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Molecular Specificity of a Medium Chain Acyl-CoA Synthetase for Substrates and Inhibitors

CONFORMATIONAL ANALYSIS

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ABSTRACT. Amino acid conjugation is an important route of detoxification of xenobiotic and endogenous carboxylic acids. The specificity of the purified medium chain acyl-CoA synthetase catalyzing the first reaction of amino acid conjugation was investigated further for substrates and inhibitors. Molecular modeling techniques were applied to derive the molecular characteristics of substrates and inhibitors for the medium chain acyl-CoA synthetase. The purified enzyme accepted not only straight medium chain fatty acids but also aromatic acids. Of the arylacetic acids, activity was obtained with naphthylacetic acids, whereas introduction of a methyl group at the α-position caused loss of activity. High activity was also observed with cyclohexanoic acid. Diflunisal, 2-hydroxydodecanoic acid, and nalidixic acid inhibited the medium chain acyl-CoA synthetase activity for hexanoic acid, with K_i values of 0.8, 4.4, and 12.3 μM, respectively. The inhibitory carboxylic acids were competitive with respect to hexanoic acid. The hydroxyl or ketone (oxo) groups at the β-position of carboxylic acids were an important determinant for inhibitory activity. All substrates and inhibitors contained a flat hydrophobic region coplanar to the carboxylate group. In addition, the substrates had negative values for charge on the carbon in the β-position of carboxylic acids. BIOCHEM PHARMACOL **55**;11:1769–1775, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. medium chain acyl-CoA synthetase; inhibition; substrate specificity; steric conformation; amino acid conjugation

Xenobiotics containing a carboxyl group are widely used as drugs, herbicides, and insecticides. In addition, many xenobiotics are readily metabolized to carboxylic acids. Amino acid conjugation is the most important route of detoxification, not only of many xenobiotic carboxylic acids but also of endogenous acids. Although amino acid conjugation is considered to be metabolically stable and nontoxic, it has been suggested that the first reaction of amino acid conjugation in some cases involves a class of active intermediates implicated in toxicity. This pathway includes various interactions with lipid-biosynthetic processes, involving both hybrid fatty acids and hybrid triacylglycerols [1]. Xenobiotic acyl-CoA species may occasionally serve as precursors in the formation of hybrid fatty acids [2, 3] and hybrid triacylglycerols [4–6]; then, xenobiotics may be accumulated in tissue. It is important to predict the metabolic fate of xenobiotics containing a carboxyl group. Therefore, we have been investigating the determinants that regulate amino acid conjugation.

Conjugation of carboxylic acids with amino acids varies with the animal species and the structure of the carboxylic acids [7–10]. Amino acid conjugation is restricted to small carboxylic acids, such as aromatic, heteroaromatic, arylacetic, and aryloxyacetic acids. The shape of the substrate binding site appears to be of limited size. The pathway of amino acid conjugation consists of two sequential reactions [11] that involve the activation of the acid [12–14] with subsequent linkage to an amino acid at the amino functional group [15–17]. However, it is unknown whether the specificity of amino acid conjugation is exerted at the activation step and/or at the level of the amino acid transfer. We have reported that formation of acyl-CoA thioesters may be the key step in glycine conjugation of xenobiotic carboxylic acids [18]. Further, acyl-CoAs may serve as precursors in toxicity. The formation of acyl-CoAs in the first step of amino acid conjugation is catalyzed by a medium chain acyl-CoA synthetase. Information on the active site of this enzyme would be useful for predicting the metabolic fate of xenobiotics. However, little information is available on the active site of the medium chain acyl-CoA synthetase. We planned to gain information about the nature of the substrate-binding site by comparing features of substrates and inhibitors of the medium chain acyl-CoA synthetase.

To further characterize the medium chain acyl-CoA synthetase, we investigated the specificity of this enzyme for a series of carboxylic acids and the kinetics of inhibition by these acids. In addition, we used molecular modeling

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techniques to elucidate structural features of substrates and inhibitors of the medium chain acyl-CoA synthetase.

MATERIALS AND METHODS

Materials

Fresh bovine liver was obtained from the Nippon Hum Co. Ltd. Sephadex G-25 and DEAE-Sephacel were purchased from Pharmacia; hydroxyapatite was obtained from Nacarai Tesque, and Reactive-Green 19 from the Sigma Chemical Co. CoA, NADH, and ATP were obtained from the Kohjin Co. Ltd., and pyruvate kinase, lactate dehydrogenase, myokinase, and phosphoenolpyruvate were from the Oriental Yeast Co. Ltd. Other chemicals were analytical grade and were used as received.

Purification of a Medium Chain Acyl-CoA Synthetase

Medium chain acyl-CoA synthetase was purified from bovine liver mitochondria according to a previously described procedure [13]. Briefly, the enzyme was solubilized from mitochondria with a solution of Triton X-100 and precipitated subsequently by the addition of ammonium sulfate. The ammonium sulfate fractions (0.26 to 0.38 g/mL) were desalted by a Sephadex G-25 column (3.0 × 45 cm). Fractions containing the activity were applied to a DEAE-Sephacel column (3.5 × 15 cm) equilibrated with 10 mM of Tris–HCl buffer (pH 8.0, 2 mM of DTT,** 2 mM of $MgCl_2$) containing 10% glycerol. The active fractions eluted with 100 mM of KCl were subjected to a hydroxyapatite column (2.5 × 20 cm). The active fractions were eluted with a linear gradient of potassium phosphate (10–150 mM, pH 7.4, 2 mM of DTT, 2 mM of $MgCl_2$, 10% glycerol). Fractions containing the medium chain acyl-CoA synthetase were chromatographed further on a Reactive-Green 19 column (2.5 × 10 cm) equilibrated with 10 mM of Tris–HCl buffer (pH 7.8) containing 2 mM of DTT, 2 mM of $MgCl_2$ and 20% glycerol. The medium chain acyl-CoA synthetase was eluted with 10 mM of Tris–HCl buffer (pH 7.8) containing 100 mM of KCl, 2 mM of DTT, 2 mM of $MgCl_2$ and 20% glycerol. The enzyme was judged to be homogeneous by SDS–10% PAGE.

Assay of the Medium Chain Acyl-CoA Synthetase

The medium chain acyl-CoA synthetase activity was determined using the coupled enzyme assay as described previously [13]. The coupled enzyme assay monitored the formation of AMP, which was measured by coupling the reaction of myokinase, pyruvate kinase, and lactate dehydrogenase with the oxidation of NADH. Absorbance of the reaction mixture was measured at 340 nm.

To examine the inhibitory ability of a series of carboxylic acids, the purified medium chain acyl-CoA synthetase (corresponding to 1–5 μg of protein) was preincubated with a 0.25-mM concentration of selected carboxylic acids for 15 min at 37°. The reaction mixture was added and further incubated at 37° for 30 min [19]. For the coupled enzyme assay, the reaction mixture consisted of hexanoic acid as the substrate (0.32 μmol), NADH (0.36 μmol), ATP (5 μmol), CoA (0.6 μmol), $MgCl_2$ (20 μmol), phosphoenolpyruvate (1 μmol), KCl (29 μmol), myokinase (2 U), pyruvate kinase (2 U), and lactate dehydrogenase (2 U) in a final volume of 2 mL of 0.2 M of Tris–HCl buffer (pH 8.5).

The kinetics of inhibition were determined by the coupled enzyme assay as indicated above, using various concentrations of hexanoic acid and each inhibitor. The K_i values were obtained from Dixon plots that were constructed from the data.

Calculation of log P

The log P values were determined according to the method of Hansch *et al.* [20] or were obtained from the literature [8].

Molecular Modeling

To construct the theoretical model for substrates and inhibitors of the medium chain acyl-CoA synthetase, we used the AM1 and *ab initio* methods within CAChe (CAChe Scientific) and Hyper Chem (Hyper Cubic). The substrates used were hexanoic acid, benzoic acid derivatives, cyclohexanoic acid, 1- and 2-naphthoic acids, and 1- and 2-anthracenecarboxylic acids. The inhibitors were salicylic acid, 2-hydroxynaphthoic acid, 2-hydroxyphenylacetic acid, α-hydroxyphenylacetic acid, and diflunisal. All compounds in this analysis possess an aromatic ring or an aliphatic chain. The compounds are dissociated in their anionic forms under the reaction conditions (pH 8.5). The geometries of the COO^- forms of all acids were subjected to semiempirical AM1 optimization. In addition, fully optimized geometries and corresponding energies were calculated at the *ab initio* STO-3G basis set level. A fit was accepted when the energy difference between the minimal conformation energy and the energy of the molecule fitting into the model was within 10 kcal/mol. All compounds were fitted onto the carboxylate moiety. Partial charge of the minimized molecules was calculated using the AM1 program.

RESULTS

Substrate Specificity of the Medium Chain Acyl-CoA Synthetase

To further elucidate structural characteristics of carboxylic acids, we determined the substrate specificity of the medium chain acyl-CoA synthetase. The relative activities of the medium chain acyl-CoA synthetase for various substrates are shown in Table 1. The purified enzyme was specific not only for straight medium chain fatty acids but

** *Abbreviations:* DTT, dithiothreitol; and P, octanol–water partition coefficient.

TABLE 1. Substrate specificity of the medium chain acyl-CoA synthetase

Compound	Relative activity (%)
Hexanoic acid*	100
Dodecanoic acid*	39
Cyclohexanoic acid	41
Tranexamic acid	7
Benzoic acid*	19
4-Amino-*	3
4-Chloro-*	17
4-Methoxy-*	47
4-Methyl-*	59
4-Heptyl-*	66
4-Nitro-*	1
3-Amino-*	5
3-Chloro-*	12
3-Methoxy-*	48
3-Methyl-*	23
3-Nitro-*	1
2-Hydroxy-*	0
1-Naphthoic acid	11
1-Anthracenecarboxylic acid	2
2-Anthracenecarboxylic acid	16
1-Naphthylacetic acid*	18
2-Naphthylacetic acid*	21
Indomethacin	6
(−)-(*R*)-2-Phenylpropionic acid	0
(+)-(*S*)-2-Phenylpropionic acid	2
Ketoprofen	1

The specific activity for hexanoic acid was 510 nmol/min/mg of protein. Activities are expressed as percent of the specific activity of hexanoic acid. Values represent the averages of three experiments.

In the case of 1- and 2-anthracenecarboxylic acids, the concentration of substrates used was one-third the concentration of the other substrates, i.e. 165 μM.

*The activities were taken from Ref. 13.

also for aromatic and arylacetic acids. Maximal activity was found with hexanoic acid. High activities were obtained with benzoic acids having methyl, heptyl, and methoxy groups in the *para-* or *meta-*positions of the benzene ring. On the contrary, benzoic acids with amino or nitro groups in the *para-* or *meta-*positions were poor substrates. Replacing the benzene ring with a cyclohexane ring, as in cyclohexanoic acid, increased the activity. However, tranexamic acid was a very poor substrate. In the case of arylacetic acids, 1- and 2-naphthylacetic acids exhibited the same activity as benzoic acid, whereas indomethacin was a poor substrate. Introduction of the methyl group at the α-position, as in phenylpropionic acid and ketoprofen, abolished the activity.

The enzyme displayed high activities toward compounds having the carboxyl group in the 2-position (e.g. 2-anthracenecarboxylic acid and 2-naphthylacetic acid) rather than in the 1-position of the aromatic ring.

Role of Substrate Hydrophobicity in the Activity of the Medium Chain Acyl-CoA Synthetase

The acids with alkyl or alkoxyl groups that exhibited high activities were more lipophilic than those having amino or nitro groups as poor substrates [8]. Cyclohexanoic acid was 2-fold more active than benzoic acid, and was more lipophilic (log P = 2.19) than benzoic acid (log P = 1.87). However, tranexamic acid (log P = 1.15), which was less lipophilic than cyclohexanoic acid, was a very poor substrate. These results indicate that hydrophobic character is one of the determinants of enzymatic activity.

TABLE 2. Inhibition of the medium chain acyl-CoA synthetase by carboxylic acids

Inhibitor	K_i (μM)
2-Hydroxyphenylacetic acid	370 ± 35
Nalidixic acid	12.3 ± 0.9
2-Hydroxydodecanoic acid	4.4 ± 0.3
Diflunisal	0.8 ± 0.1

Inhibition constants were determined at pH 8.5, using various concentrations of the inhibitors with varied concentrations of the substrate hexanoic acid. Values are means ± SD (N = 3).

Inhibition of the Medium Chain Acyl-CoA Synthetase by Carboxylic Acids

In our previous work, the hydroxyl group at the β-position of carboxylic acids was shown to be required for inhibitory activity; salicylic acid (K_i = 37 μM), 4-methylsalicylic acid (K_i = 10.2 μM), and 2-hydroxynaphthoic acid (K_i = 5.2 μM) were potent inhibitors [19]. To further explore the interactions of inhibitors with the medium chain acyl-CoA synthetase, we determined the kinetics of inhibition by a series of carboxylic acids, which did not serve as substrates. Table 2 shows inhibition of the medium chain acyl-CoA synthetase by carboxylic acids. α-Hydroxyphenylacetic acid caused no inhibition, whereas 2-hydroxyphenylacetic acid had a weak inhibitory activity (K_i = 370 μM). The salicylic acid derivatives were relatively potent inhibitors. The order of inhibitory activity was diflunisal (K_i = 0.8 μM) > 4-methylsalicylic acid > salicylic acid > 4-aminosalicylic acid (K_i = 1675 μM). In addition, the aliphatic 2-hydroxydodecanoic acid displayed strong inhibitory activity. Substitution of a ketone (oxo) group for the hydroxyl group, as in nalidixic acid, also caused strong inhibition.

Figure 1 shows the Lineweaver–Burk plots for inhibition of the medium chain acyl-CoA synthetase activity for hexanoic acid by diflunisal and nalidixic acid. Formation of hexanoyl-CoA was inhibited competitively by diflunisal and nalidixic acid. Other inhibitory carboxylic acids were also competitive with respect to hexanoic acid.

Steric Conformation of Substrates and Inhibitors

To explore a predictive model of the active site, we studied the conformational behavior of substrates and inhibitors, using the AM1 and *ab initio* methods.

Aromatic acids. In the case of aromatic acids, all substrates used had one rigid molecule, i.e. the aromatic

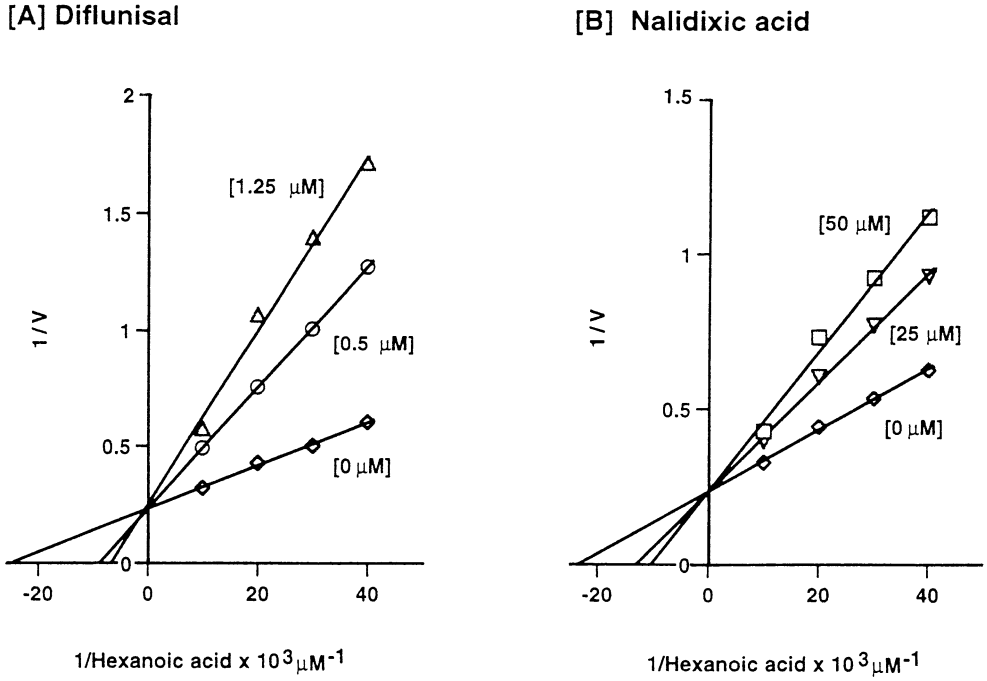

FIG. 1. Inhibition of the medium chain acyl-CoA synthetase activity for hexanoic acid by diflunisal and nalidixic acid. (A) Effect of diflunisal at concentrations of 0 μM (◇), 0.5 μM (○), and 1.25 μM (△). (B) Effect of nalidixic acid at concentrations of 0 μM (◇), 25 μM (▽), and 50 μM (□).

ring linking to the carboxylate group. In the calculated low-energy conformation of aromatic acids such as benzoic, 4-methylbenzoic, 1-anthracenecarboxylic, and 2-anthracenecarboxylic acids, a plane through the carboxylate group was kept coplanar with a plane through the aromatic ring. Although the carboxylate group was rotated about the benzene ring of benzoic acid, the changes of the conformation energies were small, within 4.3 kcal/mol above the minimum conformation energy obtained with the *ab initio* program. 2-Anthracenecarboxylic acid was more active than the 1-isomer. The low-energy conformation of 2-anthracenecarboxylic acid in the superimposition was extended to the direction of *para*-substituents of the benzene ring as compared with the conformation of the 1-isomer. This is consistent with the fact that high activities were obtained for benzoic acids having *para*-substituents.

Hexanoic acid. Hexanoic acid, which is the best substrate, is a fairly flexible molecule. Therefore, only the torsional angles O-C(1)-C(2)-C(3), C(1)-C(2)-C(3)-C(4), and C(2)-C(3)-C(4)-C(5) were rotated in 30° steps, while keeping other torsional angles fixed. The calculations indicated the existence of five low-energy conformers. The differences in conformational energy calculated for five models were fairly small (within 0.7 kcal/mol). Although the carboxylate group was rotated about the C(2)—C(3) bond of hexanoic acid, the changes of the conformation energies were small (within 0.6 kcal/mol). We proposed the conformation of hexanoic acid in which the four carbon atoms rotated are coplanar.

2-Phenylpropionic acid. (−)-(*R*)- and (+)-(*S*)-2-Phenylpropionic acids could not be superimposed with hexanoic acid in a low-energy conformation. As shown in Fig. 2, a plane through the carboxylate group became approximately orthogonal to a plane through the benzene ring of the molecule.

2-Methoxybenzoic acid. Figure 3 shows the low-energy conformation of 2-methoxybenzoic acid. In the case of the conformation with the methoxy group in the "carboxylate group" direction (A), a plane through the carboxylate group was perpendicular to the benzene plane. In the conformation with the methoxy group in the opposite direction (B), a 35° rotation of the carboxylate group about the benzene plane was required to produce a minimum-energy conformation. Because of the steric repulsion of the *ortho*-substituents, the degree of the rotation of the carbox-

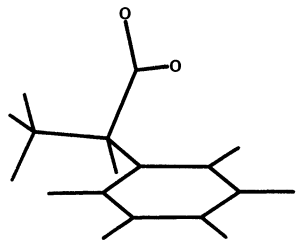

FIG. 2. Low-energy conformation of (−)-(*R*)-2-phenylpropionic acid. The geometry of the COO^- form was subjected to AM1 optimization. The energy calculated by the *ab initio* program was −309379.32 kcal/mol.

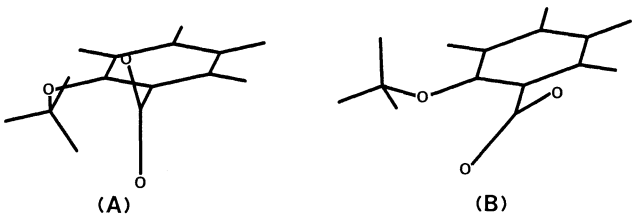

FIG. 3. Low-energy conformation of 2-methoxybenzoic acid. The geometry of the COO^- form was subjected to AM1 optimization. The energies calculated by the *ab initio* program were (A) −3292103.50 and (B) −329138.20 kcal/mol, respectively.

ylate group about the benzene plane increased with an increase of the size of the *ortho*-substituent.

Diflunisal, and salicylic, 2-hydroxynaphthoic, 2-hydroxyphenylacetic, and α-hydroxyphenylacetic acids. To gain insight into some of the parameters important in the recognition of inhibitors by the active site of the enzyme, we examined the conformation of inhibitors oriented to the active site. In the lowest-energy conformation of salicylic acid, diflunisal, and 2-hydroxynaphthoic acid as potent inhibitors, a plane through the carboxylate group of each inhibitor was kept coplanar with the planes through the benzene or naphthalene rings. Figure 4 shows the low-energy conformations of 2-hydroxyphenylacetic and α-hydroxyphenylacetic acids. The energy of fitted 2-hydroxyphenylacetic acid was no more than 10 kcal/mol above the minimal conformation energy obtained with the *ab initio* method. On the contrary, a plane through the carboxylate group of α-hydroxyphenylacetic acid could not keep the benzene plate coplanar.

Electronic Properties of Substrates and Inhibitors

Of the benzoic acid derivatives, *ortho*-substituted derivatives exhibited no activity, whereas activities were observed for *meta*- and *para*-substituted derivatives. Therefore, the electronic properties of substrates and inhibitors were examined. Table 3 shows a partial charge on the carbon atom in the 2-position of aromatic acids in the substrates, non-substrates, and inhibitors. *Ortho*-substituted benzoic acids had positive values for the charge on the carbon atom

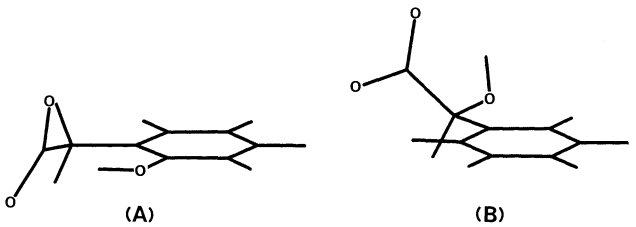

FIG. 4. Low-energy conformation of 2-hydroxyphenylacetic acid (A) and α-hydroxyphenylacetic acid (B). The geometry of the COO^- form was subjected to AM1 optimization. The energies calculated by the *ab initio* program were (A) −329241.10 and (B) −331743.61 kcal/mol, respectively.

TABLE 3. Partial charge on the carbon atom in the 2-position of aromatic ring in substrates, non-substrates, and inhibitors obtained with the AM1 program

Compound	Charge (C2)
Benzoic acid	−0.108
4-Amino-	−0.084
4-Chloro-	−0.103
4-Methoxy-	−0.081
4-Methyl-	−0.108
4-Nitro-	−0.125
3-Amino-	−0.149
3-Chloro-	−0.098
3-Methoxy-	−0.122
3-Methyl-	−0.102
3-Nitro-	−0.037
2-Amino-	0.065
2-Methoxy-	0.092
2-Hydroxy-	0.171
2-Hydroxyphenylacetic acid	0.050
1-Naphthoic acid	−0.134
2-Naphthoic acid	−0.080 (C3)*
2-Hydroxynaphthoic acid	0.166
1-Anthracenecarboxylic acid	−0.146
2-Anthracenecarboxylic acid	−0.066 (C3)*

*Partial charge on the carbon atom in the 3-position of 2-naphthoic acid and 2-anthracenecarboxylic acid.

in the *ortho*-position, whereas *meta*- and *para*-substituted benzoic acids had negative values in the *ortho*-position. Anthracenecarboxylic acids also had negative values for the charge on the carbon atom in the β-position of carboxylic acids. In the case of 2-hydroxyphenylacetic and 2-hydroxynaphthoic acids as the inhibitors, the charges of the carbon atom in the 2-position of the benzene or naphthalene rings were positive. There were no differences in the charge on the carbon and oxygen atoms except on the carbon atom in the β-position of carboxylic acids.

DISCUSSION

Amino acid conjugation is an important detoxification pathway of various carboxylic acids. Benzoic acid derivatives generally undergo glycine conjugation *in vivo* in most animals. 1-Naphthylacetic acid is also conjugated with glycine *in vivo* in the rat, mouse, rabbit, and guinea pig [21, 22]. On the contrary, 2-naphthylacetic acid undergoes three amino acid conjugations with glycine, taurine, and glutamine *in vivo* in the rat and rabbit [23]. In addition, the amino acids utilized in the *in vivo* conjugation of 2-naphthylacetic acid are glycine in the guinea pig, and taurine and glycine in the mouse [22–24]. The total amino acid conjugation of the 2-isomer was generally 1.6- to 7-fold greater than that of the 1-isomer *in vivo* in four species. Introduction of a methyl group at the α-position, as in 2-phenylpropionic acid, significantly decreases amino acid conjugation *in vivo* [25], which is quantitatively minor in the human [26], mouse and rat [27]. However, the only exceptions were the ferret [28] and the dog [29, 30], which excreted taurine conjugate *in vivo* as the major metabolite.

The purified medium chain acyl-CoA synthetase catalyzes the first step of amino acid conjugation. The substrate specificities of the present enzyme were similar to those of the enzyme partially purified (12-fold) from bovine liver mitochondria by Mahler *et al.* [12]. The extent to which the purified medium chain acyl-CoA synthetase is involved in the initial step of amino acid conjugation is unclear. We have shown recently that the present medium chain acyl-CoA synthetase is a major enzyme for glycine conjugation of substituted benzoic acids with electron-donating groups in bovine liver mitochondria [18]. On the contrary, in the case of 1- and 2-naphthylacetic acids, differences between the substrate specificity of the purified enzyme and overall *in vivo* amino acid conjugation were apparent. The present medium chain acyl-CoA synthetase does not appear to be responsible for amino acid conjugation of 2-arylpropionic acid derivatives. However, (−)-(*R*)-2-phenylpropionic acid must be activated by another isoform because it undergoes chiral inversion via the acyl-CoA. These results suggest that there are some enzymes with overlapping substrate specificities. However, the number of medium chain acyl-CoA synthetases that catalyze amino acid conjugation has not been determined. Another enzyme activating salicylate has been reported [14].

The hydroxyl or ketone (oxo) groups at the β-position of carboxylic acids were an important determinant for inhibitory activity. This finding confirmed our previous report that the hydroxyl group at the β-position of carboxylic acids is a major factor of inhibitory activity [19]. Although the inhibitors used did not serve as substrates, they were competitive with respect to hexanoic acid. Therefore, the inhibitors are accessible to the active site in a manner similar to that of the substrates; orientation of the hydroxyl or oxo groups of the inhibitors might result in formation of a hydrogen bond with one thiol group, which has been proven to be important in the catalytic activity of the medium chain acyl-CoA synthetase [13]. On the other hand, octanoic acid, and 4-chloro- and 4-nitrobenzoic acids have been reported to inhibit the formation of benzoyl-CoA by mitochondrial extracts [31]. Octanoic, benzoic, and 4-chlorobenzoic acids served as substrates for the purified medium chain acyl-CoA synthetase. Therefore, octanoic or 4-chlorobenzoic acids compete with benzoic acid as substrates for the same binding site on the medium chain acyl-CoA synthetase and, therefore, may inhibit the formation of benzoyl-CoA.

The hydrophobic character of the substrates was one of the significant factors influencing the enzymatic activity. This finding is in agreement with the finding that the lipophilicity of carboxylic acids is essential for inhibition of the medium chain acyl-CoA synthetase [19].

Further, investigation on the specificity of the medium chain acyl-CoA synthetase for structurally diverse substrates and inhibitors yields valuable insight into some of the structural features important for fitting into the active site of the enzyme. The results obtained from the molecular modeling methods of substrates and inhibitors indicate that the structural feature of the active conformation is a flat region coplanar to the carboxylate group. In the case of *ortho*-substituted derivatives and phenylpropionic acids that were inactive as substrates, the plane through the carboxylate group resulted in the benzene plane not being kept coplanar. The lack of enzyme activity toward these compounds can be explained in terms of difficulty of access to the active site of the enzyme. This explanation holds in the case of α-hydroxyphenylacetic acid, which caused no inhibition as well. The plane through the carboxylate group of α-hydroxyphenylacetic acid could not keep the benzene plate coplanar. The hydroxy group at the α-position could not render a conformation that fits to the active model. On the contrary, the molecular structure of 2-hydroxyphenylacetic acid is similar to that of α-hydroxyphenylacetic acid. However, 2-hydroxyphenylacetic acid was a weak inhibitor. One possible explanation for the decrease in inhibitory activity for 2-hydroxyphenylacetic acid is that a slightly higher conformation energy is required to bring this compound into the active conformation. The conformation seems to be greatly affected by the substituents near the carboxylate group.

In addition to steric and hydrophobic factors, the electronic factor is another property that might influence the activity of the medium chain acyl-CoA synthetase. There was a difference in the charge on the carbon in the β-position of carboxylic acids between substrates and non-substrates. The substrates had negative values for the charge on the carbon in the β-position, whereas the non-substrates had positive values in the β-position. This negativity of the charge on the carbon atom in the β-position of carboxylic acids is assumed to be important for the enzymatic activity.

The mitochondrial binding site for arylacetic acid has been reported to have three regions: a cationic region for the carboxylate group, a hydrophobic region for the aryl group, and a region for the methylene group linking the aromatic ring and the carboxylate group [25]. However, the results obtained from the molecular modeling methods did not support the idea that the present purified medium chain acyl-CoA synthetase, which displayed no activity toward (−)-(*R*)- and (+)-(*S*)-2-phenylpropionic acids, should have a specific binding site for the methylene group.

All substrates activated by the medium chain acyl-CoA synthetase contain a flat hydrophobic region coplanar to the carboxylate group and have negative values for the charge on the carbon atom in the β-position of carboxylic acids. The negatively charged position of the substrates requires a corresponding positively charged site in the protein for binding. There is a "pocket" formed by lipophilic parts of amino acids in the protein because all of the substrates contain an aromatic ring or an aliphatic chain adjacent to the carboxylate group.

Studies on the structural features of the active site of the medium chain acyl-CoA synthetase would be useful for predicting the formation of active acyl-CoAs in metabolic pathways. These results may also provide information for

exploring the rational design of more effective drugs with reduced toxicity to humans.

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