-Ala were investigated using the following buffered solutions:

a) 50 mM carbonate buffer, pH 10.2 adjusted with 25 mM $NaHCO_3$,
b) 6 mM solution of 3-F-L-Ala or 3-F-[2-^{2}H]-L-Ala,
c) 15 mM solution of NAD^+,
d) L-alanine dehydrogenase solution (1.1 U mL^{-1}).

Each kinetic experiment consisted of six runs for varying concentrations of 3-F-L-Ala or 3-F-[2-^{2}H]-L-Ala. All measurements were performed using UV–Vis spectroscopy in 3 mL quartz cuvettes, filled with appropriate volumes of the buffered solutions of reagents. The final concentrations of the substrate ranged from 0.4 to 0.9 mM with 0.1 mM intervals. Afterwards, the exact volumes of NAD^+ and AlaDH solutions were added to acquire 1.0 mM and 0.2 U mL^{-1} concentrations, respectively. Finally, each cuvette was filled with carbonate buffer up to 3 mL. The progress of oxidative deamination was registered according to the increasing absorbance measured spectrophotometrically at λ_{max} = 340 nm for 40 min (1 min intervals) as a result of forming NADH cofactor.

2.5. SIE and KIE assays

The enzymatic oxidative deamination of 3-F-L-Ala (Fig. 1) and its deuterium derivative (Fig. 2) was carried out at room temperature, and its kinetics was studied spectrophotometrically as described in Section 2.4.

The kinetic parameters of Michaelis-Menten Eq. (3.1): V_{max} and K_M in all experiments were obtained using an indirect spectrophotometric method by measuring the increasing absorbance of reduced form of cofactor NADH showing maximum at λ_{max} = 340 nm, according to oxidative deamination of 3-F-L-Ala during the progress of reaction. SIE's and KIE's for enzymatic reaction catalysed by AlaDH (Fig. 1) were determined by a non-competitive method (Parkin, 1991). Data analysis was carried out using graphical representation of Lineweaver-Burk equation of enzyme kinetics. Parameters V_{max} and K_M were calculated using Lineweaver-Burk double reciprocal plot from the experimentally obtained reaction rates at varied concentrations of each substrate.

The values of isotope effects were determined from initial rates (v)