A Carbonyl Reductase-Catalyzing Reduction of N^3 -Phenacyluridine in Rabbit Liver

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Carbonyl reductase activity for a novel hypnotic, N^3 -phenacyluridine, was mainly localized in the cytosol fraction of rabbit liver. The enzyme (N^3 -phenacyluridine reductase) which catalyzes the reduction of N^3 -phenacyluridine to N^3 - α -hydroxy- β -phenethyluridine was purified from the cytosolic fraction of rabbit liver by various chromatographic techniques (DEAE-Sephacel, Red Sepharose CL-6B and hydroxylapatite). N^3 -Phenacyluridine reductase had a minimum molecular weight of 39 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis. This enzyme required reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor and its optimal pH was 7.5. Flavonoids (quercetin and quercitrin) were potent inhibitors of the enzyme, but pyrazole or barbitol had little effect. The apparent K_m and V_{max} values of the enzyme for the reduction of N^3 -phenacyluridine were 0.32 mM and 8.7 units/mg protein, respectively. A variety of carbonyl compounds, including N^3 -phenacyluridine, were effectively reduced by the enzyme. However, the enzyme purified from rabbit liver differs in several respects from known carbonyl reductases in rabbit liver.

Key words N^3 -phenacyluridine reductase; carbonyl reductase; rabbit liver; enzyme purification; cofactor requirement; substrate specificity

Uridine is a sleep-promoting substance which was isolated from the brainstem of sleep-deprived rats.¹⁾ The nocturnal infusion of uridine promoted the rat's natural sleep. We showed for the first time that N^3 -benzyluridine exhibits hypnotic activity in mice.²⁾ From the structure–activity relationship of N^3 -substituted uridines as hypnotics, we previously reported that a uridine analogue, N^3 -phenacyluridine (N^3 -PhAcUd), had the strongest hypnotic activity in mice among the compounds tested so far.³⁾

It is important to know whether the parent compound or its metabolites are responsible for the hypnotic activity. Metabolic studies of N^3 -benzyluridine and N^3 -benzylthymidine demonstrated that the parent compound rather than the metabolites exerted hypnotic activity.^{4,5)} N^3 -PhAcUd was stereoselectively metabolized to (S)-(+)- N^3 - α -hydroxy- β -phenethyluridine (S-OH) in mice, and the metabolite was more potent than the parent compound as far as hypnotic activity is concerned (Fig. 1).⁶⁾ Biotransformation of carbonyl compounds to corresponding alcohols is catalyzed by a group of enzymes which are mainly localized in cytosol fractions of mammalian tissues.⁷⁾ Daunorubicin,⁸⁾ loxoprofen,⁹⁾ naloxone,¹⁰⁾ befunolol¹¹⁾ and acetohexamide¹²⁾ are known to be biotransformed to alcohol metabolites by carbonyl reductases.

The present study was undertaken to purify N^3 -PhAcUd reductase from the cytosol fraction of rabbit liver and to elucidate its properties.

MATERIALS AND METHODS

Chemicals Pyridine-3-aldehyde, 2,6-diacetylpyridine, benzoylacetone and pyrazole were supplied by Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Warfarin was provided by Sigma Chemical Co. (St. Louis, U.S.A.). Other substrates and inhibitors were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Pyridine nucleotides were purchased

from Oriental Yeast Co. (Tokyo, Japan). Diethylaminoethyl (DEAE)-Sephacel and Red Sepharose CL-6B were obtained from Pharmacia Biotech. AB (Uppsala, Sweden) and hydroxylapatite was from Bio-Rad Laboratories (Richmond, U.S.A.). N^3 -PhAcUd and α -OH were synthesized by the method described previously.⁶⁾

Assay of Enzyme Activity N^3 -PhAcUd reductase activity was determined spectrophotometrically in a reaction mixture consisting of enzyme, 1.0 mM N^3 -PhAcUd, 0.125 mM NADPH and 0.1 M Na,K-phosphate buffer (pH 7.4) in a final volume of 0.8 ml. In the case of determination of the optimal pH, the following 0.1 M buffer was used (pH 4.5 to 5.5, citrate–Na₂HPO₄ buffer; pH 5.5 to 8.0, Na,K-phosphate buffer; pH 8.0 to 8.5, Tris–HCl buffer). The reaction was started by the addition of NADPH, and the decrease in absorbance at 340 nm was monitored with a Shimadzu UV-3000 spectrophotometer. One unit of enzyme activity was defined as the oxidation of 1 μ mol NADPH/min at 30 °C. The K_m and V_{max} values were determined by Lineweaver–Burk plots. Protein concentration was determined by a modification of the method of Lowry *et al.*¹³⁾ using bovine serum albumin as the standard.

Enzyme Purification For the purification of N^3 -PhAcUd reductase from rabbit liver, all procedures were performed at 4 °C. Female rabbits (New Zealand white, 2.5–3.0 kg body weight) were exsanguinated from the carotid artery and the liver (160 g) was homogenized in 3 volumes 10 mM phosphate buffer (pH 7.4) containing 0.15 M KCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 10000 $\times g$ for 20 min and the resulting supernatant was centrifuged at 105000 $\times g$ for 60 min. The 105000 $\times g$ supernatant, defined as cytosol, was fractionated by adding ammonium sulfate. The proteins which precipitated between 20 and 65% saturation were collected by centrifugation and dissolved in a minimal volume of 10 mM phosphate buffer (pH

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Fig. 1. Metabolism of N^3 -PhAcUd by N^3 -PhAcUd Reductase

7.4) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer A) and dialyzed overnight against about 100 volumes of the same buffer. The dialysate was then centrifuged. The supernatant was applied to a DEAE-Sephacel column (2.5×32 cm) equilibrated with buffer A and washed with the same buffer until the eluate showed almost no absorbance at 280 nm. A linear salt gradient (1500 ml) of 0 to 0.4 M NaCl in the above buffer was passed through the column to elute the bound proteins. The peak N^3 -PhAcUd reductase activity appeared in the eluate at approximately 200 mM NaCl. The fractions containing high enzyme activity were combined, thoroughly dialyzed against 10 mM phosphate buffer (pH 6.8) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer B), and applied to a Red Sepharose CL-6B column (1.5×23 cm) previously equilibrated with the same buffer. The column was initially washed with buffer B, followed by a 300 ml linear salt gradient of 0 to 1.2 M KCl to elute the bound proteins. The peak N^3 -PhAcUd reductase activity was eluted at approximately 0.4 M KCl. The fractions containing high enzyme activity were combined, thoroughly dialyzed against buffer A without EDTA, and applied to a hydroxylapatite column (1.5×10 cm) previously equilibrated with the above buffer. The column was eluted with 5 mM phosphate buffer. The combined enzyme-active fractions were concentrated by ultracentrifugation (UK-20, Toyo Roshi, Tokyo, Japan) to about 2.0 mg/ml and stored at -80°C until use. The enzyme was stable under these conditions for at least several months.

Polyacrylamide Gel Electrophoresis (PAGE) and HPLC

The purified enzyme was electrophoresed on 10% (w/v) acrylamide gels by the method of Laemmli.¹⁴⁾ Proteins were stained with Coomassie brilliant blue. The standard proteins used for molecular weight makers were α -lactalbumin from bovine milk (14.2 kDa), trypsin inhibitor from soybean (20 kDa), trypsinogen from bovine pancreas (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa) and bovine serum albumin (66 kDa). HPLC was performed using a solvent system of 0.05% NaN_3 in 0.1 M potassium phosphate buffer (pH

Table 1. Subcellular Localization of NADPH-Dependent N^3 -PhAcUd Reducing Activity in Rabbit Liver

Subcellular fractions	Activity (m units/mg protein)
Nuclei ^{a)}	4.42 ± 0.74 (15)
Mitochondria ^{b)}	9.89 ± 1.38 (34)
Microsomes	6.30 ± 0.08 (22)
Cytosol	28.75 ± 1.42 (100)

^{a)} $600 \times g$ pellets. ^{b)} $9000 \times g$ pellets. Numbers in parentheses are expressed as a % of the activity in cytosol. Each value represents the mean $\pm$ S.E.M. ($n=3$).

6.8) and 0.1 M Na_2SO_4 using a Toso G3000SW_{XL} column under non-denaturing conditions.

RESULTS

Subcellular Distribution The N^3 -PhAcUd reducing activities in the subcellular fraction of rabbit liver are summarized in Table 1. The enzymatic activity was dependent on NADPH, but not on NADH. The $105000 \times g$ supernatant showed the highest activity, and $600 \times g$ pellets, $9000 \times g$ pellets and microsomal fraction exhibited 15–34% of the activity compared with the $105000 \times g$ supernatant.

Enzyme Purification The proteins were precipitated from the $105000 \times g$ supernatant fractions of rabbit liver (160 g) by ammonium sulfate fractionation. N^3 -PhAcUd reductase was purified by column chromatography on DEAE-Sephacel, Red Sepharose CL-6B and hydroxylapatite. Fig. 2 shows a typical elution profile of the enzyme activity from a DEAE-Sephacel column. The enzyme activity eluted mainly at about 200 mM NaCl. The main peak (fraction No. 85–100) was further purified on Red Sepharose CL-6B and hydroxylapatite columns. The recovery of the enzyme was 4.0% from the $105000 \times g$ supernatant fractions (Table 2).

SDS-PAGE and HPLC The minimum molecular weight of the purified enzyme was estimated to be 39 kDa by SDS-PAGE (Fig. 3). The retention volumes of the purified enzyme, egg albumin and bovine serum albumin were 12.3, 8.58, and 9.22 ml, respectively, under the conditions used.

Fig. 2. Elution Profile of N^3 -PhAcUd Reductase from Rabbit Liver Cytosol by DEAE-Sephacel Column Chromatography
Fractions (10 ml each) were analyzed for protein (○) and for enzyme activity (●).

Table 2. Purification of N^3 -PhAcUd Reductase from Rabbit Liver Cytosol

Purification step	Protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification (-fold)	Recovery (%)
Cytosol	9024	238.8	0.0265	1	100
Ammonium sulfate fraction 20—65%	4128	171.6	0.0416	1.6	71.6
DEAE-Sephacel	321	32.4	0.1009	3.8	13.6
Red Sepharose CL-6B	66	12.0	0.1818	6.9	5.0
Hydroxylapatite	9	9.6	1.0667	40.3	4.0

Fig. 3. SDS-PAGE of N^3 -PhAcUd Reductase Purified from Rabbit Liver Cytosol

The enzyme (10 μ g) was run on 10% (w/v) acrylamide gel, and the gel was stained by Coomassie brilliant blue.

Amino Acid Sequence The N-terminal amino acid sequence could not be determined suggesting that the N-terminal amino acid of the enzyme might be modified. A peptide fragment (about 10 kDa) of the enzyme was obtained by BrCN treatment.¹⁵⁾ The N-terminal amino acid sequence of this peptide fragment was GVTAENVAKQ—V—(—; not identified). The amino acid sequence had no homology with those of carbonyl reductases previously reported.^{16,17)}

Optimal pH and Temperature The purified enzyme showed optimal catalytic activity at pH 7.5 and 37 °C (data not shown).

Inhibition Studies Table 3 summarizes the effects of various inhibitors on the purified N^3 -PhAcUd reductase.

Table 3. Effects of Various Inhibitors on N^3 -PhAcUd Reductase Purified from Rabbit Liver Cytosol

Inhibitor	Concentration (mM)	Enzyme activity (% of control)
Control	—	100
Pyrazole	1.0	93 ^{a)}
Barbital	1.0	91
Phenobarbital	1.0	96
Quercetin	0.1	6
Quercitrin	0.1	8
DTNB	0.1	35
PCMB	0.1	20
EDTA	1.0	92
<i>o</i> -Phenanthroline	1.0	96
HgCl ₂	0.01	75
CuSO ₄	0.01	50
FeSO ₄	0.01	70
Indomethacin	0.1	72
Furosemide	0.1	70
Warfarin	0.1	80
Dicumarol	0.1	90
NADP ⁺	1.0	40
NAD ⁺	1.0	100

The concentration of N^3 -PhAcUd was 2.0 mM. ^{a)} The data are means of duplicate incubations. DTNB: 5,5'-dithiobis(2-nitrobenzoic acid); PCMB: sodium *p*-chloromercuribenzoate.

Among the inhibitors examined, quercetin and quercitrin were potent inhibitors. Both flavonoids inhibited the activity by over 90% at 0.1 mM. 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuribenzoate (PCMB), both of which are known to be sulfhydryl reagents, showed 65—80% inhibition at 0.1 mM. Hg²⁺, Cu²⁺ and Fe²⁺ exhibited some inhibition (30—50%) even at 0.01 mM. On the other hand, pyrazole, barbiturates, EDTA, *o*-phenanthroline, warfarin and dicumarol did not show any significant inhibitory effect at

0.1—1.0 mM.

Substrate Specificity The K_m (mM) and V_{max} (units/mg protein) of the purified enzyme for N^3 -PhAcUd were 0.32 ± 0.04 and 8.7 ± 1.1 , respectively. The purified enzyme exhibited catalytic activity for various ketones (benzoylpyridine, acetylpyridine, benzoylacetone, cyclohexanone and metyrapone), aldehydes (pyridine aldehydes and benzaldehyde) and a quinone (menadione) examined (Table 4), whereas capryl aldehyde and acetone were not reduced by the enzyme. Table 4 also shows that NADH was not an efficient cofactor for the enzyme.

Regioselectivity of Substrates Table 5 summarizes the substrate specificity of the enzyme in reducing *o*-, *m*- and *p*-isomers of aromatic ketones and aldehydes. The enzyme exhibited the highest catalytic activity for ortho-substituted

Table 4. Substrate Specificity of N^3 -PhAcUd Reductase, Purified from Rabbit Liver Cytosol, for Various Compounds

Substrate	K_m (mM)	V_{max} (units/mg)
N^3 -PhAcUd	$0.32 \pm 0.04^a)$	$8.7 \pm 1.09^a)$
Pyridine-3-aldehyde	0.15	4.1
Pyridine-4-aldehyde	0.22	21.7
Benzaldehyde	0.14	16.9
<i>n</i> -Caprylaldehyde	— ^{c)}	ND
4-Benzoylpyridine	0.26	26.3
4-Acetylpyridine	0.41	22.7
2,6-Diacetylpyridine	0.14	10.6
Acetone	— ^{c)}	ND
Benzoylacetone	0.27	33.3
Cyclohexanone	0.66	13.2
Menadione	0.057	28.6
Metyrapone	0.28	9.0
NADPH ^{b)}	0.0033	9.6
NADH ^{b)}	— ^{c)}	— ^{c)}

The data are means of duplicate incubations unless otherwise noted. The value in parentheses indicates the activity with 2.0 mM substrate. a) The value represents the mean $\pm$ S.E. ($n=3$). b) The activity was assayed with 2.0 mM N^3 -PhAcUd as the substrate. c) not determined. ND Not detected.

Table 5. Substrate Specificity of N^3 -PhAcUd Reductase Purified from Rabbit Liver Cytosol

Substrate	K_m (mM)	V_{max} (units/mg)	V_{max}/K_m
2-Benzoylpyridine	0.045	11.2	248.9
3-Benzoylpyridine	0.20	12.0	60.0
4-Benzoylpyridine	0.27	33.3	123.3
2-Acetylpyridine	0.0082	8.2	1000.0
3-Acetylpyridine	0.11	27.0	245.5
4-Acetylpyridine	0.41	22.7	55.4
<i>o</i> -Nitrobenzaldehyde	0.11	38.5	350.0
<i>m</i> -Nitrobenzaldehyde	0.12	23.3	194.2
<i>p</i> -Nitrobenzaldehyde	0.28	31.3	111.8

$n=2$.

Table 6. Comparison of the Characteristics of Enzymes Purified from Rabbit Liver Cytosol

Enzyme	Minimum molecular weight	Optimal pH	Cofactor	Reference
N^3 -PhAcUd reductase	39,000	7.5	NADPH	
Loxoprofen-reducing enzyme	33,000	6.2	NADPH/NADH	9)
Befunolol reductase	29,000	6.5	NADPH	11)
F1	33,000	5.0—5.4	NADPH	19)
F2	32,000	5.0—5.4	NADPH	19)
F3	29,000	5.8—6.2	NADPH	19)

derivatives (2-benzoylpyridine, 2-acetylpyridine and 2-nitrobenzaldehyde). The V_{max}/K_m values of the enzyme for the 2-substituted derivatives were 2—20 fold higher than those for the corresponding 3- or 4-substituted derivatives.

The purified enzyme stereospecifically reduced N^3 -PhAcUd to S-OH. The metabolite was identified by HPLC using a CHIRALCEL OJ column as reported for a urinary metabolite in mice.⁶⁾

DISCUSSION

The present study demonstrated that N^3 -PhAcUd, which exhibits hypnotic activity in mice, was mainly reduced to an alcohol metabolite by N^3 -PhAcUd reductase. N^3 -PhAcUd reducing activity was mainly localized in the $105000 \times g$ supernatant fraction of rabbit liver. The enzyme was purified from the $105000 \times g$ supernatant fraction of rabbit liver by column chromatography using DEAE-Sephacel, Red Sepharose CL-6B and hydroxylapatite. The purified enzyme showed a minimum molecular weight of 39 kDa and an optimal pH of 7.5. Some dihydrodiol dehydrogenases exhibit catalytic activity as aldo-keto reductase are known to be present as oligomers.¹⁸⁾ However, the purified enzyme in the present study may be a monomeric form from the gel filtration analysis data by HPLC using a Toso G3000SW_{XL} column under non-denaturing conditions. The enzyme exclusively required NADPH as a cofactor. The amino acid sequence determined had no homology with carbonyl reductases previously reported. Table 6 summarizes some characteristics of the carbonyl reductases purified from rabbit liver so far. These characteristics are different from those of the carbonyl reductase purified from rabbit liver cytosol reported previously,^{9,11,19)} suggesting that the purified enzyme is a novel enzyme hitherto unidentified. The catalytic activity of the purified enzyme was markedly inhibited by quercitrin and quercetin, which are typical inhibitors of ketone reductase.^{9,11)} The inhibition by DTNB, PCMB and Hg²⁺ suggests that SH groups in the enzyme play some role in the catalytic activity of the enzyme.

The purified enzyme stereospecifically reduced N^3 -PhAcUd to the S-OH isomer, which was identified as a major metabolite of N^3 -PhAcUd in mouse urine. The purified enzyme efficiently reduced various aromatic aldehydes and quinones together with ketones. The broad substrate specificity of this enzyme is similar to that described by Felsted *et al.*⁷⁾ and Sawada *et al.*²⁰⁾ In conclusion, the purified enzyme is a new member of the carbonyl reductase family. Whether or not the enzyme has catalytic activity on endogenous compounds and is a member of the aldo-keto reductase superfamily¹⁹⁾ remains to be seen.

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