Purification and Catalytic Properties of a Novel Acetohexamide-Reducing Enzyme from Rabbit Heart

Yorishige Imamura,*[,1] Akio Ryu,* Toshihisa Koga,* Toshiyuki Higuchi,* Masaki Otagiri,* Masako Nozawa,† and Hiroyuki Akita†

**Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862; and †School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274*

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An enzyme catalyzing the metabolic reduction of acetohexamide [4-acetyl-*N*-(cyclohexylcarbamoyl)benzenesulfonamide], an oral antidiabetic drug, was purified to homogeneity from the cytosolic fraction of rabbit heart. The molecular mass of the purified enzyme was estimated to be 110 kDa by gel filtration and nondenaturing PAGE and 28 kDa by SDS-PAGE, suggesting that the enzyme is composed of four identical-size subunits. 4-Benzoylpyridine and *p*-nitroacetophenone, typical substrates of carbonyl reductase [EC 1.1.1.184], were not reduced by the enzyme. Of drugs with a ketone group tested, only acetohexamide was a good substrate of the enzyme. The enzyme effectively reduced analogs substituted with various alkyl groups instead of the cyclohexyl group in acetohexamide, although it had little or no ability to reduce analogs substituted with various alkyl groups instead of the methyl group in acetohexamide. The enzyme was inhibited not only by quercetin, a well-known inhibitor of carbonyl reductase, but also by phenobarbital, a potent inhibitor of aldehyde reductase [EC 1.1.1.2]. These results indicate that the enzyme purified from rabbit heart is a novel enzyme responsible for the reduction of acetohexamide and its analogs.

Key words: acetohexamide analog, acetohexamide-reducing enzyme, drug metabolism, rabbit heart, tetrameric enzyme.

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Carbonyl reductase [EC 1.1.1.184] is an enzyme that catalyzes the reduction of endogenous and exogenous carbonyl compounds to the corresponding alcohol metabolites (*1-10*). The enzyme has a broad substrate specificity and contributes to the metabolism of drugs having a ketone group within their structures (*5-9*). Recently, we have purified carbonyl reductases from the cytosolic fractions of rabbit liver and kidney by using acetohexamide [4-acetyl-*N*-(cyclohexylcarbamoyl)benzenesulfonamide], an oral antidiabetic drug, as a substrate (*5, 8, 9*). The purified enzymes have the ability to reduce many drugs with a ketone group, such as befunolol, levobunolol, and daunorubicin, indicating that they play an important role in drug metabolism (*5, 8*).

Drug-metabolizing enzymes are contained mainly in the liver and kidney of mammalian species, including humans. However, our preliminary study has demonstrated that the cytosolic fraction of rabbit heart exhibits an approximately 2-fold higher activity than those of rabbit liver and kidney in the metabolic reduction of acetohexamide (*11*). It is interesting to note that a drug-metabolizing enzyme, *i.e.*, acetohexamide-reducing enzyme, exists in the heart, which essentially functions as a circulating pump for the blood. This study was undertaken to purify acetohexamide-reducing enzyme from cytosolic fraction of rabbit heart and to elucidate its catalytic properties.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials—Acetohexamide was a gift from Shionogi (Osaka). Befunolol (Kaken Pharmaceutical, Tokyo), levobunolol (Warner-Lambert, Ann Arbor, USA), daunorubicin (Meiji Seika, Tokyo), and loxoprofen (Sankyo, Tokyo) were provided by the manufacturers. Steroids were obtained from Sigma Chemical (St. Louis, USA). Other substrates and inhibitors were purchased from Wako Chemical (Osaka), Nacalai Tesque (Kyoto), and Tokyo Kasei (Tokyo). Hydroxyhexamide was synthesized from acetohexamide according to the method of Girgis-Takla and Chroneos (*12*). DEAE-Sephacel, Red Sepharose CL-6B, and Sephacryl S-300 (high resolution) were obtained from Pharmacia (Uppsala, Sweden) and hydroxylapatite from Bio-Rad Laboratories Japan (Tokyo). NADPH was purchased from Oriental Yeast (Tokyo). All other chemicals were of reagent grade.

Synthesis of Acetohexamide Analogs—Acetohexamide analogs possessing various alkyl groups instead of the methyl group were synthesized as reported previously (*13*). Furthermore, five kinds of analogs substituted with *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and *iso*-propyl groups instead of the cyclohexyl group in acetohexamide were synthesized from ethylbenzene. Commercially available ethylbenzene was treated with chlorosulfonic acid to give 4-ethylbenzenesulfonyl chloride, which was used for the next reaction without further purification. Treatment of

4-ethylbenzenesulfonyl chloride with aqueous NH_3 afforded 4-ethylbenzenesulfonamide in 57% yield. Oxidation of 4-ethylbenzenesulfonamide with chromium trioxide in acetic acid gave 4-acetylbenzenesulfonamide in 89% yield. The obtained 4-acetylbenzenesulfonamide was treated with *n*-propyl isocyanate, *n*-butyl isocyanate, *n*-pentyl isocyanate, *n*-hexyl isocyanate, and *iso*-propyl isocyanate in the presence of 1,8-diazabicyclo[5,4,0]undec-7-ene to provide the corresponding acetohexamide analogs in 85, 73, 90, 88, and 57% yields, respectively. The structures of these analogs were confirmed by elemental analysis and physical methods (IR and NMR).

Partition Coefficients—The partition coefficients of acetohexamide and its analogs in *n*-octanol/0.1 M sodium potassium phosphate buffer (pH 6.0) were determined according to the method of Hansch *et al.* (*14*).

Enzyme Purification—All procedures were performed at 3-5°C unless otherwise stated. Male rabbits (Japanese white strain) killed and the hearts (80 g) were immediately removed and homogenized in 3 volumes of 10 mM sodium potassium phosphate buffer (pH 7.4) containing 0.15 M KCl, 0.5 mM EDTA, and 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 × *g* for 20 min and the supernatant was recentrifuged at 105,000 × *g* for 60 min. The resulting supernatant (cytosolic fraction) was dialyzed against 10 mM sodium potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer A) and applied to a DEAE-Sephacel column (2.5 × 15 cm). A linear salt gradient of 0 to 0.3 M NaCl was passed through the column to elute the bound proteins. The fractions containing acetohexamide-reducing enzyme were pooled, concentrated by ultrafiltration (Funakoshi, hollow fiber membrane), dialyzed against 10 mM sodium potassium phosphate buffer (pH 6.4) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer B), and applied to a Red Sepharose CL-6B column (1.5 × 25 cm), followed by a linear salt gradient of 0 to 3.0 M KCl. The enzyme fractions were pooled, concentrated by ultrafiltration, dialyzed against buffer A without EDTA, and applied to a hydroxylapatite column (1.5 × 10 cm). The enzyme was absorbed on the hydroxylapatite column and eluted with 100 mM sodium potassium phosphate buffer (pH 7.4) containing 5 mM 2-mercaptoethanol (buffer C). The enzyme fractions were pooled, dialyzed against buffer A, concentrated by ultrafiltration, and applied to a Sephacryl S-300 (high resolution) column (1.5 × 50 cm). The enzyme fractions were pooled, concentrated by ultrafiltration and stored at 0-4°C in 20% (v/v) glycerol. The purified enzyme sample was stable for at least 1 month under these conditions.

Enzyme Assay—The enzyme activity was assayed spectrophotometrically by monitoring NADPH oxidation at 340 nm. The reaction mixture in a total volume of 0.7 ml consisted of 0.1 M sodium-potassium phosphate buffer (pH 6.0), 0.25 mM NADPH, substrates at various concentrations, and enzyme. To determine the optimal pH, the following 0.1 M buffers were used (pH 4.5 to 5.5, citrate-sodium phosphate buffer; pH 5.5 to 8.0, sodium-potassium phosphate buffer; pH 8.0 to 8.5, Tris-HCl buffer). The reaction was initiated by addition of enzyme. One unit of enzyme activity was defined as the amount causing a decrease in absorbance at 340 nm corresponding to the oxidation of 1 μmol of NADPH/min at 30°C. Protein concentration was determined by a modification of the method of Lowry *et al.* (*15*) using bovine serum albumin as a standard. The K_m and V_{max} values of the enzyme were calculated by using a computer program for least-squares linear regression of Lineweaver-Burk plots.

Gel Filtration, Nondenaturing PAGE, and SDS-PAGE—The molecular weight of the native enzyme was determined by gel filtration on a Sephacryl S-300 (high resolution) column (1.5 × 50 cm) and by nondenaturing PAGE on a Multi Gel 2/15 plate having an acrylamide concentration gradient of 2-15% (Daiichi Pure Chemicals, Tokyo). The molecular weight of the subunit was estimated by SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (*16*) using Multi Gel 10/20 (Daiichi Pure Chemicals, Tokyo). Proteins used as molecular weight markers for gel filtration, nondenaturing-PAGE and SDS-PAGE were purchased from Serva (Heidelberg, Germany) and Daiichi Pure Chemicals (Tokyo).

HPLC Analysis—The metabolite (hydroxyhexamide) of acetohexamide produced in the reaction mixture was determined by HPLC as reported previously (*17*).

RESULTS

Enzyme Purification—When the cytosolic fraction of rabbit heart was applied to a DEAE-Sephacel column, the enzyme activity appeared as a single peak. A single peak of the enzyme activity was also observed in subsequent chromatographic steps. After these chromatographic steps, the acetohexamide-reducing enzyme was purified 325-fold in terms of specific activity with a yield of 36% from the cytosolic fraction of rabbit heart (Table I). Homogeneity of the purified enzyme was confirmed by SDS-PAGE, as shown in Fig. 1.

Molecular Properties—The molecular mass of the enzyme was estimated to be 110 kDa by gel filtration on a Sephacryl S-300 (high resolution) column and by nondenaturing PAGE on a plate having an acrylamide concentration gradient of 2-15% (Fig. 2). The subunit size of the enzyme was 28 kDa, as estimated by SDS-PAGE (Fig. 1). These results suggest that the native form of the enzyme is composed of four identical-size subunits.

Substrate Specificities—The enzyme purified from rabbit heart required NADPH as a cofactor. The K_m value for the oxidation of NADPH by the enzyme was 6 μM in the

TABLE I. Purification of acetohexamide-reducing enzyme from rabbit heart.

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cytosol	1,166	15.2	0.013	1	100
DEAE-Sephacel	90.9	14.2	0.156	12	94
Red Sepharose CL-6B	2.94	9.7	3.29	253	64
Hydroxylapatite	1.48	6.0	4.03	310	39
Sephacryl S-300	1.30	5.5	4.22	325	36

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presence of 1.0 mM acetohexamide. When NADPH was replaced by NADH, no significant enzyme activity was detected. The enzyme did not catalyze the reverse reaction under the same conditions. The optimal enzyme activity was observed at around pH 6.0. Table II summarizes the activities of the enzyme towards various carbonyl compounds and steroids. The substrate specificities of carbonyl reductases purified from rabbit liver and kidney are also shown in Table II. The enzyme purified from rabbit heart had no ability to reduce 4-benzoylpyridine and *p*-nitroacetophenone, which are typical substrates of carbonyl reductase (*1-5*), or steroids such as 5α-androstan-17β-ol-3-one and 5α-androstane-3,17-dione, although rabbit liver and kidney carbonyl reductases effectively reduced these carbonyl compounds and steroids (*5, 8*). Furthermore, the activities of the enzyme purified from rabbit heart towards various drugs with a ketone group, such as befunolol, levobunolol, daunorubicin, and loxoprofen, were examined. Of drugs with a ketone group tested, only acetohexamide was a good substrate of the enzyme. The catalytic activity of the enzyme for acetohexamide was also confirmed by direct measurement of the reduction product (hydroxyhexamide) on HPLC. The V_{max}/K_m value of the enzyme purified from rabbit heart for acetohexamide was similar to those of carbonyl reductases purified from rabbit liver and kidney (*5, 8*).

Catalytic Properties with Acetohexamide Analogs—In order to establish the structural requirements of the enzyme purified from rabbit heart, acetohexamide analogs were synthesized (*13*) and the activities of the enzyme towards them were examined (Table III). The enzyme had little or no ability to reduce the analogs substituted with ethyl, *n*-propyl, *n*-butyl, and *iso*-propyl groups instead of the methyl group in acetohexamide. On the other hand, the analogs substituted with *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and *iso*-propyl groups instead of the cyclohexyl group in acetohexamide were effectively reduced by the enzyme. The V_{max}/K_m values of the enzyme for these analogs increased with increasing number of carbon atoms in straight-chain alkyl groups. The V_{max}/K_m value of the enzyme for the analog substituted with an *n*-hexyl group

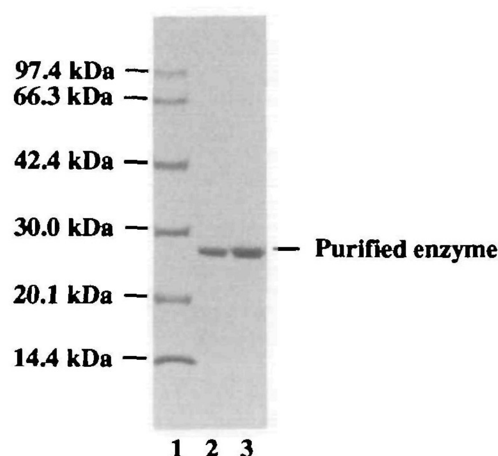

Fig. 1. **SDS-PAGE of purified enzyme.** 1, molecular weight markers; 2, purified enzyme (3 μg); 3, purified enzyme (6 μg). Molecular weight markers used were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.3 kDa), aldolase (42.4 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa).

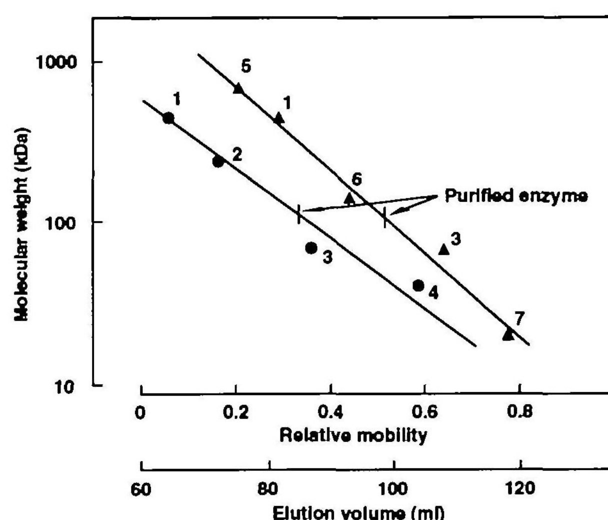

Fig. 2. **Estimation of molecular weight of the purified enzyme by gel filtration and nondenaturing PAGE.** ●: gel filtration, ▲: nondenaturing PAGE. Molecular weight markers used were (1) ferritin (443 kDa), (2) catalase (240 kDa), (3) bovine serum albumin (66.3 kDa), (4) egg albumin (45.0 kDa), (5) thyroglobulin (669 kDa), (6) lactate dehydrogenase (140 kDa), and (7) trypsin inhibitor (20.1 kDa).

TABLE II. **Substrate specificities of acetohexamide-reducing enzyme from rabbit heart and carbonyl reductases from rabbit liver and kidney for carbonyl compounds and steroids.** The values are the mean of at least two experiments. The values in parentheses indicate the activity with 0.1 or 1.0 mM substrate. Data with superscripts a and b were cited from Refs. *5* and *8*, respectively.

Substrate	Heart		Liver[a]		Kidney[b]	
	K_m (mM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)
D-Xylose	—	(0)	—	(0)	—	(0)
D-Glucuronic acid	—	(0)	—	(0)	—	(0)
DL-Glyceraldehyde	—	(0.22)	—	(0.05)	—	(0)
Pyridine-4-aldehyde	—	(0)	2.6	24.8	1.7	9.6
4-Benzoylpyridine	—	(0)	0.04	14.3	0.45	10.7
4-Acetylpyridine	—	(0)	1.8	10.1	2.9	5.4
p-Nitroacetophenone	—	(0)	0.79	4.0	0.42	3.4
4-Androstene-3,17-dione	—	(0)	0.14	4.6	0.094	1.2
5α-Androstan-17-β-ol-3-one	—	(0)	0.21	22.8	0.013	12.3
5α-Androstane-3,17-dione	—	(0)	0.10	16.0	0.15	18.3
5β-Androstan-17-β-ol-3-one	—	(0)	0.06	6.1	0.28	6.1

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TABLE III. **Catalytic properties of acetohexamide-reducing enzyme from rabbit heart for acetohexamide and its analogs with various alkyl groups instead of the methyl or cyclohexyl group.** The values are the means of at least two experiments. The values in parentheses indicate the specific activity with 0.5 mM substrate. Asterisk shows acetohexamide. PC: partition coefficient.

$R_1-C(=O)-C_6H_4-SO_2NHCONH-R_2$

R_1	R_2	K_m (mM)	V_{max} (units/mg)	V_{max}/K_m (units/mg/mM)	PC
Methyl*	Cyclohexyl*	0.74	3.86	5.2	9.1
Ethyl	Cyclohexyl	—	(0.09)	—	38.1
n-Propyl	Cyclohexyl	—	(0.06)	—	108
n-Butyl	Cyclohexyl	—	(0)	—	249
iso-Propyl	Cyclohexyl	—	(0.05)	—	125
Methyl	*n*-Propyl	3.48	5.45	1.6	0.9
Methyl	*n*-Butyl	0.45	6.44	14.3	2.9
Methyl	*n*-Pentyl	0.07	4.13	59.0	10.4
Methyl	*n*-Hexyl	0.05	3.63	72.6	44.5
Methyl	*iso*-Propyl	2.15	1.08	0.5	0.8

TABLE IV. **Effect of inhibitors on acetohexamide-reducing enzyme from rabbit heart.** The values are the mean of at least two experiments.

Inhibitor	Concentration (mM)	Inhibition (%)
Pyrazole	10	5
Quercetin	0.05	45
Phenobarbital	1	42
p-CMB	0.1	93
$HgCl_2$	0.01	23
$CuSO_2$	0.01	12

(six carbon atoms) was much higher than that for acetohexamide, having a cyclohexyl group (six carbon atoms).

Inhibitory Effects—Table IV shows the effects of various inhibitors on the reduction of acetohexamide catalyzed by the enzyme purified from rabbit heart. The concentrations of inhibitors used were as reported previously (*9*). Quercetin, a well-known inhibitor of carbonyl reductase, and phenobarbital, a potent inhibitor of aldehyde reductase [EC 1.1.1.2], exhibited significant inhibitory effects against the enzyme. *p*-Chloromercuribenzoate (*p*-CMB) strongly inhibited the enzyme. $HgCl_2$ and $CuSO_4$ slightly inhibited the enzyme. However, pyrazole, a potent inhibitor of alcohol dehydrogenase, had little effect on the enzyme activity.

DISCUSSION

We purified an acetohexamide-reducing enzyme from the cytosolic fraction of rabbit heart to homogeneity. The purified enzyme was shown to be a tetrameric enzyme with a molecular mass of 110 kDa. So far, acetohexamide-reducing enzymes have been purified as monomeric carbonyl reductases from the cytosolic fractions of rabbit liver and kidney (*5, 8, 9*). The tetrameric enzyme purified from rabbit heart is distinct from these monomeric carbonyl reductases. Some tetrameric carbonyl reductases have been purified from the lungs of guinea-pig (*18*), mouse (*19*), and pig (*20*). The rabbit heart enzyme is also distinguishable from these tetrameric carbonyl reductases, which are potently inhibited by pyrazole (*18-21*). Thus, the enzyme purified from rabbit heart appears to be a novel enzyme responsible for the reduction of acetohexamide.

The specific activity of the enzyme was 4.22 units/mg in the final chromatographic step of purification, whereas the V_{max} value of the enzyme for acetohexamide was 3.86 units/mg. There is a possibility that glycerol added as a stabilizer after purification decreases the V_{max} value of the enzyme for acetohexamide. At present, however, we can not clearly explain the reason for this discrepancy.

It has been accepted that carbonyl reductases can reduce many exogenous carbonyl compounds (*1-10*). The enzyme purified from rabbit heart, unlike these carbonyl reductases, had a very limited substrate specificity for exogenous carbonyl compounds, including drugs. Recently, daunorubicinol, the alcohol metabolite of daunorubicin, has been reported to produce cardiotoxicity in rabbits (*22*). We therefore examined whether the enzyme purified from rabbit heart has the ability to reduce daunorubicin. However, daunorubicin was not reduced by the enzyme. The rabbit heart enzyme is probably involved in the reduction of endogenous carbonyl compounds, although steroids, which are typical endogenous substrates for carbonyl reductase (*5, 8*), are not reduced by the enzyme. It would be of interest to elucidate the true endogenous substrate.

Our previous papers have demonstrated that carbonyl reductases from rabbit liver and kidney effectively reduce acetohexamide and its analogs substituted with ethyl, *n*-propyl, *n*-butyl, and *iso*-propyl groups instead of the methyl group, and that the V_{max}/K_m values increase with the number of carbon atoms in these alkyl groups adjacent to the ketone group of acetohexamide (*9, 13*). These results led us to conclude that a hydrophobic pocket is located in the substrate-binding domain of the rabbit liver and kidney enzymes. The enzyme purified from rabbit heart also reduced acetohexamide. However, the rabbit heart enzyme had little or no ability to reduce the analogs substituted with ethyl, *n*-propyl, *n*-butyl, and *iso*-propyl groups instead of the methyl group in acetohexamide.

Interestingly, the rabbit heart enzyme was found to reduce the analogs substituted with *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and *iso*-propyl groups instead of the cyclohexyl group in acetohexamide. The enzyme exhibited the highest V_{max}/K_m value for the analog substituted with an *n*-hexyl group. The number of carbon atoms in straight-chain alkyl groups appears to influence the catalytic activity of the enzyme for these acetohexamide analogs. Further-

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more, the V_{max}/K_m value of the enzyme for the analog substituted with an *n*-pentyl group was much higher than that for acetohexamide, even though these substrates have similar partition coefficients. In addition to the hydrophobicity, a steric effect may contribute to the catalytic activity of the enzyme for acetohexamide and its analogs substituted with various alkyl groups instead of the cyclohexyl group.

The enzyme purified from rabbit heart was inhibited by phenobarbital. Phenobarbital at the same concentration has little effect on carbonyl reductases purified from rabbit liver and kidney (*5, 8*), though it is a potent inhibitor of aldehyde reductase (*23*). Consequently, the rabbit heart enzyme may be classified as an aldehyde reductase rather than a carbonyl reductase. However, the rabbit heart enzyme had no ability to reduce D-glucuronic acid; aldehyde reductase is characterized by having the ability to reduce D-glucuronic acid (*24*). Thus, we can not yet definitely classify the enzyme purified here into either category, carbonyl reductase or aldehyde reductase. The rabbit heart enzyme was also inhibited by *p*-CMB. Since *p*-CMB is a thiol-specific reagent, a cysteine residue may be essential for the catalytic activity of the enzyme. We are currently investigating chemical modification of functional amino acid residues, including the cysteine residue located in the active site of the enzyme.

Carbonyl reductase, based on similar broad and overlapping substrate specificities and physicochemical properties, has been regarded as a member of the aldo-keto reductase family, like aldehyde reductase and aldose reductase [EC 1.1.1.21] (*25*). Wermuth *et al.* (*26*) have recently shown that the primary structure of carbonyl reductase from human placenta differs from those of aldo-keto reductase family proteins and has a significant homology with those of short-chain alcohol dehydrogenase family proteins. To determine the appropriate classification of the acetohexamide-reducing enzyme purified from rabbit heart, detailed studies, including analysis of the amino acid sequence, are required.

In conclusion, the present study provides evidence that the acetohexamide-reducing enzyme purified from rabbit heart is a novel tetrameric enzyme with a very limited substrate specificity. Whether or not the enzyme has catalytic activity towards endogenous substrate(s) remains to be clarified.

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