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Journal of Fluorine Chemistry 124 (2003) 219–227
JOURNAL OF FLUORINE CHEMISTRY
www.elsevier.com/locate/jfluchem

Kinetic aspects involved in the simultaneous enzymatic synthesis of (*S*)-3-fluoroalanine and (*R*)-3-fluorolactic acid

Luciana P.B. Gonçalves, O.A.C. Antunes[1], Gerson F. Pinto, Enrique G. Oestreicher[*]

Instituto de Química, UFRJ, CT Bloco A Labs 641 and 536 B, Cidade Universitária, Rio de Janeiro, RJ 21945-970, Brazil

Received 5 August 2003; received in revised form 5 August 2003; accepted 20 August 2003

Abstract

Desaminative oxidation of *rac*-3-fluoroalanine (*rac*-1) catalyzed by L-alanine dehydrogenase (L-ALADH) was studied by means of initial rate experiments both in the presence and in the absence of a product. Estimates of kinetic parameters of the reaction were obtained. The reaction mechanism is the sequential ordered BiTer mechanism with inhibition by excess of (*S*)-3-fluoroalanine. This kinetic study was the basis for the development of a couple enzymatic system for the simultaneous synthesis of (*S*)-3-fluoroalanine (**1a**) and (*R*)-3-fluorolactic acid (**3**) with L-ALADH and L-lactate dehydrogenase using *rac*-1 and NAD^+. Analysis of isolated products revealed **1a** in 60% yield and 86% ee and **3** in 80% yield and over 99% ee. Compounds **1a** and **3** represent chiral building blocks for the synthesis of several products with pharmacological activity. The presence of the fluorine atom in the substrate causes a better interaction of it in the active site of the enzyme.

Keywords: L-Alanine dehydrogenase; (*S*)-3-Fluoroalanine; (*R*)-3-Fluorolactic acid; Enantiospecific desaminative oxidation; Enantioselective reduction

1. Introduction

Oxidoreductions, especially the reduction of carbonyl compounds, the aminative reduction of α-ketoacids and the oxidative deamination of *rac*-α-amino acids catalyzed by stereoselective $NAD(P)^+$-dependent dehydrogenases, are very important organic reactions in asymmetric synthesis leading to the production of valuable chiral building blocks. The use of isolated enzymes to catalyze these reactions has greatly increased in the last years [1–12].

However, the substrate specificity and the enantioselectivity of these enzymes are very variable.

For instance, alcohol dehydrogenases are enzymes that, although having a broad substrate specificity show variable enantioselectivity [10–14]. On the other hand, L-lactic dehydrogenase from rabbit muscle (L-LDH) and L-alanine dehydrogenase (L-ALADH) have proved to be highly enantioselective, but with a rather narrow range of substrate specificity, restricted to relative small modifications on the β-carbon atom of pyruvate [5,15–17].

Fluorinated chiral α-hydroxyacids, such as (*R*)-3-fluorolactic acid, are considered to be highly versatile chiral building blocks in asymmetric synthesis for the production of several compounds of pharmacological interest, such as, β-adrenergic blocking agents of the aryloxypropanolamines type, such as, (*S*)-propranolol and (*S*)-moprolol and of other products of pharmaceutical interest such (*S*)-3-hydroxypyrrolidine-2-one which in its open form, (*S*)-4-amino-2-hydroxybutanoic acid, is considered to be one of the most potent known inhibitors of the neurotransmitter GABA, also showing anticancer activity [5,18].

On the other hand, (*S*)-3-fluoroalanine has been described as an antibiotic of wide spectra [19] via irreversible inactivation of bacterial alanine racemase [20], an enzyme involved in the biosynthesis of the cell wall. This compound also acts as an inhibitor of serine palmitoyl transferase [21]

Abbreviations: L-ALADH, *Bacillus subtilis* L-alanine dehydrogenase; L-LDH, rabbit muscle L-lactate dehydrogenase; A, NAD^+; B, (*S*)-alanine or (*R*)-3-fluoroalanine; P, NH_4^+; Q, pyruvate or 3-fluoropyruvate; R, NADH; BSA, bovine serum albumine; E, free form of the enzyme; K_{ia}, dissociation constant of L-ALADH-NAD^+ complex; K_a, Michaelis constant of NAD^+; K_b, Michaelis constant of (*S*)-alanine or (*R*)-3-fluoroalanine (3-fluoro-L-alanine); K_M, apparent value of the Michaelis constant of NAD^+; K_{ii}, apparent value of the dissociation constant of L-ALADH-NAD^+-inhibitor complex; K_{is}, apparent value of L-ALADH-inhibitor complex; v_0, initial velocity; V_0, maximum velocity; V_{app}, apparent value of the maximum velocity; ee, enantiomeric excess; HPLC, high resolution liquid chromatography; IR, infrared spectrometry

[*] Corresponding author. Tel.: +55-21-25627366; fax: +55-21-2562-7266.
E-mail addresses: octavio@iq.ufrj.br (O.A.C. Antunes), enrique@iq.ufrj.br (E.G. Oestreicher).
[1] Co-corresponding author. Tel.: +55-21-2562-7248; fax: +55-21-2562-7559.

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doi:10.1016/j.jfluchem.2003.08.009

and in addition, it is a potential precursor of fluoroamine compounds [22,23].

The utilization of enzymes as catalyst for the production of chiral building blocks is widely reported in the literature [7,8]. However, the use of dehydrogenases in preparative applications requires effective processes for coenzyme regeneration since these cofactors are too expensive to be used as stoichiometric reagents. Several of these processes have already been reported by our group [4–6].

In addition, the use of fluorinated blocks in the synthesis of pharmaceuticals has been receiving considerable attention in the past years [24].

(*R*)-3-Fluorolactic acid was obtained enzymatically in our laboratory in 80% overall yield and ee > 99% in a system involving the enantioselective reduction of 3-fluoropyruvate catalyzed by L-LDH and NADH which was in situ regenerated by the oxidation of *cis*-1,2-bis(hydroxymethyl)cyclohexane catalyzed by HLADH [5,6]. (*R*)-3-Fluoroalanine was obtained by Oshima et al. [12] with L-ALADH from *Bacillus sphaericus* by reductive amination of 3-fluoropyruvate in the presence of NADH. The reduced form of the coenzyme was in situ regenerated by the oxidation of ammonium formate catalyzed by yeast formate dehydrogenase. No enantioselective process for the production of (*S*)-3-fluoroalanine has been described before our preliminary communication [4]. The only process described in the literature for the synthesis of (*S*)-3-fluoroalanine is the photofluorination of (*R*)-alanine with CF_3-OF with a conversion of 50% [19].

An enzymatic system able to produce simultaneously (*S*)-3-fluoroalanine via kinetic resolution of *rac*-3-fluoroalanine by deaminative oxidation catalyzed by L-ALADH and (*R*)-3-fluorolactic acid by enantioselective reduction of 3-fluoropyruvate generated in the former reaction catalyzed by L-LDH, is reported in the present article. In order to improve the performance of this coupled enzymatic system since the deaminative oxidation reaction, the rate limiting step of the whole process, is thermodynamically unfavorable and strong inhibition phenomena by excess of substrate and/or by the reaction products certainly are present, a kinetic study of this reaction was undertaken. This study involved initial velocity measurements in the absence of products and the analysis of the inhibition by the products of the reaction. From this study reaction conditions for coupled enzymatic system were found that allowed the simultaneous production of (*S*)-fluoroalanine in 60% yield and 86% ee and (*R*)-3-fluorolactic acid in 80% yield and over 99% ee. The presence of fluorine allowed a stronger interaction between the enzyme and the substrate.

2. Materials and methods

2.1. Materials

Bacillus subtilis L-alanine dehydrogenase (L-alanine: NAD^+ oxidoreductase, EC 1.4.1.1), suspension in 2.4 M $(NH_4)_2SO_4$, pH 7.0, L-lactate dehydrogenase from rabbit muscle (L-(+)-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), solution in 50% glycerol containing 10 mM potassium phosphate buffer, pH 7.5, L-glutamate dehydrogenase (L-GLUDH) from bovine liver type III (L-glutamate:$NAD(P)^+$ oxidoreductase, EC 1.4.1.3), lyophilized, NAD^+ (grade IIIC), NADH (grade III), bovine serum albumine (fraction V), sodium pyruvate, sodium 3-fluoropyruvate, *rac*-alanine and Dowex 50W-X8 cation exchange resin were obtained from Sigma. α-Ketoglutarate, $NaBH_4$ and the Diazald® kit (for CH_2N_2 generation) were purchased from Aldrich Chemical. All other chemicals were of analytical grade and obtained from Merck, Darmstad.

2.2. Assay of L-ALADH activity and estimation of kinetic parameters

L-ALADH activity (assayed in the direction of *rac*-3-alanine deaminative oxidation) was measured by following NADH absorption at 340 nm at pH 7.9 and 25 °C. All assays were carried out in quartz cuvettes with a 1 cm light path using a Beckman DU 70 spectrophotometer equipped with a dot-matrix printer. The temperature of the cell holder was kept at 25 °C by forced circulating water.

Reaction mixtures contained in a total volume of 1 ml:50 mM sodium phosphate buffer, pH 7.9 with varying concentrations of NAD^+ and *rac*-3-fluoroalanine and for product inhibition studies, contained also four different fixed concentrations of 3-fluoropyruvate or NH_4Cl, that are indicated in the legends of Figs. 2 and 3, respectively. NAD^+, *rac*-3-fluoroalanine and product inhibitors solutions were made up immediately before use in the same buffer and maintained in an ice bath. After addition of the substrates or of the substrates and one inhibitor, the reaction mixtures were incubated for 5 min in the spectrophotometer cell holder before the addition of the enzyme solution. Reaction was started by adding 20 μl of an enzyme solution containing in a volume of 1.0 ml:130 μg (protein basis) corresponding to 0.45 IU of a desalinized L-ALADH enzyme preparation (obtained by ultra-filtration with Centricon-10 concentrators (AMICON), through five consecutive centrifugations at 5 °C for 1 h and 2000 × *g* and reconstitution of the original volume with sodium phosphate buffer 10 mM, pH 7.7 and 50% (v/v) of glycerol. The slopes of the recording lines were kept close to 45° by varying the absorbance full scale and/or the time full scale of the spectrophotometer. The printer curves obtained were extrapolated to the time of enzyme addition and the tangents of the curves at this time were taken as initial velocities. The initial velocity of the reaction was calculated in terms of mM of NADH produced per minute, using a molar absorption coefficient for NADH of 6220 M^{-1} cm^{-1}.

2.3. Kinetic data processing

The values (symbols) appearing in the figures are the mean experimentally determined initial velocities (triplicates), and were used in determining the kinetic parameters.

Estimates of parameters and of their asymptotic standard errors were obtained by fitting the appropriate rate equations to data using a nonlinear least-squares computer program, developed in our laboratory and specifically devised for steady-state studies of enzymes kinetics [25]. Before the data for a whole experiment were analyzed with the aid of the computer program, the linearity of the individual lines was checked graphically by plotting the reciprocal velocities against the reciprocal of the varied substrate concentration at various concentrations of the fixed changing ligand. The same procedure was extended for the effect of the fixed changing ligand on intercepts and/or slopes of the Line-Weaver–Burk plots obtained. Based on these preliminary plots, the proper pattern was selected for each experiment and the appropriate equations (see below) were fitted to the mean data points.

It will be assumed now, and justified in Section 3 that the reaction mechanism is the sequential ordered BiTer [26]. Then the initial velocity equation in the absence of products is:

$$v_0 = \frac{V_0 AB}{K_{ia}K_b + K_b A + K_a B + AB} \quad (1)$$

For the inhibition studies, the rate equations used were:- linear noncompetitive inhibition,

$$v_0 = \frac{V_{app} S}{K_M(1 + I/K_{is}) + S(1 + I/K_{ii})} \quad (2)$$

linear uncompetitive inhibition,

$$v_0 = \frac{V_{app} S}{K_M + S(1 + I/K_{ii})} \quad (3)$$

2.4. Laboratory preparative enzymatic production of (S)-3-fluoroalanine and (R)-3-fluorlactate

The reaction was performed in a jacketed batch reactor of 120 ml, continuously stirred. Reaction medium contained in a total volume of 50 ml:0.05 M sodium phosphate buffer, pH 7.9, 20 mM *rac*-3-fluoroalanine, 373 IU of L-ALADH, 4250 IU of L-LDH and 0.05% (w/v) BSA. The reaction was started by adding 0.25 mM NAD^+. The reaction mixture was maintained under continuous magnetic stirring and the temperature was kept at 25 °C by circulating water through the jacket of the reactor with a thermocirculating bath.

The extent of reaction was followed by removing at different times aliquots of 10–50 μl of the reaction mixture that were immediately diluted with 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.9 and heated at 100 °C for 5 min in order to inactivate the enzymes. The concentration of NH_4^+ present in these samples was determined by "end-point" assays in the presence of 0.2 mM NADH, 0.5 mM of α-ketoglutarate and 1 mg of L-GLUDH in a total volume of 2.0 ml at 25 °C. The decrease of NADH absorption after the addition of the enzyme was followed spectrophotometrically at 340 nm until total consumption of the substrate. The concentration of NH_4^+ was calculated from the difference of NADH absorption (before and after) the addition of L-GLUDH by using the molar absorption coefficient of NADH. This method gave results that are in agreement with those obtained by chiral HPLC in Section 2.6.

2.5. Isolation of target products

Isolation and characterization of products formed was accomplished by thermal treatment of the reaction mixture (100 °C for 15 min) in order to inactivate the enzymes. After centrifugation to remove the coagulated proteins, the supernatant fluid was acidified with 3 M HCl until pH 2.0 and submitted to cation exchange column chromatography on Dowex 50W-X8 (8 cm × 2.5 cm). The column was initially eluted with glass bidistilled water in order to recover 3-fluorolactic acid and then with 1 M NH_4OH to isolate 3-fluoroalanine. The fractions containing the former product were acidified until pH 1.7 with 1 M HCl, saturated with NaCl and continuously extracted with Et_2O for 72 h to eliminate the coenzyme. The organic phase, containing 3-fluorolactic acid, was methylated with CH_2N_2/Et_2O according to Blank et al. [27]. The resulting product, 3-fluorolactic acid methyl ester was obtained with an overall yield of 80%.

The ninhydrin positive fractions that were eluted with NH_4OH containing 3-fluoroalanine were concentrated in rotatory evaporator at 40 °C until all NH_4^+ was eliminated and vacuum dried in Speed Vac (Savant). The resulting product was re-crystallized from *iso*-propanol/H_2O as follows. The dried material was re-suspended in glass bidistilled water until complete dissolution, 30 mg of active charcoal were added and the suspension was maintained under continuous stirring for 15 min. The sample was filtered and the operation repeated until a colorless solution was obtained that was heated at 60 °C with the dropwise addition of *iso*-propanol/H_2O mixture (1:3, v/v) at 60 °C. The sample was kept at room temperature for 30 min and then for 3 h in an ice bath. After initiation of the crystallization process, drops of *iso*-propanol were added and the crystals formed were collected by filtration under vacuum and were washed successively with *iso*-propanol/H_2O 90% (v/v), *iso*-propanol and hexane. Isolated yield was 60%.

2.6. Characterization of (R)-3-fluorolactic acid methyl ester and (S)-3-fluroalanine

Both target products were analyzed and characterized by: IR spectrometry with a Nicolet Magna-IR 760 spectrophotometer; ^1H NMR spectroscopy with a 200 MHz Varian (Gemini) equipment; polarimetry, carried out with a Jasco DIP-370 digital polarimeter; Chiral HPLC performed by using an ISCO 2350 chromatograph equipped with a Nucleosil Chiral-1® column (Macherey-Nagel, ET 250/8/4) by using 1 mM $CuSO_4$, pH 4.6 as eluent at a flow rate of 1 ml/min, 23 °C and UV detection at 235 nm.

2.7. Determination of enantiomeric excess

The enantiomeric excesses of both target products were determined by chiral HPLC as described in Section 2.6, *rac*-3-fluorolactic acid methyl ester and *rac*-3-fluoroalanine were used as standards.

Rac-3-fluorolactic acid methyl ester was prepared as previously described by us in [5]. *Rac*-3-fluoroalanine was obtained by reductive amination of 3-fluoropyruvate (3.49 mmol) with $NaBH_4$ (5.5 eq.) in the presence of NH_4OH (68 mmol) as described by Dolling et al. [28]. The product was isolated, purified, crystallized and identified as described in Sections 2.5 and 2.6.

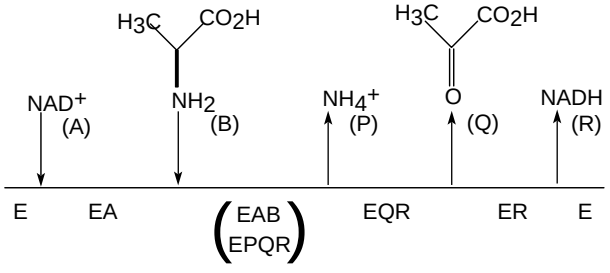
2.8. Determination of protein concentration

Protein concentration was determined by using a method suitable for the detection of low protein contents [29]. Bovine serum albumin was used as standard.

3. Results and discussion

Grimshaw and Cleland [30] determined that the kinetic mechanism for the oxidative deamination reaction of L-alanine catalyzed by L-ALADH from *B. subtilis* in the presence of NAD^+ was the sequential ordered BiTer kinetic model, with NAD^+ binding to the free form of the enzyme followed by the binding of L-alanine. The first product to be released was NH_4^+, followed by pyruvate and NADH was the last one (Scheme 1).

Considering that L-ALADH from *B. subtilis* shows an absolute stereoespecificity for the L-enantiomer of alanine [30] and that compounds harboring an halogen substituent on the β-atom carbon of alanine have not been before used to determine the kinetic mechanism of the oxidative deamination reaction of this enzyme, we decided to investigate the kinetic mechanism of the oxidative deamination reaction of *rac*-3-fluoroalanine catalyzed by L-ALADH. Also, the kinetic resolution of *rac*-3-fluoroalanine by oxidative deamination of the *R*-enantiomer of this compound will produce (*S*)-3-fluoroalanine, an important chiral building block in asymmetric synthesis for the production of several compounds of pharmacological interest [4] that in addition presents a strong antibiotic activity [19].



Scheme 1. Sequential ordered steady-state kinetic mechanism for the oxidative deamination reaction of (*S*)-alanine catalyzed by L-ALADH.

Fig. 1 shows the fitting of Eq. (1) to the initial velocity data obtained in the absence of products. In Fig. 1A *rac*-3-fluoroalanine was the variable substrate at seven fixed concentrations of NAD^+. Since an intersecting pattern of straight lines was obtained, this was the first indication that the kinetic mechanism is most probably of the sequential type involving ternary or central complexes, not necessarily kinetically important. This experiment also revealed that high concentrations of the varied substrate have inhibitory activities. In order to confirm these experimental observations, the concentration of NAD^+ was varied keeping the concentration of 3-fluoroalanine fixed at various levels. As shown in Fig. 1B the same pattern of intersecting lines was obtained, however, when concentrations of 3-fluoroalanine higher than 4.0 mM were used, parallels lines indicative of an uncompetitive inhibition by excess of 3-fluoroalanine, were obtained (Fig. 1B).

If the experimental points showing inhibition (Fig. 1A and B) by excess of substrate are eliminated and Eq. (1) is fitted to the remaining data, estimates of the parameters of this rate equation are obtained. These estimates together with the corresponding values determined by Grimshaw and Cleland [30] for the oxidative deamination of L-alanine are displayed in Table 1. On comparing the values of the kinetic parameters obtained for the oxidative deamination of 3-fluoroalanine with those for L-alanine determined by Grimshaw and Cleland [30] (Table 1), it can be seen that the values estimates for K_{ia} are not significantly different and thus, K_{ia} does not depends on the nature of the other substrate (3-fluoroalanine and L-alanine, respectively), suggesting that, in fact, this parameter is the dissociation constant of the L-ALADH-NAD^+ complex, and that for the oxidative deamination reaction of 3-fluoroalanine the order of substrate combination with the enzyme is the same as for the corresponding reaction of L-alanine catalyzed by L-ALADH [30]. On the other hand, markedly in the case of K_b, the value obtained for 3-fluoroalanine was significantly lower than the

Table 1
Estimates of kinetic parameters derived from initial velocity studies on the oxidative deamination of (*R*)-3-fluoroalanine catalyzed by L-ALADH

Parameter	Value ± S.E.[a]	Value ± S.E.[b]	95% Confidence limits[c]
V_0[d] (mM/min)	–	0.032 ± 0.002	0.029–0.036
K_{ia} (mM)	0.33 ± 0.07	0.394 ± 0.036	0.321–0.468
K_b (mM)	4.40 ± 0.70	2.499 ± 0.233	2.023–2.975
K_a (mM)	0.22 ± 0.02	0.266 ± 0.350	0.194–0.338

[a] Data from [26] for the oxidative deamination of (*S*)-alanine (L-alanine).
[b] Asymptotic standard error of parameter. Estimates of parameters obtained by nonlinear regression analysis of experimental data depicted in Fig. 1.
[c] Calculated according to Student's *t*-test adapted for nonlinear regression as described by Metzler [33].
[d] Value not supplied in [29].

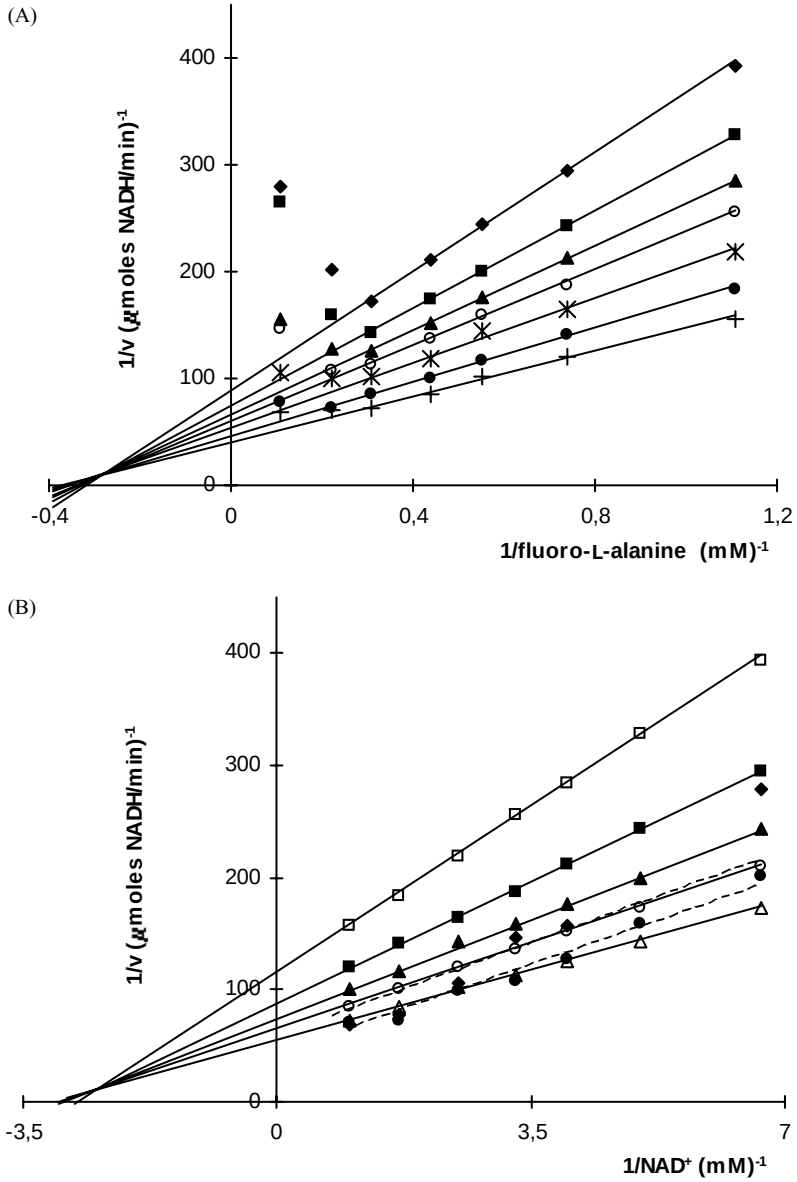


Fig. 1. Effect of 3-fluoroalanine and NAD^+ on the initial velocity of the reaction of oxidative deamination of (*R*)-3-fluoroalanine catalyzed by L-ALADH. (A) *Rac*-3-fluoroalanine was the variable substrate (its concentration is expressed as (*R*)-3-fluoroalanine considering 95% purity of the synthesized racemate). The concentrations of NAD^+ were: (◆) 0.15 mM; (■) 0.2 mM; (▲) 0.25 mM; (○) 0.3 mM; (✳) 0.4 mM; (●) 0.6 mM, and (+) 1.0 mM. (B) NAD^+ was the variable substrate. The concentrations of (*R*)-3-fluoroalanine (considering 95% purity of the synthesized racemate) were: (□) 0.9 mM; (■) 1.35 mM; (▲) 1.8 mM; (○) 2.25 mM; (△) 3.15 mM; (●) 4.5 mM; and (○) 9.0 mM. Other experimental condition are given in Sections 2.1 and 2.2.

value for the limiting Michaelis constant of the natural substrate of the enzyme ($P > 0.05$ calculated from the Student's *t*-test). *This result may indicate that the presence of the fluorine atom in the substrate causes a better interaction of it in the active site of the enzyme.*

In order to confirm the substrates binding sequence with the enzyme as well as to obtain some information concerning the order of release of products from the central complexes, a study of product inhibition was carried out. The results of these experiments by using NAD^+ as the varied substrate are illustrated in Figs. 2 and 3. When NH_4^+ was assayed as inhibitor a linear noncompetitive inhibition pattern was obtained (Fig. 2) on the other hand, 3-fluoropyruvate produced a linear uncompetitive inhibition pattern (Fig. 3). Estimates of parameters of Eqs. (2) and (3) and of their asymptotic standard errors are displayed in Table 2. The fact that NH_4^+ behaves as a noncompetitive inhibitor in relation to NAD^+ (Fig. 2), that 3-fluoropyruvate is an uncompetitive inhibitor (Fig. 3) and that NADH is a competitive inhibitor in relation to NAD^+ (results not show), strongly suggests that the desaminative oxidation of 3-fluoro-D-alanine catalyzed by *B. subtilis* L-ALADH in the presence of NAD^+ follows a sequential ordered BiTer kinetic mechanism [26], with NAD^+ being the first substrate to bind to the enzyme followed by the binding of 3-fluoro-D-alanine. Moreover, since NADH produced linear

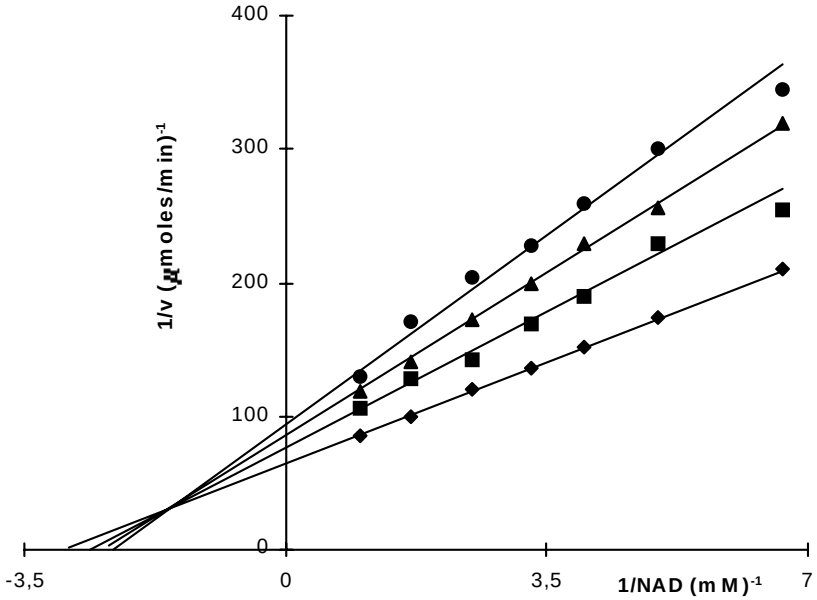


Fig. 2. Inhibition of the reaction of oxidative deamination of (*R*)-3-fluoroalanine catalyzed by L-ALADH by NH_4^+. NAD^+ was the variable substrate and (*R*)-3-fluoroalanine concentration was kept constant at 2.25 mM. Concentrations of NH_4^+ were: (◆) zero; (■) 2.0 mM; (▲) 3.5 mM; and (●) 5.0 mM. Other experimental conditions are given in Sections 2.1 and 2.2.

competitive inhibition in relation to NAD^+, the reduced coenzyme must be the last product to be release in the reaction. The mixed type inhibition produced by NH_4^+ (Fig. 2) and the linear uncompetitive inhibition found for 3-fluoropyruvate (Fig. 3), strongly suggest that NH_4^+ is the first product to be release in the reaction, followed by the release of 3-fluoropyruvate. Considering this order of products release, the uncompetitive inhibition by *rac*-3-fluoroalanine in relation to NAD^+ (Fig. 1B) can be attributed to the formation of a L-ALADH-NADH-3-fluoro-L-alanine ''dead-end'' complex. The kinetic mechanism determined with this series of experiments for the deaminative oxidation of *rac*-3-fluoroalnine catalyzed by L-ALADH is shown in Scheme 2.

Grinshaw and Cleland [30] described that D-alanine behaved as a linear competitive inhibitor with respect to L-alanine. This inhibition implies in the competition of both enantiomers of alanine for the L-ALADH-NAD^+ complex

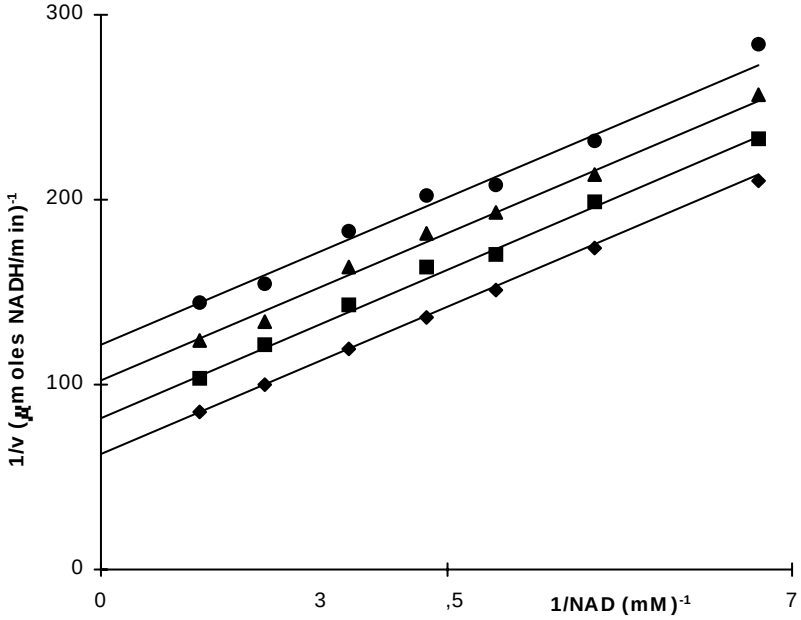


Fig. 3. Inhibition of the reaction of oxidative deamination catalyzed by L-ALADH by 3-fluoropyruvate. NAD^+ was the variable substrate and (*R*)-3-fluoroalanine concentration was kept at 2.25 mM. Concentrations of 3-fluoropyruvate were: (◆) zero; (■) 0.02 mM; (▲) 0.04 mM; and (●) 0.06 mM. Other experimental conditions are given in Sections 2.2 and 2.3.

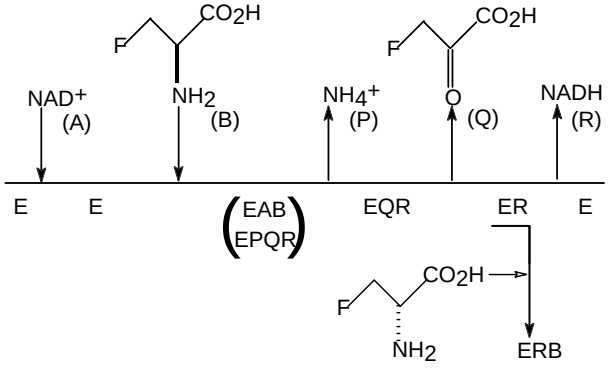
Table 2
Estimates of kinetic parameters derived from product inhibition studies on the oxidative deamination of (*R*)-3-fluoroalanine catalyzed by L-ALADH

Parameter	Estimate ± S.E.[a] (product inhibitor)	
	NH_4^+[b]	3-Fluoropyruvate[c]
V_{app} (mM/min)	0.017 ± 0.002	0.016 ± 0.002
K_M (mM)	0.338 ± 0.013	0.364 ± 0.011
K_{ii} (mM)	5.750 ± 0.355	0.063 ± 0.022
K_{is} (mM)	10.826 ± 0.458	–

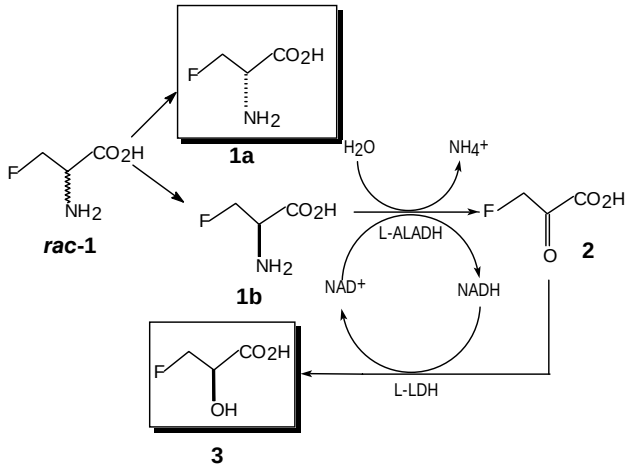
[a] Asymptotic standard error of parameter.
[b] Estimates of parameters obtained by fitting Eq. (2) to experimental data depicted in Fig. 2 by nonlinear regression analysis.
[c] Estimates of parameters obtained by fitting Eq. (3) to experimental data depicted in Fig. 3 by nonlinear regression analysis.

(Scheme 1). Since the experiments described in this work were performed with racemic 3-fluoroalanine, the interaction of 3-fluoro-D-alanine with the above mentioned complex can be discarded, otherwise, instead of the linear uncompetitive inhibition by excess of *rac*-3-fluoroalanine observed (Fig. 1B), a linear mixed type inhibition should be evident. This latter type of inhibition would arise by formation of both L-ALADH-NAD^+-3-fluoro-D-alanine and L-ALADH-NADH-3-fluoro-L-alanine dead-end complexes. The absence of the former complex is indicative of a much stronger interaction of 3-fluor-L-alanine with the L-ALADH-NAD^+ than L-alanine as suggested by the value of K_b found for the fluorinated substrate (Table 1).

As stated earlier in the present work, (*R*)-3-fluorolactic acid methyl ester and (*S*)-3-fluoroalanine can be considered as valuable chiral building blocks for the synthesis of several enantiomeric pure products with therapeutically activity. (*R*)-3-Fluorolactic acid methyl ester has been previously obtained in our laboratory [5] in 80% overall yield and ee > 99% by enantiospecific reduction of 3-fluoropyruvate catalyzed by rabbit muscle L-lactate dehydrogenase. NADH needed in this reaction was used in catalytic concentration by utilizing a NADH in situ regeneration system consisting of the oxidation of *cis*-1,2-bis(hydroxymethyl)cyclohexane to the chiral lactone (+)-(1*R*,6*S*)-*cis*-8-oxabicyclo[4.3.0] nonan-7-one catalyzed by horse liver alcohol dehydrogenase



Scheme 2. Sequential ordered BiTer kinetic mechanism for the oxidative deamination reaction of (*R*)-3-fluoroalanine catalyzed by L-ALADH.



Scheme 3. Coupled enzymatic redox system for the simultaneous production of (*S*)-3-fluoroalanine (**1a**) and (*R*)-3-fluorolactic acid (**3**) using *rac*-3-fluoroalanine (*rac*-1) as substrate. (*R*)-3-Fluoroalanine (**1b**); 3-fluoropyruvic acid (**2**).

[5]. (*S*)-3-Fluoroalanine, on the other hand, has never been before obtained enzymatically. In 1970, Kollonitsch et al. [19] obtained this compound by photofluorination of D-alanine with fluoroxytrifluoromethane with yield of 50%.

Since the kinetic properties of L-ALADH acting on *rac*-3-fluoroalanine as well as those of L-LDH catalyzing the enantioselective reduction of 3-fluoropyruvate [5,6] were studied by our group, we envisaged an enzymatic system suitable for the simultaneous production of (*S*)-3-fluoroalanine (**1a**) and (*R*)-3-fluorolactic acid (**3**). As shown in Scheme 3 this enzymatic system involves the deaminative oxidation of the *R*-enantiomer (**1b**) of *rac*-1 to produce NH_4^+, 3-fluoropyruvate (**2**) and NADH. The latter two products of the main reaction are used by L-LDH to produce (*R*)-3-fluorolactic acid (**3**) and NAD^+, then if the velocity of this reaction is not rate limiting of the whole system, just NH_4^+ and (*R*)-3-fluoralanine will accumulate in the reaction medium and NAD^+ will be continuously recycled until all (**1b**) has been consumed. Moreover, since NH_4^+ is a rather weak inhibitor of L-ALADH (Table 2) and the concentrations of NADH and of (**2**) will be almost zero in this condition, the extent of conversion must be high and (**1a**) must be obtained with a high ee and a yield of approximately 50% (total conversion of **1b**). This coupled redox system is then able to produce simultaneously two valuable chiral blocks, (**1a**) by kinetic resolution of *rac*-3-fluoroalanine catalyzed by L-ALADH and (**3**) by enantioselective reduction of (**2**) catalyzed by L-LDH.

The time–course curve for the deaminative oxidation of (**1b**) catalyzed by L-ALADH with the concomitant enantioselective reduction of (**2**) catalyzed by L-LDH that also acts as NAD^+ in situ regeneration system in a semi-preparative scale of approximately 150 mg (20 mM *rac*-3-fluoroalanine) is shown in Fig. 4. The progress of the reaction indicates that after 24 h of reaction, under the experimental conditions described in Section 2.4, 93% of (**1b**) were consumed,

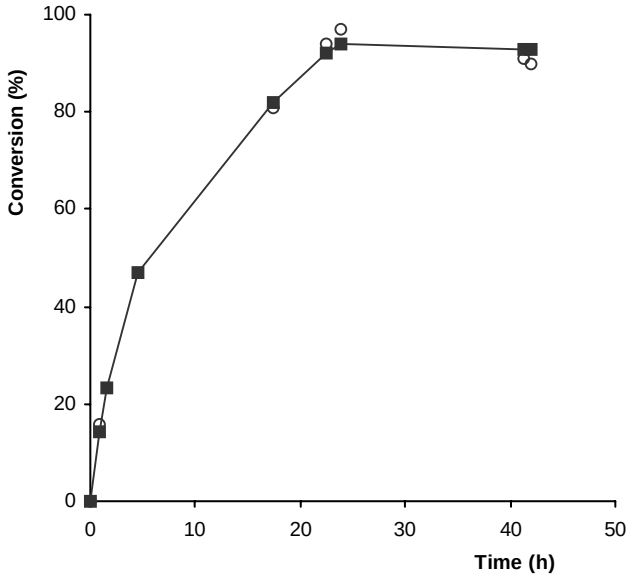


Fig. 4. Time–course curve of the coupled enzymatic system for the simultaneous production of (*S*)-3-fluoroalanine and (*R*)-3-fluorolactic acid in a laboratory preparative scale (150 mg). The progress of reaction was followed by enzymatic determination of NH_4^+ concentration at the times indicated in the figure as described in Section 2.4 (○) or by chiral HPLC determination of (*S*)-3-fluoroalanine as described in Section 2.6 (■).

suggesting that at the end of the reaction, theoretically (considering that L-ALADH is absolutely enantiospecific for (**1b**) as it has been shown for the natural substrate by Grimshaw and Cleland [30]), the reaction medium must contain 9.3 mM of (**1a**) with an ee of 86% and the same concentration of enantiopure (**3**), respectively.

At the end of the reaction, after thermal inactivation of enzymes, both target products (**1a**) and (**3**) were isolated and purified as described in Section 2.5. Compound **1a** was isolated in 60% yield, $[\alpha]_D^{25} = -8.7$ ($c = 0.8$, 1 M HCl). IR revealed the following bands of axial deformation: 3428 cm^{-1} (NH_3^+); 3061, 2987 and 2923 cm^{-1} (aliphatic C–H); 1615 cm^{-1} (C=O of carboxylate); 1356 cm^{-1} (C–N); 1022 cm^{-1} (C–F). ^1H NMR showed the following chemical shifts: δ 4.02 (ddd) $J_{H,F} = 29.5$ Hz, $J_{H,H} = 4.5$ and 3.1 Hz; δ 4.81 (ddd) $J_{H,F} = 47.4$, $J_{H,H} = 4.5$ and 3.0 Hz.

Fig. 5 shows the analysis of 3-fluoroalanine by chiral HPLC. Fig 5A shows that when *rac*-3-fluoroalanine was analyzed two peaks of the same area were obtained with retention times of 4.71 and 5.73 min, respectively. When the product obtained enzymatically was analyzed (Fig. 5B), the same two peaks were found. The one eliciting a retention time of 4.71 min representing 7% of the relative area corresponded to (*R*)-3-fluoroalanine that was not consumed by L-ALADH. The other one, with a retention time of 5.73 min, and relative area of 93%, corresponded to (*S*)-3-fluoroalanine. From these data an ee of 86% was determined for (*S*)-3-fluoroalanine. This ee determined experimentally is very similar to that calculated with the data of Fig. 4 thus confirming the absolute stereospecificity of L-ALADH for (*R*)-3-fluoroalanine.

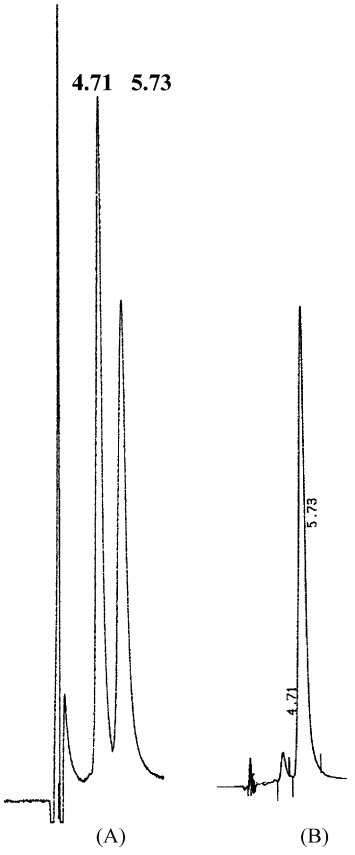


Fig. 5. Chiral HPLC analysis of 3-fluoroalanine. (A) *Rac*-3-fluoroalanine obtained by aminative reduction of 3-fluoropyruvate as described in Section 2.7. (B) Product obtained enzymatically as described in Section 2.4. The numbers appearing in the figure represent the retention times (minutes) of the peaks. Other experimental details are given in Section 2.6.

Target product (**3**) that remained stable in the reaction mixture was recovered in 80% yield and was analyzed before methylation by chiral HPLC as described for (**1a**). Just one peak with retention time of 2.84 min was obtained (results not shown). From these data an ee higher than 99% was determined for (**3**). This product was methylated as described previously [5] and analyzed by polarimetry, IR, elemental analysis (C, H, N) and ^1H NMR. The methyl ester of (**3**) showed $[\alpha]_D^{25} = -4$ ($c = 1$, EtOH) and elemental analysis, IR and ^1H NMR data that are fully consistent with our previously reported results [5]. The absolute configuration of (**3**) was considered to be (*R*) based on the fact that L-LDH presents an absolute enantioselectivity and it is considered to be a reagent to determine the absolute configuration of the α-C atom of compounds that are substrates of this enzyme [31,32].

4. Concluding remarks

This paper describes an efficient enzymatic process to produce simultaneously in the same reaction medium two important chiral blocks, enantiopure (*R*)-3-fluorolactic acid

methyl ester a 1,2,3-trisubstituted three-carbon compound with several applications in asymmetric organic synthesis and enantioenriched (*S*)-3-fluoroalanine. This latter compound as stated earlier, in addition to synthetic applications shows an interesting activity as wide spectra antibiotic. Enzymatic systems as the one reported in the present paper can be easily set up, allowing the preparation of numerous optically active products with biological or pharmacological activity. It has been observed that the presence of the fluorine atom in the substrate causes a better interaction of it in the active site of the enzyme.

Acknowledgements

Financial support from CNPq, FUJB/UFRJ, CAPES, FAPERJ, FINEP and PRONEX is acknowledged. We also thank the ''Núcleo de Pesquisas de Produtos Naturais-UFRJ'' for running NMR analysis.

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