1993

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Citation/Publisher Attribution
Shieh, W.R., & Chen, C.-S. (1993). Purification and Characterization of Novel "2-Arylpropionyl-CoA Epimerases" from Rat Liver Cytosol and Mitochondria. *Journal of Biological Chemistry, 288*(5), 3487-3493. Retrieved from http://www.jbc.org/content/268/5/3487.
Available at: http://www.jbc.org/content/268/5/3487

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 268, No. 5, Issue of February 15, pp. 3487-3493, 1993
Printed in U.S.A.

Purification and Characterization of Novel "2-Arylpropionyl-CoA Epimerases" from Rat Liver Cytosol and Mitochondria*

(Received for publication, May 1, 1992)

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Investigation on the biochemical isomerization of ibuprofen led us to the successful purification of "2-arylpropionyl-CoA epimerase" from rat liver cytosol and mitochondria. The purified enzymes from both subcellular fractions exhibit similar physical and catalytic properties and are distinctly different from rat liver methylmalonyl-CoA epimerase. Both are monomeric proteins with an apparent molecular mass of 42 kDa and show similar contents of most amino acids. Their UV spectra gave no indication of any bound cofactors, and their enzyme activities were not affected by exposure to EDTA or metal ions (except Cu^{2+}). These results suggest that the cytosolic and mitochondrial epimerases may be structurally related. The purified enzymes catalyze the epimerization of various 2-arylpropionyl-CoAs with some degree of stereochemical differentiation. For 2-(4-isobutylphenyl)propionyl-CoA, the equilibrium constant was estimated to be 1.5 in favor of the *R*-isomer. Evidence indicated that the proton exchange may be mediated by a 2-base mechanism and that a carboxylic residue in the active site may serve as a general base for proton abstraction.

The 2-arylpropionic acids are an important group of nonsteroidal antiinflammatory drugs, commonly used for the treatment of pain, fever, and inflammation, as well as other connective tissue and rheumatologic diseases. Their activity in inhibiting prostaglandin synthesis is mainly contributed by the *S*-enantiomers. With the exception of naproxen, these therapeutic agents, such as ibuprofen and fenoprofen, are administered as racemic mixtures. A unique feature regarding 2-arylpropionic acid metabolism is the stereoselective conversion of the *R*-enantiomer to its *S*-counterpart, but not vice versa (1). Such unidirectional inversion has been documented in many species of mammals, including humans (2, 3). This biochemical transformation leads to elevated serum levels of the active species and in essence accounts for a bioactivation process. Although this metabolic inversion may not represent a potential pitfall for healthy individuals, it is of concern to those with asymptomatic kidney or liver dysfunction due to the suppression of renal prostaglandin synthesis (4–6). Incidences of acute kidney failure have been reported in metabolically compromised patients after short-term use of ibuprofen (7, 8).

* This work was supported by Grant GM-39236 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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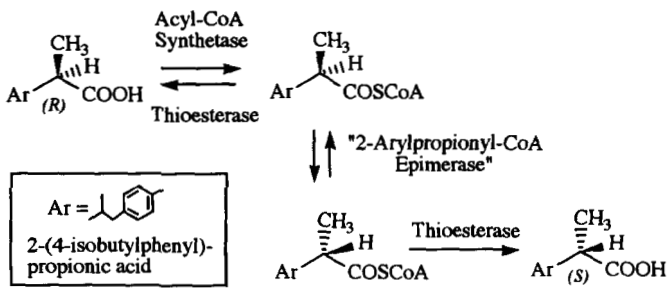


FIG. 1. **Mechanism of metabolic stereoisomeric inversion of 2-arylpropionic acids.**

A generally accepted biochemical pathway for this metabolic event is shown in Fig. 1 (9). The step of acyl-CoA formation has been shown to play a pivotal role in controlling the overall stereoselectivity, with only the *R*-enantiomer converted to its CoA-thioester (10, 11).

A putative enzyme, "2-arylpropionyl-CoA epimerase,[1]" subsequently mediates a rapid interconversion between the *R*- and *S*-epimers which, upon the action of a thioesterase, yield the free acids. Previously, using rat liver and kidney homogenates, we presented evidence indicating that the epimerization of 2-(4-isobutylphenyl)propionic acid (ibuprofen) proceeds via this CoA-dependent proton abstraction mechanism (3, 12). Yet, the identity and biological function of this putative epimerase remain to be explored. In this paper, we report the purification and characterization of 2-arylpropionyl-CoA epimerases from two subcellular fractions, cytosol and mitochondria, of rat liver.

EXPERIMENTAL PROCEDURES

Materials

Racemic 2-(4-isobutylphenyl)propionic acid (ibuprofen) was prepared from commercial ibuprofen tablets and purified by recrystallization from petroleum ether. Racemic [2-^{2}H]2-(4-isobutylphenyl)-propionic acid was synthesized via the deuterium exchange of the α-proton with excess amounts of CH_3OD in the presence of CH_3ONa (13). Optically active 2-(4-isobutylphenyl)propionic acid and its deuterated derivatives were prepared by the enzymatic resolution of the corresponding racemic methyl esters (13). The CoA thioesters were synthesized via the mixed anhydride method as described previously (3). The diastereomeric excess (*d.e.*) of chemically synthesized 2-(4-isobutylphenyl)propionyl-CoA was greater than 95%. Calculation of the diastereomeric composition was based on the equation, $d.e. = (A - B)/(A + B)$, where A and B are the concentrations of the major and minor isomers, respectively. All chromatographic materials, except hydroxylapatite, were purchased from Sigma. Hydroxylapatite gel was obtained from Spectrum Medical Industries, Inc. Other chem-

[1] As the natural function of this epimerase remains unknown, the nomenclature is tentative.

icals and biochemicals were supplied from Sigma or Aldrich unless otherwise mentioned.

Subcellular Fractionation

An adult male Charles River Wistar rat, weighing 250–300 g, was sacrificed by decapitation after light ether anesthesia. The liver was removed, minced, suspended in 2 volumes of ice-cold 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM α-toluenesulfonyl fluoride, 1 mM EDTA, 0.25 M sucrose (Buffer A), and homogenized in a mechanically driven Teflon glass homogenizer (five vertical passes at low speed). The liver homogenate was centrifuged for 10 min at 3,500 × *g* to prepare postnuclear supernatant. The mitochondrial and lysosomal fractions were prepared from postnuclear supernatant by density gradient methods (14). The postnuclear supernatant was centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant was further centrifuged at 100,000 × *g* for 1 h to yield the microsomal and cytosolic soluble fractions. All fractions were treated with Triton X-100 at a final concentration of 0.1% (w/v) before assay. The activities of the marker enzymes (lactate dehydrogenase, cytochrome *c* oxidase, and β-galactosidase) were analyzed according to the procedures described by Storrie and Madden (14).

2-Arylpropionyl-CoA Epimerase Assay

Activities of the 2-arylpropionyl-CoA epimerase in all the enzyme preparations were assayed using an enantioselective HPLC[2] method as described previously (3) with slight modifications. The incubation mixture contained 100 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA (buffer B), a suitable amount of the enzyme, and 0.5 mM (*S*)- or (*R*)-2-(4-isobutylphenyl)propionyl-CoA in a final volume of 1 ml. The enzyme reaction was started by the addition of substrate. After gentle shaking at 30 °C for 5 min, the reaction was terminated by adding 0.3 ml of 2.5 M hydroxylamine solution, pH 7.0. Extraction of the resulting hydroxamic acid with ether, followed by 6 N HCl hydrolysis, afforded free 2-(4-isobutylphenyl)propionic acid. The acid was then converted to the acyl chloride and then to the corresponding (1*R*,2*S*,5*R*)-menthyl ester. The diastereomeric composition was determined by HPLC. A control assay without enzyme addition was conducted simultaneously to determine the background value, which was subtracted. No background epimerization could be detected under these assay conditions. Enzyme activity was expressed as μmoles of (*R*)-2-(4-isobutylphenyl)propionyl-CoA formed from the *S*-substrate per min at 30 °C.

Purification of 2-Arylpropionyl-CoA Epimerase from Rat Liver Cytosol and Mitochondria

All operations were performed at 4 °C unless otherwise stated. The postnuclear supernatant was centrifuged at 10,000 × *g* for 10 min. The supernatant, after further centrifugation at 100,000 × *g* for 1 h, served as the source for the purification of the cytosolic epimerase. The 10,000 × *g* pellets that contained mostly mitochondria were washed with buffer A twice and resuspended in buffer B containing 1 mM α-toluenesulfonyl fluoride (buffer C). The mitochondrial fraction was treated with Triton X-100 at a final concentration of 0.125% and was centrifuged at 30,000 × *g* for 15 min. The supernatant was dialyzed against buffer C for 16 h and used for the isolation of the mitochondrial epimerase. Procedures for the purification of 2-arylpropionyl-CoA epimerases from both fractions were similar except that gel filtration chromatography was not required for the mitochondrial enzyme.

Ammonium Sulfate Precipitation (Step 1)—Solid ammonium sulfate was added to the supernatant to 35% saturation while the pH was maintained at 7.0 with 1 N ammonium hydroxide. After being stirred for 10 min, the preparation was centrifuged at 10,000 × *g* for 20 min. The supernatant was brought to 65% saturation with solid ammonium sulfate, stirred for 10 min, and centrifuged as described previously. The precipitate was resuspended in 20 mM imidazole buffer, pH 6.5, containing 0.2 mM α-toluenesulfonyl fluoride, and dialyzed for 16 h against the same buffer.

DEAE-Sepharose Chromatography (Step 2)—The dialyzed sample from step 1 was applied to a DEAE-Sepharose column equilibrated previously with 20 mM imidazole buffer, pH 6.5. The column sizes were 5 × 14 and 2.5 × 12.5 cm for the cytosolic and mitochondrial enzymes, respectively. The column was washed with the same buffer until the absorbance at 280 nm returned to the base line. The adsorbed protein was eluted with 50 mM NaCl and then with a linear salt gradient of 50–300 mM NaCl in the same buffer. The active fractions were pooled, concentrated over a 10,000 cutoff ultrafiltration membrane (Filtron Omega unit) and dialyzed against 15 mM potassium phosphate, pH 7.0, for 16 h.

Hydroxylapatite Chromatography (Step 3)—The dialyzed sample from step 2 was applied to the hydroxylapatite column equilibrated with 15 mM potassium phosphate buffer, pH 7.0. The column sizes were 2.5 × 13 and 2.5 × 8 cm for the cytosolic and mitochondrial enzymes, respectively. The adsorbed proteins were eluted with a linear gradient (15–100 mM) of potassium phosphate buffer, pH 7.0. Fractions exhibiting epimerase activity were collected, concentrated by ultrafiltration, and dialyzed against 20 mM sodium phosphate buffer, pH 7.0, containing ammonium sulfate with 12% saturation.

Phenyl-Sepharose CL-4B Chromatography (Step 4)—The dialyzed sample from step 3 was applied onto a phenyl-Sepharose column equilibrated with the dialysis buffer. The column sizes for the cytosolic and mitochondrial enzymes were 2.5 × 8 and 2.5 × 7 cm, respectively. A gradient of 12% saturation of ammonium sulfate to 50% ethylene glycol in 20 mM sodium phosphate, pH 7.0, was used to elute the adsorbed proteins. The active fractions were pooled, concentrated, and dialyzed against 10 mM sodium phosphate, pH 6.7, containing 50 mM NaCl.

Red Dye Affinity Chromatography (Step 5)—The dialyzed solution from step 4 was loaded onto a Reactive Red 120-agarose (type 3000-CL) column equilibrated with the dialysis buffer. The column sizes were 1.5 × 4 and 1.5 × 3.5 cm for the cytosolic and mitochondrial enzymes, respectively. Elution buffer was a gradient of 50–500 mM NaCl in 10 mM sodium phosphate, pH 6.7.

Sephacryl S-200 Chromatography (Step 6)—The cytosolic epimerase from step 5 was concentrated to about 10 ml and further purified by a Sephacryl S-200 column (2.5 × 90 cm). The equilibration and eluting buffer was 50 mM Tris/HCl, pH 7.5, containing 50 mM NaCl.

Homogeneity and Molecular Mass Determinations

The purity and molecular mass of the purified epimerase were analyzed by 12% polyacrylamide gels containing 0.1% SDS (SDS-polyacrylamide gel electrophoresis) according to the method described by Laemmli (15). The molecular mass standards were bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 kDa), trypsinogen (24,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,200 Da). Gels were stained with Coomassie Brilliant Blue after electrophoresis. The molecular mass was also estimated by gel filtration chromatography on a sephacryl S-200 column (90 × 2.5 cm) equilibrated with 50 mM Tris/HCl, pH 7.5, containing 50 mM NaCl. Blue dextran (2,000,000 *Da*) was used to determine the void volume. The proteins used as calibration standards were β-amylase (200,000 Da), alcohol dehydrogenase (150,000 Da), bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da), and cytochrome *c* (12,400 Da).

Miscellaneous

Protein determinations were performed using a Coomassie Blue dye-binding method (16) using a commercial reagent (Pierce Chemical Co.) with bovine serum albumin as a standard. Protein concentrations during purification were determined by measuring the absorbance at 280 nm. The amino acid analyses were independently performed by the Protein/Nucleic Acid Shared Facility at the Medical College of Wisconsin and by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. The results from both facilities were consistent. Low resolution electron impact and chemical ionization mass spectra were obtained on a Kratos MS-80RFA spectrometer, Chemical Instrument Center, Yale University.

RESULTS

Subcellular Distribution of 2-Arylpropionyl-CoA Epimerase Activity—Due to difficulties in analyzing the epimerase activity, previous reports on the intracellular locations of this metabolic reaction were rather inconclusive (17–19). Thus, we carried out a careful examination of the subcellular distribution in rat liver cells using an enantioselective HPLC

[2] The abbreviation used is: HPLC, high performance liquid chromatography.

method with (*S*)-2-(4-isobutylphenyl)propionyl-CoA as substrate. As indicated by the relative specific activities of the marker enzymes (Table I), each subcellular fraction was enriched after differential centrifugation. Although mitochondria and lysosome could not be completely separated by the metrizamide method (20), the epimerase activity in lysosome was negligible. The enzyme activity was located predominantly in the cytosol (51%) and mitochondria (30%), and to a lesser extent, in the microsomes (16%). It is interesting to note that intact mitochondria prior to detergent treatment also mediated the epimerization reaction at rates comparable with those of Triton X-100-treated mitochondria.

Purification of 2-Arylpropionyl-CoA Epimerase from Mitochondria and Cytosol—Enzyme purification from the cytosolic and mitochondrial fractions was similar except that the cytosolic enzyme required an additional step, Sephacryl S-200 column chromatography, to achieve homogeneity (Table II).

The overall procedures resulted in a 3,500-fold purification for the cytosolic epimerase and a 1,600-fold purification for the mitochondrial enzyme. The low increases in specific activity during the initial steps might be caused by the interference of thioesterase(s) during the assays. With (*S*)-2-(4-isobutylphenyl)propionyl-CoA as substrate, the specific activities were 410 and 600 μmol/min/mg protein for the cytosolic and mitochondria enzymes, respectively.

Purity and Molecular Mass—The degree of purity of both enzyme preparations was assessed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Both enzyme preparations gave a single protein band with a molecular mass of 42,000 Da. The native molecular masses of these two enzymes were estimated to be 40,000 Da by gel filtration on Sephacryl S-200 (data not shown), indicating that both epimerases are monomeric proteins.

Enzyme Properties—The purified enzyme, from either cytosol or mitochondria, became inactivated after freezing and thawing. Thus, the enzyme preparations were stored at 4 °C in 10 mM potassium phosphate buffer containing 5% ethylene glycol and 0.02% NaN_3. Heat treatments resulted in irreversible inactivation of the enzyme. At 50 °C, $t_{1/2}$ for inactivation was about 5 min. When the enzyme was treated at 80 °C for the same period of time, complete loss of activity was noted. Optimal pH was studied in the pH range of 4.5–9.5. Epimerase activity rose sharply between pH 4.5 and 5.0 and reached a plateau from pH 5.5 through pH 9.5. (Fig. 3). Study at pH higher than 9.5 was prohibited for two reasons. First, the thioester bond hydrolyzes spontaneously at pH values greater than 10; second, as the pK_a of the α-methine proton is about 10.3 (12), chemical epimerization becomes significant at such a high pH.

Amino Acid Composition—The amino acid compositions of both epimerase preparations are shown in Table III. Both enzymes are rich in Glx, Gly, and Leu but low in Cys, Met, Tyr, His, and Trp contents. The (Glx + Asx)/(Lys + Arg) ratios are 2.0 and 2.1 for the cytosolic and mitochondrial enzymes, respectively, indicating the acidic nature of the proteins. This is consistent with the pI value of the cytosolic epimerase, which was determined to be 5.5 by isoelectric focusing electrophoresis.

Cofactor Requirements—Our study indicated that no exogenous cofactor is required for the epimerase activity. The spectra of these two enzyme preparations showed no absorption other than that attributable to the protein. Addition of NAD(P)+, FAD, or FMN did not increase the activity. No change in activity occurred upon treatment with EDTA (0.5 mM) or various metal ions (0.5 mM) including Ni^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}. However, preincubation of cytosolic or mitochondrial 2-arylpropionyl-CoA epimerase with 0.5 mM Cu^{2+} in 100 mM potassium phosphate buffer, pH 7.0, at 30 °C for 10 min resulted in 65 and 70% loss of the activity, respectively.

Substrate Specificity and Approach Equilibrium Study—The purified epimerase, either of cytosolic or mitochondrial origin, catalyzed the epimerization of both (*S*)- and (*R*)-2-(4-isobutylphenyl)propionyl-CoA. No racemization could be detected with the corresponding free acids as substrate. Using the mitochondrial enzyme, the apparent initial rates of epimerization were 1,120 and 3,180 μmol/min/mg protein for the (*R*)- and (*S*)-2-(4-isobutylphenyl)propionyl-CoA, respectively. The reactions reached an equilibrium in favor of the (*R*)-isomer from either directions (Fig. 4). The diastereomeric excess of the product was calculated to be 0.2, which is consistent with the value determined previously using crude liver homogenates (3). Accordingly, the equilibrium constant (K_{eq}) is estimated to be 1.5.

Besides the above CoA thioesters, 2-arylpropionyl-CoA epimerase can also utilizes (*S*)-2-phenylpropionyl-CoA and (*S*)-2-(6-methoxynaphthyl)propionyl-CoA at rates comparable to that of (*S*)-2-(4-isobutylphenyl)propionyl-CoA.

α-Proton Exchange—To assess the mechanism of proton exchange in the catalytic site, (*R*)-2-[2-^{2}H]-(4-Isobutylphenyl)propionyl-CoA was used as substrate for cytosolic 2-arylpropionyl-CoA epimerase. After 20% conversion, the reaction was terminated by hydroxylamine at pH 7.0. The (*R*)- and (*S*)-isomers were separated as diastereomeric menthyl esters by HPLC and analyzed by mass spectrometry. Virtually no deuterium was found attached to the *S*-epimer product, indicating that the hydrogen atom of the new C–H bond is derived from the solvent protons.

DISCUSSION

The enzyme responsible for the epimerization of 2-arylpropionyl-CoA was purified to homogeneity from rat liver cytosol

TABLE I

Subcellular distribution of marker enzymes and 2-arylpropionyl-CoA epimerase activity in the rat liver

Subcellular fraction	Relative specific activities of marker enzymes			2-Arylpropionyl-CoA epimerase	
	Lactate dehydrogenase	Cytochrome *c* oxidase	β-Galactosidase	Total activity[a]	Distribution
				units	%
Postnuclear supernatant	1	1	1	615	
Cytosol	1.71	0.17	0.13	454	51
Mitochondria	0.16	6.0	2.6	265	30
Lysosome	0	1.5	13.3	31	3
Microsomes	0.19	0.80	0.58	144	16

[a] Total activity was measured from each subcellular fraction isolated from rat liver postnuclear supernatant containing 1430 mg of protein.

TABLE II

Purification of 2-arylpropionyl-CoA epimerases from rat liver cytosol and mitochondria

	Cytosolic epimerase				Mitochondrial epimerase			
	Total protein	Total activity	Specific activity	Purification	Total protein	Total activity	Specific activity	Purification
	mg	*units*	*units/mg*	*-fold*	*mg*	*units*	*units/mg*	*-fold*
Crude homogenate	11,420	1,366	0.12	1	428	160	0.37	1
Ammonium sulfate (35–65%)	2,844	512	0.18	1.5	96	119	1.24	3.3
DEAE-Sepharose	358	261	0.73	6	60	96	1.6	4.3
Hydroxylapatite	70	252	3.6	30	18	98	5.4	14
Phenyl-Sepharose	4.8	175	36	300	5.4	70	13	35
Red dye gel	0.26	52	200	1,700	0.036	11	600	1,600
Sephacryl S-200	0.11	46	410	3,500				

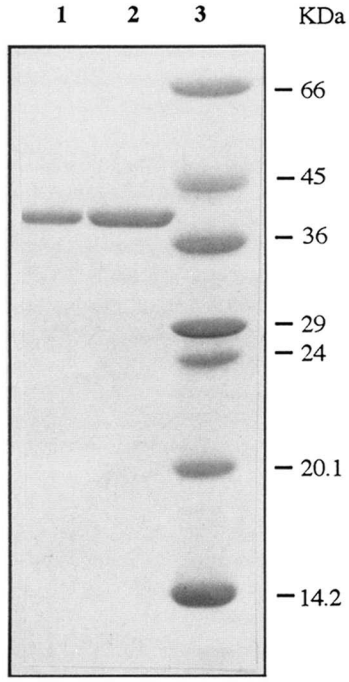


FIG. 2. **SDS-polyacrylamide gel electrophoresis of purified 2-arylpropionyl-CoA epimerase.** The proteins were analyzed on a 12% gel. *Lane 1*, the mitochondrial epimerase; *lane 2*, the cytosolic epimerase; *lane 3*, molecular mass standards.

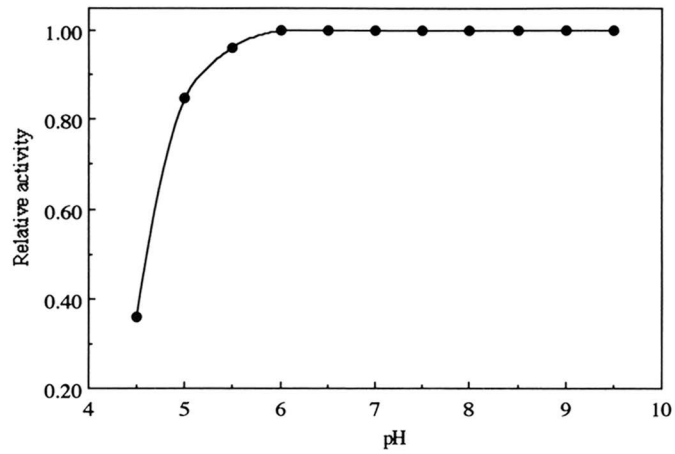


FIG. 3. **Effect of pH on 2-arylpropionyl-CoA epimerase.** The activities of the purified cytosolic epimerase were determined after 8-min preincubations at 30 °C with the following buffers: 0.1 M citrate buffer at pH 4.5–6.0; 0.1 M potassium phosphate buffer at pH 6.5, 7.0, and 7.5; 0.1 M Tris buffer at pH 8.0 and 8.5; 0.1 M glycine buffer at pH 9.0 and 9.5. No appreciable nonenzymatic epimerization was detected at these pH values.

TABLE III

Amino acid compositions of 2-arylpropionyl-CoA epimerases from rat liver cytosol and mitochondria

Amino acid	Cytosolic epimerase	Mitochondrial epimerase
	mol%	
Cysteine/cystine	1.7	1.6
Aspartic acid/asparagine	8.4	7.7
Threonine	3.9	4.3
Serine	5.7	7.6
Glutamic acid/glutamine	12.0	10.9
Proline	4.2	5.1
Glycine	11.1	14.7
Alanine	8.9	8.6
Valine	5.2	5.5
Methionine	1.9	2.1
Isoleucine	3.4	3.4
Leucine	12.8	10.3
Tyrosine	2.7	2.2
Phenylalanine	4.6	4.1
Histidine	2.7	2.3
Lysine	5.6	4.6
Tryptophan	0.7	0.8
Arginine	4.7	4.3

and mitochondria with high purification folds. This purification represents the first preparation of such an epimerase in highly purified form. Several lines of physical and mechanistic evidence indicate that this 2-arylpropionyl-CoA epimerase is distinctively different from methylmalonyl-CoA epimerase (21) (Table IV) and many other known epimerases reported in the rat liver. However, at present, one cannot exclude that the epimerase is a known protein with multiple enzymatic functions. To investigate this possibility, elucidation of the protein sequence is currently in progress.

It is interesting to note that the epimerase activity is located in both cytosol and mitochondria. The purified proteins from both fractions exhibit similar physical and catalytic properties. Their chromatographic behaviors were nearly identical throughout the purification. Both are monomeric proteins with an apparent molecular mass of 42,000 Da. Their UV spectra gave no indication of bound pyridine or flavin nucleosides, and the enzyme activity was not affected by exposure to EDTA or metal ions except Cu^{2+}. Amino acid compositions indicated that both epimerases had similar contents in most of the amino acids except Ser, Gly, and Leu. Attempts to determine the N-terminal sequences of these two proteins were unsuccessful, presumably due to the posttranslational blockage at the N termini. These results clearly suggest that the cytosolic and mitochondrial epimerases are structurally related. It is well understood that although mitochondria contain their own genetic information, most mitochondrial

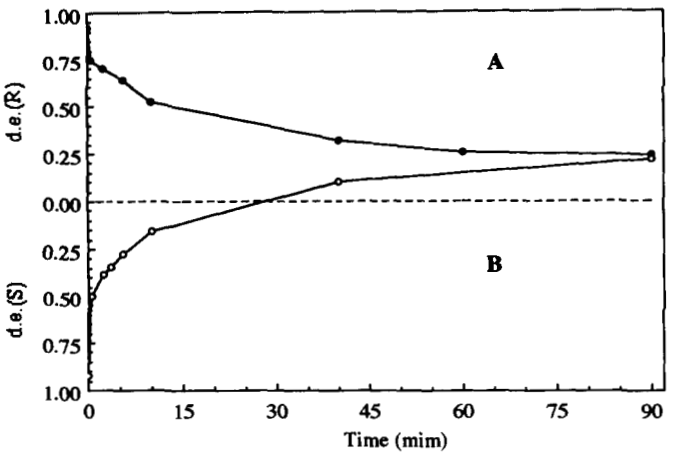


FIG. 4. **Approach equilibrium profile.** *Curves A* and *B* represent time-dependent enzymatic inversion of (*R*)- or (*S*)-2-(4-isobutylphenyl)propionyl-CoA, respectively. The reaction mixture contained 0.5 mM substrate, 1 μg of purified mitochondrial epimerase, and 100 mM potassium phosphate, pH 7.0, in a final volume of 12 ml. At various intervals, 1-ml aliquots were withdrawn and added to tubes containing 0.3 ml of 0.25 M hydroxylamine, pH 7.0. The enantiomeric composition of the resulting product was determined according to the method described under "Experimental Procedures."

TABLE IV

Enzymatic properties of 2-arylpropionyl-CoA epimerase versus methylmalonyl-CoA epimerase

Enzyme properties	2-Arylpropionyl-CoA epimerase	Methylmalonyl-CoA epimerase
Molecular weight	42,000	32,000
Subunit structure	Monomeric	Dimeric
Cofactor requirement	None	Metal ions (*e.g.* Co^{2+})
Effect of EDTA	None	Inactivation
pH optimum	5.5–9.5	7.0

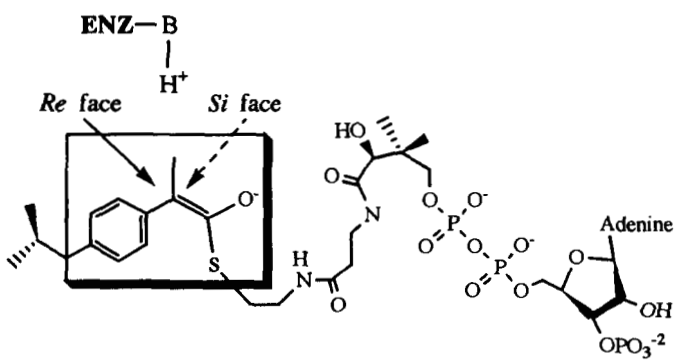


FIG. 5. **Enantiofacial differentiation in proton transfer to the *aci* thioester.**

proteins are coded for by nuclear genes and synthesized in the cytosol. The precursor proteins containing targeting sequences are posttranslationally imported into mitochondria where the targeting sequences are hydrolyzed by a signal-associated peptidase (22). Thus, it is possible that the cytosolic epimerase differs from its mitochondrial counterpart by including a short targeting sequence that may account for the discrepancy in the amino acid compositions. To clarify this speculation, the protein sequences of both enzymes are currently being examined.

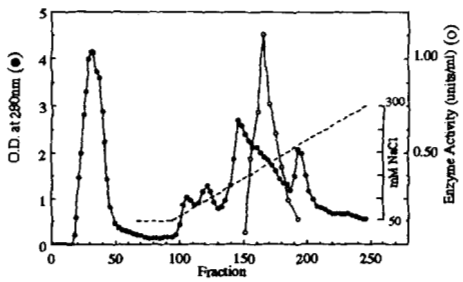
It is worth noting that mitochondria with an intact membrane structure can mediate the epimerization reaction. As acyl-CoA thioesters can readily penetrate the outer membrane but not the inner membrane of mitochondria (23), we speculate that the epimerase may reside in the intermembrane space.

The epimerization of 2-(4-isobutylphenyl)propionyl-CoA is freely reversible from both directions. Some degree of stereochemical discrimination was observed in the substrate utilization and product formation of the epimerase reaction. As shown in the approach equilibrium study (Fig. 4), the *S*-isomer is preferentially epimerized by the enzyme. The apparent initial rate for (*R*)-2-(4-isobutylphenyl)propionyl-CoA relative to that of the *S*-antipode was 35%. Based on the Haldane relationship (24), $(K_{eq} = (k_{cat}/K_m)_{(S)}/(k_{cat}/K_m)_{(R)} = [R]/[S])$, this result is consistent with the finding that the diastereomeric composition at equilibrium lies favoring the *R*-isomer (*d.e.* = 0.2). Accordingly, the equilibrium constant (K_{eq}) was estimated to be 1.5. For many chemically symmetrical reactions mediated by racemases, the equilibrium constants are found to be unity (25). However, it should be noted that for the epimerization between two diastereomers, this rule may not hold.

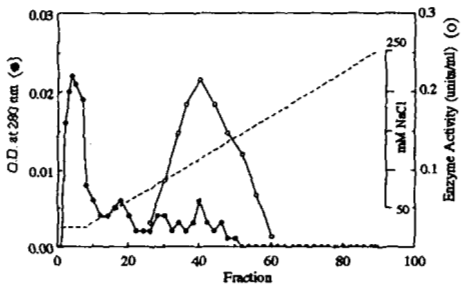
In general, epimerase- or racemase-catalyzed proton exchange can be accounted for by either single-base or 2-base acceptor mechanism (26). Our experiments indicate that partial enzymatic epimerization of (*R*)-[2-^{2}H]-2-(4-isobutylphenyl)propionyl-CoA gave the *S*-product completely depleted of the α-deuterium. This result provides supporting evidence for the 2-base mechanism. Moreover, the pH profile study shows a sharp rise in activity about pH 5.0 followed by a pH-independent plateau running from pH 5.5 to 9.5, suggesting that a carboxylic residue (Asp or Glu) may serve as a general base in the active site for proton abstraction. Currently, the nature of the amino acid residues involved in proton transfer is being investigated by the chemical modification of the active site. Because metal ions are not required for the catalysis, the unstable carbanion may be stabilized as the *aci* tautomer with an extended conjugated system (26). It appears that the reprotonation takes place in favor of the *Si*-face of the *aci* thioester, resulting in the formation of the product enriched in the *R*-isomer (Fig. 5). This enantiofacial differentiation may be attributed to the steric and/or stereoelectronic effects imposed by the CoA moiety of the molecule.

In conclusion, the significance of 2-arylpropionyl-CoA epimerase is 2-fold. First, this epimerase-mediated reaction represents a novel biocatalysis with unknown biological functions. Understanding of the molecular and mechanistic details of this new enzyme system may broaden our knowledge in the area of biochemical isomerization. Second, it plays a crucial role in the metabolic bioactivation of 2-arylpropionic acid nonsteroidal antiinflammatory drugs. The kinetic behavior of this epimerase may directly contribute to the toxicity of some members of these therapeutic agents and warrants attention. At present, the structural and kinetic properties of these epimerases are being actively investigated.

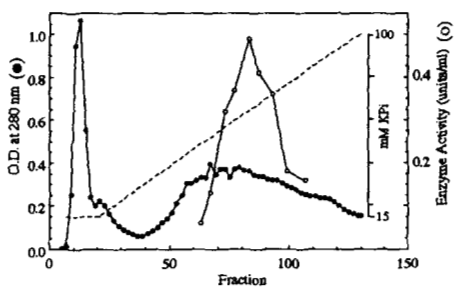
Step 2. *DEAE-Sepharose CL-6B chromatography*



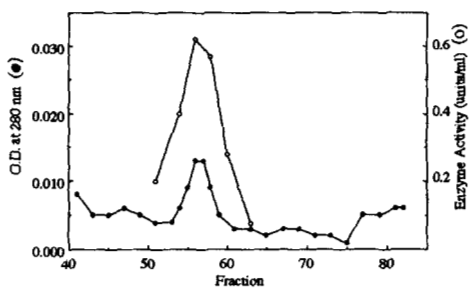
Step 5. *Red-Dye chromatography*



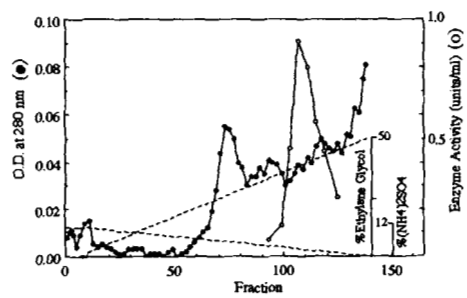
Step 3. *Hydroxyapatite chromatography*



Step 6. *Sephacryl S-200 chromatography*

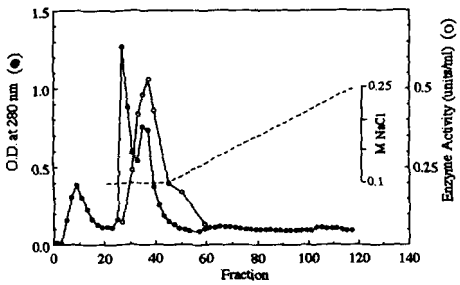


Step 4. *Phenyl-Sepharose chromatography*

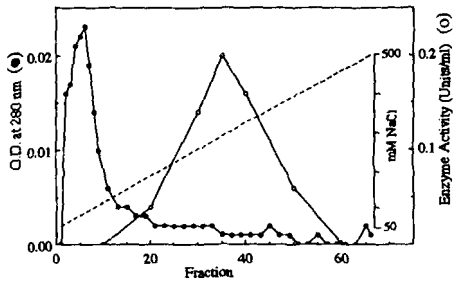


APPENDIX 1. **Chromatograms of cytosolic 2-arylpropionyl-CoA epimerase purification.**

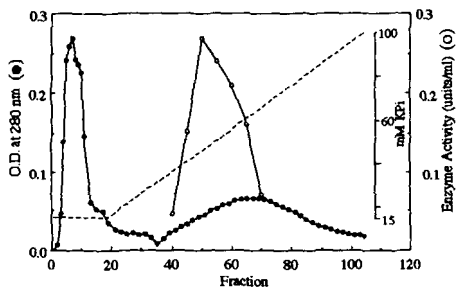
Step 2. *DEAE-Sepharose CL-6B chromatography*



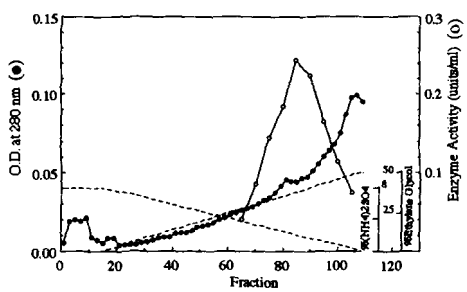
Step 5. *Red-dye chromatography*



Step 3. *Hydroxyapatite chromatography*



Step 4. *Phenyl-Sepharose chromatography*



APPENDIX 2. **Chromatograms of mitochondrial 2-arylpropionyl-CoA epimerase purification.**

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