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2-ARYLPROPIONYL-CoA EPIMERASE: PARTIAL PEPTIDE SEQUENCES AND TISSUE LOCALIZATION

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Abstract—The R-enantiomers of 2-arylpropionic acids (2-APAs) such as ibuprofen (IBU) exhibit the phenomenon of species- and substrate-dependent metabolic chiral inversion. Only R-enantiomers are activated to acyl-CoA-thioesters by an acyl-CoA-synthetase *via* an adenylate intermediate. The acyl-CoA-thioesters are substrates for an epimerase, which is responsible for chiral inversion. A 42 kDa epimerase from the cytosolic fraction of rat livers was isolated and purified to homogeneity. Polyclonal antibodies were raised against the epimerase in rabbits. The anti-epimerase antibodies were used for affinity column chromatography to separate homogeneous protein for amino acid sequence analysis. Sequence data analysis of 3 internal peptide sequences showed 50% and more homology with regions of enzymes involved in fatty acid metabolism. The polyclonal anti-epimerase antibodies were used to analyze the tissue distribution of the protein in guinea pigs and rats by Western blot analysis. Furthermore, the correlation of inversion enzyme activity in various tissues under comparable incubation conditions and cross-reactivity in Western blot analysis was investigated.

Key words: 2-arylpropionyl-CoA epimerase; ibuprofen; amino acid sequence; acyl-CoA-thioester; chiral inversion; anti-epimerase antibodies

Nonsteroidal anti-inflammatory 2-APAs† such as IBU are chiral compounds and exist as R- and S-enantiomers. A common feature of 2-APAs is metabolic chiral inversion of the *in vitro* inactive (not prostaglandin synthesis inhibiting) R(-)-enantiomers to the prostaglandin synthesis inhibiting S-forms [1]. Only the R(-)-enantiomers of 2-APAs are stereoselectively activated to acyl-CoA thioesters by an acyl-CoA-synthetase *via* an adenylate intermediate [2–5]. The acyl-CoA thioesters are substrates for an epimerase that is responsible for chiral inversion [6, 7]. The rate of inversion varies, depending on the substrate and species tested. Published results on the mechanism of inversion and the enzymes involved have been summarized recently [8, 9].

In the present investigation, epimerase was isolated and purified, and polyclonal antibodies against the enzyme were generated. Amino acid sequence analyses of the epimerase were done to gain preliminary insights into its biological function by comparison with amino acid sequences of known enzymes. Furthermore, the experiments examined the localization and activity of the epimerase in various tissue homogenates of guinea pigs and rats.

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† Abbreviations: 2-APAs, 2-arylpropionic acids; IBU, ibuprofen; R-IBU-CoA, R-ibuprofen-coenzyme A thioester; S-IBU-CoA, S-ibuprofen-coenzyme A thioester; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; PVDF-membrane, polyvinylidene-difluoride-membrane; CoA, coenzyme A; ATP, adenosine triphosphate.

MATERIALS AND METHODS

Materials

IBU enantiomers were kindly supplied by Pharma Trans Sanaq AG (Basel, Switzerland). R- and S-IBU-CoA were synthesized as described by Porubek *et al.* for flurbiprofenyl-CoA [10]. All standard chemicals were purchased from Sigma (Deisenhofen, Germany). The columns used for enzyme purification were obtained from Pharmacia LKB (Freiburg, Germany). Anti-rabbit IgG antibodies coupled to horseradish peroxidase were supplied by ICN Biomedicals (Meckenheim, Germany). Nitrocellulose paper was purchased from Sartorius (Göttingen, Germany).

Purification of the epimerase

The epimerase was purified from the cytosol fraction of rat livers (male Sprague Dawley rats, 250–300 g body weight from Savo Ivanovas, Kisslegg, Germany) as described by Shieh and Chen [6]. Epimerase activity was determined in the enzyme fractions by incubation of the chemically synthesized R-IBU-CoA thioesters and subsequent stereoselective HPLC analysis of IBU after alkaline hydrolysis [11]. The degree of inversion was estimated as the amount of S-enantiomer formed during incubation.

Antibodies and immunological techniques

Polyclonal antibodies were raised in rabbits according to standard methods. Thirty-five μg of the homogeneous enzyme in 0.5 mL PBS were suspended in 0.5 mL complete Freund's adjuvant and injected subcutaneously near the back of the neck of a rabbit. Further subcutaneous injections followed 3 and 4 weeks later. The epimerase antibodies were analyzed by SDS-PAGE and by Western immunoblotting [12, 13].

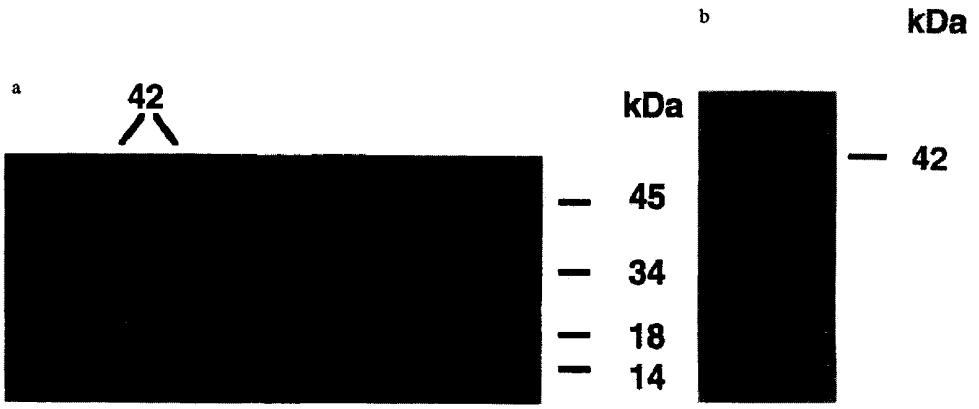

Fig. 1. The SDS-PAGE (a) (silver stained) demonstrates various fractions of active enzyme after the last step of purification. The arrows show the homogeneous enzyme fraction. These protein fractions were used for immunization. The Western blot (b) shows the reactivity of the anti-epimerase antibodies with a homogeneous epimerase fraction.

To optimize the purification procedure of the epimerase, the antibody was used for affinity-column chromatography. Anti-epimerase IgGs were affinity purified by passing serum over a protein G-superose column (Pharmacia LKB) according to the manufacturer's instructions. The purified antibodies were obtained in a volume of 83 mL and precipitated by addition of 40 g of ammonium sulfate followed by centrifugation at 30,000 g for 30 min. The antibodies were dissolved in 6 mL of 10 mM potassium phosphate, pH 7.2, dialyzed against the same buffer for 24 hr and then coupled to 750 mg of cyanogen bromide-activated Sepharose 4B (2.7 mL volume when swollen) as recommended by Pharmacia LKB Biotechnology. The dialyzed ammonium sulfate-pellet of a crude rat liver supernatant was applied to a column of antiepimerase-Sepharose. The column was then washed with 30 mL of 10 mM potassium phosphate, pH 7.2 and eluted with 3 M $MgCl_2$, pH 7.2. The eluates were dialyzed against 10 mM potassium phosphate, pH 7.2.

Determination of protein sequences

For protein sequence analysis, affinity-purified material derived from 2 rat livers was fractionated by SDS-PAGE and transferred to PVDF-membrane. Five µg of the protein transferred were visualized by Coomassie staining and the 42 kDa epimerase band excised. Because the protein was found to contain a blocked N-terminal, the membrane-bound protein was subjected to *in situ* proteolytic cleavage using 1 µg of endoproteinase LysC in 400 µL digestion-buffer (0.1 M Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 10% CH_3CN, 1% NP40) at 24°C for 8 hr. The resulting peptide mixture was fractionated by Superspher 60 RP select B (Merck, Darmstadt) reversed phase HPLC. Three dominant peak fractions from the RP-HPLC chromatography were loaded on a sequencer and analyzed (Toplab, Gesellschaft für Angewandte Biotechnologie mbH, München, Germany). Peptide sequences were compared with entries in sequence data base using the Lasergene software package (DNASTAR).

Western blot and enzyme activity analysis

Tissue homogenates of various organs of rats and guinea pigs were prepared according to standard methods [14]. Homogenates of liver, heart, lung, kidney, ileum, and several brain fractions were analyzed by Western blotting as described by Bang *et al.* [13]. Using the same homogenates (protein concentration 1 mg/mL), either R-IBU (0.1 mM), CoA (0.3 mM), and ATP (3 mM) or R-IBU-CoA (0.1 mM) were incubated in a final volume of 600 µL for 10 min (n = 3 each animal, 2 animals). The reaction was stopped by addition of 200 µL of 2 M HCl and samples were analyzed by stereoselective HPLC after alkaline hydrolysis as described previously [5, 11]. The degree of inversion was estimated as the amount of S-enantiomer formed during incubation, corrected by the amount of S-enantiomer formed during alkaline hydrolysis in control incubations.

Table 1. Comparison of the 3 epimerase sequences with the protein sequences of the fatty acid synthetase (FAS) of saccharomyces cerevisiae

		Amino acid sequences														% of Identity
Epimerase		I	Y	A	X	L	S	G	F	W	Q	S	G	I	F	S	
FAS	1571	S	Y	A	N	L	P	G	T	I	T	H	G	M	F	S	53
Epimerase		Y	G	F	S	Q	E	E	I								
FAS	1702	Y	G	F	K	Q	E		I								62.5
Epimerase		V	I	N	E	G	Y	A									
FAS	1548	S	T	N	E	P	Y	A									57

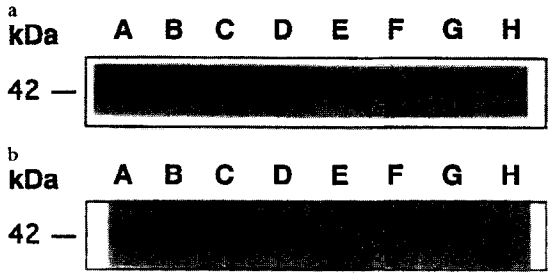

Fig. 2. The tissue distribution of epimerase in rats (a) and guinea pigs (b) was analyzed by Western blotting using polyclonal anti-epimerase antibodies. Tissue homogenates: A, heart; B, lung; C, liver; D, kidney; E, ileum; F, medulla oblongata; G, cerebellum; H, lumbar region of the spinal cord.

RESULTS AND DISCUSSION

Purification of the epimerase—polyclonal antibodies

The R- and S-IBU-CoA inverting epimerase was purified to homogeneity according to the method described by Shieh and Chen, with the modification that the purification procedure could be improved by affinity column chromatography based on a polyclonal antibody against epimerase [6]. The purity of various active enzyme fractions after the last step of the purification was demonstrated by an SDS-PAGE stained with silver nitrate (Fig. 1a). In line with reported findings, we found a molecular weight of approximately 42 kDa [6]. Only with the active fractions of the enzyme was an intensive staining in the Western blot found (Fig. 1b).

Amino acid sequence analysis—comparison with data bases

Digestion with endoproteinase LysC was necessary for sequencing because the N-terminal amino acid of the epimerase was blocked. Approximately 100 pmol of the digested material was analyzed by automated Edman degradation. In the extended sequencing runs, 3 sequences could be identified (Table 1). Comparison of these 3 sequences with sequences in protein data bases yielded no complete identity (i.e. epimerase appears to be an as yet unidentified enzyme). However, homology of 50% and more was found with peptides of the fatty acid synthetase complex in the dehydratase domain of saccharomyces cerevisiae, lending support to the hypothesis that the enzyme may be involved in fatty acid metabolism. A complete DNA sequence analysis and expression of epimerase in sufficient quantities, however, is necessary to allow valid conclusions on its normal physiological function by comparison with related enzymes. Consequently, the next experimental steps should be identification of the gene and generation of a recombinant epimerase protein.

Localization and activity of the epimerase

Furthermore, various organs of rats and guinea pigs were analyzed with respect to both the presence and activity of epimerase by means of polyclonal antibodies on the one hand, and by measuring enzyme activity in incubation experiments on the other. The Western blot analysis demonstrates the presence of epimerase in all organs of both species except the brain fractions of the guinea pigs (Fig. 2a and b). The immunoreactive signal of the polyclonal antiserum with heart, lung, liver, kidney, and ileum was extensive in the molecular weight area of 42 kDa. Additionally weak cross-reactivities with other proteins were observed.

After incubation of R-IBU, inversion activity was predominantly found in livers of rats and in livers and kidneys of guinea pigs as illustrated in Fig. 3a and b, probably due to the lack of thioester formation in other tissues *in vitro* [3]. Incubation of the thioester intermediate of R-IBU showed highest activity in the kidneys of guinea pigs and the livers and kidneys of rats, in line with previous reports [15]. Lower, but significant, activity was measured in hearts and ilea of both species. In the lungs of rats, a small amount of inversion could be measured. The lungs of the guinea pigs showed no enzyme activity under the same incubation conditions as for livers and kidneys. However, marked cross-reactivity was detected in the lung fraction of both species by Western blotting, leading to the conclusion that epimerase is present but its activity is not detectable under the experimental conditions used. The fact that polyclonal antibodies were used supports the possibility of detect-

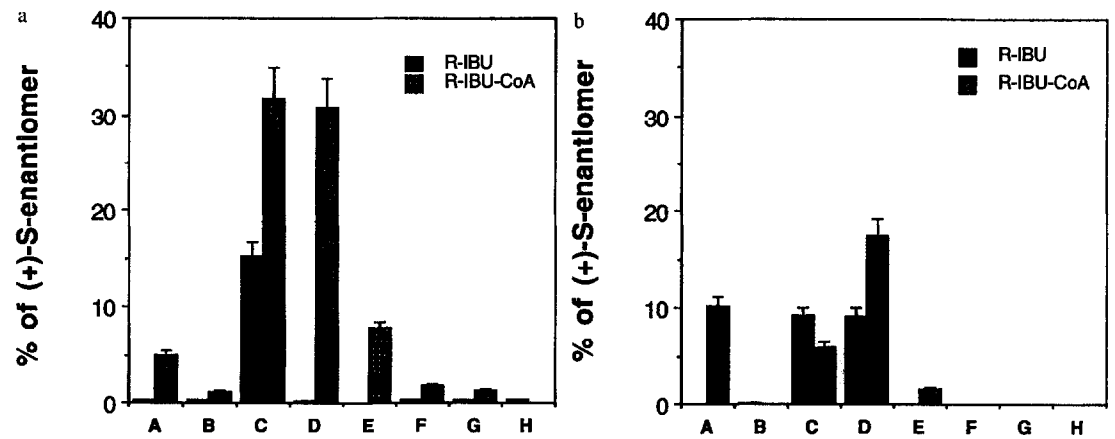

Fig. 3. Percentage of S(+)-enantiomers formed by inversion after incubation of R-IBU or R-IBU-CoA after alkaline hydrolysis in different tissue homogenates of rats (a) and guinea pigs (b). Tissue homogenates: A, heart; B, lung; C, liver; D, kidney; E, ileum; F, medulla oblongata; G, cerebellum; H, lumbar region of the spinal cord (mean data ± SD, n = 3 each animal, 2 animals).

ing isoforms of the epimerase. In addition, weak activity was found in some brain fractions of rats (*i.e.* medulla oblongata and cerebellum), corresponding to a weak signal in the Western blot.

With respect to the extent of inversion based on equal protein concentrations, the rat tissues proved to be more extensive "inverters" than those from the guinea pigs. However, the organ distribution (Western blot analysis) of the epimerase was similar in both species, except for the brain. Higher catalytic activity of the enzyme in rats or the presence of an isoform may be the reason for this discrepancy. The species specific inversion observed *in vivo* may, in addition to differences of acyl-CoA-synthetase activity, also depend on varying epimerase activity.

Apparently, the failure to demonstrate inversion of R-IBU in tissue homogenates described in the literature may result either from the lack of enzymatic acyl-CoA synthesis or from very rapid hydrolysis of the intermediates [15, 16].

From our results we conclude: (a) epimerase is an as yet unidentified enzyme that may be involved in fatty acid metabolism; (b) the liver and the kidney are the major organs of inversion *in vitro;* and (c) epimerase is also located in other organs where no or only slight inversion activity is measurable in incubation experiments.

Further investigations will be necessary to clearly define the physiological function of epimerase in the various tissues.

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