

Deuterium isotope effects in reduction of β -fluoropyruvic acid catalyzed by L-alanine dehydrogenase

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Abstract

L-alanine dehydrogenase (AlaDH, EC 1.4.1.1) from *Bacillus subtilis* catalyzes the biotransformation of β -fluoropyruvic acid into β -fluoro-L-alanine in presence of cofactor NADH and ammonia. This unnatural β -fluorinated amino acid shows antibacterial properties due to inactivation of alanine racemase - enzyme involved in peptidoglycan biosynthesis in bacterial cell wall. We report the studies on mechanism of this reaction using solvent, SIE, and kinetic, KIE, isotope methods. The kinetic of reaction was studied spectrophotometrically by measuring the decreasing absorbance of the reduced form of NADH at 340 nm. For kinetic studies the deuterium labeled NADH was obtained by reduction of NAD⁺ catalyzed by formate dehydrogenase (FDH, EC 1.2.1.2). SIE's and KIE's were determined using non-competitive spectroscopic method.

1. Introduction

Enzyme L-alanine dehydrogenase, AlaDH, (EC 1.4.1.1) catalyzes the reversible conversion of pyruvic acid into L-alanine according to the Fig.1:

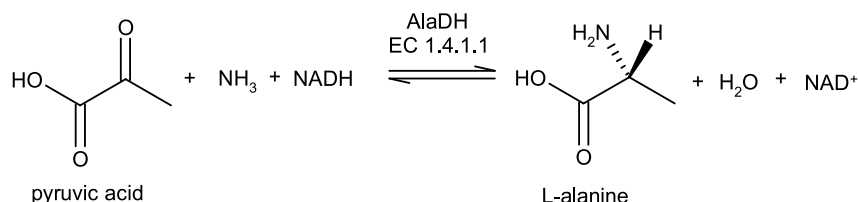


Fig.1 The reversible conversion of pyruvic acid catalyzed by L-alanine dehydrogenase

Above mentioned reaction shows the preferable reductive amination direction, however, enzyme catalyzes also the reaction of oxidative deamination of L-alanine [1]. AlaDH isolated from *Bacillus subtilis* shows *pro-R* stereospecificity and transfers the hydrogen from *pro-R* position at C-4 of nicotinamide ring of NADH to C-2 of pyruvate [2]. High enantioselectivity of AlaDH causes that it acts only on L isomer of alanine in presence of cofactor NADH. AlaDH is characterized by narrow substrate specificity, it works towards pyruvic acid, L-alanine and their isotopologues and derivatives with small modifications at β -C atom such as halogen substituents or hydroxyl group [3]. Moreover, fluorinated amino acids labeled with short-living β^+ -emitters, such as ¹⁸F and ¹¹C can be potentially used as markers in positron emission tomography (PET) for the diagnosis of cancer and neurodegenerative diseases [4].

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 couple of years ago it was discovered that compounds such as β -fluoro-L-alanine and β -fluoro-D-alanine exhibit antibacterial and antiviral properties and they are wide spectrum antibiotics in connection with irreversible inactivation of alanine racemase - enzyme involved in biosynthesis of the bacterial cell wall [5, 6]. Furthermore, fluorinated α -hydroxyacids and α -amino acids potentially can be the highly versatile chiral building blocks for the asymmetric synthesis of compounds of pharmacological interest and they can be potential precursors of fluoroamine compounds [7]. Amino acids containing fluorine can be used in bioorganic applications such as biological traces, mechanistic probes and as potential irreversible inhibitors of PLP-dependent enzymes like decarboxylases, transaminases and racemases [8].

Using β -fluoropyruvic acid as a substrate for AlaDH allows to obtain β -fluoro-L-alanine by the simple and effective method comparing to chemical photofluorination of L-alanine in liquid HF [6] or asymmetric synthesis from β -fluoropyruvate and (S)- α -methylbenzylamine catalyzed by ω -transaminase [9]. Synthesis of β -fluoro-L-alanine using AlaDH from *Bacillus sphaericus* was applied in enzymatic continuous production from β -fluoropyruvic acid in an enzyme membrane reactor with simultaneous regeneration of coenzyme [5], Fig. 2:

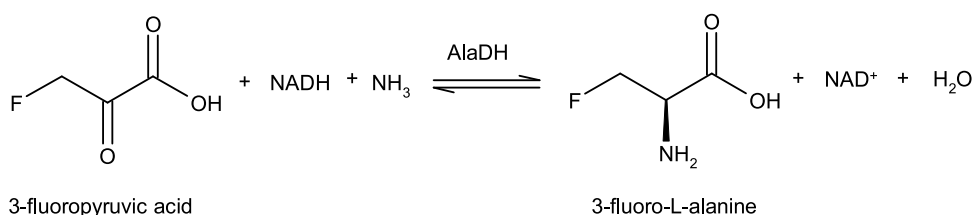


Fig. 2 The reductive amination of β -fluoropyruvic acid catalyzed by AlaDH

Although the reaction catalyzed by AlaDH has been the subject of numerous studies, still many of the intrinsic mechanism details remain unknown. We decided to synthesize β -fluoro-L-alanine and examine some specifics of the mechanism of action of AlaDH in the reductive amination of β -fluoropyruvic acid using solvent and kinetic isotope effects (SIE and KIE) methods. These studies require deuterium labeled isotopologue of cofactor [(4R)- ^2H]-NADH which was obtained by reduction of NAD^+ by formic acid- d_2 catalyzed by formate dehydrogenase (FDH, EC 1.2.1.2) [10, 11, 12]. To determine the numerical values of SIE's and KIE's a non-competitive, spectrophotometric method was applied which allows to obtain the ratios of rate constants. The values of primary and secondary isotope effects can be useful to identify the rate determining step and characterize mechanism important for kinetics such as bond breaking or forming and the structure of active complex.

2. Experimental

2.1. Materials

The enzymes formate dehydrogenase, FDH, (EC 1.2.1.2) from *Candida boidinii*, L-alanine dehydrogenase, AlaDH, (EC 1.4.1.1) from *Bacillus subtilis* and coenzymes NAD⁺ and NADH were from Sigma. Deuteriated water (99.9% ²H) and 30% KO²H/²H₂O were from Polatom, Poland. Other chemicals needed for enzymatic synthesis and trail experiments, such as 95% deuteriated formic acid in ²H₂O (²HCOO²H, 98% ²H) and β-fluoropyruvic acid were also from Sigma. Thin layer chromatography (TLC) plates with UV indicator (DC-Plastikfolien Keiselgel 60 F₂₅₄) and Amberlite IR-120 (Na⁺) were from Merck and Aldrich respectively.

2.2. Methods

The proton nuclear magnetic resonance (¹H NMR) spectra were recorded in ²H₂O 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 and the progress of reduction of NAD⁺ were measured using Shimadzu-UV-1800 spectrophotometer. The presence of the reagents was checked by TLC (thin layer chromatography) using silica gel plates and developing solvent: acetonitrile: water, 4:1; *v/v* (visualization by ninhydrin in ethanol and UV lamp).

2.3. Synthesis

I. Synthesis of β-fluoro-L-alanine

To the 10 mL vial containing 8,3 mg (0.064 mmol) of β-fluoropyruvic acid and 45 mg (0.064 mmol) of NADH about 6 U of enzyme AlaDH was added and solubilized in 3 mL of 0.025 M ammonium buffer (NH₄Cl/NH₃ · H₂O) adjusted to pH 8.8 with 30% KOH. The reaction mixture was incubated at 30°C for 24 hours and its progress was monitored by TLC. The reaction was quenched by acidification with concentrated HCl to pH~2 and denaturized enzyme was removed by centrifugation. Then, the remaining pyruvic acid was extracted with 8×1 mL portions of diethyl ether and the mixture was loaded onto an Amberlite IR 120 H⁺ column (100 × 10 mm). The column was then washed with 500 mL of water in order to remove the salts. The product - β-fluoro-L-alanine was eluted with 0.3 M NH₃(aq), and collected as 7 mL fractions. The presence of the product in fractions was confirmed by TLC as mentioned before. The fractions containing alanine were combined, concentrated under reduced pressure at 50°C and lyophilized. In effect 6 mg (0.056 mmol) of β-fluoro-L-alanine was obtained with 87% chem. yield. The chemical structure of the product was determined by the ¹H NMR spectrum [in D₂O: δ 4.05 (ddd, 1H), δ 4.88 (ddd, 2H)].

II. Synthesis of [(4R)-²H]-NADH

The samples of 60 mg (0.09 mmol) NAD⁺ and 20 U (16 mg) FDH from *Candida boidinii* were added to the vial and dissolved in 6 mL of solution 0.05 M sodium carbonate and containing 0.3 mmol deuteriated formic acid and adjusted to pH 8.5. The reaction mixture was stirred and incubated at room temperature. The progress of the reaction was monitored by measuring the absorbance ratio: A_{260}/A_{340} spectrophotometrically. When the value of ratio was < 2.3 (what took about 3 hours) the reaction was quenched by adding the equal (6 mL) volume of absolute ethanol, (99.8%), cooled to -20°C. The obtained precipitate was centrifugated off at 7000 rpm for 5 minutes at 4°C. Then other portion of cooled ethanol was added to the supernatant to reach the final volume of 40 mL. The mixture was chilled for 15 minutes in refrigerator. The precipitate of [(4R)-²H]-NADH was centrifuged at 7000 rpm for 5 minutes in 4°C, then washed a couple of times with ethanol and diethyl ether and finally dried by lyophilisation. As a result 50 mg (0.07 mmol) of [(4R)-²H]-NADH was obtained with 80% chem. yield. The 4R position of deuterium incorporation and the 100% deuterium enrichment was proved by ¹H NMR spectrum. Two geminal C-4 protons of the nicotinamide ring of NADH showed signals at δ 2.7 ppm for H_S and at δ 2.8 ppm for H_R in ¹H NMR spectrum, whereas after the labeling one signal at δ 2.7 ppm was observed only.

2.4. Kinetic assays

To study isotope effects the previously synthesized [(4R)-²H]-NADH was used. The kinetic experiments for determining of the SIE's and KIE's in reductive amination of β -fluoropyruvic acid were carried out using the following solutions:

- (a) 25 mM ammonium buffer, pH 8.8; the desired pH was adjusted with 30% KOH, cognately the fully deuteriated buffer solution was prepared and corrected to pD with 30% KO²H/²H₂O,
- (b) 5 mM solution of β -fluoropyruvic acid,
- (c) 3 mM solution of NADH,
- (d) L-alanine dehydrogenase solution (0.5 U mL⁻¹).

Depending on the measurement being performed all reagents needed for kinetic assays were solubilized in ammonium buffer or in fully deuteriated buffer medium. Each kinetic experiment consisted of six runs for varying concentrations of β -fluoropyruvic acid. The measurements were carried out in 3 mL quartz spectroscopic cuvettes which were filled with appropriate volumes of the buffered solution of β -fluoropyruvic acid. The final concentrations of substrate were ranged from 0.07 to 0.23 mM with 0.03 mM intervals. Afterwards, the adequate volumes of NADH and AlaDH solutions were added to obtain 0.2 mM and 0.02 U mL⁻¹ concentrations, respectively. Finally, each cuvette was filled with ammonium buffer up to 3 mL. Decreasing absorbance measured at 340 nm for 30 minutes (1 minute intervals) caused by oxidation of NADH allowed to monitor the progress of reaction.

3. Results and discussion

3.1. Synthesis

The unnatural fluorinated amino acid – β -fluoro-L-alanine was synthesized by enzymatic reductive amination catalyzed by L-alanine dehydrogenase from *Bacillus subtilis* according to the reaction presented at Fig. 2. The synthesis was carried out at 30°C and the source of ammonia was 25 mM ammonium buffer (pH 8.8). The chemical structure of the product was confirmed by ^1H NMR spectrum and obtained values of chemical shifts are consistent with results reported in literature [7].

The stereospecifically labeled cofactor [(4*R*)- ^2H]-NADH was synthesized according to the modified experimental procedure by reduction of NAD^+ carried out at room temperature in carbonate buffer (pH 8.5) containing $^2\text{HCOO}^2\text{H}$ as a source of deuterium, Fig. 3. The conversion of NAD^+ to reduced form was monitored by measuring the absorbancy ratio: A_{260}/A_{340} . When reaction was complete the product was isolated and dried by lyophilisation. The degree of deuterium incorporation was calculated using ^1H NMR spectrum by measurement integrations of the signals.

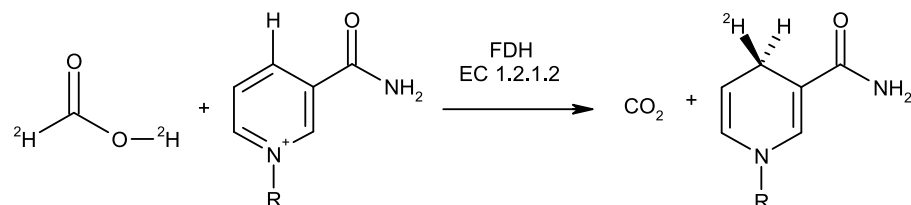


Fig. 3 Synthesis of [(4*R*)- ^2H]-NADH catalyzed by FDH

The obtained product was used to determine KIE's and SIE's of reductive amination of β -fluoropyruvic acid. The deuterium is exclusively transferred from cofactor [(4*R*)- ^2H]-NADH to C-2 position of β -fluoropyruvic acid to form [^2H]- β -fluoro-L-alanine according to the Fig. 4:

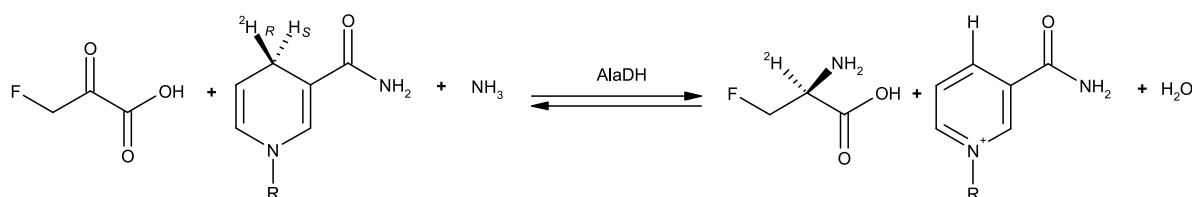


Fig. 4 Reductive amination of β -fluoropyruvic acid involving deuterium labeled [(4*R*)- ^2H]-NADH

3.2. Kinetic assay

The kinetic parameters of Michaelis-Menten equation (3.1): V_{max} and K_M in reductive amination of β -fluoropyruvic acid were obtained using the indirect spectrophotometric method by registering of NADH absorbancy. The coenzyme NADH shows a maximum of absorbance at $\lambda=340$ nm, so its decreasing allows to track the course of reaction. The deuterium KIE's and SIE's in enzymatic reduction were determined using a non-competitive method [13]. The kinetic parameters were

calculated using Lineweaver-Burk plot from the experimentally obtained reaction rates at varied concentrations of substrate.

The values of isotope effects were obtained from initial rates (v) for corresponding isotopomer calculated from Equation (3.1) [14]:

$$v = \frac{[S]}{\frac{1}{V_{\max} K_M} + [S]/V_{\max}}, \quad (3.1)$$

where: K_M - the Michaelis constant,

$[S]$ - the concentration of substrate (S is much greater than the concentration of enzyme),

V_{\max} - the maximum reaction rate,

v - the initial rate at $[S]$.

The experimental values of isotope effects are shown in Table 1. Experimental errors were calculated with Student's t-distribution for 95% confidence interval. In most cases the normal isotope effects in reaction of reductive amination were observed. Obtained results indicate that the reaction rates involving the heavier isotope are lower than with lighter isotope. That suggests that breaking of the C-²H bond is more difficult and more energy is required than for C-¹H bond. Rather high values of SIE's for NADH and [(4R)-²H]-NADH on V_{\max} =6 and 4.4 respectively, suggest that deuteriated solvent plays an important role in the transformation of an enzyme-substrate complex into an enzyme-product complex [15]. The value of SIE on V_{\max}/K_M close to unity for reaction involved labeled cofactor shows that dissociation of substrate from enzyme is much slower than converting the substrate into product. Obtained values of KIE's indicate that hydride transfer between substrate and coenzyme is partially rate limiting [16].

Table 1

Solvent and Kinetic Isotope Effects for reductive amination of β -fluoropyruvic acid

	Effects	on V_{\max}	on V_{\max}/K_M
SIE	NADH	6 ± 1	1.5 ± 0.1
	[(4R)- ² H]-NADH	4.4 ± 0.3	0.8 ± 0.09
KIE	in ¹ H ₂ O	1.9 ± 0.2	1.7 ± 0.1
	in ² H ₂ O	1.4 ± 0.2	0.8 ± 0.1

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