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ENZYMATIC ELIMINATION OF FLUORIDE FROM α-FLUORO-β-ALANINE

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Abstract—Rat liver homogenates catalyzed the elimination of fluoride from (*R,S*)-α-fluoro-β-alanine. The substrate specificity and physical properties of the defluorinating enzyme were similar to those of mitochondrial L-alanine-glyoxylate aminotransferase II (EC 2.6.1.44, AlaAT-II). Furthermore, AlaAT-II activity, measured with L-alanine and glyoxylate as substrates, copurified with the α-fluoro-β-alanine-defluorinating enzyme. The NH_2-terminal sequence (18 residues) of the enzyme did not show significant sequence similarity with any of the proteins currently listed in GenBank. The purified enzyme catalyzed the transamination of L-alanine (Ala) and glyoxylate (glyx) at pH 8.5 by a ping–pong mechanism with kinetic parameters of k_{cat} = 17 sec^{-1}, $K_{L\text{-}Ala}$ = 3.2 mM, and K_{glyx} = 0.3 mM, respectively. The kinetic parameters for the defluorination of (*R*)-α-fluoro-β-alanine and (*S*)-α-fluoro-β-alanine were k_{cat} = 6.2 and 2.6 min^{-1}, respectively, and K_m = 2.7 and 0.88 mM, respectively. L-Alanine potently inhibited the defluorination reaction with an apparent K_i of 0.024 mM. (*R,S*)-α-Fluoro-β-alanine converted the optical spectrum of the enzyme-bound cofactor from the pyridoxal form to the pyridoxamino form, which indicated that this cofactor may participate in the defluorination reaction. The product of the enzymatic reaction, malonic semialdehyde, reacted nonenzymatically with (*R,S*)-α-fluoro-β-alanine to form an adduct that was detected spectrally. AlaAT-II was not inactivated during dehalogenation of (*R,S*)-α-fluoro-β-alanine but was inactivated completely during dehalogenation of β-chloro-L-alanine.

Key words: (*R*)-α-fluoro-β-alanine; (*S*)-α-fluoro-β alanine; (*R,S*)-α-fluoro-β-alanine; α-β elimination; alanine aminotransferase; malonic semialdehyde; fluoride; pyridoxal phosphate; enamine; β-chloro-L-alanine; inactivation; (*R*)-5-fluoro-5,6-dihydrouracil; 5-fluorouracil

5-Fluorouracil, a widely used antineoplastic agent for the treatment of human solid tumors, is catabolized in humans (Scheme I) with a T½ of approximately 10 min [1, 2]. The major product (>80%) of 5-fluorouracil catabolism is (*R*)-α-fluoro-β-alanine [3]. However, fluoride is an additional product of 5-fluorouracil catabolism that could contribute to the cytotoxicity of 5-fluorouracil [4–6]. Even though it has not been established whether fluoride is generated directly from 5-fluorouracil or from one of the catabolites of 5-fluorouracil (Scheme I), it is usually assumed that (*R*)-α-fluoro-β-alanine is the immediate precursor for fluoride [4, 5].

Pyridoxal phosphate enzymes, which catalyze the α-β elimination of halides from appropriate substrate analogues [7–9], have been suggested to be involved in the defluorination of α-fluoro-β-alanine (Scheme II).

In particular, γ-aminobutyrate aminotransferase [4] and serine hydroxymethyltransferase [5] have been proposed to be physiological catalysts for this reaction. Serine hydroxymethyltransferase catalyzes the defluorination of (*R,S*)-α-fluoro-β-alanine in the presence of tetrahydrobiopterin. The K_m for (*R,S*)-α-fluoro-β-alanine is 50 mM, and the k_{cat} is similar to that for serine [5]. Because these latter determinations were made with (*R,S*)-α-fluoro-β-alanine, it is uncertain whether the enzyme defluorinated the physiologically relevant *R* enantiomer. Based on the dehalogenating activity of γ-aminobutyrate aminotransferase [8], this enzyme has also been proposed to catalyze the defluorinating reaction. However, this activity has not been demonstrated. Thus, the relevance of serine hydroxymethyltransferase and γ-aminobutyrate aminotransferase to the physiological defluorination reaction is unclear.

The studies presented herein demonstrated that rat liver homogenates catalyze the defluorination of (*R*)- and (*S*)-α-fluoro-β-alanine but do not catalyze the defluorination of 5-fluoro-5,6-dihydrouracil or 5-fluorouracil. The protein responsible for this activity was purified to homogeneity and identified as mitochondrial AlaAT-II based upon its substrate specificity and physical properties. The product of the reaction, malonic semialdehyde, reacted nonenzymatically with (*R,S*)-α-fluoro-β-alanine to form a spectrally detectable complex.

MATERIALS AND METHODS

Materials

Rat liver was from Pel-Freez (Rogers, AR). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-alanine, glyoxylate, oxalacetate, pyruvate, D-alanine, L-alanine, L-aspartate, L-glutamate, succinic semialdehyde, (D,L)-β-aminoisobutyrate, $NADP^+$, NADH, glutamate-oxalacetate aminotransferase (porcine liver), glutamate-pyruvate aminotransferase (porcine liver), alcohol dehydrogenase (horse liver), GABASE, L-ornithine, α-ketobutyrate, β-chloro-L-alanine, pyridoxal phosphate,

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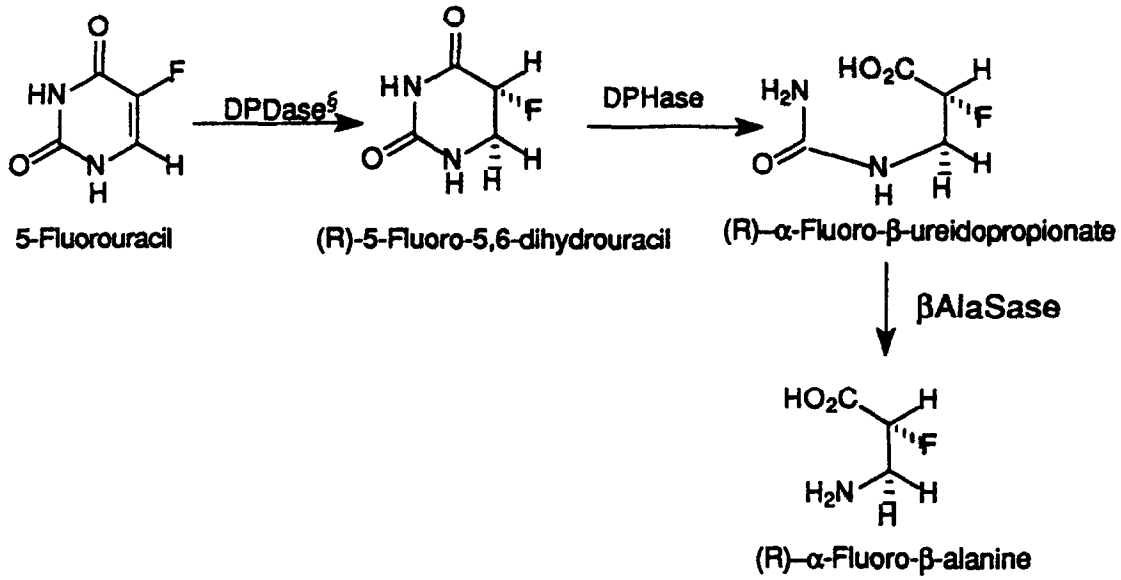
§ Abbreviations: DPHase, dihydropyrimidine aminohydrolase (EC 3.5.2.2); DPDase, dihydrophyrimidine dehydrogenase (EC 1.3.1.2); AlaAT-II L-alanine-glyoxylate aminotransferase II (EC 2.1.6.44); MES, 2-[*N*-morpholino]ethanesulfonic acid; and βAlaSase, β-alanine synthetase (EC 3.5.1.6).



Scheme 1.

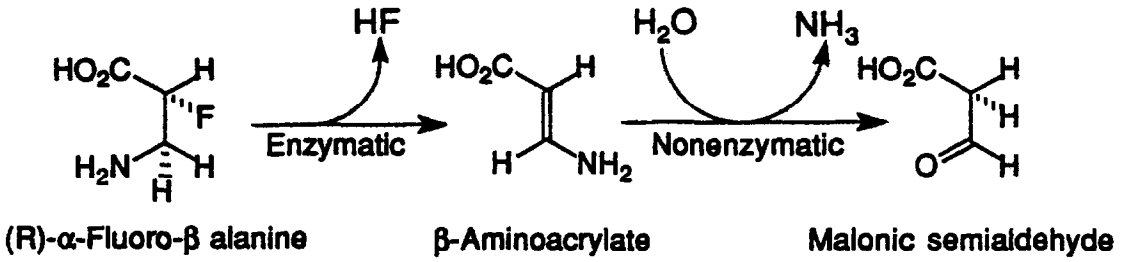
β-mercaptoethanol, 5-fluorouracil, Dowex 50W and Dowex 1 resins were from the Sigma Chemical Co. (St. Louis, MO). Sinapinic acid and 3-methyl-2-benzothiazolinone hydrazone were from the Aldrich Chemical Co. (Milwaukee, WI). (*R,S*)-α-Fluoro-β-alanine and 3,3-diethoxypropionic acid ethyl ester were purchased from TCI America (Portland, OR). Whatman DE-52 was from Bodman (Media, PA). P-6 resin and hydroxylapatite BIO-GEL HTP Gel were from Bio-Rad (Richmond, CA). Sephacryl S-200 resin and the Mono S HR5/5 column were from Pharmacia (Piscataway, NJ). Protein concentration was determined with the Protein Assay Kit from Bio-Rad, using bovine serum albumin as the standard protein. β-Alanine synthetase was purified from rat liver through the ammonium sulfate step as described by Tamaki *et al.* [10]. This preparation of enzyme was free of (*R,S*)-α-fluoro-β-alanine defluorinating activity.

Assay of the defluorination of (R,S)-α-*fluoro*-β-*alanine*

The enzymatic formation of fluoride from (*R,S*)-α-fluoro-β-alanine was monitored with a fluoride electrode (Orion) that was calibrated with five concentrations of fluoride (10–100 μM). Defluorinating activities in samples from the purification procedure were determined with 50 mM (*R,S*)-α-fluoro-β-alanine at 37° in 0.05 M potassium phosphate, 2 mM EDTA, 5 mM β-mercaptoethanol and 40 μM pyridoxal phosphate at pH 7.0. Typically, enzyme and substrate were reacted for approximately 1 hr prior to determining fluoride concentration (<100 μM). The activity of the enzyme was expressed as micromoles of fluoride generated per hour. The standard buffer for kinetics studies of fluoride generation from (*R*)- or (*S*)-α-fluoro-β-alanine by pure enzyme was 0.05 M sodium pyrophosphate at pH 8.5 and 25°.

Assay of the transamination of L-*alanine and glyoxylate*

The enzymatic transamination of L-alanine and glyoxylate was determined by monitoring pyruvate formation with lactate dehydrogenase and NADH by the method of Rowsell *et al.* [11]. Glyoxylate is not a substrate for lactate dehydrogenase in 0.3 M Tris–HCl at pH 8.3. Typically, 800 μL of 50 mM L-alanine in 0.125 M sodium pyrophosphate at pH 8.5, 2 mM EDTA, 5 mM β-mercaptoethanol and 40 μM pyridoxal phosphate were mixed at 37° with 10 μL of 200 mM glyoxylate, the enzyme sample, and sufficient H_2O to make the final volume 1 mL. After 10 min at 37°, the reaction was quenched with 0.18 mL of 2 M Tris–HCl (pH 8.5), which complexed the glyoxylate. The pyruvate content of the solution was calculated from the loss of absorbance of NADH after adding 200 μM NADH and then excess lactate dehydrogenase. The transaminase activity



Scheme 2.

was expressed as micromoles of NADH oxidized per minute.

Assay of γ-aminobutyrate aminotransferase activity

γ-Aminobutyrate aminotransferase-catalyzed transamination of 5 mM L-glutamate and 1.6 mM succinic semialdehyde in 0.05 M sodium pyrophosphate at pH 8.5 was monitored by the absorbance change associated with the conversion of L-glutamate and succinate semialdehyde to α-ketoglutarate and γ-aminobutyrate semialdehyde ($\Delta\varepsilon_{220}$ = 0.8 mM^{-1} cm^{-1}). The activity of the enzyme was expressed as micromoles of L-glutamate transaminated per minute.

Purification of (R,S)-α-fluoro-β-alanine defluorinating enzyme from rat liver

All purification steps were performed at 5° unless otherwise noted. Buffers were prepared at 25°.

Step 1: *Homogenization of liver.* Frozen rat livers (100 g) were thawed in 400 mL of 0.05 M HEPES with 40 μM pyridoxal phosphate at pH 7.0 for approximately 20 min in a 40° water bath. The liver was then homogenized at room temperature in a commercial Waring blender for 3 min.

Step 2: *Heat denaturation.* The mixture from the previous step was heated to 63–65° in a 95° water bath for 3 min and immediately cooled to 40° in ice water. The volume of the mixture was increased to approximately 700 mL with H_2O prior to centrifugation.

Step 3: *DE-52 anion exchange.* The supernatant from the previous step was mixed with 100 mL of packed DE-52 resin that had been adjusted to pH 7.5 in 0.025 μM sodium HEPES with 1 N NH_4OH. The enzyme was adsorbed onto the resin by stirring for 30 min at 5°. The resin was collected by filtration with a sintered glass funnel (600 mL, coarse). The column of resin was washed under vacuum with 500 mL of 0.025 M sodium HEPES, 1 mM EDTA, 5 mM β-mercaptoethanol and 40 μM pyridoxal phosphate at pH 7.5 (Buffer A). The enzymatic activity was eluted from the column with 0.1 M KCl in Buffer A.

Step 4: *Hydroxylapatite chromatography.* The fractions with enzymatic activity from the previous step were pooled and adjusted to pH 6.8 with 1 N phosphoric acid. The enzyme was adsorbed onto a 2.5 x 6 cm column of hydroxylapatite equilibrated in 0.1 M potassium phosphate, 1 mM EDTA, 2 mM β-mercaptoethanol and 40 μM pyridoxal phosphate at pH 6.8 (Buffer B). The column was washed with approximately 100 mL of Buffer B. The enzyme was eluted with Buffer B containing an additional 0.4 M potassium phosphate.

Step 5: $(NH_4)_2SO_4$ *fractionation.* Fractions from the previous step that contained enzymatic activity were pooled (22 mL). This solution was made 40% saturated $(NH_4)_2SO_4$ by addition of 5.3 g of solid $(NH_4)_2SO_4$. After stirring for 20 min, the mixture was centrifuged. The supernatant was brought to 70% saturated $(NH_4)_2SO_4$ by addition of 5.1 g of solid $(NH_4)_2SO_4$. The mixture was stirred for 20 min, and then the precipitate was collected by centrifugation.

Step 6: *Sephacryl S-200 chromatography.* The precipitate from the previous step was dissolved in 3.1 mL of buffer A. This solution was applied to a 90 x 1.5 cm column of Sephacryl S-200 that had been equilibrated in Buffer A. Fractions 72 through 75 were pooled (4.2 mL). Fraction 81 had maximal γ-aminobutyrate aminotransferase activity.

Step 7: *Anionic chromatography.* The pooled fractions from the previous step were adjusted to pH 5.8 with 0.5 M MES. The enzyme was adsorbed onto a Mono S HR5/5 column that had been equilibrated with 0.05 M MES, 1 mM EDTA, 2 mM β-mercaptoethanol and 40 μM pyridoxal phosphate at pH 5.8. The enzyme was eluted from the column with a 20-mL gradient of 0 to 0.3 M NaCl. The enzyme was collected in 3 mL with approximately 0.1 M NaCl.

Step 8: *Sephacryl S-200 chromatography.* The enzyme solution from the previous step was rechromatographed on the Sephacryl S-200 column as described for Step 6. The enzyme was eluted from the column with the same volume of buffer as described for Step 6. Three fractions containing maximal enzymatic activity were pooled.

Concentration of enzyme-bound pyridoxal phosphate

The concentration of enzyme-bound pyridoxal phosphate was determined by the absorbance of an enzyme solution at 390 nm in 0.1 N NaOH and ε_{390} = 6 mM^{-1} cm^{-1} [12]. The extinction coefficient for pyridoxal phosphate bound to the defluorinating enzyme at pH 7.0 was calculated from these data to be 17 ± 1 mM^{-1} cm^{-1} (N = 2).

Substrate specificity of alanine aminotransferase

The activities of the purified enzyme with different amino acid donors and glyoxylate or pyruvate were determined spectrophotometrically. The difference in absorbance at 220 nm between the α-keto acid and glyoxylate was used for α-amino acid donors with very small extinction coefficients at 220 nm (ε_{220} < 0.01 mM^{-1} cm^{-1}). Glyoxylate absorbed slightly at 220 nm in 0.05 M sodium phosphate at pH 8.5 (ε_{220} = 0.08 mM^{-1} cm^{-1}), whereas α-ketoacids absorbed significantly (pyruvate ε_{220} = 1.05 mM^{-1} cm^{-1}; α-ketoglutarate ε_{220} = 1.03 mM^{-1} cm^{-1}). Thus, $\Delta\varepsilon_{220}$ for the transamination of an alkyl α-amino acid and glyoxylate was 0.95 mM^{-1} cm^{-1}.

The transamination of β-alanine and β-aminoisobutyrate with glyoxylate or pyruvate resulted in an absorbance increase at 268 nm caused by secondary nonenzymatic reaction of products with the substrate (see Results). The difference extinction coefficients for these transamination reactions were determined by allowing a transamination reaction that contained 10 μM glyoxylate and 25 mM amino acid to go to completion. Under these conditions, the $\Delta\varepsilon_{268}$ values for the transamination reaction with β-alanine and β-aminoisobutyrate were 2.3 and 1.1 mM^{-1} cm^{-1}, respectively. These extinction coefficients and the rate of absorbance change were used to calculate the steady-state rates of aldehyde formation in the presence of 25 mM amino acid.

Amino terminal sequence analysis and laser desorption mass spectrometry

Samples for amino terminal sequence analysis were spun through a ProSpin™ (Applied Biosystems) sample preparation cartridge and then rinsed with H_2O. After drying the membrane, it was excised and placed in a Blott™ cartridge for sequence analysis. Pulsed liquid phase Edman sequencing was performed on an Applied Biosystems 477a protein sequencer with an online model 120a PTH amino acid analyzer. Data were collected and

analyzed using Applied Biosystems model 610a software on a Macintosh IIvx computer. Laser desorption mass spectrometry was performed on a Lasermat (Finnigan MAT) matrix-assisted laser desorption mass analyzer. The samples (0.75 μL) were applied to a nitrocellulose-coated target, dried, and rinsed once with H_2O. The samples were then overlaid with 0.6 μL of a 10 mg/mL solution of sinapinic acid in 60% acetonitrile/0.4% trifluoroacetic acid.

Ammonia determination

Ammonia was assayed enzymatically with glutamate dehydrogenase (82 units/mL), 5 mM α-ketobutyrate and 200 μM NADH in 0.05 M sodium pyrophosphate at pH 8.5. The reaction was monitored by the absorbance decrease resulting from the oxidation of NADH ($\Delta\varepsilon_{340}$ = 6.22 mM^{-1} cm^{-1}). Glutamate dehydrogenase and NADH were added to the reference cuvette and to the sample cuvette, which contained the unknown amount of ammonia. After equilibration to 25°, α-ketoglutarate was added to both cuvettes, and the decrease in absorbance was monitored. The concentration of ammonia was calculated from the absorbance change associated with the unknown sample and that associated with a known amount of ammonia (20 μM).

Synthesis of malonic semialdehyde

Malonic semialdehyde was synthesized by sequential alkaline and acid hydrolysis of 3,3-diethoxypropionic acid ethyl ester as described by Hayaishi *et al.* [13]. The total aldehyde in the solution at the end of the reaction was estimated from the absorbance increase at 305 nm ($\Delta\varepsilon_{305}$ = 13.3 mM^{-1} cm^{-1}) after reaction with acidic 3-methyl-2-benzothiazolinone hydrazone [14]. Acetaldehyde, a product of decarboxylation of malonic semialdehyde, was estimated from the extent of oxidation of NADH by horse liver alcohol dehydrogenase. Acetaldehyde was less than 1% of the total aldehyde determined with 3-methyl-2-benzothiazolinone hydrazone. The yield of malonic semialdehyde from 3,3-diethoxypropionic acid ethyl ester was approximately 70%.

Synthesis of (R)-and (S)-α-fluoro-β-alanine

(*R*)-α-Fluoro-β-alanine was synthesized enzymatically from (*R*)-5-fluoro-5,6-dihydrouracil, which was prepared from 5-fluorouracil as previously described [15]. (*R*)-5-Fluoro-5,6-dihydrouracil (132 mg) was dissolved in 25 mL of H_2O and hydrolyzed nonenzymatically to (*R*)-β-carbamoyl-α-fluoropropionate at pH 10. After 14 min at room temperature, the pH of the solution was adjusted to 7 with 1 N H_3PO_4. This solution was added to 100 mL of 25 mM potassium phosphate containing 10 mM β-mercaptoethanol at pH 7. The enzymatic hydrolysis of (*R*)-β-carbamoyl-α-fluoropropionate to (*R*)-α-fluoro-β-alanine was initiated by the addition of 5 units of β-alanine synthetase. Hydrolysis, which was monitored by ammonia formation, was complete after incubating overnight at 37°. The solution was adjusted to pH 2 with 6 N HCl, and the precipitated protein was removed by centrifugation. (*R*)-α-Fluoro-β-alanine was purified chromatographically from this solution by a modification of the method of Mukherjee and Heidelberger [16]. (*R*)-α-Fluoro-β-alanine was absorbed onto a 1.5 x 12 cm column of Dowex-50W resin in the chloride form at pH 2. The resin was washed with a column volume of 10 mM HCl, and the product was eluted with a 200-mL gradient from 10 mM HCl to 2 M HCl. (*R*)-α-Fluoro-β-alanine was eluted between 80 and 120 mL of buffer. These fractions were pooled and lyophilized. The powder was dissolved in 10 mL H_2O and chromatographed on a 1.5 x 12 cm column of Dowex-1 formate. (*R*)-α-Fluoro-β-alanine was eluted with H_2O and lyophilized. The powder was dissolved in 1.6 mL of H_2O and crystallized by addition of 9 mL of absolute ethanol to yield 61 mg of crystalline (*R*)-α-fluoro-β-alanine (57% yield). ^1H NMR (300 MHz, D_2O): δ 4.91 (dm, J = 50 Hz, 1 H), 3.38 (m, 1 H), 3.29 (m, 1 H). Analysis calculated (found) for $C_3H_6N_1O_2F_1 \cdot 0.12H_2O$: C, 32.97 (32.98); H, 5.76 (5.58); N, 12.82 (12.70). Optical rotation: $[\alpha]_{589}^{20}$ = +29.1° (c 1.33, H_2O). (*S*)-α-Fluoro-β-alanine was synthesized chemically by the method of Somekh and Shanzer [17]. (*S*)-α-Fluoro-β-alanine had properties similar to those of (*R*)-α-fluoro-β-alanine except that $[\alpha]_{589}^{20}$ = −29.1° (c 0.78, H_2O).

Reaction of (R,S)-α-fluoro-β-alanine with malonic semialdehyde

The nonenzymatic reaction of (*R,S*)-α-fluoro-β-alanine with malonic semialdehyde was monitored by the absorbance increase at 268 nm (see Results). Equation 1 was fitted to the magnitude of the absorbance change as a function of the concentration of (*R,S*)-α-fluoro-β-alanine ([FBAL]) to yield the apparent dissociation constant (K_d) and the maximal absorbance change at 268 nm (A_{268}).

$$\Delta A_{268}\,([\mathrm{FBAL}]) = \frac{A_{268}[\mathrm{FBAL}]}{[\mathrm{FBAL}] + K_d} \qquad (1)$$

Equation 2 was fitted to the time–course for the absorbance changes at 268 nm (A(t)) resulting from the reaction of (*R,S*)-α-fluoro-β-alanine with malonic semialdehyde. The pseudo-first-order rate constant for the reaction was k_{obs}, the initial absorbance was A_0, and the final absorbance was A_∞.

$$A(t) = A_\infty + (A_0 - A_\infty)\exp(-k_{obs} \cdot t) \qquad (2)$$

Assay of the dechlorination of β-chloro-L-alanine

The dechlorination of β-chloro-L-alanine to pyruvate the purified enzyme was monitored by the absorbance increase at 220 nm ($\Delta\varepsilon_{220}$ = 1.0 mM^{-1} cm^{-1}) associated with the formation of pyruvate. Equation 2 was fitted to the time–course for absorbance changes at 220 nm (A(t)) resulting from the time-dependent decrease in the rate of pyruvate production from β-chloro-L-alanine catalyzed by the purified enzyme. k_{obs} was the pseudo-first-order rate constant for onset of inhibition. A_0 was the initial absorbance of the solution, and A_∞ was the final absorbance of the solution.

Analysis of steady-state kinetic data

Initial velocity data for defluorination of (*R*)- and (*S*)-α-fluoro-β-alanine were analyzed by the Michaelis–Menten equation. Initial velocity data for the transamination of L-alanine (L-Ala) by glyoxylate (glyx) were analyzed by Equation 3, which describes the substrate dependence of the initial velocity for product formation for a ping–pong kinetic mechanism [18].

$$v/E_t = \frac{k_{cat}}{1 + \dfrac{K_{L\text{-}Ala}}{[\mathrm{L\text{-}Ala}]} + \dfrac{K_{glyx}}{[\mathrm{glyx}]}} \qquad (3)$$

Results are reported as means ± SD (range) for the indicated value of N. If a value for N is not indicated, the results are reported as means ± SEM.

RESULTS

Comparison of 5-fluorouracil, 5-fluoro-5, 6-dihydrouracil and (R,S)-α-fluoro-β-alanine as substrates for the defluorinating enzyme in rat liver homogenates

We tested 5-fluorouracil and two catabolites of 5-fluorouracil as substrates for the defluorinating enzyme in rat liver homogenates. A liver homogenate that was prepared as described for Step 1 of the purification procedure (see Materials and Methods) catalyzed fluoride formation in 0.05 M sodium HEPES, 1 mM EDTA, 2 mM β-mercaptoethanol and 40 μM pyridoxal phosphate at pH 7 with 1.0 mM 5-fluorouracil, 5-fluoro-5,6-dihydrouracil and (*R,S*)-α-fluoro-β-alanine with relative rates of <0.01, 0.6, and 1.0, respectively. (*R,S*)-α-Fluoro-β-alanine was the most efficient substrate, and 5-fluorouracil was not a detectable substrate. Because the liver has large amounts of dihydropyrimidinase and β-alanine synthetase (Scheme I), fluoride that was generated from 5-fluoro-5,6-dihydrouracil probably occurred after enzymatic hydrolysis of 5-fluoro-5,6-dihydrouracil to α-fluoro-β-alanine [10, 19]. Consequently, we purified the enzyme catalyzing the defluorination of (*R,S*)-α-fluoro-β-alanine.

Purification of the defluorinating activity from rat liver

The purification of the defluorinating enzyme from rat liver is summarized in Table I. β-Mercaptoethanol (2 mM), EDTA (1 mM), and pyridoxal phosphate (40 μM) were included in the purification buffers. The enzyme appeared as a single Coomassie blue staining band on 12% SDS–PAGE with a relative molecular weight of 53,000. Because the recovery of defluorination activity could be quantitatively accounted for by summing the activity discarded in side fractions in each purification step, the defluorinating activity in rat liver homogenates was probably a single enzymatic species. For kinetic studies, free pyridoxal phosphate was removed from the purified enzyme by size exclusion chromatography on a 10 × 1.5 cm column of P-6 resin equilibrated in 0.05 M sodium phosphate with 1 mM β-mercaptoethanol at pH 7.0. In this buffer, the enzyme lost approximately 20% of its activity after 2 days at 5°.

Physical properties of purified enzyme

Laser desorption mass spectrometry and SDS–PAGE yielded a subunit molecular weight for the enzyme of 53,000 ± 600 (N = 2). The native molecular weight was determined to be 170,000 ± 10,000 (N = 2) by chromatography on Sephacryl S-200 resin calibrated with Gel Filtration Molecular Weight Markers (12,000–200,000) from the Sigma Chemical Co. This value was slightly less than the value expected for a tetrameric protein with a subunit molecular weight of 53,000. A partial sequence of the NH_2-terminal end of the protein was determined to be LHTKHNMPPPDFSPEKYQ. This N-terminal amino acid sequence had no similarity with sequences in the current GenBank data base when searched by GCG sequence comparison software [20].

The procedure that we developed for purifying the (*R,S*)-α-fluoro-β-alanine defluorinating activity was similar to those for several amino acid aminotransferases [21–28]. Consequently, the substrate specificity of the defluorinating enzyme as an amino acid aminotransferase was studied. The enzyme catalyzed the transamination of L-alanine to glyoxylate very efficiently (Table 2). The enzyme also catalyzed the transamination of numerous α- and β-amino acids (Table 2). The enzyme did not catalyze the transamination of D-alanine, the transamination of L-glutamate or L-aspartate with glyoxylate. The purified enzyme did not catalyze the defluorination of 5-fluorouracil or 5-fluoro-5,6-dihydrouracil [<1% the rate with (*R,S*)-α-fluoro-β-alanine].

The subunit and native molecular weights of the enzyme and the substrate specificity suggested that the defluorinating enzyme was mitochondrial AlaAT-II. To establish that the defluorinating activity and L-alanine:glyoxylate transaminating activity were due to the same enzyme, these activities were compared at each step of the purification procedure (Table I). The ratio of the defluorinating activity to the transaminating activity was constant for steps 4 through 8 (Table I). The change in this ratio observed during the initial purification steps was probably due to the removal of L-alanine:glyoxylate aminotransferase I from the preparation.

Table 1. Summary of the purification of α-fluoro-β-alanine defluorinase and alanine transaminase from rat liver

Step*	Volume (mL)	Protein (mg)	Defluorinase activity† (μmol/hr)	Transaminase activity† (μmol/min)	Defluorinase Transaminase‡
1. Homogenate	500	24,600	28 (0.0011)§	810 (0.033)§	0.034
2. Heat	620	2,300	29.5 (0.013)	498 (0.22)	0.059
3. DE-52	50	225	18.3 (0.081)	290 (1.29)	0.063
4. Hydroxylapatite	22	85.4	19 (0.22)	202 (2.4)	0.094
5. $(NH_4)_2SO_4$	3.1	26.9	14.2 (0.53)	175 (6.5)	0.081
6. Sephacryl S-200	4	5.92	7.6 (1.3)	84 (14)	0.090
7. Mono S	3	1.53	4.7 (3.1)	52 (34)	0.090
8. Sephacryl S-200	3.2	0.72	2.1 (2.9)	20 (28)	0.105

* Details of each purification step are given in Materials and Methods.

† The enzyme was assayed at 37°C. Defluorinating activity was determined from the formation of fluoride from 50 mM (*R,S*)-fluoro-β-alanine. Alanine aminotransferase activity was determined from the formation of pyruvate with 40 mM L-alanine and 2 mM glyoxylate as described in Materials and Methods.

‡ Ratio of defluorinating activity to transaminating activity.

§ Specific activity values (units/mg) are given in parentheses.

Table 2. Transaminase activity of the α-fluoro-β-alanine defluorinating enzyme with selected amino-donor substrates*

Amino-donor substrate	Amino-acceptor substrate	Catalytic activity (v/E_t, sec^{-1})
L-Alanine	Glyoxylate	11.9 ± 0.8†
L-α-Aminobutyrate	Glyoxylate	7.1 ± 0.2
L-Ornithine	Glyoxylate	0.28 ± 0.03
β-Aminoisobutyrate	Glyoxylate	0.27 ± 0.01
β-Alanine	Glyoxylate	0.177 ± 0.007
L-Arginine	Glyoxylate	0.10 ± 0.03
L-Glutamate	Glyoxylate	< 0.05‡
L-Aspartate	Glyoxylate	< 0.05‡
D-Alanine	Glyoxylate	< 0.05‡
β-Alanine	Pyruvate	0.172 ± 0.007
β-Aminoisobutyrate	Pyruvate	0.221 ± 0.004
γ-Aminobutyrate	Pyruvate	< 0.05‡

* Transaminase activity was determined with 25 mM amino-donor substrate and 1 mM amino-acceptor substrate. The transfer reaction was monitored spectrophotometrically as described in Materials and Methods.

† Values are means ± SD, N = 3.

‡ Lower limit for detection of catalytic activity under the conditions of this assay.

Effect of L-alanine and (R,S)-α-fluoro-β-alanine on the spectrum of the enzyme

The spectrum of the purified enzyme had λ_{max} values of 408 and 278 nm. The ratio of the absorbance at 278 to that at 408 was 2.7 (Fig. 1). These spectral properties are characteristic of pyridoxal phosphate enzymes [7, 27]. The spectrum of a pyridoxal phosphate enzyme is dependent upon whether the coenzyme is in the pyridoxal form (λ_{max} ~ 420 nm) or in the pyridoxamino form (λ_{max} ~330 nm). The coenzyme can be converted from the pyridoxal form to the pyridoxamino form by incubation of the enzyme with an amino-donor substrate. L-Alanine and (*R,S*)-α-fluoro-β-alanine caused a decrease in the absorbance of the enzyme at 408 nm and an increase in absorbance at 340 nm (Fig. 1), which suggested that the purified protein with absorbance at 408 nm, and not a minor contaminating protein, was responsible for the observed catalytic activity.

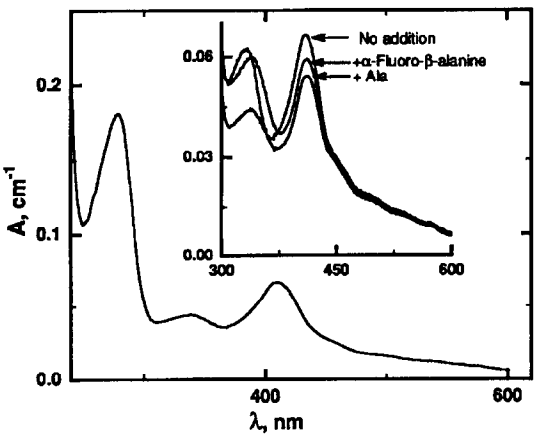


Fig. 1. Spectra of purified AlaAT-II. The concentration of enzyme-bound pyridoxal phosphate was 4.2 μM. Inset: Effect of 10 mM L-alanine or 10 mM (*R,S*)-α-fluoro-β-alanine on the spectrum of the enzyme. The enzyme was in 0.05 M sodium pyrophosphate at pH 8.5 and 25°.

Steady-state kinetics with alanine and glyoxylate as substrate

Initial velocity data for the transamination of glyoxylate and L-alanine at pH 8.5 and 25° were collected spectrophotometrically by monitoring the formation of pyruvate at 220 nm. Equation 3, which describes the dependence of the initial velocity on substrate concentrations for a ping–pong kinetic mechanism, was fitted to these data to give values for k_{cat}, $k_{L\text{-}Ala}$, and K_{glyx} of 17 ± 1 sec^{-1}, 3.2 ± 0.4 mM and 0.3 ± 0.3 mM, respectively.

Steady-state kinetics for fluoride production from (R)- and (S)-α-fluoro-β-alanine

The steady-state kinetic parameters (k_{cat}, K_m) for the defluorination of (*R*)-α-fluoro-β-alanine and (*S*)-α-fluoro-β-alanine were calculated from the initial velocity of fluoride formation (Fig. 2). The values of these parameters were $k_{cat} = 6.2 \pm 0.2$ min^{-1} and $K_m = 2.7 \pm 0.1$ mM for (*R*)-α-fluoro-β-alanine, and $k_{cat} = 2.6 \pm 0.1$ min^{-1} and $K_m = 0.88 \pm 0.02$ mM for (*S*)-α-fluoro-β-alanine. Because L-alanine converted AlaAT-II from the pyridoxal to the pyridoxamino form of the enzyme, it potently inhibited the defluorination reaction. We determined that the inhibition by L-alanine was competitive with an apparent K_i of 0.024 ± 0.002 mM for inhibition of the defluorination of 5 mM (*R,S*)-α-fluoro-β-alanine.

Pyridoxal phosphate (300 μM) did not catalyze the nonenzymatic release of fluoride (<5 μM) from 45 mM (*R,S*)-α-fluoro-β-alanine after a 1-hr incubation in 0.05 M sodium pyrophosphate at pH 8.5 and 37°. Under similar conditions, 1 μM AlaTA-II catalyzed the formation of over 300 μM fluoride.

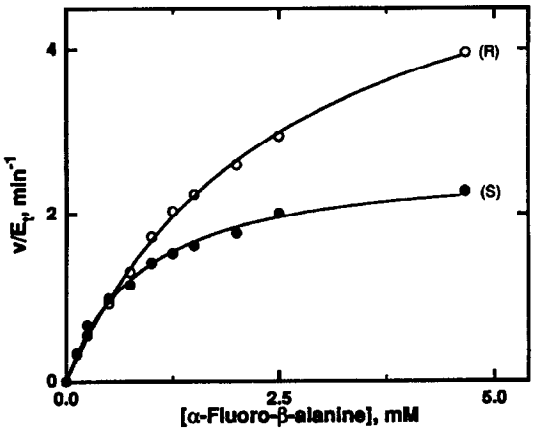


Fig. 2. Steady-state kinetics for the release of fluoride from (*R*)-α-fluoro-β-alanine and (*S*)-α-fluoro-β-alanine catalyzed by AlaAT-II. Initial velocities of fluoride production were determined with 0.61 μM enzyme. The abscissa values are the total concentration of the *R* and *S* enantiomers of α-fluoro-β-alanine. The solid line for the initial velocity for defluorination of (*R*)-α-fluoro-β-alanine was calculated with the Michaelis–Menten equation and values for K_m and k_{cat} of 2.6 mM and 6.2 min^{-1}, respectively. The solid line for (*S*)-α-fluoro-β-alanine was calculated with values for these parameters of 0.88 mM and 2.7 min^{-1}, respectively.

Time–course for the reaction of (R,S)-α-fluoro-β-alanine with enzyme

AlaAT-II catalyzed the defluorination of (*R,S*)-α-fluoro-β-alanine. Because halogenated amino-donor substrates are inactivators of numerous pyridoxal phosphate enzymes, (*R,S*)-α-fluoro-β-alanine was tested as an inactivator of AlaAT-II. However, fluoride production by 0.8 μM AlaAT-II from 25 mM (*R,S*)-α-fluoro-β-alanine was linear for 1 hr (Fig. 3), which demonstrates that the enzyme was not inactivated significantly during this time. Under these conditions, the minimal value for the partition ratio (moles of product formed/mole of enzyme inactivated) was greater than 1000. The concentration of ammonia was determined at the end of this reaction to be 104 μM. The ratio of ammonia to fluoride generated in this experiment was 1.46. In a repeat experiment, the ratio of ammonia to fluoride was 1.36. The stoichiometry of the defluorination reaction predicts that equal amounts of fluoride and ammonia should have been formed (Scheme II). We do not have an explanation for this apparent discrepancy.

During turnover of AlaAT-II with (*R,S*)-α-fluoro-β-alanine, a time-dependent increase in absorbance at 268 nm was observed (Fig. 3). The time–course for the absorbance change paralleled that for fluoride production only after an initial burst. Because malonic semialdehyde, fluoride and ammonia, the expected products of the defluorination reaction (Scheme II), have insignificant absorbance at 268 nm (see below), other products were generated during reaction. Furthermore, if the initial velocity of the absorbance change at 268 nm was used to monitor the reaction of (*R,S*)-α-fluoro-β-alanine with AlaAT-II, the K_m of (*R,S*)-α-fluoro-β-alanine was calculated to be 28 ± 3 mM, which was approximately 20-fold larger than the K_m based on initial velocity data for fluoride production. These results demonstrated that the reaction of (*R,S*)-α-fluoro-β-alanine with AlaAT-II involved reactions other than those outlined in Scheme II.

Nonenzymatic reaction of malonic semialdehyde with (R,S)-α-fluoro-β-alanine

In the absence of AlaAT-II, malonic semialdehyde (100 μM) formed an adduct with (*R,S*)-α-fluoro-β-alanine (40 mM) that absorbed at 268 nm (Fig. 4). The spectrum of this adduct was identical to that generated during the enzymatic defluorination of 10 mM (*R,S*)-α-fluoro-β-alanine. The apparent dissociation constant of the adduct was 13.4 ± 0.9 mM and the extinction coefficient at 268 nm was 12.2 mM^{-1} cm^{-1} (Fig. 4). The pseudo-first-order rate constant for the reaction of 10 mM (*R,S*)-α-fluoro-β-alanine with 100 μM malonic semialdehyde was (2.39 ± 0.02) × 10^{-2} sec^{-1} ($T_{1/2}$ = 29 sec).

Inactivation of AlaAT-II by β-chloro-L-alanine

A typical time–course for inhibition of 0.14 μM AlaAT-II by 25 mM β-chloro-L-alanine is presented in Fig. 5. The formation of product was monitored by the absorbance increase at 220 nm due to pyruvate formation. The rate of product formation decreased in a first-order manner with a k_{obs} of (2.44 ± 0.05) × 10^{-3} sec^{-1}. Addition of an equal amount of enzyme to the spent reaction resulted in a similar burst in pyruvate formation. The K_m of AlaAT-II for β-chloro-L-alanine was 70 ± 10 μM and the k_{cat} was 3.9 ± 0.3 sec^{-1}. Similar results were observed if pyruvate was monitored by the oxidation of NADH by lactate dehydrogenase. If L-alanine and glyoxylate were substrates, the rate of pyruvate formation was constant over the duration of the experiment (Fig. 5). These results were consistent with β-chloro-L-alanine inactivating AlaAT-II. The ratio of the moles of product formed per mole of enzyme inactivated was 900.

DISCUSSION

Fluoride is a minor catabolite of 5-fluorouracil metabolism that could contribute to the cytotoxicity of this chemotherapeutic agent [4–6]. However, neither the substrate nor the enzyme responsible for the defluorination reaction has been unequivocally identified. γ-Aminobutyrate aminotransferase and serine hydoxymethyltrans-

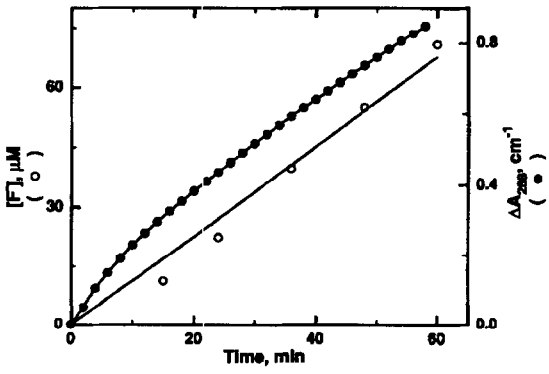


Fig. 3. Time–courses for AlaAT-II-catalyzed release of fluoride and for the increase in absorbance at 268 nm with (*R,S*)-α-fluoro-β-alanine as substrate. The reaction was initiated by the addition of 0.8 μM enzyme to a solution containing 25 mM (*R,S*)-α-fluoro-β-alanine. Other conditions are given in the legend to Fig. 1.

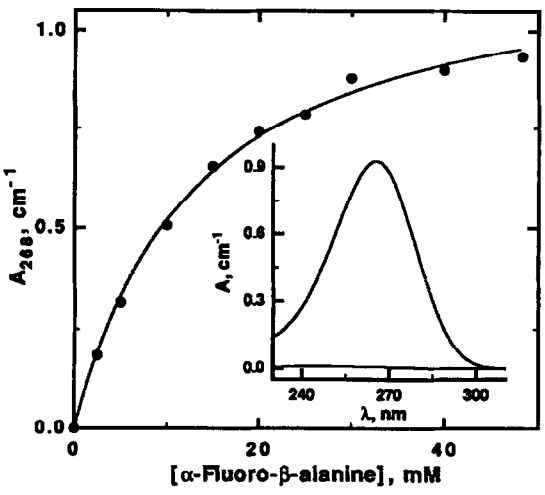


Fig. 4. Nonenzymatic reaction of malonic semialdehyde with (*R,S*)-α-fluoro-β-alanine. Titration of 100 μM malonic semialdehyde with (*R,S*)-α-fluoro-β-alanine was monitored at 268 nm. The solid line was calculated with Equation 1 and ΔA_{268} = 1.22 cm^{-1} and K_d = 13.4 mM. Inset: Spectra of 100 μM malonic semialdehyde (lower trace) and equilibrium mixture of 100 μM malonic semialdehyde and 40 mM (*R,S*)-α-fluoro-β-alanine (upper trace).

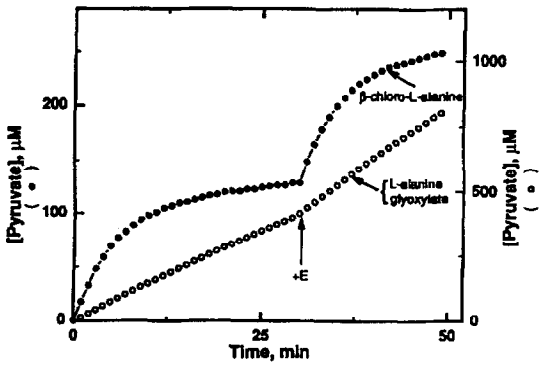


Fig. 5. Time–course for inhibition of AlaAT-II by β-chloro-L-alanine. The reaction of 0.14 μM AlaAT-II with 25 mM β-chloro-L-alanine was monitored at 220 nm (●). A second addition of enzyme (0.14 μM) was made at the indicated time. For comparison, the time–course for the transamination of 5 mM L-alanine and 2 mM glyoxylate to glycine and pyruvate by 0.027 μM AlaAT-II was followed (○). A second addition of enzyme (0.027 μM) was made at the indicated time.

ferase, pyridoxal phosphate enzymes, have been suggested to generate fluoride from (*R*)-α-fluoro-β-alanine, which is a major catabolite of 5-fluorouracil [4, 5]. We have shown herein that (*R*)-5-fluoro-5,6-dihydrouracil and (*R*)- and (*S*)-α-fluoro-β-alanine were substrates for fluoride generation by rat liver homogenates, whereas 5-fluorouracil was not. Because rat liver contains large amounts of dihydropyrimidinase and β-alanine synthetase activities, the defluorination of (*R*)-5-fluoro-5,6-dihydrouracil probably occurred after its enzymatic hydrolysis to α-fluoro-β-alanine [10, 19]. The absence of fluoride information from 5-fluorouracil under these conditions demonstrated that 5-fluorouracil was not a substrate for defluorinating enzyme. Furthermore, these results demonstrated that reduction of 5-fluorouracil to (*R*)-5-fluoro-5,6-dihydrouracil by dihydropyrimidine dehydrogenase (Scheme I) did not occur in rat liver homogenates in the absence of added NADPH. It appeared that (*R*)-α-fluoro-β-alanine was the preferred substrate for the defluorinating activity in rat liver homogenates. We subsequently found that this defluorinating activity in rat liver homogenates was primarily the result of AlaAT-II. The purified enzyme did not catalyze the defluorination of 5-fluorouracil or (*R*)-5-fluoro-5,6-dihydrouracil. AlaAT-II was concluded to be the enzyme responsible for the defluorinating activity because (1) AlaAT-II and the defluorinating enzyme copurified, (2) the molecular weights of AlaAT-II and the defluorinating enzyme were similar, and (3) the transaminating substrate specificities of AlaAT-II and the defluorinating enzyme were similar.

Both the *R* and *S* enantiomers of α-fluoro-β-alanine were defluorinated by AlaAT-II with catalytic efficiencies of 0.038 and 0.050 mM^{-1} sec^{-1}, respectively. For comparison, the catalytic efficiency for the transamination of L-alanine at a saturating concentration of glyoxylate was 5.3 mM^{-1} sec^{-1}. Thus, the catalytic efficiency of AlaAT-II for defluorinating α-fluoro-β-alanine was approximately 1% of the value for transaminating L-alanine, and the k_{cat} for the defluorination of (*R*)-α-fluoro-β-alanine by AlaAT-II was 0.5% of the value for transaminating L-alanine. Because AlaAT-II is a pyridoxal phosphate enzyme, the substrate should cause perturbations in the optical spectrum of the enzyme due to changes in the oxidation state of the coenzyme. For example, the absorbance of the pyridoxal phosphate enzyme γ-aminobutyrate aminotransferase is decreased at 420 nm and increased at 330 nm after addition of the substrate γ-aminobutyrate [27]. (*R,S*)-α-Fluoro-β-alanine and L-alanine had similar effects on the spectrum of AlaAT-II that corresponded to the conversion of the enzyme-bound pyridoxal phosphate from the pyridoxal form to the pyridoxamino form.

AlaAT-II catalyzes numerous reactions that could be of physiological importance. For instance, AlaAT-II catalyzes the transamination of (1) D-β-aminoisobutyrate, the catabolite of thymine [25, 28], (2) dimenthylarginine, found in proteins methylated post-translationally [23], (3) 2-aminobutyrate, found in mammalian blood [24], and (4) δ-aminolevulinic acid, a precursor of heme biosynthesis [22, 28]. The defluorinating activity of AlaAT-II reported herein is analogous to the dehalogenating activity of other pyridoxal phosphate enzymes [7–9]. For example, γ-ketoglutarate aminotransferase catalyzes the elimination of halide from 4-amino-5-halopentanoic acids [8], and L-alanine: α-ketoglutarate aminotransferase catalyzes the elimination of halide from β-halo-L-alanine [9].

β-Chloro-L-alanine was a time-dependent inhibitor of AlaAT-II that had the properties of an inactivator. The partition ratio (molar ratio of product generated to enzyme inactivated) was 900. These results were analogous to those for inactivation of alanine:2-oxoglutarate aminotransferase by β-chloro-L-alanine that occurred with a partition ratio of 500 [9]. Alanine:2-oxoglutarate aminotransferase was inactivated by β-chloro-L-alanine by covalent incorporation of the carbon moiety of the substrate into the enzyme. It was proposed that the enzymatically bound intermediate generated during the elimination reaction reacted with a nucleophilic amino acid residue at the active site [9]. The analogous enamine would be generated during the elimination of fluoride from α-fluoro-β-alanine. Consequently, AlaAT-II could be inactivated during the elimination of fluoride from α-fluoro-β-alanine. However, the rate of fluoride production from 25 mM α-fluoro-β-alanine was constant for 1 hr. Because the enzyme had completed 100 catalytic cycles at the end of this incubation and was not inactivated significantly, more than 1000 turnovers must be required for complete inactivation of the enzyme. Studies on the inactivation of γ-aminobutyrate aminotransferase by halo-substrate analogues have demonstrated that primary halo-amino acid analogues are more efficient inactivators of the enzyme than secondary halo-amino acid analogues. For example, the enzyme is stoichiometrically inactivated by 4-amino-5-halopentanoic acids with the formation of one enzyme equivalent of halide, whereas the enzyme catalyzes the elimination of chloride from 4-amino-3-chlorobutyric acid without inactivation [8]. This result is analogous to the inefficient inactivation of AlaAT-II by α-fluoro-β-alanine relative to that by β-chloro-L-alanine. In the former case, the substrate is a primary halo amino acid analogue, whereas in the latter case the substrate is a secondary halo amino acid analogue. The enamine resulting from halide elimination from a secondary halo amino acid analogue would be less reactive toward nucleophilic attack because of steric hindrance and the electron-donating properties of the alkyl group.

AlaAT-II-catalyzed defluorination of (*R,S*)-α-fluoro-β-alanine (Fig. 3) was associated with large absorbance

increases at 268 nm that were due to formation of an adduct between malonic semialdehyde and (*R,S*)-α-fluoro-β-alanine. The apparent K_m value of AlaAT-II for (*R,S*)-α-fluoro-β-alanine calculated from data monitoring the absorbance change at 268 nm was 20-fold larger than that calculated from data monitoring fluoride release. Thus, the steady-state kinetic parameters for the reaction cannot be estimated from initial velocity data monitoring these absorbance changes. This discrepancy can be explained as follows. The time–course for adduct formation during the enzymatic defluorination of (*R,S*)-α-fluoro-β-alanine is dependent on the K_m of the enzyme for (*R,S*)-α-fluoro-β-alanine and on the K_d of malonic semialdehyde for (*R,S*)-α-fluoro-β-alanine. Because the K_m of AlaAT-II for (*R,S*)-α-fluoro-β-alanine is approximately 1 mM, the rate of enzymatic formation of malonic semialdehyde does not increase significantly for (*R,S*)-α-fluoro-β-alanine concentrations greater than 4 mM. In contrast, because the dissociation constant for adduct formation is 13.4 mM, the amount of adduct increases significantly with increasing concentrations of (*R,S*)-α-fluoro-β-alanine. Consequently, when the velocity of the enzymatic defluorination reaction is monitored by the rate of adduct formation, the apparent K_m of the enzyme for (*R,S*)-α-fluoro-β-alanine (28 mM) is similar to the dissociation constant of malonic semialdehyde for (*R,S*)-α-fluoro-β-alanine (13 mM).

The goal of the present studies was to purify and characterize the enzyme in rat liver that was responsible for the generation of fluoride from 5-fluorouracil or one of the catabolites of 5-fluorouracil. AlaAT-II could account for most of the (*R,S*)-α-fluoro-β-alanine defluorinating activity in rat liver. These results are obviously relevant for understanding the catabolism of 5-fluorouracil in the rat. However, the relevance of AlaAT-II for fluoride formation from 5-fluorouracil catabolites in humans is not certain due to the report that this enzyme is not found in human liver [29]. Recently, D-3-aminoisobutyrate aminotransferase (EC 2.6.1.40) and AlaAT-II were shown to be the same enzyme [30]. D-3-Aminoisobutyrate aminotransferase activity is present in the liver of humans who are low D-3-aminoisobutyrate excretors, but it is absent in individuals who are high D-3-aminoisobutyrate excretors [31]. Consequently, AlaAT-II may be responsible for fluoride formation in some humans during 5-fluorouracil chemotherapy. (*R,S*)-α-Fluoro-β-alanine has been demonstrated to be an ineffective inhibitor of the transamination reaction catalyzed by D-3-aminoisobutyrate aminotransferase (K_i = 8 mM), but the defluorination of (*R,S*)-α-fluoro-β-alanine by rat liver D-3-aminoisubutyrate aminotransferase was not reported in these studies [32]. The importance of AlaAT-II in fluoride production from 5-fluorouracil catabolites in human tissue remains to be studied.

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