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Purification and Some Properties of Ketone Reductase Forming an Active Metabolite of Sodium 2-[4-(2-Oxocyclopentylmethyl)-phenyl]propionate Dihydrate (Loxoprofen Sodium), a New Anti-inflammatory Agent, in Rabbit Liver Cytosol

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The enzyme which catalyzes the reduction of the cyclopentanone moiety of sodium 2-[4-(2-oxocyclopentylmethyl)phenyl]propionate dihydrate (loxoprofen sodium) was purified from rabbit liver cytosol to homogeneity as judged by polyacrylamide gel electrophoresis. The purified enzyme had a molecular weight of 33000, showed a preference for nicotineamide adenine dinucleotide phosphate as a cofactor and had an optimal pH of 6.2. The K_m and V_{max} values for the reduction of loxoprofen were 0.45 mM and 0.81 μmol/min/mg protein, and the *trans*-alcohol was the main product. The reducing activity was inhibited by *p*-chloromercuribenzoate, *N*-ethylmaleimide and quercitrin.

The enzyme efficiently catalyzed reduction of various aromatic aldehydes and ketones, cyclohexanones and 5α-3-ketosteroids. Cyclopentanone and its methylsubstituted derivatives were not reduced at all. However, 2-ethyl- and 2-*n*-propylcyclopentanone were reduced, and 2-benzylcyclopentanone was a good substrate, comparable to loxoprofen itself.

These results strongly suggest that the loxoprofen reducing enzyme is probably identical with the aromatic aldehyde-ketone reductase (F_3) of rabbit liver cytosol, identified as 3α-hydroxysteroid dehydrogenase by Sawada *et al.*[1]

Keywords——anti-inflammatory agent; sodium 2-[4-(2-oxocyclopentylmethyl)phenyl]-propionate dihydrate; loxoprofen sodium; α-arylpropionic acid derivative; enzyme purification; enzyme characterization; rabbit liver; aromatic aldehyde-ketone reductase; 3α-hydroxysteroid dehydrogenase; substrate specificity

Sodium 2-[4-(2-oxocyclopentylmethyl)phenyl]propionate dihydrate (loxoprofen sodium) is a potent anti-inflammatory agent with a relatively weak gastro-intestinal ulcerogenicity.[2] It has been established that loxoprofen sodium exerts its pharmacological activities after conversion to an active metabolite, the *trans*-alcohol, (2*S*)-2-[4-(1*R*,2*S*)-2-hydroxycyclopentylmethyl)phenyl]propionic acid, which has a strong inhibitory effect on prostaglandin (PG) synthetase.[3] Metabolic studies of loxoprofen sodium have dealt with the structural determination of rat urinary metabolites,[4] the species differences in urinary metabolites[5] and the optical inversion of (2*R*)- to (2*S*)-configuration at the carboxylic acid side chain.[6] The main metabolite in rat plasma has also been found to be the active metabolite, the (2*S*,1′*R*,2′*S*)-*trans*-alcohol.[3] This metabolic conversion involves the stereospecific reduction of the α-substituted cyclopentanone moiety in the loxoprofen molecule (Chart 1).

Cyclopentanone is reportedly a poor substrate for enzymatic reductions with horse liver alcohol dehydrogenase and aromatic aldehyde-ketone reductases, in contrast to cyclohexanone and related compounds, which serve as good substrates for these enzymes.[7,8] Therefore, it seemed important to clarify the characteristics of the loxoprofen reducing

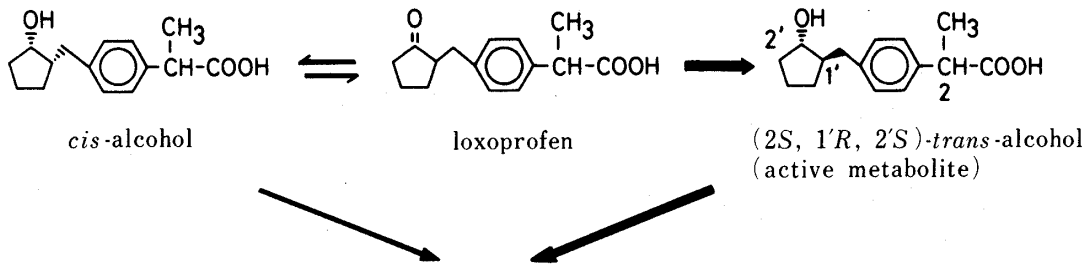

Chart 1. Main Metabolic Pathways of Loxoprofen

enzyme in order to understand the pharmacological action of loxoprofen sodium.

This paper describes the purification and some properties of the loxoprofen reducing enzyme of rabbit liver cytosol, with emphasis on the substrate specificity.

Materials and Methods

Chemicals——Loxoprofen and its metabolites (*trans*- and *cis*-alcohol) were all synthesized by Naruto *et al.* in the Chemical Research Laboratories of Sankyo Co., Ltd.

2-Methyl- and 2-benzylcyclopentanone were prepared by oxidation of the corresponding alcohols with chromic acid-8 N sulfuric acid in acetone solution at 0 °C. 2-Ethyl- and 2-*n*-propylcyclopentanone were synthesized by the reaction of 2-ethoxycarbonylcyclopentanone with ethyl bromide and propyl bromide according to the method of Philips.[9] 2-Methyl-, 2-ethyl- and 2-*n*-propylcyclopentanones thus prepared were purified by distillation at 60 °C under reduced pressure (50 mmHg), at 39 °C (24 mmHg) and at 68 °C (14 mmHg), respectively. 2-Benzylcyclopentanone was purified by column chromatography on silica gel with hexane–EtOAc (10 : 1). The chemical structures and purities of these synthesized samples were confirmed by nuclear magnetic resonance spectroscopy and thin-layer chromatography.

Nicotineamide adenine dinucleotide phosphate (NADPH), nicotineamide adenine dinucleotide (NADP), prostaglandin E_2 and steroidal compounds were purchased from Sigma (St. Louis, U.S.A.) and other chemical compounds of analytical reagent grade were obtained from Tokyo Kasei (Tokyo). Sephadex G-100, DEAE-Sephacel and Blue Sepharose CL-6B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and Biogel HT was from Bio-Rad Laboratories (Richmond, U.S.A.).

Enzyme Assay——Method A: Reaction mixtures consisted of 80 mM potassium phosphate buffer (pH 6.2), an enzyme solution, 0.20 mM NADPH and 1 mM substrate (0.1 mM for steroids) in a total volume of 3.0 ml. Reactions were initiated by the addition of enzyme solution and the decrease in absorbance at 340 nm was monitored with a Shimadzu MPS-5000 recording spectrophotometer (Kyoto) at 25 °C. Background rates of NADPH oxidation without substrate were subtracted from the rates observed with the substrates. One enzyme unit is defined as the change in absorbance at 340 nm corresponding to the oxidation of 1 μmol of NADPH per minute.

Method B: Each reaction mixture (4 ml) contained 0.1 M potassium phosphate buffer (pH 7.4), 0.32 mM NADP, 3 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 200 mg of liver preparation (or purified enzyme) and 1.0 mM loxoprofen sodium. Incubations were carried out for 30 min at 37 °C with shaking and were stopped by addition of EtOH (20 ml). After centrifugation at 2500 rpm for 10 min, the supernatant was concentrated *in vacuo*. The residue was dissolved in EtOAc and subjected to high performance liquid chromatography (HPLC) as described below.

Protein concentration was determined by a modification of the method of Lowry *et al.*[10] with bovine serum albumin as the standard.

HPLC Analysis——A TRIROTAR high-performance liquid chromatography (JASCO, Tokyo) equipped with a μPorasil column (3.9 mm × 30 cm) (Waters Assoc., Milford, U.S.A.) and a UVIDEC II ultraviolet (UV) monitor (225 nm, JASCO) was used. Samples were applied using a Waters model U6K sample loop injector. Hexane–isoPrOH–AcOH (95 : 5 : 0.3) was employed as a mobile phase at a flow rate of 1.0 ml/min. For the measurements of retention time and peak area, a Hewlett Packard 3390A integrator was used. Under these conditions, loxoprofen, the *cis*-alcohol and the *trans*-alcohol were eluted at t_R 5.7, 7.3 and 8.9 min, respectively. There was no interference peak due to components of the incubation mixture. The calibration curve obtained by plotting peak area *vs.* concentration of each compound showed good linearity in the range of 50—1000 ng and passed through the origin. The recoveries

of these compounds from liver homogenates were more than 95%.

Polyacrylamide Gel Electrophoresis and Gel Filtration——Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed on 12% gel prepared in 0.3 M Tris-HCl buffer (pH 8.9) containing 0.1% sodium dodecyl sulfate according to the method of Weber and Osborn.[11] Protein bands were stained with Coomassie brilliant blue. Polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate was accomplished with 7.5% acrylamide gel using the same buffer system. Analytical gel filtration was performed on a Sephadex G-100 column (2 × 90 cm). The standard proteins used for determination of the molecular weight of the purified enzyme were bovine serum albumin (MW 66000), ovalbumin (45000), trypsinogen (24000) and lysozyme (14300).

Preparation of Subcellular Fractions——Liver was obtained from adult male Wistar-Imamichi rats (*ca.* 200 g), Hartley guinea pigs (*ca.* 250 g) and Japanese white rabbits (*ca.* 2.5 kg) after sacrifice by decapitation. All subsequent manipulations were performed at 0—4 °C. Finely chopped livers were homogenized in 3 volumes of 10 mM potassium phosphate buffer (pH 7.4) containing 1.15% KCl in a Waring blender for 90 s. The homogenates were centrifuged at $9000 \times g$ for 20 min and the resulting supernatant was centrifuged at $105000 \times g$ for 60 min. For studies on the subcellular localization of the loxoprofen reducing activity, the livers were homogenized in a Potter-Elvehjem homogenizer with three strokes of a Teflon pestle. The homogenates were centrifuged at $500 \times g$ for 10 min. The resulting supernatants were centrifuged at $9000 \times g$ for 20 min to sediment a mitochondrial fraction and finally at $105000 \times g$ for 60 min to obtain microsomal pellets and the cytoplasmic supernatant. The sedimented pellets at each step were washed twice with the same homogenizing buffer by use of a Teflon homogenizer.

Purification of Loxoprofen Reducing Enzyme——The rabbit liver (300 g) was homogenized and fractionated as described above. The $105000 \times g$ supernatant fluid was mixed portionwise with powdered ammonium sulfate to give 30% saturation and the resulting precipitate was separated by centrifugation at $9000 \times g$ for 20 min. Powdered ammonium sulfate was added to the supernatant to give 65% saturation. The protein precipitates, which contained most of the reducing activity, were collected by centrifugation, dissolved in a minimum volume (*ca.* 10 ml) of 10 mM potassium phosphate buffer (pH 7.4) and dialyzed overnight against about 100 volumes of the same buffer. The dialysate was concentrated by ultrafiltration using a YM-10 membrane (Toyo, Japan), applied to a Sephadex G-100 column (4 × 100 cm) and eluted with 10 mM potassium phosphate buffer (pH 7.4). Fractions (each 10 ml) containing the enzyme activity were pooled. The pooled fraction was applied to a DEAE-Sephacel column (2 × 25 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM β-mercaptoethanol and washed with the same buffer until the eluate showed no absorbance at 280 nm. The adsorbed enzyme activity was eluted with a linear salt gradient (700 ml) of 0 to 0.10 M NaCl in the above buffer and 10-ml fractions were collected. The loxoprofen reducing activities were resolved into a main peak eluted with *ca.* 40 mM NaCl and several other minor components. The main peak fraction was then subjected to affinity chromatography on a Blue Sepharose CL-6B column (2 × 20 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 6.4) containing 1 mM β-mercaptoethanol and 0.5 mM ethylenediaminetetra acetic acid (EDTA). The column was washed with the same buffer solution until A_{280} of the eluate became nearly zero and then eluted with a linear salt gradient (350 ml) of 0 to 0.60 M KCl in the same buffer solution. The combined fractions (from 0.20 to 0.30 M KCl) containing the enzyme activity were applied to a column (1 × 12 cm) of Biogel HT (hydroxylapatite) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM β-mercaptoethanol and washed with the same buffer. The loxoprofen reducing enzyme was not adsorbed on the Biogel HT column and was recovered in the washings. The combined enzyme-active fractions were concentrated to give a solution of about 1 mg protein/ml and stored frozen in small portions at −80 °C. The enzyme activity was stable under these conditions for at least several weeks.

Results

Subcellular Localization of Loxoprofen Reducing Activity

The loxoprofen reducing activity in the subcellular fractions of liver of rat, guinea pig and rabbit are shown in Table I. The activity was localized only in $105000 \times g$ supernatant and was not detected in the microsomal fraction. The livers of rabbit and guinea pig showed high activity (about ten times that of rat liver). The $105000 \times g$ supernatant from rat and rabbit liver gave the *trans*-alcohol in two to five times larger amounts than the *cis*-alcohol, but the supernatant of guinea pig liver afforded approximately equal amounts of the two reduction products.

Purification of Loxoprofen Reducing Enzyme from Rabbit Liver Cytosol

Rabbit liver was used as an enzyme source because of its high reducing activity. Table II summarizes the results of typical purification of the enzyme. The DEAE-Sephacel column chromatography (Fig. 1) resolved the loxoprofen reducing activities into a main component

Table I. Loxoprofen Reducing Activity in Liver Cell Fractions of Rat, Guinea Pig and Rabbit

Species	Fractions	Activity (μmol/30 min/200 mg liver tissue)		
		trans-Alcohol	*cis*-Alcohol	Total
Rat	Whole homogenate	0.12±0.01	0.02±0.002	0.14±0.01
	Mitochondria	n.d.[a]	n.d.	n.d.
	Microsomes	n.d.	n.d.	n.d.
	Cytosol	0.16±0.01	0.03±0.003	0.19±0.01
Guinea pig	Whole homogenate	0.83±0.03	0.75±0.04	1.58±0.06
	Mitochondria	n.d.	n.d.	n.d.
	Microsomes	n.d.	n.d.	n.d.
	Cytosol	1.01±0.04	0.97±0.06	1.98±0.06
Rabbit	Whole homogenate	1.25±0.05	0.58±0.04	1.83±0.10
	Mitochondria	n.d.	n.d.	n.d.
	Microsomes	n.d.	n.d.	n.d.
	Cytosol	1.44±0.06	0.73±0.02	2.17±0.08

Cell fractions from 200 mg of liver were incubated with 4 μmol of loxoprofen sodium for 30 min at 37 °C in the presence of the NADPH-generating systems. Enzyme activity was assayed by Method B described in the text. Values represent means±S.E. ($n=4$).
a) Not detectable (<4 nmol per 30 min per 200 mg liver tissue).

Table II. Purification of the Rabbit Liver Loxoprofen Reducing Enzyme

Fraction	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
105000×*g* sup	0.0019	1	100
$(NH_4)_2SO_4$ 30—65%	0.0028	1.5	94
Sephadex G-100	0.0100	5.8	85
DEAE-Sephacel	0.121	63.7	53
Blue Sepharose CL-6B	0.429	226	32
Biogel HT (hydroxylapatite)	0.675	355	28

The loxoprofen reducing activity was assayed by Method A described in the text. One unit is defined as the change in absorbance at 340 nm corresponding to the oxidation of 1 μmol of NADPH per minute.

and several other minor components. The activities with *p*-nitroacetophenone and pyridine-4-carbaldehyde as substrates were separated into four to five peaks, of which the main peak coincided with that of the loxoprofen reducing activity. The loxoprofen reducing enzyme was finally purified about 350-fold in 28% yield starting from the activity of the 105000×*g* supernatant, showing a single protein band (Fig. 2) on polyacrylamide gel electrophoresis. The molecular weight of the purified enzyme was estimated to be 38000 by gel filtration on a column of Sephadex G-100 or 33000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 12% polyacrylamide gel.

Properties of the Purified Enzyme

pH Optimum and Cofactor Requirements——Loxoprofen, *p*-nitroacetophenone and 5α-dihydrotestosterone were reduced optimally at pH 6.2 in the presence of NADPH or NADH. NADPH was a better cofactor than NADH, being 5 times more efficient for the reduction of loxoprofen and 5α-dihydrotestosterone and 10 times more efficient for *p*-nitroacetophenone (Table III). The apparent K_m for NADPH with loxoprofen as the substrate was estimated to

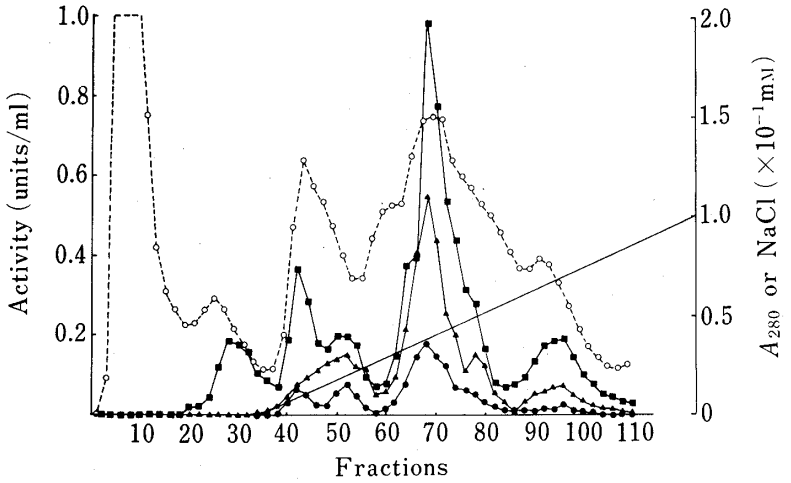

Fig. 1. DEAE-Sephacel Column Chromatography of Loxoprofen Reducing Enzyme of Rabbit Liver

The column (2 × 25 cm) was eluted with a linear gradient from 0 to 0.1 M NaCl (——), and 10 ml fractions were collected. ○, A_{280}; ●, reduction activity with loxoprofen; ▲, reduction activity with *p*-nitroacetophenone; ■, reduction activity with pyridine-4-carbaldehyde.

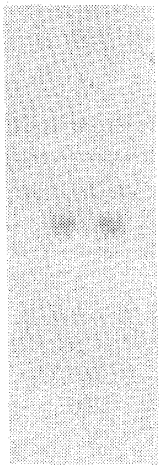

Fig. 2. Polyacrylamide Gel Electrophoresis of Loxoprofen Reducing Enzyme of Rabbit Liver

About 10 μg of the enzyme was applied per well. Electrophoresis and staining were carried out as described under Experimental.

TABLE III. Cofactor Requirement of Loxoprofen Reducing Enzyme Purified from Rabbit Liver Cytosol

Substrate	Activity (μmol/min/mg protein)	
	NADPH	NADH
Loxoprofen	0.56	0.11
p-Nitroacetophenone	6.70	1.48
5α-Dihydrotestosterone	2.26	0.23

Enzyme activity was assayed by Method A described in the text.

be 10 μM.

Effect of Inhibitors——The effects of various inhibitors on the reduction of loxoprofen are summarized in Table IV. *p*-Chloromercuribenzoate (PCMB), *N*-ethylmaleimide and quercitrin exhibited strong inhibitory effects. Pyrazole, phenobarbital and metal chelating agents all had little effect on the reduction rate.

Substrate Specificity——The apparent K_m and V_{max} for loxoprofen were 0.45 mM and

TABLE IV. Effects of Various Inhibitors on the Purified Loxoprofen Reducing Enzyme Activity

Inhibitors	Concentration (mM)	Inhibition (%)
Phenobarbital	1	0
Barbital	1	0
PCMB	0.1	87
N-Ethylmaleimide	1	28
2,2′-Dipyridyl	1	0
Pyrazole	1	0
Disulfiram	0.05	0
Quercitrin	0.05	85
Quercetin	0.05	95

Inhibition studies were carried out as described in the text using 1.0 mM loxoprofen sodium as the substrate. After preincubation for 1 min, the reaction was initiated by adding NADPH.

TABLE V. Apparent K_m and V_{max} Values for Various Aldehydes and Ketones with the Purified Loxoprofen Reducing Enzyme

Substrates	K_m (mM)	V_{max} (μmol/min/mg protein)
Loxoprofen	0.45	0.81
p-Chlorobenzaldehyde	2.88	2.85
p-Nitrobenzaldehyde	0.30	2.65
Pyridine-3-carbaldehyde	3.61	2.47
Pyridine-4-carbaldehyde	0.50	3.90
Acetophenone	7.67	0.45
p-Chloroacetophenone	3.73	1.26
p-Nitroacetophenone	0.71	3.20
4-Benzoylpyridine	0.30	5.58
2-Methyl-1,2-di(3-pyridyl)-1-propanone (metyrapone)	0.49	0.28

The following compounds showed less than 10% reduction with respect to loxoprofen: benzylidene acetone, α- and β-ionone. The following compounds showed no detectable reduction: acetone, methyl ethyl ketone, acetaldehyde, butyraldehyde, γ-D-glucuronolactone and D-glucuronate.

TABLE VI. Apparent K_m and V_{max} Values of the Purified Loxoprofen Reducing Enzyme for Steroidal Ketones

Steroids	K_m (mM)	V_{max} (μmol/min/mg protein)
5α-Dihydrotestosterone	0.046	4.16
5β-Dihydrotestosterone	0.140	3.39
5α-Androstan-3,17-dione	0.031	4.63
5β-Androstan-3,17-dione	0.174	2.58
Epiandrosterone	0.100	2.08

Various concentrations of substrates were added to the reaction mixture described in the text. K_m and V_{max} values were determined from Lineweaver–Burk plots.

0.81 μmol/min/mg protein, respectively. The substrate specificity of the purified loxoprofen reducing enzyme was examined for various aldehydes and ketones as described in assay method A (Table V). Aromatic aldehydes and ketones such as *p*-nitrobenzaldehyde and *p*-

Table VII. Relative Activity of the Purified Loxoprofen Reducing Enzyme towards Various Cyclic Ketones

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Loxoprofen	100	2-[4-(3-Hydroxy-2-oxocyclopentan-1-ylmethyl)phenyl] propionic acid	73
Cyclopentanone	0		
2-Methylcyclopentanone	0		
3-Methylcyclopentanone	0	2-[4-(2-Oxocyclohexan-1-ylmethyl)phenyl]propionic acid	120
2-Ethylcyclopentanone	20		
2-*n*-Propylcyclopentanone	27	Ketoprofen	19
2-Methoxycarbonylhexylcyclopentanone	17	PGE_2	53
		Cyclohexanone	302
2-Benzylcyclopentanone	115	2-Methylcyclohexanone	64
2-Ethoxycarbonylcyclopentanone	135	3-Methylcyclohexanone	165
2-Ethoxycarbonylmethylcyclopentanone	45	4-Methylcyclohexanone	293
		Tetralin-1-one (α-Tetralone)	65
2-[4-(2-Oxocyclopent-3-en-1-ylmethyl)phenyl]propionic acid	11	Tetralin-2-one (β-Tetralone)	76
		Indan-1-one	0
		Indan-2-one	44
		Adamantanone	202

Relative activity is expressed by percent activity relative to that obtained when loxoprofen was used as the substrate under the same conditions.

nitroacetophenone served as efficient substrates, whereas simple aliphatic aldehydes and ketones such as acetone, methyl ethyl ketone, acetaldehyde and butyraldehyde were not reduced at all. α,β-Unsaturated ketones, *e.g.*, benzylidene acetone and α- and β-ionone, were poor substrates. Sugars and steroids were tested as possible naturally occurring substrates (Table VI). γ-D-Glucuronolactone, D-glucuronate and D-glucose were not reduced. Steroidal 3-ketones such as 5α- and 5β-dihydrotestosterones, especially 5α-androstan-3,17-dione, were good substrates. On the other hand, the 17-keto groups of androsterone, etiocholanolone, epietiocholanolone and estrone were all poor substrates. Epiandrosterone was reduced at an exceptionally high rate.

The reduction rates of a number of alicyclic ketones are summarized in Table VII. Cyclohexanone and its methyl substituted derivatives all served as good substrates. In contrast, cyclopentanone, 2-methyl- and 3-methylcyclopentanone were not reduced at all. However, 2-ethyl- and 2-*n*-propylcyclopentanone showed limited but significant rates of reduction, and 2-benzylcyclopentanone was a good substrate, comparable to loxoprofen. Unexpectedly, cyclopentanone-2-carboxylic acid ethyl ester was reduced rapidly. Introduction of an α,β-double bond with respect to the cyclopentanone moiety of the loxoprofen molecule decreased the reduction rate markedly, but a hydroxyl group at the β-position only slightly reduced the rate. Prostaglandin E_2 (PGE_2) which has an analogous β-hydroxy cyclopentanone moiety was reduced at a moderate rate. The loxoprofen analogue having a cyclohexanone ring showed a similar reduction rate. Ketoprofen with a keto group flanked by two aromatic rings was a poor substrate for this enzyme. Both tetralin-1- and -2-one served as good substrates but only indan-2-one, not indan-1-one, was reduced moderately.

HPLC Determination of the Reduction Products of Loxoprofen

The reaction products with the purified enzyme were analyzed by HPLC. Loxoprofen afforded mainly the *trans*-alcohol accompanied by a small amount of the *cis*-alcohol after

incubation for 30 min, the ratio of *trans*-alcohol to *cis*-alcohol being about 13.

Discussion

Loxoprofen reducing activity of the liver was localized in the 105000 × *g* supernatant in all animals examined, and was highest in the rabbit and lowest in the rat. These interspecies differences in activity are in accord with the findings on ketone reductases reported by Ahmed *et al.*[12] and Coutts *et al.*[13] Equal amounts of *trans*- and *cis*-alcohols were produced by the liver of guinea pig, indicating that the guinea pig liver might contain some enzyme other than the enzyme purified from rabbit liver.

The loxoprofen reducing enzyme purified from the cytoplasmic fraction of rabbit liver homogenates had a molecular weight of 33000 and an optimal pH of 6.2, and showed a preference for NADPH as a cofactor. The enzymic reduction of loxoprofen was inhibited markedly by quercitrin, which is an effective inhibitor of ketone reductases.[14] Neither pyrazole (a classical inhibitor of alcohol dehydrogenase) nor phenobarbital (a potent inhibitor of aldehyde reductases[14]) had any significant effect on the enzyme activity. The purified enzyme efficiently catalyzed the reduction of aromatic (but not simple aliphatic) aldehydes and ketones examined. Cyclohexanone and tetralin-1-one were good substrates. In contrast, cyclopentanone and indan-1-one were not reduced at all. All of these results strongly suggest that the purified loxoprofen reducing enzyme is identical with the aromatic aldehyde ketone reductases described by Culp *et al.*[8] and Sawada *et al.*[15]

Cyclopentanone was essentially inactive as a substrate for the purified enzyme, as mentioned above. This was also the case for 2- and 3-methylcyclopentanone. However, the 2-alkylhomologues such as 2-ethyl- and 2-*n*-propylcyclopentanone along with 2-ethoxycarbonylcyclopentanone were all reduced at rather limited but significant rates. 2-Benzylcyclopentanone, which corresponds to the skeletal moiety of the loxoprofen molecule without the propionic acid side chain, was reduced at a high rate comparable to that of loxoprofen. Thus, introduction of some lipophilic substituents larger than the methyl group seems to allow the 2-substituted cyclopentanones to be satisfactory substrates for the ketone reductase. It is interesting to note that the purified ketone reductase effectively catalyzes the reduction of PGE_2 in view of the substrate specificity with substituted cyclopentanones, and also the possible physiological significance of the proposed reduction pathway of PGE_2 to $PGF_{2\alpha}$.[16]

The purified loxoprofen reducing enzyme catalyzed the reduction of steroidal 3-ketones with high efficiency, especially that of 5α-steroid-3-ketones. However, it showed only moderate reduction rates for the 17-ketones, except for epiandrosterone. These results suggest that the purified enzyme may be a 3α-hydroxysteroid dehydrogenase. This is further supported by the following facts. (1) In the purification procedure, DEAE-Sephacel column chromatography separated the loxoprofen reducing enzyme activity from that of testosterone 17β-dehydrogenase and (2) loxoprofen served as a good substrate for bacterial 3-hydroxysteroid dehydrogenase with NADPH as a cofactor, giving rise to the *trans*-alcohol as the main reduction product. Recently, Sawada *et al.* claimed that their two aromatic aldehyde–ketone reductases, F_1 and F_3, purified from rabbit liver are identical with 3(17)β- and 3α-hydroxysteroid dehydrogenases respectively.[1] The loxoprofen reducing enzyme seems to be coincident with the F_3 enzyme.

The purified enzyme reduced loxoprofen to give mainly the *trans*- alcohol, the amount of the *cis*-alcohol being only 7% of that of the *trans*-alcohol. This *trans*-alcohol can reasonably be expected to have (1′*R*, 2′*S*)-configuration, as already established in rats.[4] This should be confirmed by the development of an analytical method for separating the four stereoisomers of the *trans*-alcohol, which is now under investigation.

References and Notes

1) H. Sawada, A. Hara, T. Nakayama and F. Kato, *J. Biochem.* (Tokyo), **87**, 1153 (1980).
2) E. Misaka, T. Yamaguchi, T. Iizuka, K. Kamoshida, T. Kojima, K. Kobayashi, Y. Endo, Y. Misawa, S. Kobayashi and K. Tanaka, *Oyo Yakuri*, **21**, 753 (1981).
3) K. Matsuda, K. Ohnishi, Y. Tanaka, S. Ushiyama and M. Yamazaki, *Biochem. Pharmacol.*, in press.
4) S. Naruto, Y. Tanaka, R. Hayashi and A. Terada, *Chem. Pharm. Bull.*, **32**, 258 (1984).
5) Y. Tanaka, Y. Nishikawa and R. Hayashi, *Chem. Pharm. Bull.*, **31**, 3656 (1983).
6) H. Nagashima, Y. Tanaka, H. Watanabe, R. Hayashi and K. Kawada, *Chem. Pharm. Bull.*, **32**, 251 (1984).
7) A. D. Merrit and G. M. Tomkins, *J. Biol. Chem.*, **234**, 2778 (1959).
8) H. W. Culp and R. E. McMahon, *J. Biol. Chem.*, **243**, 848 (1968).
9) A. P. Philips and J. Mentha, *J. Am. Chem. Soc.*, **78**, 140 (1956).
10) E. F. Hartree, *Anal. Biochem.*, **48**, 422 (1972).
11) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
12) N. K. Ahmed, R. L. Felsted and N. R. Bachur, *J. Pharmacol. Exp. Ther.*, **209**, 12 (1979).
13) R. T. Coutts, D. B. Prelusky and G. R. Jones, *Can. J. Physiol. Pharmacol.*, **59**, 195 (1980).
14) N. K. Ahmed, R. L. Felsted and N. R. Bachur, *J. Pharmacol. Exp. Ther.*, **209**, 12 (1979).
15) H. Sawada and A. Hara, *Biochem. Pharmacol.*, **28**, 1089 (1979).
16) S. C. Lee and L. Levine, *J. Biol. Chem.*, **249**, 1369 (1974).