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Characterization of Acetohexamide Reductases Purified from Rabbit Liver, Kidney, and Heart: Structural Requirements for Substrates and Inhibitors

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The structural requirements of acetohexamide reductases purified from rabbit liver, kidney, and heart for substrates and inhibitors were examined. Acetohexamide, an oral antidiabetic drug with a ketone group, and analogs of it with various alkyl groups instead of the cyclohexyl group were used as substrates for these three enzymes. The results obtained as to substrate specificity suggested that the nature of the substrate-binding region of the heart enzyme is markedly different from those of the substrate-binding regions of the liver and kidney enzymes. Tolbutamide, which has no ketone group within its chemical structure, strongly inhibited the heart enzyme, whereas it had little ability to inhibit the liver or kidney enzyme. The inhibition of the heart enzyme by tolbutamide was competitive with respect to acetohexamide and uncompetitive with respect to NADPH. Furthermore, tolbutamide analogs with *n*-pentyl and *n*-hexyl groups instead of the *n*-butyl group exhibited very pronounced inhibition of only the heart enzyme. Therefore, it is reasonable to postulate that the heart enzyme, unlike the liver and kidney ones, has a cleft of a strongly hydrophobic nature near its substrate-binding region, and that this hydrophobic cleft plays a critical role in the interaction of the heart enzyme with the cyclohexyl group of acetohexamide.

Key words: acetohexamide analog, acetohexamide reductase, drug-metabolizing enzyme, structural requirement, substrate-binding region.

Carbonyl reductase [EC 1.1.1.184] is a member of the aldo-keto reductase superfamily similar to aldehyde reductase [EC 1.1.1.2] and aldose reductase [EC 1.1.1.21] (*1, 2*). Carbonyl reductase can catalyze the metabolic reduction of drugs having a ketone group within their chemical structures to the corresponding alcohol metabolites (*3, 4*). Many carbonyl reductases have been purified from various tissues of mammalian species, including man (*5-9*). Furthermore, the primary structures of some carbonyl reductases have been determined (*10-13*). However, the structural and functional properties of the active center of carbonyl reductase are poorly understood.

We recently purified three reductases from rabbit liver, kidney, and heart using acetohexamide, an oral antidiabetic drug with a ketone group, as a substrate (*14-17*). Among these acetohexamide reductases, the liver and kidney enzymes, based on their substrate specificities and inhibitor sensitivities, were classified as carbonyl reductases, because they effectively reduced drugs with a ketone group such as befunolol, metyrapone, and daunorubicin. On the other hand, the heart enzyme had no ability to reduce drugs with a ketone group, except acetohexamide and analogs of it with various alkyl groups instead of the cyclohexyl group (*17*). In the present study, we compared the structural requirements of these three enzymes for substrates (acetohexamide and its analogs) and inhibitors (tolbutamide and its analogs), and propose models for their substrate-binding regions.

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MATERIALS AND METHODS

Materials—Acetohexamide was a gift from Shionogi (Osaka). Tolbutamide (Hoechest Japan, Tokyo), carbutamide (Sumitomo Chemical, Osaka), and chlorpropamide (Ono Pharmaceutical, Osaka) were provided by the respective manufacturers. Cibacron Blue (F3GA) was obtained from Sigma Chemicals (St. Louis, MO, USA) and NADPH was from Oriental Yeast (Tokyo). Other chemicals were purchased from Wako Pure Chemicals (Osaka) or Nacalai Tesque (Kyoto). Three acetohexamide reductases were purified to homogeneity from the cytosolic fractions of rabbit liver, kidney, and heart according to the methods reported previously (*14-17*).

Synthesis of Acetohexamide Analogs—Acetohexamide analogs with various alkyl groups instead of the methyl group were synthesized from ethylbenzene as described previously (*17, 18*).

Synthesis of Tolbutamide Analogs—Tolbutamide analogs were synthesized from commercially available *p*-toluenesulfonamide. To a solution of *p*-toluenesulfonamide (2.04 g in benzene), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 2.46 g, 1.3 equivalents), and *n*-propyl isocyanate

(1.28 g, 1.3 equivalents) were added. The reaction mixture was stirred for 12 h at room temperature, diluted with aqueous 6 M HCl and then extracted with ethyl acetate. The organic layer was washed with saturated brine and then dried over anhydrous magnesium sulfate. Evaporation of the organic solvent under reduced pressure gave an oily product, which was chromatographed on silica gel to afford 4-methyl-*N*-(*n*-propylcarbamoyl)benzenesulfonamide (tolbutamide analog with *n*-propyl group instead of the *n*-butyl group) from the *n*-hexane/ethyl acetate (2 : 1, v/v) eluate. Recrystallization of the crude product gave colorless needles (2.9 g, 95% yield). 4-Methyl-*N*-(*n*-pentylcarbamonyl)benzenesulfonamide (90% yield) and 4-methyl-*N*-(*n*-hexylcarbamonyl)benzenesulfonamide (68% yield) were also synthesized in the same way to as mentioned above, respectively. The structures of these tolbutamide analogs were confirmed by elemental analysis and spectroscopic methods.

Enzyme Assay—The activities of the three acetohexamide reductases were assayed spectrophotometrically by monitoring the oxidation of NADPH at 340 nm. The reaction mixture, with 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 the enzyme. Tolbutamide and its analogs used as inhibitors, being insufficiently soluble in the buffer, were dissolved in methanol and then added to the reaction mixture to give a final methanol concentration of below 5.0%, which has no effect on the enzyme activity. The reaction was initiated by addition of the enzyme. One unit of enzyme activity was defined as the amount catalyzing the oxidation of 1 μmol of NADPH/min at 30°C. Protein concentrations were determined by a modification of the method of Lowry *et al.* (*19*) using bovine serum albumin as the standard.

Kinetic Analysis—The K_m and V_{max} values of the enzyme for acetohexamide and its analog were determined by the least-squares linear regression of double reciprocal plots. The inhibition constants, K_{Is} and K_{Ii}, were determined from replots of the slopes and intercepts, respectively, of double-reciprocal plots in the presence of inhibitors. The initial velocities in the asymptotic region which follow Michaelis kinetics were fitted to Eq. 1 by using a computer program for least-squares linear regression;

$$v = VAB/(K_{IA}K_B + K_AB + K_BA + AB) \quad (1)$$

where A and B are the concentrations of the substrates, and K_A and K_B are the Michaelis constants for the substrates. K_{IA} is the dissociation constant of the binary enzyme-substrate complex, and v and V are the initial and maximum velocities, respectively.

RESULTS

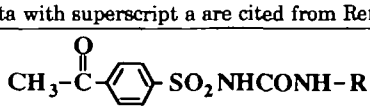
Substrate Specificities for Acetohexamide and Its Analogs—Table I summarizes the substrate specificities of acetohexamide reductases purified from liver, kidney, and heart for acetohexamide and its analogs with various alkyl groups instead of the cyclohexyl group. All the V_{max}/K_m values of the liver, kidney, and heart enzymes for acetohexamide analogs increased with increasing numbers of carbon atoms in straight-chain alkyl groups. Interestingly, the magnitude of the increase in the V_{max}/K_m values was found to be much larger for the heart enzyme than for the liver and kidney ones. Furthermore, the heart enzyme exhibited a higher V_{max}/K_m value for the analog substituted with an *n*-propyl group than that with an *iso*-propyl group. The V_{max}/K_m value of the heart enzyme for the acetohexamide analog substituted with an *n*-pentyl group was much higher than that for acetohexamide, which has a cyclohexyl group; these substrates exhibited similar partition coefficients.

Inhibition by Sulfonylurea Drugs—The inhibitory effects of tolbutamide, carbutamide, and chlorpropamide, each at a final concentration of 0.5 mM, on the reduction of acetohexamide catalyzed by the liver, kidney, and heart enzymes were examined. These sulfonylurea drugs, unlike acetohexamide, have no ketone group within their chemical structures. As shown in Table II, tolbutamide and carbutamide were potent inhibitors of the heart enzyme. Chlorpropamide also slightly inhibited the heart enzyme. Carbutamide and chlorpropamide slightly inhibited the kidney enzyme. These sulfonylurea drugs, however, had little effect on the liver enzyme. The mechanism of inhibition of the heart enzyme by tolbutamide was further examined. As is evident from Fig. 1, tolbutamide inhibited the heart

TABLE II. **Inhibition of acetohexamide reductases from rabbit liver, kidney, and heart by sulfonylurea drugs.** The concentrations of acetohexamide (substrate) and sulfonylurea drugs (inhibitors) were 1.0 and 0.5 mM, respectively. The values are the means±SD of three experiments.

	R_1	R_2	Inhibition (%)		
			Liver	Kidney	Heart
Tolbutamide	Methyl	*n*-Butyl	4.0±3.6	5.4±0.3	60.0±2.0
Carbutamide	Amino	*n*-Butyl	1.0±1.7	13.3±3.4	61.7±2.1
Chlorpropamide	Chloro	*n*-Propyl	0.3±0.6	12.8±4.2	10.7±2.3

TABLE I. **Catalytic properties of acetohexamide reductases from rabbit liver, kidney, and heart as to acetohexamide and its analogs.** The asterisk denotes acetohexamide. Data with superscript a are cited from Ref. *17*. PC: partition coefficient.



R	K_m (mM)			V_{max} (units/mg)			V_{max}/K_m (units/mg/mM)			PC[a]
	Liver	Kidney	Heart[a]	Liver	Kidney	Heart[a]	Liver	Kidney	Heart[a]	
Cyclohexyl[a]	0.72	0.41	0.74	2.20	1.11	3.86	3.1	2.7	5.2	9.1
n-Propyl	0.92	1.82	3.48	0.56	0.88	5.45	0.6	0.5	1.6	0.9
n-Butyl	1.35	0.77	0.45	1.36	1.00	6.44	1.0	1.3	14.3	2.9
n-Pentyl	0.43	0.49	0.07	0.92	1.51	4.13	2.1	3.1	59.0	10.4
n-Hexyl	0.28	0.14	0.05	2.00	1.66	3.63	7.1	11.9	72.6	44.5
iso-Propyl	0.69	1.05	2.15	0.54	0.51	1.08	0.8	0.5	0.5	0.8

enzyme competitively with respect to acetohexamide and uncompetitively with respect to NADPH. The inhibition patterns were confirmed by comparing the values of the two inhibition constants, K_{is} and K_{ii} (Table III). These results suggest that tolbutamide competes with acetohexamide at the substrate-binding region of the heart enzyme. The inhibition constants for the liver and kidney enzymes could not be determined, because tolbutamide caused little inhibition of these enzymes, even at a final concentration of 0.8 mM (liver, 6.7±3.2%; kidney, 7.3±4.8%).

Inhibition by Tolbutamide and Its Analogs—Table IV shows the inhibitory effects of tolubutamide and its analogs on the reduction of acetohexamide catalyzed by the liver, kidney, and heart enzymes. Tolbutamide analogs substituted with *n*-pentyl and *n*-hexyl groups could not be dissolved sufficiently at a final concentration of 0.5 mM in the buffer used for the enzyme assay. Thus the inhibition experiments were performed with a final concentration of 0.2 mM instead of 0.5 mM. Tolbutamide analogs substituted with *n*-pentyl and *n*-hexyl groups exhibited very pronounced inhibition of only the heart enzyme. In addition, the inhibi-

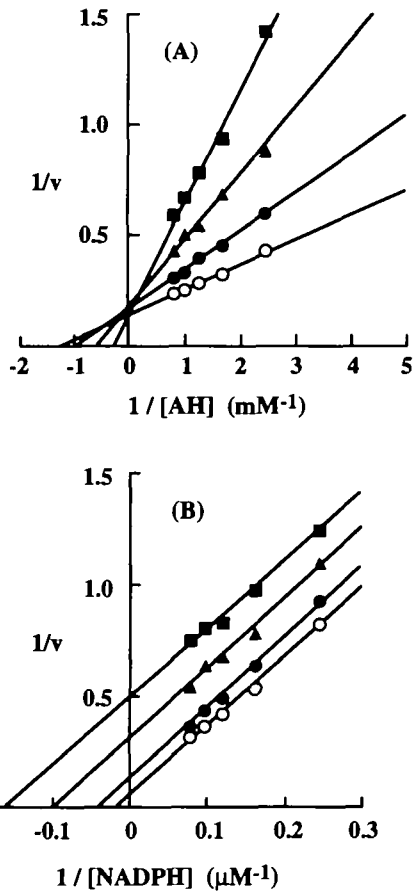


Fig. 1. **Inhibitory effect of tolbutamide on acetohexamide reductase from rabbit heart.** (A) The concentrations of tolbutamide were 0 mM (○), 0.1 mM (●), 0.3 mM (▲), and 0.5 mM (■). The concentration of NADPH was 0.25 mM. (B) The concentrations of tolbutamide were 0 mM (○), 0.1 mM (●), 0.3 mM (▲), and 0.5 mM (■). The concentration of acetohexamide (AH) was 1.0 mM. Velocity is expressed as units/mg.

TABLE III. **Inhibition patterns of rabbit heart acetohexamide reductase with tolbutamide.** AH: acetohexamide.

Varied substrate	Fixed substrate	Inhibition pattern	Inhibition constant	
			K_{is} (μM)	K_{ii} (μM)
AH	0.25 mM NADPH	Competitive	138	–
NADPH	1.0 mM AH	Uncompetitive	–	38

TABLE IV. **Inhibition of acetohexamide reductases from rabbit liver, kidney, and heart by tolbutamide and its analogs.** The asterisk denotes tolbutamide. The concentrations of the substrate (acetohexamide) and inhibitors were 1.0 and 0.2 mM, respectively. The values are the means±SD of three experiments.

CH_3-⟨⟩-$SO_2NHCONH$-R

R	Inhibition (%)		
	Liver	Kidney	Heart
n-Propyl	1.9±1.4	2.1±1.4	13.1±1.5
n-Butyl*	6.0±5.0	4.6±3.5	35.5±1.8
n-Pentyl	7.7±4.9	7.0±2.3	78.4±0.9
n-Hexyl	12.2±5.9	9.3±2.1	83.7±0.7

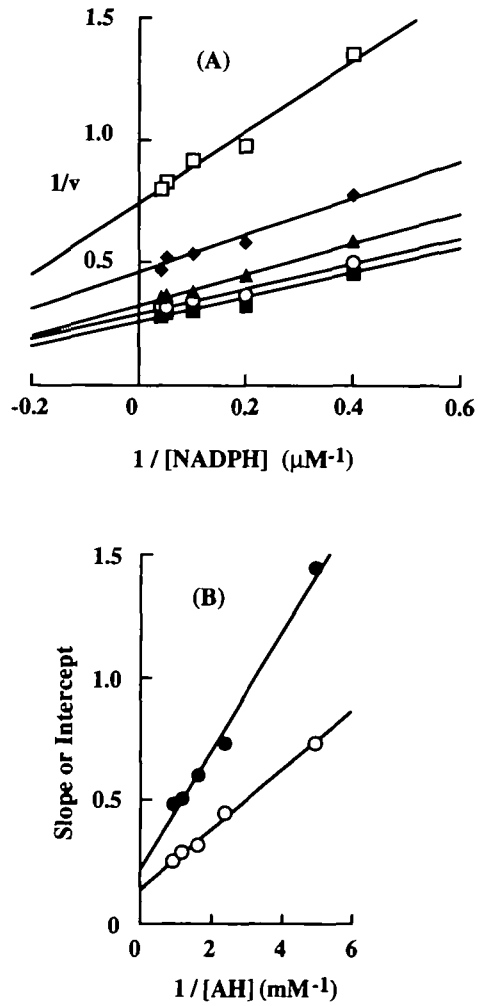


Fig. 2. **Initial velocity analysis of acetohexamide reductase from rabbit heart.** (A) Double-reciprocal plots against the NADPH concentration at several acetohexamide (AH) concentrations. The concentrations of AH were 0.2 mM (□), 0.4 mM (◆), 0.6 mM (▲), 0.8 mM (○), and 1.0 mM (■). (B) Replots of the slopes (●) and intercepts (○). Velocity is expressed as units/mg.

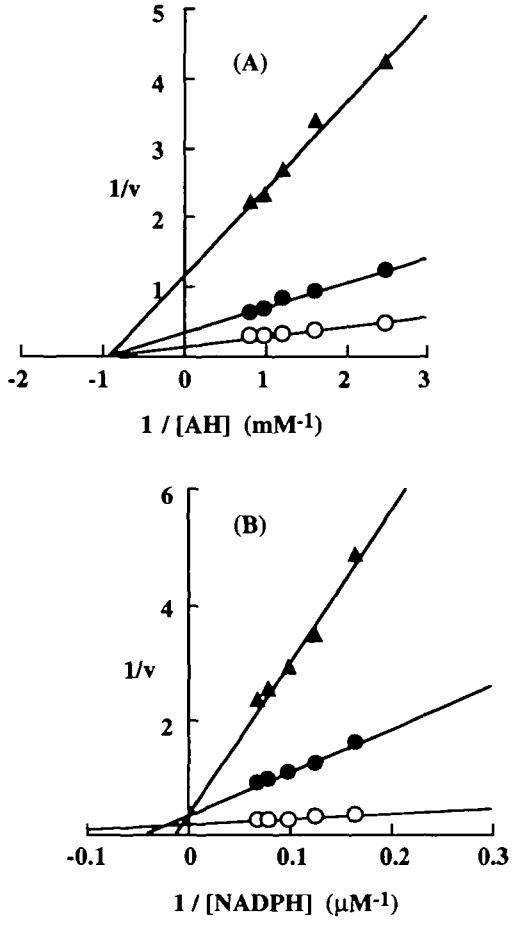


Fig. 3. **Inhibitory effect of Cibacron Blue on acetohexamide reductase from rabbit heart.** (A) The concentrations of Cibacron Blue were 0 μM (○), 0.5 μM (●), and 1.0 μM (▲). The concentration of NADPH was 0.25 mM. (B) The concentrations of Cibacron Blue were 0 μM (○), 1.0 μM (●), and 2.0 μM (▲). The concentration of acetohexamide (AH) was 1.0 mM. Velocity is expressed as units/mg.

tion of the heart enzyme by tolbutamide and its analogs was enhanced with increasing numbers of carbon atoms in straight-chain alkyl groups.

Kinetic Mechanism of Acetohexamide Reduction—To determine kinetically the mechanism underlying the reduction of acetohexamide catalyzed by the heart enzyme, the initial velocity analysis was performed as a function of NADPH concentration. Double-reciprocal plots against variable NADPH concentrations at fixed acetohexamide concentrations gave a series of intersecting lines, as shown in Fig. 2A. The slopes and intercepts of the lines were replotted against reciprocals of the fixed acetohexamide concentrations and were linear in each case (Fig. 2B). The values of V, K_{NADPH}, $K_{acetohexamide}$, and K_{iNADPH} obtained on kinetic analysis were 7.14 units/mg, 1.49 μM, 864 μM, and 2.01 μM, respectively. These results were consistent with a reaction mechanism that proceeds in a sequential manner.

Cibacron Blue is known to interact with the coenzyme-binding sites of many NADP(H)-dependent enzymes and is widely used as a dead-end type inhibitor (*20-22*). As shown in Fig. 3, Cibacron Blue inhibited the heart enzyme noncompetitively with respect to acetohexamide and competitively with respect to NADPH. The results as to the dead-end inhibition by Cibacron Blue indicate that the reduction of acetohexamide catalyzed by the heart enzyme follows an ordered Bi Bi mechanism (*23*), in which NADPH binds to the enzyme first and NADP leaves from the enzyme last. A similar kinetic mechanism has been proposed for the reduction of acetohexamide catalyzed by the liver and kidney enzymes (*24, 25*).

DISCUSSION

A variety of carbonyl reductases have been purified from liver (*7, 26*), kidney (*8, 27*), lung (*6, 28*), testis (*29*), and brain (*5*) of mammalian species. We have also purified acetohexamide reductases, which belong to carbonyl reductases, from rabbit liver and kidney (*14-16*), and examined the catalytic activities of these enzymes toward analogs with various alkyl groups instead of the methyl group adjacent to the ketone group in acetohexamide. As expected, the liver and kidney enzymes effectively reduced these analogs (*15, 18*). Furthermore, the results obtained led us to conclude that the liver and kidney enzymes have a hydrophobic pocket of five carbon atoms in length, which interacts with alkyl groups adjacent to the ketone group in acetohexamide analogs (*15, 18*). On the other hand, acetohexamide reductase purified from rabbit heart exhibited little ability to reduce these analogs (*17*). Therefore, it is likely that the region of the heart enzyme interacting with the methyl group adjacent to the ketone group in acetohexamide comprises a very limited cavity.

In the present study, we further examined the catalytic activities of the liver, kidney, and heart enzymes toward analogs with various alkyl groups instead of the cyclohexyl group in acetohexamide. The V_{max}/K_m values of the heart enzyme for these analogs markedly increased with increasing numbers of carbon atoms in straight-chain alkyl groups, indicating that the heart enzyme has a strongly hydrophobic region responsible for the binding to the cyclohexyl group of acetohexamide. Interestingly, the V_{max}/K_m value of the heart enzyme for the acetohexamide analog substituted with an *n*-pentyl group was much higher than that for acetohexamide itself, even though these two substrates exhibit similar partition coefficients. It is reasonable to assume that the strongly hydrophobic region of the heart enzyme forms a cleft with steric hindrance. The V_{max}/K_m values of the liver and kidney enzymes for acetohexamide and its analogs slightly increased with increasing numbers of carbon atoms in straight-chain alkyl groups. The liver and kidney enzymes, unlike the heart one, are likely to have a weakly hydrophobic region that interacts with the cyclohexyl group of acetohexamide.

To establish the characteristics of the substrate-binding regions of the liver, kidney, and heart enzymes, the inhibitory effects of sulfonylurea drugs on the reduction of acetohexamide catalyzed by these enzymes were compared. Tolbutamide and carbutamide, which have an *n*-butyl group of four carbon atoms, were potent inhibitors of the heart enzyme. In contrast, chlorpropamide, which has an *n*-propyl group of three carbon atoms, was a weak inhibitor of the heart enzyme. Tolbutamide also inhibited the heart enzyme competitively with respect to acetohexamide and uncompetitively with respect to NADPH. Since the reduction of acetohexamide catalyzed by the heart enzyme

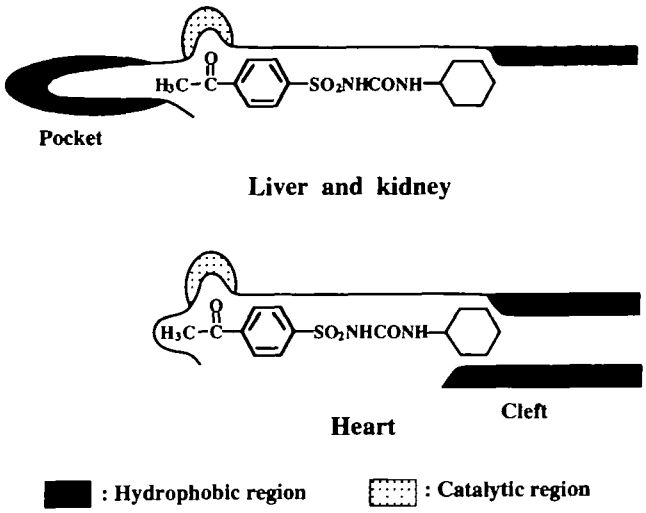


Fig. 4. **Proposed models for substrate-binding regions of acetohexamide reductases from rabbit liver, kidney, and heart.**

follows an ordered Bi Bi mechanism, tolbutamide probably only binds to the complex of the heart enzyme with NADPH (*23*). Furthermore, the heart enzyme was found to be more strongly inhibited by the analogs substituted with *n*-pentyl and *n*-hexyl groups than by tolbutamide itself. We conclude that these two alkyl groups of tolbutamide analogs are firmly inserted into the strongly hydrophobic cleft neighboring the substrate-binding region of the heart enzyme. The acetohexamide reductase from rabbit heart, unlike the monomeric acetohexamide reductases from rabbit liver and kidney, is a tetrameric enzyme, being composed of four identical-size subunits (*17*). Thus, the hydrophobic cleft of the heart enzyme may be formed at the junction of subunits. The inhibitory effects of tolbutamide and its analogs on the reduction of acetohexamide catalyzed by the liver and kidney enzymes were also examined. Judging from the inhibitory effects, it is evident that the liver or kidney enzyme lacks such a strongly hydrophobic cleft, but has a weakly hydrophobic region. On the basis of all the results obtained in our studies (*15, 17, 18*), proposed models for the substrate-binding regions of acetohexamide reductases purified from rabbit liver, kidney, and heart are shown in Fig. 4.

Acetohexamide reductases from rabbit liver and kidney exhibit broad substrate specificities for drugs with a ketone group and function as drug-metabolizing enzymes, whereas the enzyme purified from rabbit heart exhibits a high substrate specificity and probably plays a role in the metabolism of endogenous aldehydes and ketones. Of the drugs with a ketone group tested, only acetohexamide was effectively reduced by the heart enzyme. However, there is no endogenous compound which shows structural resemblance to acetohexamide or analogs of it with various alkyl groups instead of the cyclohexyl group. It is of interest to determine biochemically and pharmacologically the reason why the heart enzyme can catalyze the reduction of acetohexamide and its analogs. Our preliminary work demonstrated that the amino acid sequences of some peptides obtained on hydrolysis by the heart enzyme exhibit homology with the deduced amino acid sequence of the cDNA for pig lung carbonyl reductase, which is a tetrameric enzyme similar to the heart enzyme (*30*). We are currently analyzing the primary structure of the heart enzyme.

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