Thermodynamics and Kinetic Aspects Involved in the Enzymatic Resolution of (*R,S*)-3-Fluoroalanine in a Coupled System of Redox Reactions Catalyzed by Dehydrogenases

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Abstract:

Two systems of redox enzymatic reactions were tested, looking forward to the preparation of (*S*)-3-fluoroalanine, a potent antibiotic, by kinetic resolution of *rac*-3-fluoroalanine. This starting material was the main substrate for the deaminative oxidation reaction catalyzed by L-alanine dehydrogenase (L-AlaDH) in the presence of NAD$^+$. One system was formed by coupling this reaction (main reaction) to the reduction of 3-fluoropyruvate (a cascade system) produced in the main reaction catalyzed by L-lactate dehydrogenase (L-LDH) in the presence of NADH, also formed in the main reaction. This system, that was able to achieve 92% of conversion, allows the accumulation of NH$_4^+$, one of the secondary products of the main reaction. The other coupled redox system involved the coupling to the L-AlaDH reaction to the aminative reduction reaction of α-ketoglutarate in the presence of NADH and NH$_4^+$ (both side products of the main reaction) catalyzed by L-glutamate dehydrogenase (L-GluDH), that allows accumulation of 3-fluoropyruvate. With this system, the extent of the reaction in the coupled system was only 22%. This big difference in the efficiency of both systems was identified as being the result of a different potency of the products that accumulates in both systems, acting as inhibitors of L-AlaDH. It was demonstrated that 3-fluoropyruvate is a much stronger inhibitor of L-AlaDH than NH$_4^+$. This fact, and not thermodynamic considerations, explains the results obtained with both systems.

1. Introduction

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, for example, β-adrenergic blocking agents, such as (*S*)-propranolol and (*S*)-moprolol, and other products of pharmaceutical interest such as (*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 and also shows anticancer activity.[1,2] In addition, such a compound is the ideal precursor for either (*S*)- or (*R*)-3-fluoroalanine, via triflate formation and one inversion or chloride formation and two inversions.
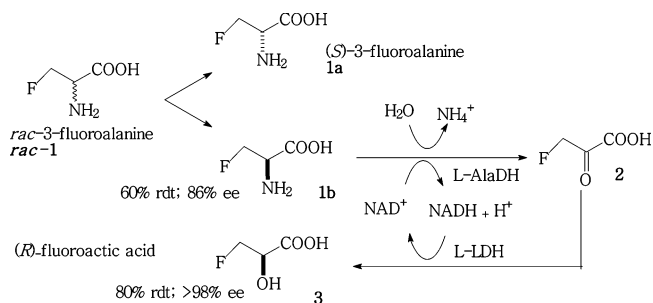

Figure 1. Coupled enzymatic redox system for the simultaneous production of (*S*)-3-fluoroalanine (1a) and (*R*)-3-fluorolactic acid (3) using *rac*-3-fluoroalanine (*rac*-1), (*R*)-3-fluoroalanine (1b), 3-fluoropyruvic acid (2). The regeneration reaction was constituted by the enzymatic reduction of 2 catalyzed by L-LDH in the presence of NADH both produced in the main reaction (oxidative deamination of (*R*)-fluoroalanine).

On the other hand, (*S*)-3-fluoroalanine has been described as a potent antibiotic of wide spectrum.[3] Its mechanism of action involves the irreversible inactivation of the typical bacterial enzyme, alanine racemase,[4] an enzyme involved in the biosynthesis of the cell wall. This nonproteinogenic amino acid also acts as an inhibitor of serine palmitoyl transferase;[5] in addition, it is a potential precursor of fluoroamine compounds.[6,7]

Both fluorinated compounds can be produced simultaneously by using a coupled enzymatic system involving dehydrogenases. However, an enzymatic system of this type necessarily involves the regeneration of the redox active form of the nicotinamide coenzyme, since it cannot be used in stoichiometric concentration with the other substrate of the reaction system.

Our group has been involved in the production of (*S*)-3-fluoroalanine[8] via kinetic resolution of *rac*-3-fluoroalanine by deaminative oxidation catalyzed by L-AlaDH. (*R*)-3-Fluorolactic acid was simultaneously produced via L-LDH-catalyzed enantioselective reduction of 3-fluoropyruvate, generated in the former reaction (Figure 1).

In the development of the process leading to a possible industrial use, a more thermodynamically suitable redox enzymatic system was tested. This coenzyme regeneration

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(1) Gonçalves, L. P. B.; Antunes, O. A. C.; Pinto, G. F.; Oestreicher, E. G. *J. Mol. Catal. B: Enzym.* **1998**, *4*, 67.
(2) Bentley, J. M.; Wadsword, H. J.; Willis, C. L. *J. Chem. Soc., Chem. Commun.* **1995**, 231.
(3) (a) Kollonitsch, J.; Barash, L.; Kahan, F. M.; Kropp, H. *J. Am. Chem. Soc.* **1988**, *110*, 2959. (b) Grabowski, E. J. J. *Chirality* **2005**, *17*, S249.
(4) Esaki, N.; Walsh, C. T. *Biochemistry* **1986**, *25*, 3261.
(5) Sandvig, K.; Barred, O.; van Helvoort, A.; van Meers G.; van Deurs, B. *Mol. Biol. Cell.* **1996**, *7*, 1391−404.
(6) Schengrund, C. L.; Kovac, P. *Carbohydr. Res.* **1999**, *319*, 24.
(7) Hutton, C. A. *Org. Lett.* **1999**, *1*, 295.
(8) Gonçalves, L. P. B.; Pinto, G. F.; Antunes, O. A. C.; Oestreicher, E. G. *Tetrahedron: Asymmetry* **2000**, *11*, 1465.
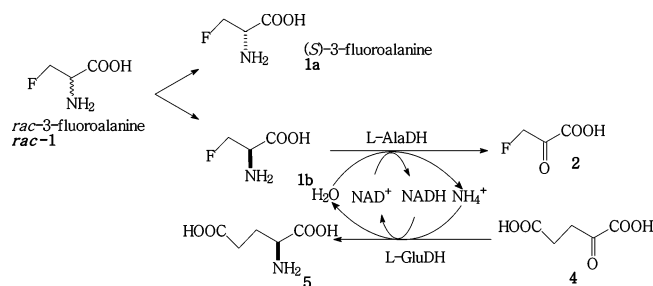
Figure 2. Alternative coupled redox system for the enzymatic production of (*S*)-3-fluoroalanine. The substrate for AlaDH was *rac*-3-fluoroalanine (*rac-1*) which deaminated oxidatively the (*R*)-enantiomer of the racemic amino acid (**1b**) producing 3-fluoropyruvic acid (**2**) and leaving unreacted (*S*)-3-fluoroalanine (**1a**), α-ketoglutarate (**3**), (*S*)-glutamate (**4**).

system was constituted by L-GluDH and α-ketoglutarate (Figure 2). This enzymatic system has the advantage over the former one (L-LDH and 3-fluoropyruvate) that the chemical reaction involved has a greater thermodynamic driving force ($\Delta G'_o$) than the reduction of 3-fluoropyruvate to 3-fluorolactate (supposing E'_o equals that of the half-reaction pyruvate/lactate).

In the present paper the performances of both recycling systems were compared, and the higher efficiency of the 3-fluoropyruvate/3-fluorolactate recycling system was attributed to a kinetic effect, strong inhibition of L-AlaDH by 3-fluropyruvate, a product accumulating just with the α-ketoglutarate/glutamate system. This inhibition of the enzyme catalyzing the main reaction, deaminative oxidation of (*L*)-3-fluoroalanine, by a reaction product that it is not eliminated by the recycling reaction is a much more important factor than the higher thermodynamic driving force supplied by the other coenzyme recycling system. This, therefore, explains the success of the cascade reaction employed.

2. Experimental Section

Bacillus subtilis L-alanine dehydrogenase (L-AlaDH) (L-alanine:NAD+ oxidoreductase, EC 1.4.1.1), suspension in 2.4 M $(NH_4)_2SO_4$, pH 7.0, L-lactate dehydrogenase (L-LDH) 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) (L-glutamate:NAD(P)+ oxidoreductase, EC 1.4.1.3), lyophilized, NAD+ (grade III), NADH (grade III), bovine serum albumin (fraction V), sodium pyruvate, sodium 3-fluoropyruvate, 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 purchase from Aldrich Chemical. All other chemicals were of analytical grade and were obtained from Merck Darmstad.

2.1. Assay of L-AlaDH Activity. L-AlaDH activity (measured in the direction of *rac*-3-fluoroalanine deaminative oxidation) was assayed 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, 20 mM *rac*-3-fluoroalanine, and 0.25 mM NAD+. Reaction was started by adding 20 μL of an enzyme solution containing in a volume of 1.0 mL: 130 μg (protein corresponding to 0.45 IU of a desalinized L-AlaDH enzyme preparation (obtained by ultrafiltration 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 velocity. The initial velocity of the reaction was calculated in terms of millimoles of NADH produced per minute, using a molar absorption coefficient for NADH of 6220 M^{-1} cm^{-1}.

2.2. Inibition of AlaDH by 3-Fluoropyruvate and NH_4^+. Assays of AlaDH activity under conditions of initial rate were performed as described in section 2.1 using a constant concentration of both *rac*-3-fluoroalanine (4.5 mM) and of NAD+ (0.25 mM). Each product inhibitor (3-fluoropyruvate and NH_4^+) was added to the respective reaction mixture in concentrations ranging from zero to 5.0 mM, as indicated in the legend of Figure 4.

2.3. Coupled Enzymatic Redox Systems for NAD+ Regeneration. Reaction was performed in a jacketed batch reactor of 30 mL, continuously stirred. The reaction medium contained, in a total volume of 12.5 mL, 0.05 mM of rac-3-fluoroalanine, sodium phosphate buffer, pH 7.9, 20 mM *rac*-3-fluoroalanine, 90 IU of L-AlaDH, 1000 IU of L-LDH (or 50 mM α-ketoglutarate and 950 IU of L-GluDH). Reactions were started by adding 0.25 mM NAD+. Reaction mixtures were maintained under continuous magnetic stirring, and the temperature was maintained at 25 °C by circulating water through the jacket of the reactor with a thermocirculating bath.

The extent of reaction was determined by removing aliquots of the reaction medium (10−50 μL) at different times. These aliquots were immediately diluted with 0.05 M sodium phosphate buffer, pH 7.9, and heated at 100 °C for 5 min to inactivate the enzymes. The concentration of NH_4^+ (reaction system represented by Figure 1) or of 3-fluoropyruvate (reaction system shown in Figure 2) present in the corresponding samples was determined by "end-point" assays. NH_4^+ was assayed in a solution 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 concentration of 3-fluoropyruvate that accumulated in the reaction medium was determined in the presence of 0.25 mM NADH and 14 IU of L-LDH in a total volume of 2.0 mL at 25 °C.

2.4. Determination of Enantiomeric Excesses. The enantiomeric excesses of the target products were determined by chiral HPLC which was performed with an ISCO 2350 chromatograph equipped with a Nucleosil Chiral-1 column (Macherey-Nagel, ET 250/8/4) by using 1 mM $CuSO_4$, pH

redox reaction	E_o' (v)	$\Delta G_o'$ (cal/mol)
alanine + H_2O + NAD^+ ↔ pyruvate + NH_4^+ + NADH[a]	−0.19	
pyruvate + NADH ↔ lactate + NAD^+ [b]	0.13	2760[a]
[b]α-ketoglutarate + NH_4^+ + NADH ↔ glutamate + H_2O + NAD^+ [b]	0.18	466[b]

[a] Considering reactions a and b and $\Delta G_o' = -nF\Delta E_o'$ where $n = 2$, $F = 23\,000$ (cal/absolute volt equivalent) and $\Delta E_o'$ the difference of standard redox potentials between the half reactions considered. [b] Considering reactions a and c, $\Delta G_o' = -nF\Delta E_o'$ and the values of n, F, and the corresponding $\Delta E_o'$ value.
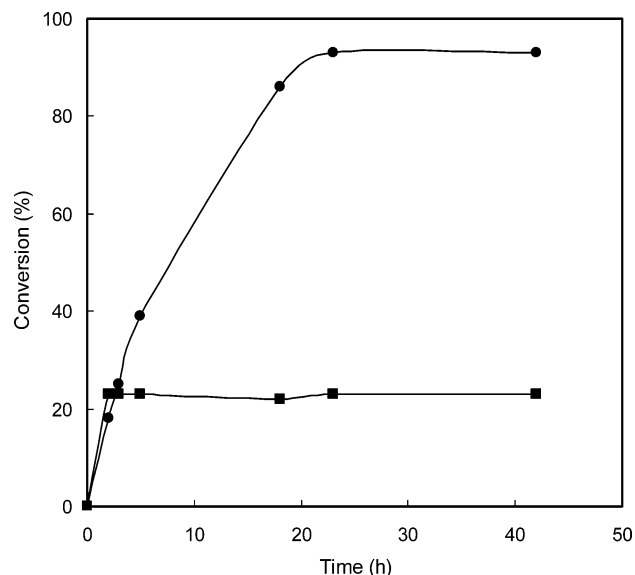

Figure 3. Time-course curves of the coupled enzymatic systems for the production of (*S*)-3-fluoroalanine in a laboratory preparative scale (150 mg). The progress of reactions was followed by chiral HPLC determination of (*S*)-3-fluoroalanine as described in section 2.3.

4.6, as eluent at a flow rate of 1 mL/min, 23 °C, and UV detection at 235 nm. Chemically synthesized *rac*-3-fluoro-lactic acid and *rac*-3-fluoroalanine were used as standards. The latter was obtained by reductive amination of 3-fluoropyruvate. In this procedure 3-fluoropyruvate (3.5 mmol) was treated with $NABH_4$ (5.5 equiv) in the presence of NH_4-OH (68 mmol) as described in the literature.[10] This product as well as the target ones was isolated, purified, crystallized, and identified as described previously by us.[1,8,9]

2.5. Determination of Protein Concentration. Protein concentration was determined by using a method suitable for the detection of low protein contents.[11] Bovine serum albumin (Fraction V) was used as the standard.

3. Results and Discussion

Oxidoreductions, especially the reduction of carbonyl compounds, the aminative reduction of α-ketoacids, the oxidation of hydroxyl groups, and the oxidative deamination of rac-α-amino acids catalyzed by stereoselective $NAD(P)^+$-dependent dehydrogenases, are very important organic reactions for the preparation of enantiopure or highly enantioenriched chiral compounds. Reduction reactions are important in asymmetric synthesis and oxidation reactions and in kinetic resolutions of racemates.

Our research group has used both types of applications with good results.[1,8,9,12−17] However, the use of dehydrogenases in preparative applications requires effective processes of coenzyme regeneration since these cofactors are too expensive to be used as stoichiometric reagents. Moreover, since oxidation (or oxidative deamination) reactions are thermodynamically unfavorable, coenzyme regeneration by coupling to these reactions (strongly exergonic reduction reactions) drives the main reaction in the desired direction, making the whole system of coupled reactions to behave exergonically, allowing the main or desired reaction to proceed with high yields. On the other hand, with reduction (or aminative reduction) reactions, coupling of an oxidation coenzyme regeneration reaction allows the kinetic resolution of racemate of oxidized compounds.[1,8]

Two coenzyme regeneration systems were envisaged to allow the production of (*S*)-3-fluoroalanine. The first one, shown in Figure 1, allowed the production of two chiral compounds, our target product ((*S*)-3-fluoroalanine) (**1a**), a potent antibiotic agent (**3**, **8−9**), and (*R*)-3-fluorolactic acid (**3**), a versatile chiral building block (**1,12**). The (*S*)-enantiomer of the nonproteinogenic amino acid is obtained by 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^+. If the velocity of the coenzyme recycling reaction is not rate limiting of the whole process, just NH_4^+ and (*R*)-3-fluoroalanine will accumulate in the reaction medium, and NAD^+ will be continuously recycled until all **1b** has been consumed. Of course this picture will only be observed if the coenzyme-recycling reaction has enough driving force ($\Delta G_o'$) to direct the system in this direction.

(9) Gonçalves, L. P. B.; Antunes, O. A. C.; Pinto, G. F.; Oestreicher, E. G. *J. Fluorine Chem.* **2003**, *124*, 219.

(10) Dolling, U. H.; Douglas, A. W.; Grabowski, E. J. J.; Schonewaldt, E. F.; Sohar, P.; Sletzinger, M. *J. Org. Chem.* **1978**, *43*, 1634.

(11) Hartree, E. F. *Anal. Biochem.* **1972**, *48*, 422.

(12) Menezes, R. R.; Santos, A. S.; Oestreicher, E. G.; Pinto, G. F. *Biotechnol. Tech.* **1998**, *12*, 35.

(13) Gonçalves, L. P. B.; Antunes, O. A. C.; Pinto, G. F.; Oestreicher, E. G. *Tetrahedron: Asymmetry* **1996**, *5*, 1237.

(14) Machado, G. D. C.; Gomes, M., Jr.; Antunes, O. A. C.; Oestreicher, E. G. *Proc. Biochem.* **2005**, *40*, 3186.

(15) Arcuri, M. B.; Sabino, S. J.; Antunes, O. A. C.; Oestreicher, E. G. *J. Fluorine Chem.* **2003**, *121*, 55.

(16) Arcuri, M. B.; Antunes, O. A. C.; Machado, S. P.; Almeida, C. H. F.; Oestreicher, E. G. *Amino Acids* **2004**, *27*, 69.

(17) Machado, G. D.; Paiva, L. M. C.; Pinto, G. F.; Oestreicher, E. G. *Enzyme Microb. Technol.* **2001**, *28*, 322.

Table 2. Experimental data used to plot Figure 3

	conversion (%)	
time (h)	L-LDH[b]	GluDH[c]
0	0	0
2	18	22
3	25	21.8
5	39	22
18	86	21.5
23	92	22
42	92	22

[a] The progress of reaction was followed by chiral HPLC determination of (S)-3-fluoroalanine as described in section 2.3. [b] Coupled enzymatic system formed with the L-LDH coenzyme recycling reaction (See Figure 1). [c] Coupled enzymatic system formed with the GluDH/α-ketoglutarate NAD$^+$ recycling reaction (See Figure 2).
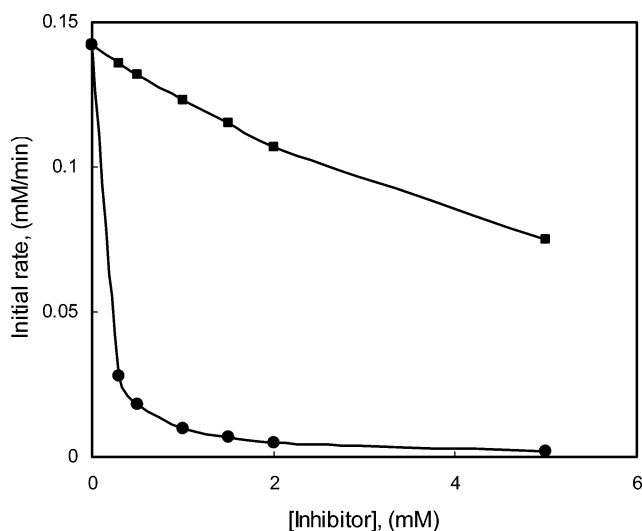

Figure 4. Inhibition of AlaDH by 3-fluoropyruvate and NH$_4$$^+$. Experimental conditions for the measurement of initial rate of AlaDH-catalyzed reaction of oxidative deamination of *rac*-3-fluoroalanine were given in section 2.1. Experimental details for the inhibition of AlaDH by products of the main reaction remaining with the L-LDH coenzyme recycling system (NH$_4$$^+$) and the GluDH/α-ketoglutarate NAD$^+$ recycling system, respectively, were given in section 2.2. The concentration of each product inhibitor, (■) NH$_4$$^+$ and (●) 3-fluoropyruvate, varied from zero to 5.0 mM.

The other coenzyme regeneration system is shown in Figure 2. In this system NAD$^+$ produced in the main reaction is regenerated by coupling to the deaminative oxidation reaction of (R)-3-fluoroalanine catalyzed by L-AlaDH, the aminative reduction of α-ketoglutarate is catalyzed by L-GluDH in the presence of NH$_4$$^+$, and NADH is produced in the main reaction. This system is also able to produce two chiral compounds (Figure 1), (S)-3-fluoroalanine and (S)-glutamate.[19] This latter system must be considered since, as shown in Table 1, it has a much smaller positive $\Delta G_o'$ than the former one. This is an important issue to be considered since L-AlaDH being absolutely stereospecific for the (L or S)-enantiomer of alanine[18] and also for the (L or R)-enantiomer of 3-fluoroalanine,[8,9] the enantiomeric excess

(18) Grimshaw, C. E.; Cleland, W. W. *Biochemistry* **1981**, *20*, 5650.
(19) Ödman, P.; Wellborn, W. B.; Bommarius, A. S. *Tetrahedron: Asymmetry* **2004**, *15*, 2933.

Table 3. Experimental data[a] of the inhibition of L-AlaDH by 3-fluoropyruvate and NH$_4$$^+$

	residual activity (mM/min)	
inhibitor concentration (mM)	3-fluoropyruvate	NH$_4$$^+$
0	0.142	0.142
0.3	0.028	0.136
0.5	0.018	0.132
1.0	0.010	0.123
1.5	0.007	0.115
2.0	0.005	0.107
5.0	0.002	0.075

[a] These experimental data were used to plot Figure 4. Experimental details are given in the caption of Figure 4 and in the Experimental Section.
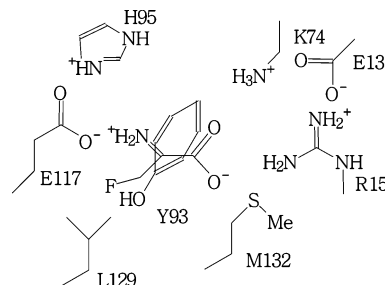

Figure 5. Active site of L-AlaDH showing the interactions of the Shiff base with the amino acid residues.

of unreacted (S)-3-fluoroalanine, our target product, will be a direct function of the degree of conversion which, in turn, will depend on the resulting driving force of the whole system which is determined by the $\Delta G_o'$ of the coenzyme recycling reaction.

However $\Delta G_o'$ is not the unique thermodynamic factor involved since, in the first redox system (Figure 1 and Table 1), both NADH and pyruvate, two products of the main reaction, are consumed by the recycling reaction, thus increasing the driving force of the latter redox reaction. The same picture happens with the recycling reaction shown in Figure 2, where the products of the main reaction, NADH and NH$_4$$^+$, are also removed by this recycling reaction. An extra effect of the removal of these products of the main reaction is the elimination or removal of an inhibitor since it is a well-known fact that in enzymic reactions, the products formed are inhibitors of the reaction. Then, associated with the thermodynamic effect exists a very important kinetic effect that can influence the extent of the process.

Figure 3 shows the progress curve of the desaminative oxidation reaction of *rac*-3-fluoroalanine with both recycling reactions. With L-LDH as recycling enzyme, the system reached 92% of conversion, whereas with the L-GluDH/α-ketoglutarate system, the extent of the main reaction was only of 22% (Figure 3) although this latter redox-coupled system is thermodynamically more suitable than the former one (Table 1). In Table 2 are displayed the experimental data corresponding to these time-course curves. The enantiomeric excess of (S)-3-fluoroalanine determined by chiral HPLC as described in section 2.4 was of 86%. Since as shown in Figure 1 with L-LDH as recycling enzyme, the only product of the main reaction that accumulates is NH$_4$$^+$, whereas with L-GluDH and α-ketoglutarate an accumulation of 3-fluoro-
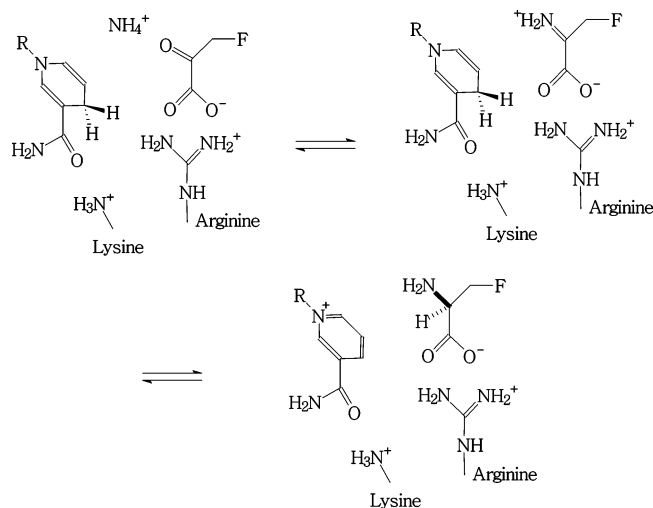
Figure 6. Mechanistic proposal of the inhibition of the enzyme by 3-fluoropyruvate.

pyruvate occurs, the possibility exits than these two products inhibit the enzyme AlaDH with different strengths, 3-fluoropyruvate being a stronger inhibitor than NH_4^+. To verify this possibility inhibition studies of L-AlaDH by 3-fluoropyruvate and NH_4^+ were undertaken.

Figure 4 shows the results obtained when NH_4^+ and 3-fluoropyruvate were tested as inhibitors, separately, of the enzyme L-AlaDH. As shown in Figure 4, clearly 3-fluoropyruvate is a much stronger inhibitor of L-AlaDH than NH_4^+; for instance with a concentration of 1.5 mM of the product inhibitor, 3-fluoropyruvate caused almost a total inhibition of the enzyme, whereas the same concentration of NH_4^+ produced less than 20% of inhibition of L-AlaDH. The actual values of residual activity of the enzyme at each concentra-

tion of each product inhibitor are given in Table 3. This experiment then clearly indicated that the main reason for the L-LDH coenzyme recycling reaction, although having a lower thermodynamic driving force than the GluDH/α-ketoglutarate system, is more efficient due to kinetic reasons rather than to thermodynamic ones.

On the basis of the active site of a model L-AlaDH[20] it is reasonable to suppose that the intermediate, the Shiff base complex, should interact with different amino acid residues, such as a tyrosine residue (via π-stacking), that protects one face from the hydride attack, a glutamic acid residue and an arginine residue, as ionic pair interactions (Figure 5). Therefore, inhibition of L-AlaDH, of the uncompetitive type, by 3-fluoropyruvate is explained by the formation of an NADH/3-fluoropyruvate complex. This complex binds NH_4^+, the first product of the reaction and the complex thus resulting (due to the equilibrium), then is changed (via NADH−Schiff base complex) back to the intermediate complex L-AlaDH−NADH-L-3-fluoroalanine (Figure 6).

Conclusions

The difference in the efficiency in two recycle systems, using L-LDH and L-GluDH, was identified as being the result of different potencies of the products that accumulate in both systems, acting as inhibitors of L-AlaDH. It was demonstrated that 3-fluoropyruvate is a much stronger inhibitor of L-AlaDH than NH_4^+. This fact, and not thermodynamic considerations, explains the results obtained with both systems. The cascade system developed which afforded the simultaneous production of two fluorinated chiral products is strongly favored.

(20) Baker, P. J.; Sawa, Y.; Shibata, H.; Sedelnikova, S. E.; Rice, D. W. *Nat. Struct. Biol.* **1998**, *5*, 561.