Selective transport of amino acids across a crosslinked poly(L-glutamic acid) membrane

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Poly(L-glutamic acid) (PLG) membranes were prepared by crosslinking poly[γ-(2-chloroethyl) L-glutamate] with diethylenetriamine and subsequently hydrolysing the side-chain esters. Selective amino acid transport across the membrane was examined using L-phenylglycine, L-phenylalanine and L-tryptophan. Transport selectivity increased with increasing pH of the solute solution, i.e. from 2.0 to 5.7. When pH was raised to 8.0 or when $CaCl_2$ was added, transport selectivity was lost. Conformational changes of PLG and electrostatic interactions between the solute and the membrane are suggested to cause the observed permselectivity behaviour.

(Keywords: poly(L-glutamic acid); crosslinking; amino acids; permselectivity; pH dependence)

INTRODUCTION

Synthetic polypeptides have been examined as models for membrane proteins in living cells[1-4]. In most of these studies, changes of the secondary structure of polypeptides induced by pH changes or other external stimuli have been intensively looked at in view of potential application for drug delivery or for selective transport of solutes through the membrane. There are, however, few reports on selective transport of amino acids through polypeptide membranes. Yoshimura *et al.* have examined selective transport of amino acids through a membrane made of a naturally occurring polypeptide, fibroin[5]. In the present study, we prepared a poly(L-glutamic acid) (PLG) membrane by crosslinking poly[γ-(2-chloroethyl) L-glutamate] (PCELG) with diethylenetriamine and subsequently hydrolysing the remaining 2-chloroethyl esters and examined transport of selected amino acids, i.e. L-phenylglycine (Pgly), L-phenylalanine (Phe) and L-tryptophan (Trp), across the membrane at various pHs and in the presence and absence of a divalent salt, $CaCl_2$.

EXPERIMENTAL

Poly(γ-methyl L-glutamate) (PMLG, $M_v = 1.1 \times 10^5$), supplied by Ajinomoto Co., Japan, was treated with ethylene chlorohydrin in dichloromethane in the presence of a catalytic amount of sulfuric acid[6]. The polymer was precipitated in methanol. The conversion of the side-chain methyl ester to 2-chloroethyl ester was determined to be ~90% by ^1H n.m.r. To a 3 wt% dimethylformamide solution (2.0 ml) of the above polymer was added diethylenetriamine (1.5 μl, 10 mol% with respect to the 2-chloroethyl ester group). The mixture was stirred at 25°C for 30 min, and then 100 μl were spread over a 3 cm^2 glass plate. After drying in air and subsequently *in vacuo*, the membrane was removed from the plate and immersed in a 1:3 mixture of concentrated HBr and acetic acid at 40°C. The hydrolysed membrane was washed thoroughly and stored at 5°C in an aqueous HCl solution (pH 2.0). I.r. measurements were made with samples dried in air and subsequently *in vacuo*. Complete hydrolysis of the 2-chloroethyl ester group was confirmed by the disappearance of the 660 cm^{-1} peak of the C–Cl stretching vibration. The hydrolysed membrane, unlike a membrane prepared in the absence of diethylenetriamine, did not dissolve in water even at pH 8.0. A membrane, whose thickness is 1/100 that of the above membrane, was prepared on a quartz plate, hydrolysed, conditioned at appropriate pH, and subjected to circular dichroic measurement.

The hydrolysed membrane was inserted between the two compartments of a U-shaped cell using silicone packing and conditioned in water of appropriate pH with stirring at 5°C for 2 days. The water in one compartment was then replaced with a pH-adjusted aqueous solution containing two kinds of amino acids, the concentration of each being 1×10^{-2} mol l^{-1}. The permeation area of the membrane was 0.785 cm^2. No apparent flow of water across the membrane due to the osmotic pressure was noted during permeation experiments. A portion of the solution (0.4 ml) in each compartment was withdrawn at intervals and, after appropriate dilution, subjected to h.p.l.c. analysis using a 25 cm Enantio L1 column (4.6 mm inner diameter, Tosoh Co., Japan) and a 254 nm u.v. detector. A $CuSO_4$ solution (2.0×10^{-3} mol l^{-1}, pH 4.6) was used as a mobile phase. Each time the pH was changed, the membrane was reconditioned for 3 h prior to permeation measurement. Trp, Phe and Pgly were all commercially available high-grade reagents and were

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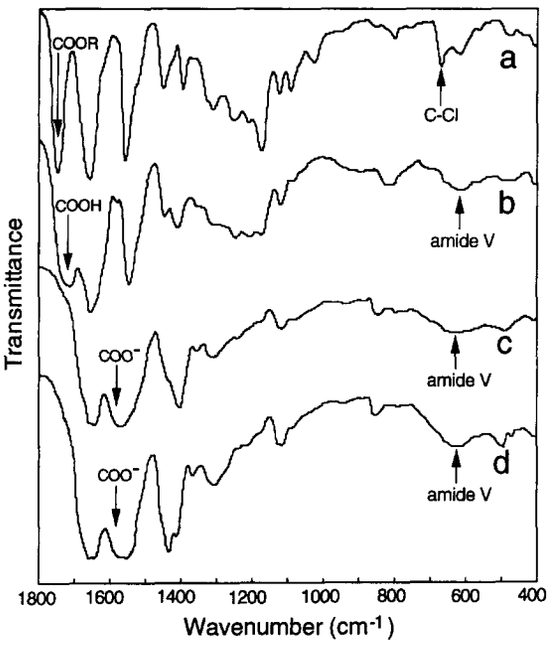

Figure 1 I.r. spectra of PCELG membrane (a) and PLG membrane conditioned at pH 2.0 for 2 days (b), at pH 8.0 for 2 h (c) and in aqueous $CaCl_2$ solution (2×10^{-2} mol l^{-1}) for 2 h (d)

used as received. Water was deionized and subsequently distilled.

RESULTS AND DISCUSSION

The i.r. spectra of the crosslinked membrane before and after hydrolysis are shown in *Figures 1a* and *b*. Disappearance of the C–Cl and C=O stretching vibrations of the 2-chloroethyl ester at 660 and 1740 cm^{-1}, together with the appearance of the C=O stretching vibration of the COOH group at 1720 cm^{-1}, shows virtually complete hydrolysis of the 2-chloroethyl ester in the membrane. The amide V band, which is assigned to out-of-plane N–H bending vibrations, centres around 620 cm^{-1}, suggesting that the conformation of the polypeptide in the membrane is mostly the α-helix[7]. The circular dichroism spectrum of a membrane prepared and hydrolysed on a quartz plate shows two negative ellipticities at 206 and 222 nm and a positive ellipticity at 190 nm, confirming the α-helical conformation.

The i.r. spectrum of the membrane did not change when the membrane was treated with an aqueous solution of at least up to pH 6. (In aqueous solution, PLG is known to undergo transition from α-helix to random coil at pH ~5.5[8].) Its treatment with dilute aqueous NaOH solution (pH 8), however, causes the carbonyl stretching vibration to shift from 1720 cm^{-1} (COOH) to 1580 cm^{-1} (COO^-) and the amide V band to broaden and shift to a higher frequency, indicating that PLG in the membrane requires a pH somewhere between 6 and 8 to cause extensive dissociation of the side-chain carboxyl group and bring about conformational transition to random coils (*Figure 1c*).

The results of the transport experiments for Trp and Pgly are shown in *Figure 2*. At pH 2.0, Trp is transported slightly faster than Pgly, the flux ratio being ~1.2. When the pH was raised to 4.0, transport of Trp became twice as fast, while that of Pgly increased only by ~40%, giving a larger flux ratio of ~1.8. At pH 5.7, the flux ratio improved to ~2.0. The change of transport selectivity with pH is reversible, as shown in *Figure 2*.

Phe, on the other hand, is transported at a rate nearly equal to that of Pgly at pH 2.0. At pH 4.0, Phe is transported slightly faster than Pgly, the flux ratio being 1.2. No apparent increase of selectivity was observed at pH 5.7. Again, the dependence on pH was reversible.

From the pK_1 (1.83, 2.16 and 2.38) and pK_2 (4.39, 9.31 and 9.39) values of Pgly, Phe and Trp, the amino acids are in part in the zwitterion form ($NH_3^+CHRCOO^-$) and in part in the cationic form ($NH_3^+CHRCOOH$) at pH 2.0, the ratios of the former to the latter being ~1.5, 0.7 and 0.4 for Pgly, Phe and Trp, respectively. The pK_a value of the γ-carboxyl group of L-glutamic acid, i.e. 4.31, suggests that the side-chain carboxyl group of PLG is not dissociated in an aqueous solution of pH 2.0. Thus, through the uncharged membrane, an amino acid which has a smaller zwitterion/cation ratio is expected to dissolve more easily into the membrane and be subsequently transported more quickly across the membrane. Some specific interactions between the polypeptide and the amino acid may also be responsible for the observed permselectivity.

At higher pHs, i.e. pH 4.0 and pH 5.7, the carboxyl groups of PLG are expected to dissociate partially and to allow the membrane to swell, accelerating the amino acid transport. While at these pHs Phe and Trp are virtually all in the zwitterion form, Pgly is partially in the anionic form ($NH_2CHRCOO^-$), suggesting the transport of Pgly to be suppressed by unfavourable electrostatic interactions.

Even though Trp and Phe were not directly compared, their relative transport rates may be evaluated from the results obtained for the Trp/Pgly and Phe/Pgly pairs: Trp would be transported faster than Phe at pH 2.0 with a flux ratio of ~1.2. The selectivity would increase to ~1.5 and ~1.7 at pH 4.0 and pH 5.7, respectively.

When the pH was raised to 8.0, the membrane swelled markedly and gave an ~10-fold increase in amino acid transport rate with complete loss of selectivity. Even when the membrane was brought back to pH 4.0 and stored at that pH for ~30 h, no apparent suppression of transport rates or recovery of selectivity was observed,

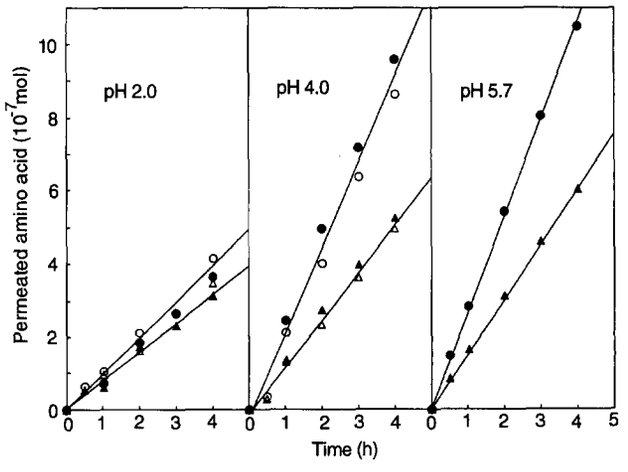

Figure 2 Permeation of Trp (○, ●) and Pgly (△, ▲) across the PLG membrane. Initial concentrations: [Trp] = [Pgly] = 1×10^{-4} mol per 10 ml; temperature: 5°C. pH changes were made in the following order: 2.0→4.0→2.0→4.0→5.7. The open and filled symbols represent the first and second cycles, respectively. The amount of permeated amino acid is corrected for reduced solution volume due to each sampling

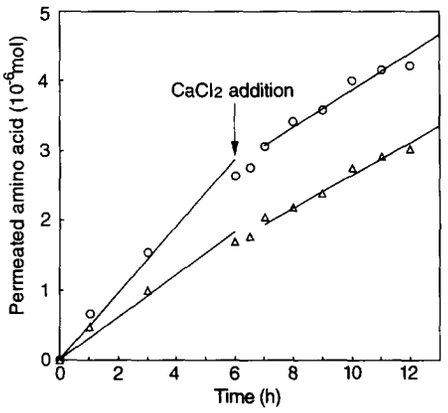

Figure 3 Permeation of Trp (○) and Pgly (△) at pH 5.7 and that after addition of $CaCl_2$ (2×10^{-3} mol per 10 ml) to both sides of the membrane. The conditions are the same as those for *Figure 2*

suggesting the irreversibility of the conformational change in the membrane.

When $CaCl_2$ was added to both sides of the membrane, we found a decrease of transport rates and loss of transport selectivity, as shown in *Figure 3*. The presence of metallic salts is known to affect polypeptide conformation in film as well as in solution[8]. Noguchi and Yang reported that monovalent and divalent salts cause crosslinked PLG membranes to contract and attributed it, without giving evidence, to the salting-out effect in the case of monovalent salts and to the increase of the α-helix content in the case of divalent salts[9]. Maeda *et al.* have reported that phenyl-2-ethanediol transport through a membrane made of poly(L-glutamic acid)-grafted vinyl polymers is suppressed in the presence of the Ca^{2+} ion[10]. The Ca^{2+} ion most likely forms a complex with two carboxylate ions, causes PLG to contract and reduces transport rates.

The i.r. spectrum of the PLG membrane treated with an aqueous $CaCl_2$ solution shows extensive dissociation of the carboxyl group to the carboxylate ion together with a shift of the amide V band from 620 to 645 cm^{-1} (*Figure 1d*). Thus, we may conclude that reduced transport rates as well as loss of transport selectivity are caused by membrane contraction accompanied by a conformational change to random coils by the above complexation.

We have thus shown that the conformation of PLG and electrostatic interactions between the amino acid and the membrane are important for selective amino acid transport through the PLG membrane.

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