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Lipoamidase is a multiple hydrolase

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The substrate specificity of lipoamidase, purified from the pig brain membrane with lipoyl 4-aminobenzoate (LPAB) as a substrate, was extensively studied. This single polypeptide was found to hydrolyse the bonding between amide, ester and peptide compounds. However, stringent structural requirements were found in the substrates, e.g. LPAB was hydrolysed, whereas biotinyl 4-aminobenzoate was not, as stated in our previous paper [Oizmui & Hayakawa (1990) Biochem. J. **266**, 427–434]. The enzyme specifically recognized the whole molecular structure of the substrate, whereas it loosely recognized the bond structure of the substrate; e.g. the dipeptide Asp-Phe was not hydrolysed, whereas the methyl ester of Asp-Phe (aspartame) was. The exopeptidase activity was demonstrated by lipoamidase; however, longer peptides than the hexamer seemed not to be substrates. Lipoyl esters, which were electrically neutral, exhibited higher specificity with longer acyl groups. Molecular mass and molecular hydrophobicity (hydropathy) seemed to determine the substrate specificity. Lipoyl-lysine, acetylcholine and oligopeptides were hydrolysed at similar K_m values; however, acetylcholine was hydrolysed at a velocity 100 times higher. Although many similar specificities were found between electric eel acetylcholinesterase and lipoamidase, distinctly different specificity was demonstrated with lipoyl compounds. The role of lipoamidase, which resides on the brain membrane and possesses higher specificity for hydrophobic molecules, remains to be elucidated.

INTRODUCTION

Lipoamidase (lipoyl-X hydrolase, EC number not given) has been found in bacterial cells as a hydrolase which liberates lipoic acid from lipoyl proteins [α-oxoglutarate dehydrogenase complex (KGDH) or pyruvate dehydrogenase complex (PDH)], lipoyl-lysine, lipoyl methyl ester, lipoamide and lipoyl 4-aminobenzoate (LPAB) [1]. Because mitochondrial KGDH and PDH complexes were also present in the cells of mammalian tissues, together with the glycine-cleavage system [2], the cofactor or coenzyme of lipoic acid was also expected to be present in mammalian cells as a lipoyl-protein or lipoyl-lysine. Therefore, we examined the LPAB hydrolase activity in various mammalian tissues, and recently found the activity in various tissues, i.e. human serum [3], human breast milk [4] and guinea-pig liver [5].

We found that strong lipoamidase activity was present in pig brain tissues (cerebellum, cerebrum and medulla) and that the enzyme was enriched in membrane subfractions (nuclear, mitochondrial and microsomal fractions) [6]. The role of lipoic acid in the brain has not yet been established; however, lipoate-responsive brain disease was present [7].

It is of interest that the lipoamidase in the brain recognizes and acts exclusively on the vitamin lipoate moiety. Therefore, we decided to study the substrate specificity with various natural and synthetic substrates. This type of experiment contributes to increased understanding of the role of lipoamidase in the brain.

MATERIALS AND METHODS

Chemicals and reagents

The following dipeptides were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: Asp-Lys, Asp-Phe, Asp-Phe-OMe, Ser-Phe, Ser-Tyr, γ-Glu-Gln, Glu-Lys, Gly-Gly, Gly-Glu, Gly-Leu, Gly-Lys, Ala-Phe, Val-Lys, Val-Phe, Leu-Arg, Leu-Asp, Tyr-Phe, Phe-Ala, Lys-Leu, Lys-Lys, Lys-Phe, His-Lys, His-Phe, Arg-Ala, Arg-Asp, Arg-Glu, Arg-Gly, Arg-Ile, Arg-Leu, Arg-Phe, Arg-Val. The following physiological peptides were from Peninsula Laboratories, Belmont, CA, U.S.A.: acetyl-Asp-Glu, Arg-Phe-NH_2, Tyr-Arg (kyotorphin), Leu-Gly (morphine tolerance peptide), Gly-His-Lys (liver cell growth factor), < Glu-His-Pro-NH_2 (thyrotropin-releasing hormone, TRH), Phe-Met-Arg-Phe-NH_2 (FMRF amide), Tyr-Pro-Phe-Pro-NH_2 (morphiceptin), Arg-Tyr-Leu-Pro-Thr (proctolin), Tyr-D-Ser-Gly-Phe-Leu-Thr (delta-receptor peptide), Tyr-Pro-Phe-Pro-Gly-Pro-Ile (β-casomorphin), Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser (*Hu*-ras^{HA}), < Glu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH_2 (adipokinetic hormone), substance P (Arg-Pro-Lys-Pro-Glu-Glu-Phe-Phe-Gly-Leu-Met-NH_2), neurotensin (< Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu). Insulin (from bovine pancreas), and aprotinin were from Takara Shuzo Co., Kyoto, Japan. Pepstatin A (pepsin inhibitor), chymostatin (chymotrypsin inhibitor), leupeptin (serine- and thiol-protease inhibitor), eserine (physostigmine; acetylcholinesterase inhibitor), BW (acetylcholinesterase inhibitor; free base), THA (acetylcholinesterase inhibitor), arecoline (methyl l-methyl-1,2,5,6-tetrahydronicotinate hydrobromide; crystalline; acetylcholinesterase inhibitor), edrophonium chloride [ethyl (*m*-hydroxyphenyl) + dimethylammonium chloride, acetylcholinesterase inhibitor], ebelactone A (3,11-dihydroxy-2,4,6,8,10,12-hexamethyl-9-oxo-6-tetradecenoic acid 1,3-lactone), ebelactone B (2-ethyl-3,11-dihydroxy-4,6,8,10,12-pentamethyl-9-oxo-6-tetradecenoic acid 1,3-lactone; inhibitor of esterase, lipase, *N*-formylmethionine aminopeptidase), acetylcholine chloride, butyrylcholine chloride, acetylcholinesterase (EC 3.1.1.7; from electric eel; Type V-S), lipoamide, choline acetyltransferase (EC 2.3.1.6; from bovine brain), n-propionyl-CoA, n-butyryl-CoA, n-valeryl-CoA, n-hexanoyl-CoA, cholesteryl oleate, cholesteryl acetate and biotin methyl ester were also from Sigma. GSSG and GSH were from Boehringer-Mannheim Yamanouchi, Tokyo, Japan. ADAM was from Funakoshi Pharmaceutical Co., Tokyo, Japan. Reduced and oxidized forms of LPAB and lipoyl-lysine and the reduced form of lipoamide were synthesized by us

Abbreviations used: LPAB, lipoyl 4-aminobenzoate; KGDH, α-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; < Glu, pyroglutamic acid; TRH, thyrotropin-releasing hormone; THA, 9-amino-1,2,3,4-tetrahydroacridine hydrochloride; BW, 1,5-bis-[4-(allyldimethylammonium)-phenyl]pentan-3-one; DFP, di-isopropyl fluorophosphate; PMSF, phenylmethanesulphonyl fluoride; PCMB, *p*-chloromercuribenzoate; ADAM, 9-anthryldiazomethane

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according to refs. [3] and [8]. Lipoyl acyl esters were synthesized by us from lipoic acid and corresponding acyl alcohols with a catalytic amount of HCl by incubating at 70 °C for 3 h [6]. Enzymic synthesis of various acetylcholine analogues was performed by incubating choline acetyltransferase, choline and the corresponding n-fatty acyl-CoA at 37 °C for 1 h. The product was assessed by fatty acid analysis by using the method of ADAM derivative formation as described previously [9].

Lipoamidase

Pig brain lipoamidase was prepared by us [6]. Membrane was prepared from pig brain (82 g wet wt.; Pel-freez Biologicals, Rogers, AR, U.S.A.). After an eight-step purification procedure [6], lipoamidase was purified 601-fold.

Enzyme assays

The hydrolysing activity was assayed in an assay buffer of 0.1 M-sodium phosphate buffer (pH 7.0) containing 1 mM-EDTA and 10 mM-2-mercaptoethanol at 37 °C. Hydrolysis of LPAB (LPAB hydrolase activity) was assayed by the h.p.l.c. fluorimetric method described previously by us [3]. Hydrolyses of acetylcholine and ethyl acetate were assayed by directly measuring liberated acetic acid by h.p.l.c. and detecting with a refractive-index detector (Erma Co., Tokyo, Japan), because purified enzyme did not interfere with the substrate and product. Liberated choline and residual acetylcholine were also measured, and the enzyme assay was shown to be stoichiometric for purified enzyme. The liberated acetic acid was separated on a reversed-phase h.p.l.c. column (Nucleosil 5C18; 250 mm × 4.6 mm internal diam.; Macherey–Nagel, Düren, Germany) with a mobile phase of 0.1 M-sodium phosphate buffer (pH 2.1) containing 0.5 M-Na_2SO_4 at a flow rate of 1.0 ml/min. Retention times of acetic acid, ethanol, ethyl acetate and acetylcholine were 6.4, 7.1, 8.7 and 9.0 min respectively. The detection limit of acetic acid was 200 ng [signal-to-noise ratio (S/N) = 3]. Acetylcholinesterase activity was also assayed by a Cholinesterase Diagnostic Kit no. 420 (colorimetric, Kit No. 420 MC, Sigma). Hydrolysis of acetylcholine analogues were also determined by fatty acid analysis by ADAM derivative formation [9].

Hydrolyses of lipoamide and lipoyl acyl esters were assayed by reversed-phase h.p.l.c. with specific lipoate detection at 330 nm, and hydrolysis of biotin methyl ester was detected at 210 nm. Hydrolyses of peptides were assayed by a h.p.l.c. *o*-phthaldialdehyde amino acid analyser (Hitachi, Tokyo, Japan).

Protein content

Protein concentrations were determined by the method of Lowry *et al.* [10], with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, U.S.A.). BSA was used as a standard protein.

RESULTS AND DISCUSSION

The substrate specificity of lipoamidase from pig brain [6] was extensively studied. The results for the lipoyl-amide compounds are shown in Table 1. In such compounds (lipoyl-X), it seems that the smaller the molecular mass of the X moiety, the faster the reaction rate.

Electrically neutral lipoyl acyl esters were then synthesized and tested. As shown in Table 2, the longer the acyl moiety, the smaller the K_m value. Neutral lipoyl-acyl compounds with longer acyl chains seemed more specific substrates. However, the neutral esters ethyl acetate, cholesteryl acetate and cholesteryl oleate were not hydrolysed. The presence of lipoate in neutral esters seemed necessary for them to be substrates.

Because acetylcholine is abundant in the brain and has an ester bond, its hydrolysis by lipoamidase was studied, together with that of various synthetic acetylcholine analogues. As shown in Table 3, acetylcholine was hydrolysed with a high velocity. The time course of liberation of acetic acid from the substrate acetylcholine by lipoamidase was linear for 2.5 h (results not shown). The acetylcholinesterase-assay kit also gave a similar result for the hydrolysis of acetylcholine by lipoamidase. Further, the longer the acyl moiety in acylcholine, the higher the specificity, as measured by $k_{cat.}/K_m$ (Table 3, column 3). This finding with regard to acyl chain length is similar to the results obtained for lipoyl-acyl esters (Table 2); however, the increase in specificity ($k_{cat.}/K_m$ values) in acyl-choline esters is more gradual than for lipoyl-acyl esters. This different increment in specificity seems to be caused by the presence of a positive charge (tertiary amine group) in choline moiety.

As acetylcholine is a special ester which is present in nervous systems, we examined the inhibitory effects of the specific acetylcholinesterase inhibitors on the hydrolysis of acetylcholine by lipoamidase. As shown in Table 4, all the specific acetylcholinesterase inhibitors except arecoline inhibited acetylcholine hydrolysis almost completely. Serine inhibitors such as DFP and PMSF also completely inhibited the enzyme activity, whereas the thiol inhibitor PCMB did not. These findings strongly suggest that lipoamidase has a very similar active centre to that of acetylcholinesterase. However, 50% inhibition by 50 μM-PCMB was demonstrated for LPAB (amide compound) hydrolysis by lipoamidase [6].

Table 1. Lipoyl substrate (lipoyl amide compounds) specificity of pig brain lipoamidase

Purified enzyme (1.8 μg/0.1 ml of the reaction mixture) was used. Means of three determinations ± S.D. are shown. The data for lipoyl-lysine, LPAB, reduced LPAB and lipoamide had been presented in a previous paper [6].

Lipoyl substrate	K_m (μM)	$V_{max.}$ (nmol/min per mg of protein)	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Lipoyl-lysine*	333 ± 38	3.4 ± 0.22	0.022 ± 0.005
Reduced lipoyl-lysine*	105 ± 12	13.8 ± 1.56	0.307 ± 0.071
LPAB†	31 ± 2.6	8.5 ± 0.6	0.639 ± 0.099
Reduced LPAB†	12 ± 1.8	25.5 ± 1.2	4.96 ± 1.00
Lipoamide‡	502 ± 46	98.8 ± 13	0.460 ± 0.103
Reduced lipoamide‡	164 ± 18	326 ± 32	4.64 ± 0.977

* Determined with an h.p.l.c. amino acid analyser.
† Determined by the h.p.l.c.-fluorimetric PAB assay method [3].
† Lipoate and reduced lipoate determined by h.p.l.c. method [8] with u.v. detection at 330 nm and by refractive-index detector respectively.

Table 2. Effect of acyl groups of lipoyl esters on lipoamidase activity

Liberated lipoic acid from the ester was measured as described in the Materials and methods section. The amount of enzyme used and data processing were the same as in Table 1. The data for lipoyl methyl and ethyl esters are presented in a previous paper [6].

Lipoyl substrate	K_m (μM)	$V_{max.}$ (nmol/min per mg of protein)	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Lipoyl methyl ester	64±3.7	166.0±11	6.25±0.79
Lipoyl ethyl ester	37±1.4	129.2±13	8.16±1.13
Lipoyl n-propyl ester	26±2.1	208.5±21	18.7±3.40
Lipoyl isopropyl ester	23±3.2	212.6±23	21.7±5.45
Lipoyl n-butyl ester	18±2.3	366.6±58	47.5±13.8
Lipoyl isoamyl ester	11±1.9	456.8±73	97.3±33.2

Table 3. Effect of various acetylcholine analogues as substrates on lipoamidase activity

The amount of enzyme used and data processing were the same as in Table 1. Liberation of fatty acids was assayed as described in the Materials and methods section.

Substrate	K_m (μM)	$V_{max.}$ (nmol/min per mg of protein)	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Acetylcholine	465±41	2.05±0.25	10.3±2.2
Propionylcholine*	432±48	2.23±0.27	12.1±1.5
n-Butyrylcholine	403±35	2.68±024	15.5±1.4
n-Valerylcholine*	368±14	3.04±0.31	19.3±2.1
n-Hexanoylcholine*	334±39	3.76±0.48	26.3±2.7

* Enzymic syntheses were performed as described in the Materials and methods section.

Table 4. Effect of acetylcholinesterase inhibitors on hydrolysis of acetylcholine by lipoamidase

The amount of enzyme used was 1.8 μg per 0.1 ml of the reaction mixture. Liberation of acetic acid was measured as described in the Materials and methods section. Specific activity without inhibitor was 1.95 μmol of acetic acid liberated/min per mg of protein.

Inhibitor	Concn. (mM)	Inhibition (%)
BW	0.01	100
Edrophonium chloride	0.01	85
Eserine	0.01	89
THA	0.01	90
Arecoline	1.0	0
Atropine	10.0	45
Acetylcholine	10.0	0
Butyrylcholine	10.0	0
DFP	0.01	100
PMSF	0.01	100
PCMB	0.05	0
Ebelactone A	1.0	33
Ebelactone B	1.0	7

As previously shown by Chubb *et al.* [11], purified acetylcholinesterase exhibited peptidase activity. The replacement of lipoate by amino acid in lipoyl-lysine produces a dipeptide. Therefore, we considered whether or not lipoamidase could also hydrolyse peptide bonds. Possible liberation of amino acids from the physiological oligopeptides (containing 2–58 amino acids) by brain lipoamidase was monitored by using the h.p.l.c. amino acid analyser. Although considerable numbers of dipeptides (15 out of 35 tested dipeptides) could not be hydrolysed by the enzyme, such a dipeptide as Leu-Arg was hydrolysed (Table 5). This observation is similar to that by Chubb *et al.* [11]. Considerable numbers of hydrolysable physiological di-, tri-, tetra- and penta-peptides were found, as shown in Table 5, but no specific conclusions regarding the substrate specificity could be drawn from these results. However, as indicated by arrows in Table 5 (column 1), the *N*-terminal amino acid is mainly liberated by lipoamidase. Hydrolysis of oligopeptides ranging from di- to penta-mers was observed; however, hydrolysis of more than hexamers (except the octomer Hu-ras^{HA}) seemed to be difficult. Moreover, lipoamidase exhibited no exo-oligopeptidase activity on the oligopeptides of 9 to 58 residues (aprotinin). Oligopeptides having a molecular mass less than 2000 seemed to be the substrates of lipoamidase.

In a more simple case of Arg-Xaa-type dipeptide, the substrate specificity characteristics were clearly shown (Table 6). Substrate specificity (Table 6, column 3) coincided well with the hydrophobicity calculated according by the method of Nozaki & Tanford [12]. This increase in specificity with hydrophobicity seemed valid when molecular masses and charges in dipeptides were similar.

Lipoamidase has all three (amidase, esterase and peptidase) hydrolysing activities, and this feature is very similar to acetylcholinesterase [11,13]. Therefore, it is of interest whether or not there is a correlation between brain lipoamidase and the

Table 5. Exopeptidase activity of lipoamidase on various physiological dipeptides and peptides

The amount of enzyme used and data processing were the same as in Table 1. –, Not hydrolysed within 18 h incubation time.

Peptide		K_m (μM)	$V_{max.}$ (nmol/min per mg of protein)
Structure	Name		
Leu-Arg	Dipeptide	833±74	0.34±0.026
Tyr-Arg	Kyotorphin	250±15	0.26±0.018
Asp-Phe-OMe	Aspartame	1670±184	0.8±0.072
Arg-Phe-NH_2	Neuropeptide	208±8	0.2±0.016
γ-Glu-Gln	Dipeptide	909±80	8.5±1.02
γ-Glu-Cys-Gly ↑	GSH (3-mer)	59±6.5	0.7±0.056
Gly-His-Lys ↑	Liver cell growth factor (3-mer)	909±45	2.7±0.189
Phe-Met-Arg-Phe-NH_2 ↑	FMRF-amide (4-mer)	625±50	0.3±0.018
Arg-Tyr-Leu-Pro-Thr ↑	Proctolin (5-mer)	182±13	0.36±0.047
↓ γ-Glu-Cys-Gly | γ-Glu-Cys-Gly ↑	GSSG (6-mer)	417±38	0.3±0.020
Tyr-D-Ser-Gly-Phe-Leu-Thr	Delta-receptor peptide (6-mer)	–	–
Tyr-Pro-Phe-Pro-Gly-Pro-Ile	β-Casomorphine (7-mer)	–	–
Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser ↑ ↑	*Hu*-ras^{HA} (8-mer)	833±58	1.4±0.126
<Glu-Leu---Trp-Gly-NH_2	Adipokinetic hormone (9-mer)	–	–
Arg-Pro---Leu-Met-NH_2	Substance P (11-mer)	–	–
<Glu-Leu---Ile-Leu	Neurotensin (13-mer)	–	–
A: Gly-Ile---Cys-Asn B: Phe-Val---Lys-Ala	Insulin (51-mer)	–	–

Table 6. Substrate specificity of Arg-Xaa dipeptide with respect to hydrophobicity

The amount of enzyme used and the data processing were the same as in Table 1. Hydrophobicity was calculated in accordance with Nozaki & Tanford [12]. Key: –, not hydrolysed; ND, not determined.

Arg-Xaa	$V_{max.}$ (pmol/min per mg of protein)	K_m (μM)	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	Hydrophobicity
Arg-Glu	–	–	ND	0.0
Arg-Asp	–	–	ND	0.0
Arg-Ala	339±20	2500±195	0.32±0.026	0.3
Arg-Val	338±24	1176±94	0.67±0.034	0.8
Arg-Leu	358±16	1111±49	0.75±0.083	0.9
Arg-Ile	823±49	2111±162	0.91±0.070	0.9
Arg-Phe	2089±181	3333±398	1.46±0.175	1.3
Arg-Phe-Phe	1976±178	667±58	6.9±0.62	2.5
Arg-Gly	1355±81	3125±141	1.0±0.089	0.0
Arg-Phe-NH_2	195±14	208±12	2.1±0.095	ND

prominent acetylcholinesterase. There has been no previous report on the purified pig brain acetylcholinesterase, and no comparison is available at present. However, highly purified acetylcholinesterase was obtained from the electric eel. The molecular mass of this acetylcholinesterase was 70000 Da [14], whereas that of lipoamidase was 140000 Da [6]. As shown in Table 7, acetylcholinesterase from the electric eel hydrolysed dipeptides, although it did not hydrolyse either LPAB or lipoyllysine. The substrate-specificity characteristics of lipoamidase were distinctly different from those of electric eel acetylcholinesterase with respect to lipoyl compounds.

The results of Tables 4 and 7 suggest the presence of different active centre(s) for LPAB hydrolysis and for acetylcholine hydrolysis. Active centre(s) of lipoamidase seemed to be composed of complex sites and structures. On the other hand, purified acetylcholinesterase in the electric eel [13] has a subpopulation of acetylcholinesterase responsible for the peptidase activity. In the subpopulation of purified acetylcholinesterase, which has a molecular mass of 70000 Da, Small & Chubb [13] have described the two reactive centre(s), i.e. the acetylcholinesterase site and exo- and endo-peptidase sites. Purified lipoamidase, which is composed of a single polypeptide

Table 7. Comparison of K_m values between electric-eel acetylcholinesterase and lipoamidase on various substrates

The data processing was the same as in Table 1. The concentration of acetylcholinesterase (AChE) used was 50 units/ml. For lipoamidase, the amount of enzyme used was the same as in Table 1.

	K_m (μM)	
Substrate	Eel AChE	Lipoamidase
Acetylcholine	368±28	465±41
Asp-Phe-OMe (aspartame)	1264±134	1670±184
Arg-Phe	2867±143	3333±378
LPAB	Not hydrolysed*	29±2.6
Lipoyl-lysine	Not hydrolysed*	333±38

* Hydrolysis was assessed after incubation for 18 h at 37 °C.

chain, was found to have multiple enzymic functions in hydrolysing acetylcholine and as an oligoexopeptidase, other than in hydrolysing lipoyl-X compounds. This may be caused by another mechanism than an enzyme subpopulation, because chemical homogeneity of lipoamidase was already shown [6].

Lipoamidase is abundantly present in brain membranes, where various hydrophobic molecules are enriched. However, hydrophobic molecules in membranes must be metabolized promptly, because such molecules are not easily diffusible. Then membrane lipoamidase, which has a relatively wide range of substrate specificity and has higher specificity for hydrophobic compounds, might play some indispensable role(s) in brain membrane metabolism.

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