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Substrate Selectivity of Artificial Transaminase Constituted with Functionalized Bilayer Membrane†

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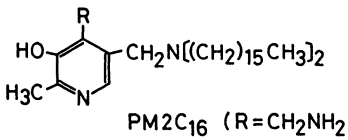
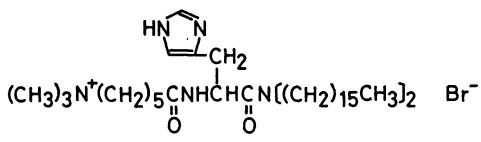
The transamination reactions of α-amino acid derivatives with α-keto acids as catalyzed by an artificial transaminase were examined in aqueous media under mild conditions. The present artificial holoenzyme was constituted with a combination of a cationic peptide lipid having a L-histidyl residue, a hydrophobic pyridoxamine derivative, and copper(II) ions, and found to exercise marked substrate selectivity which came from the molecular recognition in the course of formation of the aldimine Schiff-base chelates composed of the hydrophobic pyridoxal derivative, α-amino acids, and copper(II) ions. The transamination reaction of the hydrophobic β-benzyl L-aspartate with the hydrophilic pyruvate was most effectively catalyzed among 18 combinations of substrate species employed here. Enantioselectivity was observed for the catalytic transamination of phenylalanine with pyruvate.

Biomimetic approaches to constitution of artificial enzymes have been attracting current attention.[1] Nature has adopted α-amino acids as the main building blocks for enzymes and often cofactors, such as coenzymes and metal ions, are incorporated into their catalytic sites. Meanwhile, lipids are major constituents of biomembranes but do not directly participate in enzymatic functions. In our current approaches, we have been using the synthetic lipids having an α-amino acid residue as molecular building blocks for artificial enzymes, and demonstrated that functionalized bilayer membranes formed with the peptide lipids provide appropriate reaction sites for simulation of various enzymatic reactions.[2-4]

When a hydrophobic vitamin B_6 derivative was introduced into the bilayer membrane, the transamination reaction of an α-amino acid with an α-keto acid proceeded catalytically in the presence of copper(II) ions under mild conditions.[5] In a previous study,[6] we have modified the active site of our artificial transaminase by employing various combinations of peptide lipids and hydrophobic vitamin B_6 derivatives and investigated their catalytic performance in the transamination reaction of L-phenylalanine with pyruvate. As a result, the highest catalytic activity has been observed with the bilayer aggregates composed of a peptide lipid having a L-histidyl residue (N^+C_5-His2C_{16}) and a hydrophobic pyridoxamine derivative (PM2C_{16}) in the presence of copper(II) ions. In the present work, we studied on catalytic efficiency of the identical artificial transaminase in detail with primary emphasis on the substrate selectivity.



PM2C_{16} (R=CH_2NH_2)
PL2C_{16} (R=CHO)



N^+C_5His2C_{16}

Experimental

Materials. The following α-amino acids and their derivatives were obtained from commercial sources as guaranteed reagents and used without further purification: DL-2-aminobutanoic acid, DL-norvaline, DL-norleucine, DL-2-aminooctanoic acid, D-phenylalanine, methyl L-phenylalaninate, L- and D-glutamic acid, L-aspartic acid (these were from Nakarai Chemicals), L-phenylalanine, benzyl L-phenylalaninate, γ-benzyl L-glutamate, α,γ-dibenzyl L-glutamate, β-benzyl L-aspartate, and α,β-dibenzyl L-aspartate (these were from Protein Research Foundation). α-Benzyl L-aspartate was prepared by selective hydrolysis of α,β-dibenzyl L-aspartate in a manner similar to that reported previously[7] (Found: C, 58.96; H, 5.91; N, 6.29%. Calcd for $C_{11}H_{13}NO_4$: C, 59.19; H, 5.87; N, 6.27%). Three kinds of α-keto acids which were coupled with the above α-amino acids were commercially available as guaranteed reagents: sodium pyruvate (Wako Pure Chemical Industries), 2-oxobutanoic acid (Nakarai Chemicals), and 3-hydroxypyruvic acid (Sigma Chemical Co., Missouri, U.S.A.). Preparation and characterization of 4-aminomethyl-5-[(dihexadecylamino)methyl]-2-methyl-3-pyridinol (PM2C_{16})[6] and *N,N*-dihexadecyl-N^{α}-[6-(trimethylammonio)hexanoyl]-L-histidinamide bromide (N^+C_5-His2C_{16})[8] have been reported elsewhere. Dansyl chloride [5-(dimethylamino)-1-naphthalenesulfonyl chloride] for fluorescent labeling of amino acids was obtained from Nakarai Chemicals as a guaranteed reagent. Copper(II) perchlorate (Kishida Chemical Co.) was dissolved in deionized water and standardized by conventional chelatometric titration.

Analyses and Measurements. Elemental analyses were performed at the Microanalysis Center of Kyushu Univer-

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sity. A Beckman Φ71 pH meter equipped with a Beckman 39505 combined electrode was used for pH measurements after calibration with a combination of appropriate standard aqueous buffers. Single-walled covesicles composed of the peptide lipid and the hydrophobic vitamin B_6 derivative were prepared upon sonication of an aqueous dispersion sample of these species by a probe-type sonicator (W-220F, Heat Systems-Ultrasonics) in a manner similar to that reported previously.[5] Progress of the transamination reaction of an α-amino acid with an α-keto acid, as catalyzed by the vesicular catalyst composed of $N^+C_5His2C_{16}$, $PM2C_{16}$, and copper(II) ions in an aqueous acetate buffer (25 mmol dm^{-3}) at pH 5.0, μ 0.10 with KCl, and 30.0 °C, was monitored by measuring amounts of an amino acid produced. An amino acid derived from the corresponding α-keto acid was dansylated, separated by liquid chromatography on a column of TSK gel ODS-120T (length, 250 mm; internal diameter, 4.6 mm) with a Hitachi 655A high performance liquid chromatograph, and eluting fractions were monitored by a Hitachi F-1000 fluorescence spectrophotometer: flow rate, 1.0 mL min^{-1}; mobile phase, mixtures of Tris buffer (10 mmol dm^{-3}, pH 8.0) and methanol at volume ratios of 2:1, 13:7, and 7:3 for the dansylated alanine, 2-aminobutanoic acid, and serine, respectively. The analytical procedure in detail was similar to that reported elsewhere.[5]

Results and Discussion

On the basis of our current studies on the functionalized bilayer vesicles having vitamin B_6 activity,[5,6,8,9] the reaction cycle for the catalytic transamination of an α-amino acid with an α-keto acid in the $PM2C_{16}$-$N^+C_5His2C_{16}$-Cu^{II} vesicular system is schematically shown in Fig. 1. In order to clarify the mechanistic implication in substrate selectivity exercised by the present artificial transaminase, its catalytic activity for various sets of substrates was examined under comparable kinetic conditions: $PM2C_{16}$, 5.0×10^{-5} mol dm^{-3}; $N^+C_5His2C_{16}$, 1.0×10^{-3} mol dm^{-3}; $Cu(ClO_4)_2$, 5.0×10^{-5} mol dm^{-3}; α-amino acid derivatives, 5.0×10^{-3} mol dm^{-3}; α-keto acids, 5.0×10^{-3} mol dm^{-3}; in an aqueous acetate buffer (25 mmol dm^{-3}) at pH 5.0, μ 0.10 with KCl, and 30.0 °C.

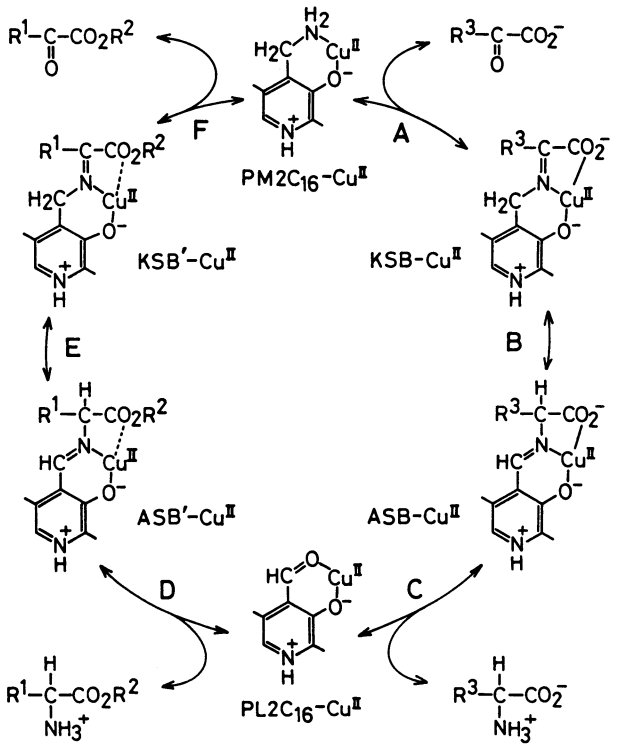


Fig. 1. Schematic representation of transamination cycle with an α-amino acid and an α-keto acid as substrates, as catalyzed by $PM2C_{16}$ and copper(II) ions in functionalized bilayer membrane.

Time courses for the transamination reactions of various DL-α-amino acids, having different normal alkyl side chains, with pyruvate are shown in Fig. 2. The reactivity increased sensitively along with increase in hydrophobicity of the amino acid. Thus, the reaction cycle for the amino group transfer from DL-2-aminooctanoic acid to pyruvate proceeded 47 times faster than that from DL-2-aminobutanoic acid to pyruvate in the present catalytic system (Table 1, Entries 2 and 16, respectively). The transamination reaction of L-phenylalanine (L-Phe) with the hydrophilic pyruvate was also effectively catalyzed by the present vesicular catalyst, as previously reported.[6] However, for a combination of L-Phe and a slightly

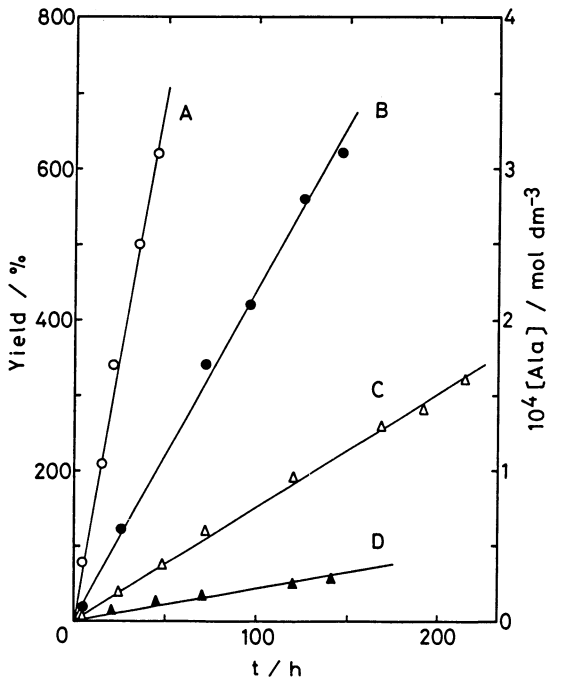


Fig. 2. Time courses for transamination catalyzed by an artificial transaminase formed with $PM2C_{16}$ (5.0×10^{-5} mol dm^{-3}), $N^+C_5His2C_{16}$ (1.0×10^{-3} mol dm^{-3}), and copper(II) ions (5.0×10^{-5} mol dm^{-3}) in an aqueous acetate buffer (25 mmol dm^{-3}) at pH 5.0, μ 0.10 with KCl, and 30.0 °C with combinations of pyruvic acid and the following α-amino acids (5.0×10^{-3} mol dm^{-3} each): A, DL-2-aminooctanoic acid; B, DL-norleucine; C, DL-norvaline; D, DL-2-aminobutanoic acid.

Table 1. Substrate Selectivity of an Artificial Transaminase Formed with $PM2C_{16}$ (5.0×10^{-5} mol dm^{-3}), $N^+C_5His2C_{16}$ (1.0×10^{-3} mol dm^{-3}), and Copper(II) Ions (5.0×10^{-5} mol dm^{-3}) at 30.0 °C[a)]

Entry	α-Amino acid derivative [$R^1CH(NH_2)CO_2R^2$]			α-Keto acid (R^3COCO_2H)	Reactivity	
					h	
	R^1	R^2	Chirality	R^3	Turnover	Relative rate
1	$PhCH_2O_2CCH_2$	H	L	CH_3	5	100
2	$CH_3(CH_2)_5$	H	DL	CH_3	6	83
3	$PhCH_2O_2C(CH_2)_2$	H	L	CH_3	9	56
4	$PhCH_2$	H	D	CH_3	12	42
5	$PhCH_2$	H	L	CH_3	16	31
6	$CH_3(CH_2)_3$	H	DL	CH_3	22	23
7	$PhCH_2$	H	L	CH_3CH_2	44	11
8	$PhCH_2O_2CCH_2$	CH_2Ph	L	CH_3	52	9.6
9	$CH_3(CH_2)_2$	H	DL	CH_3	65	7.7
10	$PhCH_2O_2C(CH_2)_2$	CH_2Ph	L	CH_3	65	7.7
11	$PhCH_2$	CH_2Ph	L	CH_3	84	6.0
12	$PhCH_2$	CH_3	L	CH_3	90	5.6
13	HO_2CCH_2	H	L	CH_3	100	5.0
14	$HO_2C(CH_2)_2$	H	L	CH_3	190	2.6
15	$HO_2C(CH_2)_2$	H	D	CH_3	190	2.6
16	CH_3CH_2	H	DL	CH_3	280	1.8
17	HO_2CCH_2	CH_2Ph	L	CH_3	322	1.6
18	$PhCH_2$	H	L	$HOCH_2$	—[b)]	0

a) In an aqueous acetate buffer (25 mmol dm^{-3}) at pH 5.0 and μ 0.10 with KCl. b) Turnover behavior was not observed.

more hydrophobic α-keto acid, 2-oxobutanoate, the reactivity was ca. one-third of that for a combination of L-Phe and pyruvate (Fig. 3). Thus, the present vesicular system requires a combination of a hydrophobic α-amino acid and a hydrophilic α-keto acid to achieve high catalytic performance. Such marked substrate selectivity supports our mechanistic concept previously proposed: the overall transamination reaction is controlled by the distribution behavior of substrates between bilayer and bulk aqueous phases and not by the isomerization rate between the aldimine Schiff-base chelate and the ketimine Schiff-base chelate; the former chelate is composed of the hydrophobic pyridoxal derivative ($PL2C_{16}$), an α-amino acid, and the copper(II) ion, while the latter composed of $PM2C_{16}$, an α-keto acid, and the copper(II) ion.[6] In addition, the substrate selectivity evidently comes from the molecular recognition exercised during the course of forming the aldimine Schiff-base chelates (steps C and D in Fig. 1) and the reaction steps for formation of the ketimine Schiff-base chelates (steps A and F in Fig. 1) are not responsible for the selectivity in the overall reaction cycle. On the other hand, the transamination reaction of L-Phe with 3-hydroxypyruvate, a more hydrophilic α-keto acid than pyruvate, did not proceed catalytically (Fig. 3, line C). In this case, the reaction behavior, exhibiting high reactivity in the initial stage followed by gradual inactivation, suggests consumption of the α-keto acid via a certain side reaction because of its labile 3-hydroxyl moiety.

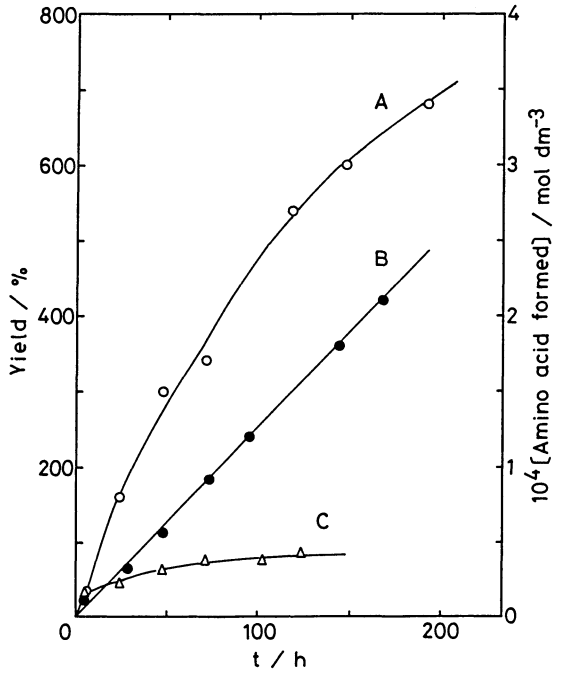


Fig. 3. Time courses for transamination catalyzed by an artificial transaminase formed with $PM2C_{16}$ (5.0×10^{-5} mol dm^{-3}), $N^+C_5His2C_{16}$ (1.0×10^{-3} mol dm^{-3}), and copper(II) ions (5.0×10^{-5} mol dm^{-3}) in an aqueous acetate buffer (25 mmol dm^{-3}) at pH 5.0, μ 0.10 with KCl, and 30.0 °C with combinations of L-Phe and the following α-keto acids (5.0×10^{-3} mol dm^{-3} each): A, pyruvic acid; B, 2-oxobutanoic acid; C, 3-hydroxypyruvic acid.

The substrate selectivity was also much affected by modification of the α-carboxyl moiety of an α-amino acid. As shown in Fig. 4, the transamination reactivity of L-Phe with pyruvate was much depressed upon conversion of the α-carboxylato group into its methyl

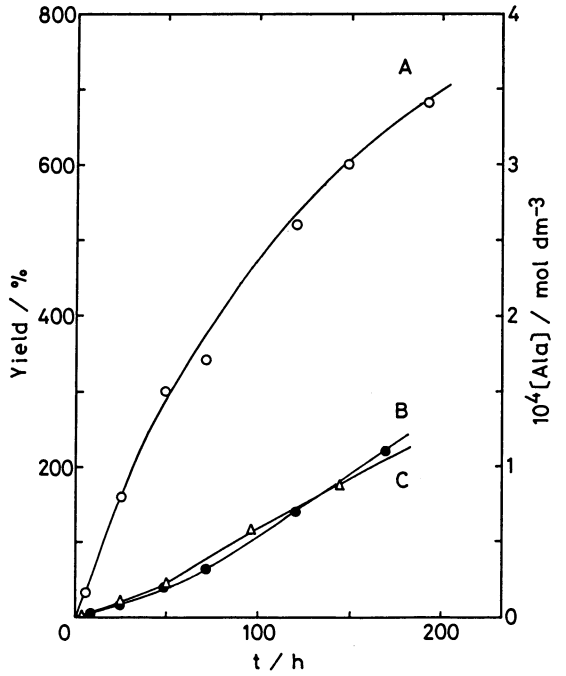


Fig. 4. Time courses for transamination catalyzed by an artificial transaminase formed with PM2C$_{16}$ (5.0×10^{-5} mol dm^{-3}), N^{+}C$_5$His2C$_{16}$ (1.0×10^{-3} mol dm^{-3}), and copper(II) ions (5.0×10^{-5} mol dm^{-3}) in an aqueous acetate buffer (25 mmol dm^{-3}) at pH 5.0, μ 0.10 with KCl, and 30.0 °C with combinations of pyruvic acid and the following L-Phe analogs (5.0×10^{-3} mol dm^{-3} each): A, L-Phe; B, methyl L-phenylalaninate; C, benzyl L-phenylalaninate.

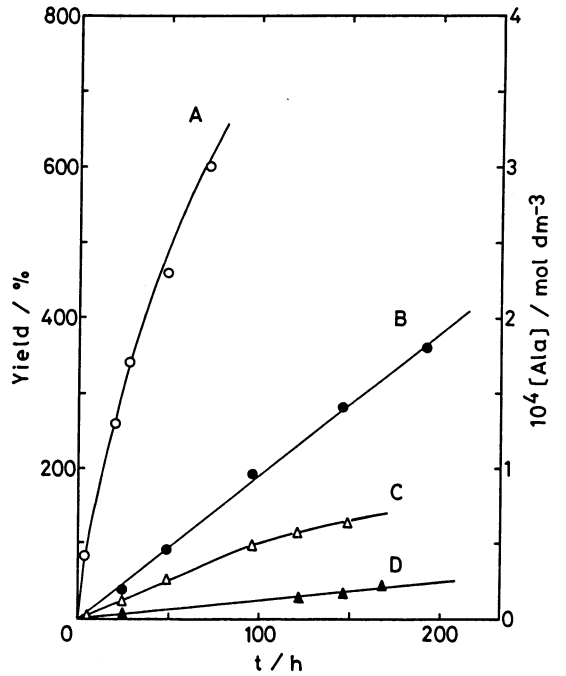
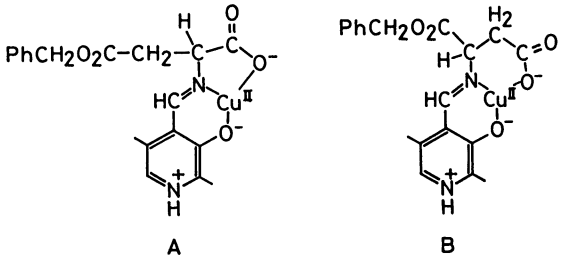


Fig. 5. Time courses for transamination catalyzed by an artificial transaminase formed with PM2C$_{16}$ (5.0×10^{-5} mol dm^{-3}), N^{+}C$_5$His2C$_{16}$ (1.0×10^{-3} mol dm^{-3}), and copper(II) ions (5.0×10^{-5} mol dm^{-3}) in an aqueous acetate buffer (25 mmol dm^{-3}) at pH 5.0, μ 0.10 with KCl, and 30.0 °C with combinations of pyruvic acid and the following L-aspartate analogs (5.0×10^{-3} mol dm^{-3} each): A, β-benzyl L-aspartate; B, α,β-dibenzyl L-aspartate; C, L-aspartate; D, α-benzyl L-aspartate.

or benzyl ester, in spite of increased hydrophobicity of the α-amino acid species. This seems to reflect the fact that the stability of the copper(II) complex of the terdentate aldimine Schiff-base formed with the former α-amino acid and PL2C$_{16}$ is much enhanced relative to that of the Schiff-base chelate derived from the latter amino acid ester; the alkoxycarbonyl group is a weaker ligand than the carboxylato for copper(II) ions. Consequently, the substrate selectivity is of thermodynamic origin; the higher stability the aldimine Schiff-base chelate attains, the greater overall reactivity is observed.

To confirm this mechanistic implication on the substrate selectivity, the transamination reactions of various L-aspartate and L-glutamate analogs with pyruvate were investigated. Time courses for the transamination of four L-aspartate analogs with pyruvate are shown in Fig. 5. The L-asparate-pyruvate couple is 2.8 times more reactive than the DL-2-aminobutanoate-pyruvate couple (Table 1, Entries 13 and 16, respectively). Although the transamination reactivity was increased by two-fold upon conversion of L-aspartic acid into the corresponding dibenzyl ester due to increased hydrophobicity of the latter substrate, the highest reactivity was observed for the β-benzyl L-aspartate-pyruvate couple among these L-aspartate analogs. In contrast, the α-benzyl L-aspartate-pyruvate couple is markedly less reactive than the β-benzyl L-aspartate-pyruvate couple, even though both α-amino acid derivatives possess comparable hydrophobicity (Table 1, Entries 1 and 17, respectively). This seems to come from the difference in thermodynamic stability between the aldimine Schiff-base chelate composed of β-benzyl L-aspartate, PL2C$_{16}$, and the copper(II) ion (**A**) and the corresponding aldimine chelate of α-benzyl L-aspartate (**B**); the former forms a five-membered chelate ring, while the latter a six-membered one with the carboxylate moiety. Figure 6 shows time courses for the transamination reactions of three L-glutamate analogs with pyruvate. The reaction behavior is analogous to that for combinations of the L-aspartate analogs with pyruvate. Lower reactivity of the L-glutamate analog-pyruvate couples in comparison with the corresponding L-aspartate



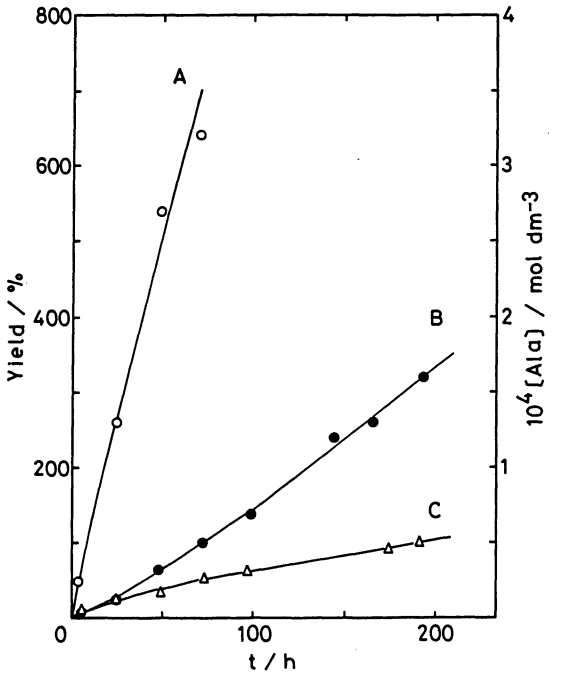


Fig. 6. Time courses for transamination catalyzed by an artificial transaminase formed with PM2C$_{16}$ (5.0×10^{-5} mol dm^{-3}), N$^+$C$_5$His2C$_{16}$ (1.0×10^{-3} mol dm^{-3}), and copper(II) ions (5.0×10^{-5} mol dm^{-3}) in an aqueous acetate buffer (25 mmol dm^{-3}) at pH 5.0, μ 0.10 with KCl, and 30.0 °C with combinations of pyruvic acid and the following L-glutamate analogs (5.0×10^{-3} mol dm^{-3} each): A, γ-benzyl L-glutamate; B, α,γ-dibenzyl L-glutamate; C, L-glutamate.

analog-pyruvate couples are also explained primarily from the viewpoint of the chelate effect; the aldimine Schiff-base chelates formed with the former couples are lower in stability relative to those formed with the latter.

As regards the transamination reaction catalyzed by the present vesicular system, the general acid-base catalysis by the imidazolyl group introduced into the peptide lipid is essential to exhibiting the turnover behavior, since the sequential reactions shown in Fig. 1 did not cycle in the vesicle of *N,N*-dihexadecyl-*N*$^{\alpha}$-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide in which the L-histidyl residue of N$^+$-C$_5$His2C$_{16}$ is replaced by the L-alanyl residue.[6] In addition, the reaction site is provided in the hydrogen-belt domain of the highly organized and chiral bilayer membrane.[8] We have previously observed enantioselective catalysis by the chiral L-histidyl moiety of an analogous synthetic peptide lipid in ester hydrolysis.[2] Thus, one can expect to observe enantioselectivity for the transamination reaction catalyzed by the PM2C$_{16}$-N$^+$C$_5$His2C$_{16}$-Cu^{II} vesicular system. In fact, we observed that the amino group transfer from D-Phe to pyruvate was accelerated to a 33% extent relative to that from L-Phe to pyruvate (Table 1, Entries 4 and 5, respectively), presumably due to favorable thermodynamic stability of the aldimine Schiff-base chelate of D-Phe. On the other hand, there was no significant difference in transamination reactivity between the L-glutamate-pyruvate and D-glutamate-pyruvate couples (Table 1, Entries 14 and 15, respectively). This seems to arise from the weaker coordination effect by the γ-carboxylato moiety of glutamate for the development of enantioselectivity as discussed above.

In conclusion, it became apparent that the artificial transaminase composed of PM2C$_{16}$, N$^+$C$_5$His2C$_{16}$, and copper(II) ions exhibits marked substrate selectivity in the catalytic transamination reactions of α-amino acids with α-keto acids under mild conditions. The substrate recognition exercised in the course of formation of the aldimine Schiff-base chelates in the pseudo-hydrophobic domain of the bilayer membrane is responsible for such selectivity. Although further modification of the present artificial holoenzyme is required for performing high enantioselectivity, it is noteworthy that a simple system composed of three molecular components, a synthetic peptide lipid, a hydrophobic vitamin B$_6$ derivative, and metal ions, readily undergoes self-organization in aqueous media to form the supramolecular bilayer assembly capable of mimicking the catalytic functions of vitamin B$_6$-dependent transaminase.

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