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Kinetic aspects of the enantiospecific reduction of sodium 3-fluoropyruvate catalyzed by rabbit muscle L-lactate dehydrogenase: Production of homochiral (*R*)-3-fluorolactic acid methyl ester

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Abstract

In the present work the reduction of sodium 3-fluoropyruvate catalyzed by rabbit muscle L-lactate dehydrogenase (L-LDH) was studied by means of initial rate experiments. Estimates of the limiting values of the kinetic parameters of the reaction were obtained. A kinetic mechanism involving a compulsory order of substrate binding to L-LDH, with NADH being the first substrate, is proposed. In addition, a simple procedure for the enantiospecific reduction of 3-fluoropyruvate catalyzed by this enzyme in a laboratory preparative scale is described. NADH was used in catalytic concentration by utilizing a NADH in situ regeneration system consisting of the oxidation of *cis*-1,2-bis(hydroxymethyl)cyclohexane (BHMC, **7**) to chiral lactone (+)-(1*R*, 6*S*)-*cis*-8-oxabicyclo[4.3.0]nonan-7-one (**8**) catalyzed by horse liver alcohol dehydrogenase (HLADH). Analysis of the isolated product (100% conversion), after methylation, revealed the presence of (*R*)-3-fluorolactic acid methyl ester as the unique product that was obtained in 80% overall yield and ee > 99%. This compound represents an important chiral building block for the synthesis of several products with biological and/or pharmacological activity. © 1998 Elsevier Science B.V.

Keywords: L-lactate dehydrogenase; (*R*)-3-fluorolactic acid methyl ester; Enantiospecific reduction; Enzyme kinetics; Chiral building block

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Abbreviations: L-LDH, rabbit muscle L-lactate dehydrogenase; HLADH, horse liver alcohol dehydrogenase; BHMC, *cis*-1,2-bis(hydroxymethyl)cyclohexane; A, NADH; B, sodium pyruvate or sodium 3-fluoropyruvate; P, (*S*)-lactate; Q, NAD^+; E, free form of the enzyme; K_a, Michaelis constant of NADH; K_b, Michaelis constant of pyruvate or 3-fluoropyruvate; K_{ia}, dissociation constant of L-LDH–NADH complex; k_{cat}, catalytic constant; K_m, apparent value of the Michaelis constant of pyruvate or 3-fluoropyruvate; v, initial velocity; V, maximum velocity; V_m, apparent value of the maximum velocity; ee, enantiomeric excess; HRGC, high resolution gas chromatography; IR, infrared spectrometry; MS, mass spectroscopy

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1. Introduction

Oxidoreductions, especially the stereoselective oxidation of *meso* diols and reduction of carbonyl compounds, are very important organic reactions in asymmetric synthesis allowing the transformation of prochiral substrates into valuable chiral building blocks. The use of isolated enzymes to catalyze these reactions has greatly increased in the last years, especially those catalyzed by alcohol dehydrogenases from different sources [1–10]. Alcohol dehydrogenases are enzymes that, although having a broad substrate specificity show variable enantioselectivity [6,7,10,11]. On the other hand, L-lactic dehydrogenase from rabbit muscle (L-LDH) has 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 [12,13]. Among the unnatural substrates of L-LDH, 3-halopyruvates, 3-hydroxypyruvate, 3-mercaptopyruvate and 2-oxobutanoate were considered, on the basis of the apparent kinetic parameter k_{cat}/K_m, to be the best non physiological substrates of this enzyme [12]. Within the group of 3-halopyruvates, 3-fluoropyruvate is the best substrate of L-LDH, characterized by an apparent k_{cat}/K_m value that is 22% of that determined for pyruvate [12,13]. In spite of the kinetic preference of this enzyme, only 3-chloropyruvate which has an apparent k_{cat}/K_m value of 3% of that of the natural substrate [12] has been previously used in the preparative scale production of (*R*)-3-chlorolactic acid (ee > 97%), by using the formate/formate dehydrogenase system to recycle NADH [14].

Kim and Whitesides [12] with their classical screening work determined apparent values for the kinetic parameters V_m and K_m for the reduction of several α-keto acids, including 3-fluoropyruvate, catalyzed by L-LDH. Since the determination of apparent values of kinetic parameters for a bisubstrate enzyme reaction has only a very restricted validity, the objective of the present work was the determination of the limiting values of the kinetic parameters for the reduction of 3-fluoropyruvate catalyzed by this enzyme, comparing these values with those determined by Zewe and Fromm for the reduction of pyruvate catalyzed by L-LDH [15], as well as, the preparative scale production of (*R*)-3-fluorolactic acid methyl ester via enantiospecific reduction of sodium 3-fluoropyruvate catalyzed by L-LDH.

(*R*)-3-fluorolactic acid methyl ester represents an important three carbon chiral building block 1,2,3-trisubstituted for the synthesis, for instance, of β-adrenergic blocking agents of the aryloxypropanolamines group such as, (*S*)-propranolol and (*S*)-moprolol and of 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, also showing anticancer activity [16,17].

2. Experimental

2.1. Materials

Horse liver alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1), crystalline, rabbit muscle L-lactate dehydrogenase (L-(+)-lactate: NAD^+ oxidoreductase, EC 1.1.1.27, crystalline suspension in 3.2 M $(NH_4)_2SO_4$, pH 6.1, NAD^+ (grade III-C), NADH (grade III), bovine serum albumin (fraction V) and sodium 3-fluoropyruvate were obtained from Sigma Chemical. Ethanol (chromatographic grade), *cis*-1,2-bis(hydroxymethyl)cyclohexane and the Diazald® kit (for CH_2N_2 generation) were purchased from Aldrich Chemical. All other chemicals used were of analytical grade and obtained from Merck, Darmstadt.

2.2. Methods

2.2.1. Assay of L-LDH activity and estimation of kinetic parameters

L-LDH activity (assayed in the direction of 3-fluoropyruvate reduction) was measured by

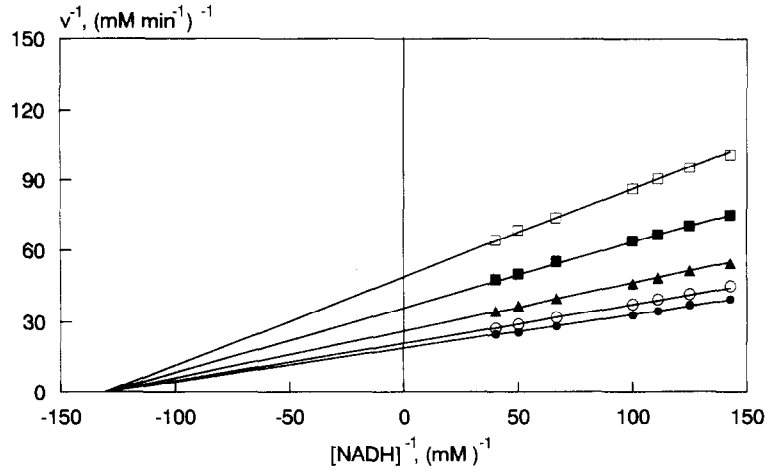


Fig. 1. Effect of sodium 3-fluoropyruvate on the initial velocity of the reaction of 3-fluoropyruvate reduction catalyzed by L-LDH with NADH as the variable substrate. The concentration of 3-fluoropyruvate were: □, 0.05 mM; ■, 0.07 mM; ▲, 0.10 mM; ○, 0.13 mM; and ●, 0.15 mM. Other experimental conditions are given in Section 2.2.

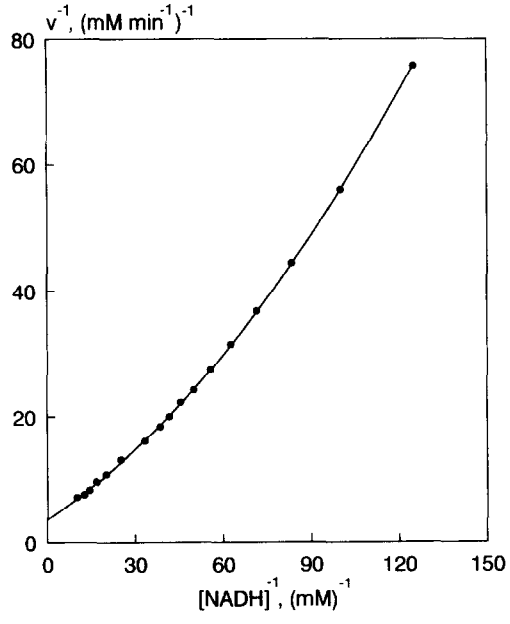


Fig. 2. Lineweaver–Burk plot where the concentration of 3-fluoropyruvate was varied along with the concentration of NADH at [3-fluoropyruvate] = 8[NADH]. NADH concentration was varied between 0.008 mM and 0.1 mM, 3-fluoropyruvate concentration was consequently varied between 0.064 mM and 0.8 mM. The curve represents the 'best-fit' curve obtained by fitting Eq. (2) to the experimental data with a computer program based on the Hooke Jeeves method of direct search with acceleration in distance ([19]). Other experimental details are given in Section 2.2.

following NADH absorption at 340 nm at pH 7.2 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 3 ml: 0.1 M sodium phosphate buffer, pH 7.2 with varying concentrations of NADH and 3-fluoropyruvate that are indicated in the legends of Figs. 1 and 2. NADH and 3-fluoropyruvate solutions were made up immediately before use in the same buffer and maintained in an ice bath. After addition of the substrates 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 30 μl of an enzyme solution containing in a volume of 2.0 ml: 99 μg of L-LDH (protein basis), 0.1 M sodium phosphate buffer, pH 7.2 and 0.1% (w/v) bovine serum albumin. 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 to the curves at this time were taken as initial velocities. The initial velocity of the reaction was calculated in terms of mM of

NADH consumed per min, using a molar absorption coefficient for NADH of 6220 M^{-1} cm^{-1}.

2.2.2. Kinetic data processing

Estimates of parameters and of their asymptotic standard errors were obtained by fitting Eq. (1) to data using a nonlinear least-squares computer program, developed in our laboratory and specifically devised for steady-state studies of enzyme kinetics [18]. The kinetic parameters of Eq. (2) were estimated by fitting this rate equation to experimental data with a computer program based on the Hooke–Jeeves methods of direct search with acceleration in distance [19], also developed in our laboratory [20].

2.2.3. Laboratory preparative scale reduction of 3-fluoropyruvate catalyzed by L-LDH

Reduction of 3-fluoropyruvate, in a preparative scale of 200 mg was performed in a jacketed batch reactor of 230 ml, continuously stirred. The reaction medium contained in a total volume of 123 ml: 0.1 M sodium phosphate buffer, pH 7.2, 26.3 mM sodium 3-fluoropyruvate, 15 mM BHMC, 61 μg of L-LDH (protein basis, corresponding to 154 IU assayed with 1 mM 3-fluoropyruvate and 0.1 mM NADH), 21.8 mg of HLADH (protein basis which corresponds to 37 IU measured with 10 mM BHMC and 0.1 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 reaction was started by adding 0.1 mM NAD^+.

2.2.4. Determination of the time–course curve for the reduction of 3-fluoropyruvate

The extent of reaction was followed by removing at different times aliquots of 10–50 μl of the reaction mixture (containing approximately 0.2 μmol of unreacted 3-fluoropyruvate). These samples were immediately diluted with 1 ml of 0.1 M sodium phosphate buffer, pH 7.2 and heated at 100°C for 5 min in order to inactivate the enzymes. The concentration of 3-fluoropyruvate present in the samples was determined by 'end-point assays' in the presence of 0.25 mM NADH and 14 IU of L-LDH in a total volume of 2.5 ml at 25°C. The decrease of NADH absorption after the addition of the enzyme was followed at 340 nm until total consumption of the substrate. The concentration of 3-fluoropyruvate was calculated from the difference of NADH absorption (before and after the addition of L-LDH) by using the molar absorption coefficient of NADH.

2.2.5. Isolation and methylation of 3-fluorolactic acid

Isolation and characterization of the product formed was accomplished by thermal treatment of the reaction mixture (100°C for 5 min) in order to inactivate the enzymes. After filtration to remove the coagulated proteins, the filtrate was extracted with $CHCl_3$ (v/v) during 5 min to eliminate unreacted BHMC (**7**) and the lactone (**8**) produced by the NADH recycling system. The aqueous phase, which in addition to the target product, contained the coenzyme, was acidified to pH 1.5 with 1 M HCl, saturated with NaCl and continuously extracted with Et_2O for 72 h. The organic phase, containing 3-fluorolactic acid, was methylated with CH_2N_2/Et_2O according to Blank et al. [21]. The resulting product, 3-fluorolactic acid methyl ester was obtained with an overall isolated yield of 80%.

2.2.6. Characterization of 3-fluorolactic acid methyl ester

The product obtained was analyzed and characterized by: IR spectrophotometry with a Perkin–Elmer 467 spectrophotometer; ^{1}H-NMR spectroscopy with a 200 MHz Varian (Gemini) equipment; GC–MS analyses that were performed with a mass spectrometer HP-5987A coupled to a HP-5880 gas chromatograph equipped with a 30 m J&W DB-5 column; determination of the $[\alpha]_D^{25}$ value, carried out by using a Jasco DIP-370 digital polarimeter.

2.2.7. Determination of enantiomeric excess

The product obtained was analyzed by chiral HRGC which was performed on a capillary column (30 m) coated with 2,3,6-tri-*O*-methyl-β-cyclodextrin (PMCD/OV 1701-OH), isothermally at 70°C by using a HP-5890 (series II) chromatograph. *Rac*-3-fluorolactic acid methyl ester was used as standard.

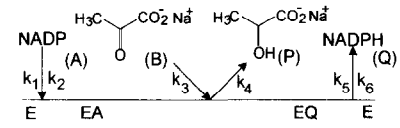
Rac-3-fluorolactic acid methyl ester was prepared by reduction of 100 mg of sodium 3-fluoropyruvate with 100 mg of $NaBH_4$ in 4 ml of glass bidistilled water. The reaction mixture was maintained under continuous stirring at 0°C for 75 min. The reaction was stopped by the addition of 0.1 M HCl until a pH near 1.0. The product was then extracted and methylated with CH_2N_2 as already described for the enzymatically generated product.

2.2.8. Determination of protein concentration

Protein concentration was determined by using a method suitable for the detection of low protein contents [22]. Bovine serum albumin was used as standard.

3. Results and discussion

Zewe and Fromm [15] determined that the kinetic mechanism for the reduction of pyruvate catalyzed by L-LDH in the presence of NADH was the Theorell–Chance BiBi kinetic model [23], with NADH binding to the free form of the enzyme followed by the binding of pyruvate. The first product to be liberated was L-(+)-lactate and NAD^+ was the last product to be released (Scheme 1).



Scheme 1. Theorell–Chance steady-state kinetic mechanism for the reduction of pyruvate catalyzed by L-LDH.

Kim and Whitesides [12] showed that 3-halopyruvates were relatively good substrates of L-LDH and that among these, 3-fluoropyruvate was the best substrate of the enzyme. These authors arrived to this conclusion on the basis of initial rate measurements performed in the presence of a single fixed concentration of NADH (0.2 mM) and varied concentrations of 3-halopyruvates. Under these experimental conditions only apparent values of the kinetic parameters V_m and K_m can be determined and these are only valid for that specific experimental situation. A more realistic picture of the kinetic behavior of the enzyme can be obtained from initial rate experiments performed at various concentrations of both NADH and 3-fluoropyruvate since in these latter condition, limiting values of the kinetic parameters are obtained. Fig. 1 shows the double reciprocal-type plots for a series of experiments in which the concen-

Table 1
Estimates of kinetic parameters derived from initial velocity studies on the reduction of sodium 3-fluoropyruvate catalyzed by L-LDH

Parameter	Value [a]	Value ± SE [b]	95% confidence limits	Value [c]
V [d] (mM min^{-1})	0.001	0.277 ± 0.007	0.263–0.292	0.275
K_{ia} (μM)	8.14	7.60 ± 0.30	7.00–8.20	7.10
K_a (μM)	10.70	8.00 ± 1.10	5.80–10.20	7.10
K_b (mM)	0.164	0.628 ± 0.022	0.582–0.673	0.647

[a] Data from Ref. [15] for the reduction of sodium pyruvate.
[b] Asymptotic standard error of parameter. Estimates of parameters obtained by nonlinear regression analysis of the experimental data depicted in Fig. 1.
[c] Estimates of parameters obtained by fitting Eq. (2) to the experimental data shown in Fig. 2 with a computer program based on a direct search algorithm [19].
[d] No comparison between the value determined by Ref. [15] and the estimate for 3-fluoropyruvate is possible since the L-LDH concentration used by these authors was not specified.

tration of NADH was varied at five fixed concentrations of sodium 3-fluoropyruvate. Since a family of straight lines having a point of common intersection to the left of the v^{-1} axis was obtained, this pattern is indicative of a sequential combination of the substrates with the enzyme [23], and thus confirming this characteristic of the kinetic mechanism proposed by Zewe and Fromm [15] for the reduction of pyruvate catalyzed by L-LDH. Eq. (1) is the initial rate function for this type of kinetic mechanism,

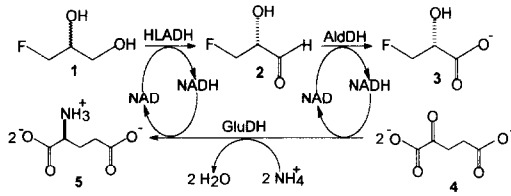
$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad (1)$$

Fitting of Eq. (1) to these experimental data allowed the estimation of the kinetic parameters of this rate equation. These estimates are displayed in Table 1 together with the corresponding values determined by Zewe and Fromm [15] for the reduction of pyruvate. The limiting values of the kinetic parameters for the reduction of 3-fluoropyruvate catalyzed by L-LDH were also obtained by varying together the concentrations of both substrates in a constant ratio. According to the values obtained for K_a and K_b on fitting Eq. (1) (Table 1), a ratio [B]/[A] = 8 was chosen. Under these experimental conditions Eq. (1) is transformed into Eq. (2),

$$v = \frac{8V[A]^2}{K_{ia}K_b + K_b[A] + 8K_a[A] + 8[A]^2} \quad (2)$$

The double reciprocal form of Eq. (2) describes a parabolic curve. Fig. 2 shows the double reciprocal plot obtained under these experimental conditions. The estimates of the kinetic parameters of Eq. (2) are also displayed in Table 1. Since the Hooke–Jeeves method was used to fit Eq. (2) and this method does not give the asymptotic standard errors of the parameter estimates, the 95% confidence limits for the estimates of parameters of Eq. (1) were calculated according to Student's t-test adapted for nonlinear regression as described by Metzler [24]. As shown in Table 1 the confidence limits for the four kinetic parameters of Eq. (1) include the values of the respective parameters obtained on fitting Eq. (2) to the aforementioned experimental data set. This analysis reveals that both sets of kinetic parameter estimates are not significantly different and that both experimental designs used to determine the limiting value of the kinetic parameters, gave essentially the same results. On comparing the values of the kinetic parameters obtained for the reduction of 3-fluoropyruvate with those for pyruvate determined graphically by Zewe and Fromm [15] (Table 1), it can be seen that the values estimated for K_{ia} are not significantly different and thus, K_{ia} does not depend on the nature of the other substrate (3-fluoropyruvate and pyruvate, respectively), suggesting that, in fact, this parameter is the dissociation constant of the L-LDH–NADH complex, and that for the reduction of 3-fluoropyruvate the order of substrate combination with the enzyme is the same as for the reaction of reduction of pyruvate catalyzed by L-LDH [15]. On the other hand, markedly in the case of K_b, the value obtained for 3-fluoropyruvate was approximately four times higher than the value for the limiting Michaelis constant of the natural substrate of the enzyme. No comparison was possible in the case of parameter V because Zewe and Fromm [15] have not specified the concentration of L-LDH used.

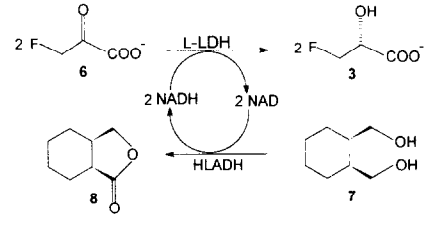
As stated earlier in the present work, (*R*)-3-fluorolactic acid methyl ester can be considered as a valuable chiral building block giving access to the synthesis of several enantiomeric pure products with therapeutical activity. This chiral building block has been previously obtained by Matos et al. [25] using 3-fluoro-1,2-propane diol (**1**) as starting material that was kinetically resolved with HLADH. The resulting hydroxyaldehyde (**2**) was further oxidized by yeast aldehyde dehydrogenase (EC 1.2.1.5) yielding the desired (*R*)-3-fluorolactic acid (**3**). The system used to regenerate NAD^+ from the NADH produced by the two sequential reactions of oxidation consisted on the reductive amination of α-ketoglutarate (**4**) to (*S*)-glutamate (**5**) catalyzed by glutamate dehydrogenase (GluDH) (EC 1.4.1.3) according to Scheme 2.



Scheme 2. Coupled enzymatic redox system for the production of (*R*)-3-fluorolactate. *Rac*-3-fluoropropane-1,2-diol (**1**); (*R*)-3-fluorolactaldehyde (**2**); (*R*)-3-fluorolactate (**3**); α-ketoglutarate (**4**); (*S*)-glutamate (**5**); AldDH, Baker's yeast aldehyde dehydrogenase (EC 1.2.1.5); GluDH, L-glutamate dehydrogenase (EC 1.4.1.3).

This process presents a priori the inconvenience that the theoretical yield of (*R*)-3-fluorolactic acid (**3**) can only be 50% based on the racemic diol (**1**). In fact, Matos et al. [25] in order to improve the ee of **3**, since HLADH does not present an absolute stereospecificity, controlled the reaction so that only 70% of the favorable enantiomer of substrate (**1**) could be oxidized to the acid (**3**). In this way, the maximum yield of (*R*)-3-fluorolactic acid (**3**) could only be of 35%. On the other hand, by the fact reported in the present work, the process utilizes L-LDH as catalyst, which is an enantiospecific enzyme [12,25] and 3-fluoropyruvate as the starting material, the theoretical yield of **3** must be 100%. The system used to recycle NAD^+ to its reduced form was the oxidation of BHMC (**7**) catalyzed by HLADH to the chiral lactone (+)-(1*R*,6*S*)-*cis*-8-oxabicyclo[4.3.0]nonan-7-one (**8**). The reaction involved in this NADH recycling system has been shown to occur in three steps [26,27]. Initially the enzyme oxidizes the (*S*)-hydroxymethyl group of the monocyclic *meso* diol using one equivalent of NAD^+ and producing one equivalent of NADH and the corresponding hydroxyaldehyde. This latter compound undergoes a spontaneous cyclization reaction forming the respective lactol which is finally oxidized by the enzyme in the presence of a second molecule of NAD^+, producing another equivalent of NADH and the corresponding chiral lactone. This recycling system for in situ NADH regeneration from NAD^+ coupled to the reduction of 3-fluoropyruvate catalyzed by L-LDH is shown in Scheme 3. Since for each mol of BHMC (**7**) being oxidized two equivalents of NADH are produced, an additional driving force is provided to the process. In addition, the chiral γ-lactones, products of the oxidation of aliphatic monocyclic *meso* diols catalyzed by HLADH are considered of potential or recognized value as chiral starting materials in natural product synthesis [27]. This coupled redox system is then potentially able to provide simultaneously two valuable chiral building blocks.

The time–course curve for the reduction of sodium 3-fluoropyruvate catalyzed by L-LDH, using the BHMC (**7**)/HLADH NADH regeneration system in a semi-preparative scale of 200 mg is shown in Fig. 3. The progress of the reaction indicates that in approximately 9 h of reaction, under the experimental conditions described in Section 2.2, all 3-fluoropyruvate (**3**) has been consumed. As shown in Fig. 3 the time course curve has a sigmoidal aspect, i.e., at the beginning of the reaction when the concentration of 3-fluoropyruvate was high, the slope to the curve was lower than the slope described by the curve between approximately 25 and 80% of conversion. This initially diminished rate of consumption of 3-fluoropyruvate can be explained on the basis of the existence of a phenomenon of inhibition of L-LDH by excess of



Scheme 3. NADH in situ regeneration system for the reduction of sodium pyruvate catalyzed by L-LDH. Sodium 3-fluoropyruvate (**6**); sodium (*R*)-3-fluorolactate (**3**); BHMC (**7**); (+)-(1*R*,6*S*)-*cis*-8-oxabicyclo[4.3.0]nonan-7-one (**8**).

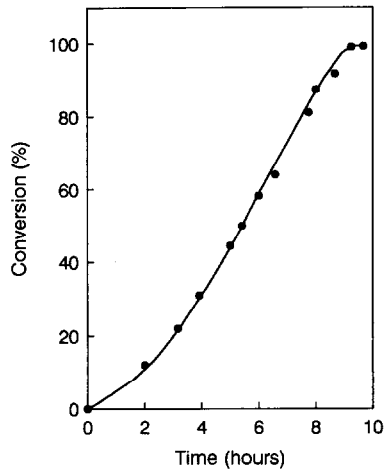


Fig. 3. Progress curve for the reduction of 3-fluoropyruvate catalyzed by L-LDH. The course of reaction was followed by enzymatic determination of the residual concentration of 3-fluoropyruvate at the times indicated in the figure as described in Section 2.2.

substrate that becomes more evident between zero and approximately 25% of consumption of 3-fluoropyruvate. As the reaction time increases the inhibition of the system is less evident since the concentration of substrate present in the reaction medium decays concomitantly. This phenomenon of inhibition of L-LDH by 3-fluoropyruvate was not observed in the experiments shown in Figs. 1 and 2, since in both the highest concentration of this substrate (0.15 and 0.80 mM, respectively) was substantially lower than that used for the experiment shown in Fig. 3 (26.3 mM). Experiments designed to evidence this type of inhibition were conducted by Zewe and Fromm [15] with the natural substrate of this enzyme. An uncompetitive substrate inhibition model was determined and explained by the formation of a dead-end ternary complex L-LDH–NAD^+–pyruvate, with an inhibition constant of 0.202 mM [15].

After total consumption of 3-fluoropyruvate the reaction medium was heated at 100°C for five minutes in order to eliminate the denatured enzymes. The filtrate was submitted to $CHCl_3$ extraction to eliminate BHMC (**7**) and the corresponding lactone (**8**) from the aqueous phase which was then acidified until pH 1.5, saturated with NaCl and continuously extracted with Et_2O for 72 h. The chiral 3-fluorolactic acid (**3**) present in the organic phase was finally methylated with CH_2N_2/Et_2O. Chiral 3-fluorolactic acid methyl ester was obtained in 80% overall yield. This product showed $[\alpha]_D^{25} = -5$ ($c = 1$, EtOH). IR showed the following bands of axial deformation: 3450 cm^{-1} (OH of alcohol); 1740 cm^{-1} (C=O of ester); 1125 and 1230 cm^{-1} (C–O of ester); and, 1000 cm^{-1} (C–F). ^{1}H-NMR revealed the following chemical shifts: $\delta = 3.85$ (3H, s, CH_3); 4.40 (1H, dt, $J_{CHCF} = 30$ Hz, $J_{CHCH_2} = 3$ Hz); 4.70 (2H, ddd, $J_{CH_2F} = 47$ Hz, $J_{CH_2(gem)} = 0.5$ Hz, $J_{CH_2CH} = 3.0$ Hz). HRGC/MS analyses, performed both with *rac*-3-fluorolactic acid methyl ester and the product obtained via L-LDH reduction of 3-fluoropyruvate, revealed identical chromatograms of total ions, showing the presence of the molecular ion m/z 122, characteristic of the target product.

Fig. 4 shows the analysis of 3-fluorolactic acid methyl ester by HRGC. Fig. 4A shows that

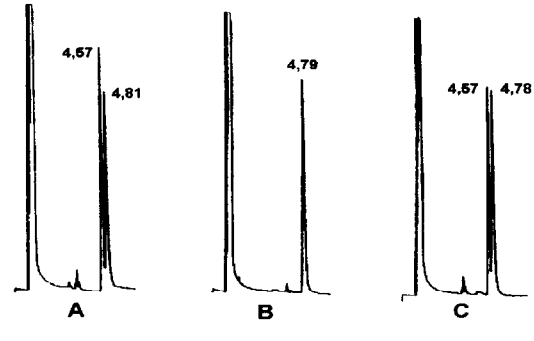


Fig. 4. Chiral high resolution gas chromatography of 3-fluorolactic acid methyl ester. (A) racemate obtained by reduction of 3-fluoropyruvate sodium salt with $NaBH_4$. (B) product obtained via L-LDH catalyzed reduction of 3-fluoropyruvate sodium salt. (C) co-injection of the racemate with the enantiomerically pure product. The capillary column (30 m) was coated with 2,3,6-tri-*O*-methyl-β-cyclodextrin (PMCD/OV 1701-OH). The numbers appearing in the figure represent the retention times (minutes) of the peaks. Other experimental details are given in Section 2.2.

when *rac*-3-fluorolactic acid methyl ester was analyzed two peaks of the same area were obtained with retention times of 4.57 and 4.81 min, respectively. When the product obtained via enzymatic reduction was analyzed, only one peak with a retention time of 4.79 min was detected (Fig. 4B). Moreover, when the racemate obtained by $NaBH_4$ reduction (Fig. 4A) was co-injected with a diluted solution of the enzymatically synthesized product, a significant increase of the area of the enantiomer presenting a higher retention time (4.78 min) was obtained (Fig. 4C), thus indicating this to be the enantiomer resulting from the action of L-LDH. This result together with the fact that in Fig. 4B no trace of the first peak was detected, is indicative of an ee > 99% of the chiral 3-fluorolactic acid methyl ester produced via L-LDH reduction of sodium 3-fluoropyruvate. In addition, since it has been shown that the reduction of pyruvic acid and of their derivatives catalyzed by L-LDH occurs enantiospecifically, the absolute configuration of the α-C atom must be the same for all the products [12,14]. Based on this fact the absolute configuration of homochiral 3-fluorolactic acid methyl ester prepared enzymatically was considered to be (*R*). This statement is supported by the fact that L-LDH, because of its absolute enantioselectivity, has been commonly used as a reagent to determine the absolute configuration of the α-C atom of products that are substrates of this enzyme [25,28].

This paper describes a very efficient enzymatic process to produce (*R*)-3-fluorolactic acid methyl ester in holemic form, a 1,2,3-trisubstituted three carbon atoms chiral building block with several applications in asymmetric organic synthesis. The process hereon reported can be easily executed in any synthetic laboratory, allowing the preparation of numerous optically active products with biological or pharmacological activity. Although it was not the purpose of the present work to obtain chiral monocyclic lactones, the enzymatic system developed is suitable for the simultaneous preparation of both chiral building blocks.

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