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Interaction of α-lactalbumin with phospholipid vesicles as studied by photoactivated hydrophobic labeling

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The α-lactalbumin segment which penetrates into phosphatidylserine / phosphatidylethanolamine vesicle bilayer under acidic condition was photoactively labeled with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) which had been partitioned into the hydrophobic interior of the bilayer. The hydrophobically labeled amino acid residues were identified by trypsin digestion of the α-lactalbumin / vesicle complex, extraction and Edman degradation of the membrane embedded fragment. The results are consistent with a notion that the segment exists in the membrane as an α-helix and that only one surface of this α-helix is exposed to the hydrophobic interior of the bilayer. Possible models are: (a) a loop of tightly held α-helix penetrating deep into the bilayer and (b) the helix being located on the interface between bilayer and the aqueous solution. The time-dependent [^{125}I]TID labeling process revealed that the middle part of this segment goes into the bilayer first and is then followed by both ends. The penetration rate is comparable to that of the fusion of the lipid vesicles of the same composition by α-lactalbumin at the same pH, which further supports that the penetration is the cause of fusion.

Introduction

Recently, the penetration of soluble proteins into phospholipid bilayers under diverse conditions has become a subject of increased interest. This stems from the fact that the process is important for membrane fusion, translocation of proteins across the membranes, incorporation of membrane integral proteins, and the pore formation in the membrane. A variety of techniques including hydrophobic labeling, proteolytic digestion, and electron spin resonance spectroscopy have been employed for this study.

In order to correlate the relationship between α-lactalbumin-induced fusion of PS/PE vesicles and irreversible binding of this protein to the vesicles, we employed proteolytic digestion and hydrophobic labeling by dansyl chloride and observed that a particular segment of this protein penetrates the bilayer [1]. In the present investigation, we employed [^{125}I]TID, a hydrophobic label that can be photoactivated, to study the structure and topology of the α-lactalbumin segment within the bilayer. Hoppe et al. [2] demonstrated that combination of this hydrophobic labeling and Edman degradation gives a lucid picture of the structure of integral protein segments within the membrane. These authors found that the subunit b of the *Escherichia coli* ATP synthase F_0 complex is anchored to the membrane by a short segment as judged from the accessibility of all the hydrophobic amino acid residues. Subunit c, however, forms a tightly packed α-helix. This conclusion was drawn from the inaccessibility of the label to certain residues. The one-sided labeling with [^{125}I]TID was also observed for the case of a segment of bacteriorhodopsin in the membrane [3]. In the present investigation, we found that the [^{125}I]TID-labeling and Edman degradation technique of Brunner et al. [3] is also useful for studying soluble protein–bilayer interactions.

Brunner et al. [3] also suggested that this technique may be used to study the kinetics of protein penetration into the bilayer. We have studied the time-dependent

Abbreviations: TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PTH, phenylthiohydantoin; PMSF, phenylmethanesulfonyl fluoride; TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ESR, electron spin resonance; ATZ, anilinothiazolinone; DMAA, *N*,*N*-dimethyl-*N*-allylamine; TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate.

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α-lactalbumin penetration into the bilayer and found that the rate of penetration closely parallels that of fusion.

Materials and Methods

Materials

PS (from bovine brain), PE (from bovine brain), PMSF, trypsin (from bovine pancrease) and α-lactalbumin (from bovine milk) were all purchased from Sigma. Phospholipids migrated as single spots upon thin-layer chromatography on silica gel. The purification procedure for α-lactalbumin has already been described [1]. All the necessary reagents for the amino acid sequence determination and the PTH-amino acid standard kit were obtained from Pierce. [^{125}I]TID was from Amersham.

Photoactivated hydrophobic labeling of bilayer penetrating segment of α-lactalbumin

PS/PE (1 : 1) vesicles in HCl/glycine buffer (50 mM glycine, 11 mM HCl, 48 mM NaCl, pH 3) were formed by the ether injection technique [4] as described previously [1]. 10 ml of this PS/PE vesicle suspension containing 2 mM of P_i was mixed with 250 μCi of [^{125}I]TID in 0.05 ml ethanol and incubated for 15 min at room temperature in the dark. 1 ml of buffer solution (50 mM glycine, 11 mM HCl, 48 mM NaCl, pH 3) containing 4 mg of α-lactalbumin was added to the suspension and incubated for 30 min at 18°C. This was illuminated with a 300 W mercury lamp or 5 s. The vesicle-protein complex was separated from unbound α-lactalbumin by ultracentrifugation at 35 000 rpm for 90 min in a Beckman SW 41 rotor. After suspending the pellets in pH 7.5 buffer (150 mM KCl, 25 mM imidazole), 500 μg of trypsin was added and the solution was incubated for 2 h at 37°C. The proteolysis was terminated by the addition of PMSF in ethanol, the final concentration of this inhibitor being 3 mM. The extraction of the protein from the vesicle was done as described elsewhere [1] and lyophilized. The recovery of this fragment was found to be 80 ± 4%. About 2% of the [^{125}I]TID labels α-lactalbumin while the rest of the compound labels phospholipid.

The lyophilized [^{125}I]TID-labeled α-lactalbumin segment was dissolved in 2 ml of 0.1 M Tris buffer (pH 7), containing 5 M guanidinium chloride and 0.2% EDTA. After the pH was adjusted to 8, 100 μl of β-mercaptoethanol was added and the solution was incubated for 4 h. Next, 150 mg of iodoacetic acid in 1 M NaOH solution was slowly added. This solution was dialysed against 1 M formic acid and the protein segment which precipitated was lyophilized.

The carboxymethylated polypeptide was dissolved again in Tes buffer (pH 7.4) and passed through a Sephadex G-50 column (1.8 × 180 cm). The eluted protein fractions were monitored by absorbance at 280 nm as well as by γ-ray counting. Fig. 1 presents this elution profile which shows two protein peaks. It can be seen only the leading peak was hydrophobically labeled. The polypeptide fractions were collected and lyophilized.

Edman degradation of the α-lactalbumin segment

In order to facilitate the manual sequencing, the [^{125}I]TID-labeled fraction was cleaved at the position of Met-90 with cyanogen bromide. The polypeptide from the leading peak (Fig. 1) was dissolved in 70% formic acid (5 ml). A 50-fold excess of crystalline cyanogen bromide was added to the solution, and the resulting mixture was allowed to stand at room temperature for 16 h in a tightly stoppered flask. A 10-fold volume of distilled water was then added to the solution and the protein fragments were lyophilized. Next, these fragments were dissolved in a 0.2 M pyridine acetate buffer, (pH 3.1) and the resulting solution was passed through a Dowex 50-X2 column (1.5 × 180 cm). Two well-resolved [^{125}I]TID-labeled fractions were obtained. The protein determination of the separated fractions was accomplished using the ninhydrin reaction monitored at the wavelength of 570 nm and a γ-count was employed to determine the [^{125}I]TID-labeled fraction. Each fraction collected was lyophilized.

The sequence of each segment was obtained by going through Edman degradation and the [^{125}I]TID-labeling of a particular residue was determined by γ-ray counting. 50 nmol of extracted polypeptide in 0.1 ml of buffer solution was freeze-dried in an Edman tube. After the addition of 0.1 ml of buffer A (0.4 M DMAA/TFA in pyridine/water (3 : 2, v/v), pH 9.5) to the above Edman tube, the mixture was vortexed. 10 μl of PITC was added slowly to this solution as it was stirred and the Edman tube was heated for 5 min on a heating block maintained at 50°C. DMAA and excess PITC were removed by extracting three times, each time with 0.5 ml of *n*-heptane/ethyl acetate (10 : 1, v/v). N_2 gas was passed over the aqueous solution in order to remove residual organic solvents. The protein solution in the tube was lyophilized again. The cleavage of the PITC-labeled terminal amino acid was achieved by slowly adding 10 μl of TFA to the lyophilized polypeptide and incubating for 5 min at 50°C. The TFA was then completely expelled with N_2 gas and the cleaved ATZ-amino acid was extracted twice, each time with 1 ml of ethylene chloride. After removing the ethylene chloride with N_2 gas, the dried extract was dissolved in 0.1 ml of 1 M HCl and shaken gently for 3 min. This was incubated for 10 min at 80°C and 1 ml of ethyl acetate was added followed by a vigorous shaking. When this was centrifuged at 3000 rpm for 5 min, two phases were separated and a UV-absorption spectrophotometer was used to decide which phase contained a particular PTH-amino acid. The identification

of the PTH-amino acids was done by HPTLC. The polypeptides remaining in the Edman tube after the ethylene chloride extraction of the cleaved ATZ-amino acid were subjected to the next Edman degradation cycle.

Each amino acid released by the Edman degradation was also subjected to TLC in chloroform/ethanol (98 : 2, v/v) solvent and then autoradiographed at −80 °C for 1 week. From among the above cleavage degradation products, those with negligible γ-counts were compared with standard PTH-amino acids on TLC. The PTH-amino acid from each of these Edman degradation cycles was found to be identical to the one expected from the known amino acid sequence of this segment. The [^{125}I]TID-labeled amino acid residues between these non-labeled residues can then be readily identified.

Time-dependent TID labeling

In the previous paper [1], a quantitative relationship between the extent of binding and the initial fusion rate by α-lactalbumin could not be established because of the entirely different nature of these two methods. By taking advantage of the fact that only one second or less is required to photoactivate the labeling by [^{125}I]TID, we monitored the course of α-lactalbumin penetration by following the extent of labeling with the incubation time after the PS/PE vesicles and α-lactalbumin were mixed. For this purpose we employed a fraction collector since its tray can be moved with a fixed rate in a 'stop-go' manner.

Holes with 0.8 cm diameter were drilled on one side of a rack on the ISCO Model 328 fraction collector so that light could reach the test tubes in the rack. 0.1 ml each of PS/PE vesicle suspension was introduced into the test tubes, the amount of [^{125}I]TID partitioned into vesicles in each test tube being fixed. At time zero, 10 μl each of α-lactalbumin solution (10 mg/ml) was introduced into 10 test tubes simultaneously from ten micropipettes connected to a glass manifold through rubber tubing. This glass manifold was, in turn, connected to a nitrogen cylinder. This device enables one to apply identical volumes of α-lactalbumin solution, preloaded in the pipettes, into ten test tubes all at once by applying the N_2 gas. The N_2 bubbles also help mix the vesicle with α-lactalbumin. The time required for this process is less than 1 s. When the α-lactalbumin solution was introduction into the vesicle suspension, the fraction collector was activated. After 6 s has elapsed, the first tube moved past the UV light beam in the direction normal to the light pathway for 1 s and then stopped for another 6 s before the next shift. This photoactivation of the vesicle/α-lactalbumin solution in the test tubes during the rack advancement was repeated automatically for a fraction of a second each with a 6 s interval between each repetition. Subsequent trypsin digestion and sequence determination were as before.

Fusion of PS/PE vesicles induced by α-lactalbumin fragment

The α-lactalbumin segment protected from tryptic digestion when present with vesicles was extracted and tested for its fusogenic property for PS/PE vesicles at pH 3. The experimental procedure for the fusion was the same as discussed earlier [1].

SDS-PAGE of vesicle-bound fragment of α-lactalbumin in the absence of β-mercaptoethanol

In order to see if any other fragment of the α-lactalbumin is attached to the membrane-penetrating fragment through disulfide linkage after the trypsin treatment of vesicle-α-lactalbumin complex, the SDS-PAGE described in the previous communication [1] was repeated in the absence of β-mercaptoethanol.

Results

Tryptic digestion

In our previous communication [1], we reported that the molecular weight of the α-lactalbumin fragment which remains attached to the vesicles after the tryptic digestion of α-lactalbumin/vesicle suspension is about 4000 as determined by SDS-PAGE in the presence of β-mercaptoethanol. This segment was found to be the polypeptide chain from amino acid residues 80 to 108 (we will refer to this as the α-lactalbumin (80–108) fragment). The Cys-91 within this fragment forms a disulfide linkage with Cys-73. Since the binding and trypsin treatment were done in the absence of β-mercaptoethanol while subsequent SDS-PAGE was performed in the presence of this agent, it occurred to us that another segment might be attached to this membrane binding fragment through this disulfide linkage.

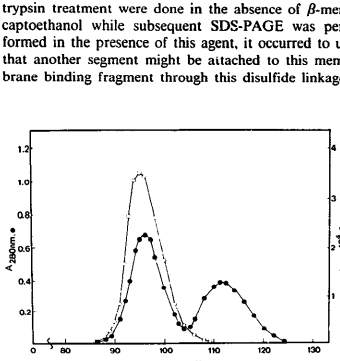

Fig. 1. Elution profile of the α-lactalbumin fragments extracted from the vesicles on Sephadex G-50 chromatography after going through the [^{125}I]TID labeling, trypsin digestion and then carboxymethylation. The eluents were monitored by the absorbance at 280 nm as well as by γ-counting.

It was possible that this segment may not have been detected when SDS-PAGE was performed in the presence of β-mercaptoethanol. We, therefore, performed the electrophoretic experiment in the absence of β-mercaptoethanol; the result is shown in Fig. 2. It can be seen that the estimated molecular weight of the band is about 5000, larger than the unit obtained in the presence of the reducing agent. This indicates that a small piece of α-lactalbumin is attached to the α-lactalbumin (80–108) fragment, a result consistent with the result of the extraction of the α-lactalbumin segment protected from tryptic digestion and subsequent carboxymethylation in the presence of β-mercaptoethanol. Fig. 1 shows the gel filtration profile of the carboxymethylated polypeptides where the leading peak is the α-lactalbumin (80–108) fragment and the trailing peak represents the segment attached to the α-lactalbumin (80–108) fragment through a disulfide linkage.

[^{125}I]TID-labeled amino acid residues

The γ-ray count of each amino acid after Edman degradation of the α-lactalbumin (80–108) fragment which was cleaved with cyanogen bromide at Met-90 is given in Fig. 3. A prior Edman degradation of the same α-lactalbumin fragment which was not labeled with [^{125}I]TID established that this is indeed the α-lactalbumin (80–108) fragment. This result confirms our previous finding that the protection of this fragment from tryptic digestion is due to its penetration into the bilayer. It is noteworthy that not all the hydrophobic residues with a potential of covalent labeling are affected. Obviously Leu-81, Ile-101 and Leu-105 are not accessible to [^{125}I]TID which is presumably is dissolved in the hydrophobic core of the bilayer. The autoradiograms of these amino acids shown in Fig. 4 essentially confirm the above results. Also, the extent of labeling for one kind of residue tends to be larger in the central part of the α-lactalbumin (80–108) fragment. The helical wheel diagram of this segment is given in Fig. 5 identifying the [^{125}I]TID-labeled amino acid residues. It is of interest to note that only amino acids on one side of the helix are labeled. One would expect these results if this segment forms an α-helix when bound to the vesicles and that only one side is accessible to the hydrophobic labeling. However, further studies will be required to confirm this. The trailing peak in Fig. 1 which is not labeled with [^{125}I]TID is probably located outside of the bilayer even if this segment is still connected to the membrane-penetrating segment by a disulfide link. The amino acid sequence of this segment was determined and it was found that this is the polypeptide chain spanning from Asn-63 to Lys-79 of the α-lactalbumin.

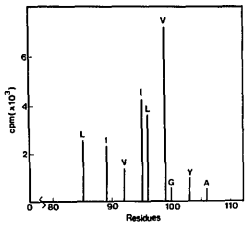

Fig. 3. Histogram of the distribution of [^{125}I]TID-label among individual amino acid residues within the α-lactalbumin (80–108) fragment. See text for the experimental details. The values indicated are averages of two independent experiments. Average deviation from the mean is 4% when the total γ-counts of the labeled α-lactalbumin (80–108) segments are normalized.

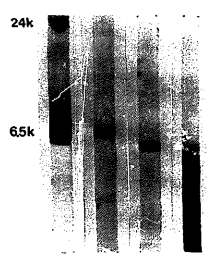

Fig. 2. SDS-PAGE of the protected segment of α-lactalbumin after trypsin digestion of α-lactalbumin-vesicle suspension that had been incubated at pH 3 and then brought to pH 7.5 before the trypsin treatment at 37°C. (a) The marker proteins. 24K unit trypsinogen and 6.5K, aprotinin. (b) SDS-PAGE in the absence of β-mercaptoethanol. (c) SDS-PAGE in the presence of β-mercaptoethanol. (d) Control patterns (in the presence of β-mercaptoethanol) obtained by incubating α-lactalbumin at pH 3 in the absence of PS/PE vesicles and then digesting with trypsin at 37°C after bringing the pH to 7.5.

Fig. 4. Autoradiogram of the TLC patterns of the radioactive derivatives of PTH amino acids derived from α-lactalbumin (80–108) fragment. The experimental procedures are given in the text.

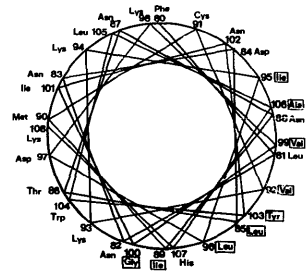

Fig. 5. Axial projection helical wheel diagram of the amino acid sequence within the α-lactalbumin (80–108) fragment. Amino acid residues in boxes are those labeled by [^{125}I]TID.

Kinetics of penetration

Fig. 6 gives the γ-ray count of the [^{125}I]TID-labeled α-lactalbumin, plotted against the time between the mixing of α-lactalbumin and vesicles and the photoactivation. The photolabeled α-lactalbumin was extracted without proteolysis using a procedure identical to that for the isolation of the α-lactalbumin (80–108) fragment [1]. The maximum labeling was achieved in 1 or 2 min which indicates that the penetration of the protein is complete within this time limit. This time-course of labeling corresponds well with the time-course of α-lactalbumin induced fusion [1]. In Fig. 6, the % of maximum Tb fluorescence is corrected for the leakage according to the method described by Bentz et al. [5].

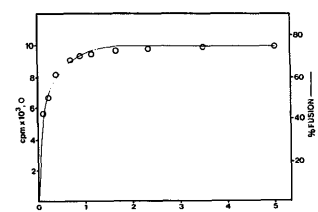

Fig. 6. Comparison of time-dependent [^{125}I]TID-labeling of the intact α-lactalbumin after the α-lactalbumin and vesicles were mixed prior to the photoactivation, with the time-course of α-lactalbumin induced fusion at pH 3, 18°C. Time-dependent [^{125}I]TID-labeling of the α-lactalbumin was performed as described in the text. The time course of α-lactalbumin induced fusion corrected for the leakage was obtained by using the method of Benz et al. [5]. ———, corrected fusion rate; ○, extent of [^{125}I]TID-labeling. The γ-counts indicated are average values of four experiments with a mean deviation of 4%.

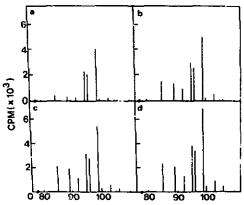

Fig. 7. Time-dependent γ-counts for individual amino acids in the α-lactalbumin (80–108) fragment labeled with [^{125}I]TID. Time-dependent TID labeling was performed as described in the text. Incubation times after α-lactalbumin and vesicles were mixed preceding the photoactivation. (a) 13 s, (b) 27 s, (c) 41 s, and (d) 69 s, pH 3, 18°C. The values indicated are average of two independent experiments. Average deviation from the mean is 4% when the total γ-counts of the labeled α-lactalbumin (80–108) segments are normalized.

This provides more convincing support that the penetration is indeed the cause of fusion.

Fig. 7 gives the histograms of the extent of [^{125}I]TID labeling of individual amino acid residues at several different durations of the incubation of α-lactalbumin/vesicle suspension before photoactivation. It appears that the middle portion of the segment (Ile-95, Leu-96, Val-99) enters membrane first and is then followed by both ends of the segment. The autoradiograms shown in Fig. 8 support this observation. Table I gives the reduced γ-ray count of individual amino acid residues at different times. Here again the percentage labeling for Ile-95, Leu-96, and Val-99 decreases with time while those for other residues increase. However they tend to reach a steady state after 27 s although minor increases

TABLE I

Reduced γ-ray count as a function of incubation time

Amino acid residue	Reduced counts [a]				
	13 s	27 s	41 s	69 s	equilibrium
Leu-85	4.1	9.8	11.5	10.7	11.4
Ile-89	3.0	8.9	10.9	9.7	9.9
Val-92	2.5	6.5	6.3	5.8	5.9
Ile-95	24.0	19.6	17.9	17.7	18.6
Leu-96	20.4	16.6	15.8	16.0	15.7
Val-99	42.9	33.7	30.7	32.3	31.2
Gly-100	1.4	1.4	1.7	2.4	2.3
Tyr-103	2.2	3.4	3.6	4.2	4.4
Ala-106	0.1	1.2	1.7	2.4	2.3

[a] For each time, the γ-ray counts for each amino acid was divided by the total counts which is equal to the sum of the counts for individual amino acid. These are average value of two experiments with a mean deviation of 4%.

afterwards can be seen for Gly-100, Tyr-103, and Ala-106. The overall picture emerging from these results is that the central portion enters the bilayer first and is followed by both ends. Although this differential penetration ends at about 27 s, the penetration persists until about 90 s after incubation is commenced.

For a given type of amino acid residue, such as Leu-85, or Leu-96 for example, the centrally located residue is labeled to a greater extent. This may be due to the restraint exerted by the rest of the polypeptide

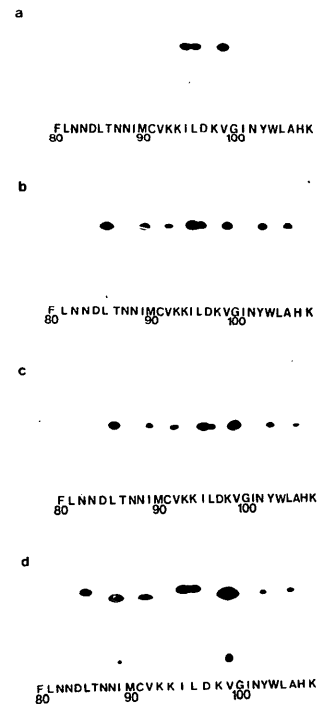

Fig. 8. Autoradiograms demonstrating the time dependent TLC patterns of the radioactive derivatives of PTH amino acids within α-lactalbumin (80–108) fragments. See text for experimental details.

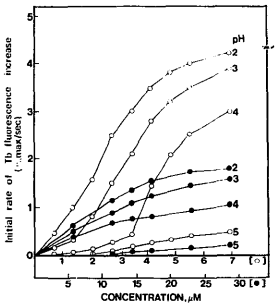

Fig. 9. Comparison of the initial rate of fusion induced by intact α-lactalbumin and α-lactalbumin (80–108) fragment. The experimental procedure for the fusion is given in the previous publication [1]. ○——○, intact α-lactalbumin; ●——●, α-lactalbumin fragment. pH 3, 18 °C.

chain on the marginally located residues within the α-lactalbumin (80–108) fragment.

Fusion of the vesicles induced by the membrane-bound α-lactalbumin fragment

Fig. 9 compares the initial fusion rate of PS/PE vesicles induced by intact α-lactalbumin and the α-lactalbumin (80–108) fragment. On the basis of weight the fragment is a more potent fusogen but based on the mole concentration it lags behind the intact protein. Still, the fusogenic activity of the fragment is substantial.

Discussion

In the previous communication [1], evidence of α-lactalbumin penetration into the bilayer was presented. The involvement of hydrophobic interaction between α-lactalbumin and PC vesicles at low pH was also observed by Hanssens et al. calorimetrically [6] and spectroscopically [7]. The present investigation of [^{125}I]TID labeling confirms the earlier result that the α-lactalbumin sequence between Leu-81 and Ala-106 enters the bilayer.

The utility of the [^{125}I]TID labeling and subsequent identification of labeled amino acid residues in determining the structure of the membrane-embedded portion of integral proteins was amply demonstrated by Hoppe et al. [2] and Brunner et al. [3]. These workers elucidated the structures and topologies of subunits of the F_0 part of ATP synthase of *E. coli* as well as a

membrane-spanning sequence of bacteriorhodopsin within the membrane. The present work demonstrates that this general method is also applicable to soluble protein-bilayer interactions.

The hydrophobic amino acid residues within the α-lactalbumin (80–108) fragment which was not labeled with [^{125}I]TID are Leu-81, Ile-101, and Leu-105. The α-helical wheel diagram given in Fig. 5 shows that the labeled residues are distributed only on one side of the diagram. As was argued by Brunner et al. [3], this may mean that the segment assumes an α-helical configuration within the bilayer and that only one side of the helix is accessible to the hydrophobic label. The only unlabeled residue which lies within this labeled surface is Leu-81 and this is possible if it is located outside the bilayer.

Assuming that the membrane-penetrating segment exists as an α-helix, there are two possible topologies whereby one side of the helix is inaccessible to [^{125}I]TID: either it forms a tight loop reaching deep inside the hydrophobic core of the bilayer or it is located on the surface of the bilayer with one side of the α-helix facing the aqueous solution while the other side is in contact with the interior of the membrane. The difficulty with the model of tight loop formation, however, is that the membrane-penetrating segment forms a disulfide linkage in the middle of this loop which requires that the rest of the protein be closely located to this segment. It has already been shown that the 63–79 segment, although attached to the α-lactalbumin (80–108) fragment through a disulfide linkage, is situated outside of the bilayer. The [^{125}I]TID does not label the 63–79 segment while it does the α-lactalbumin (80–108) fragment. The dansyl chloride labeling in the previous study also showed that there is only one membrane penetrating segment. These observations indicate the plausibility of the surface topology of the α-lactalbumin segment. In this configuration, some of the hydrophobic amino acid residues will be in contact with the aqueous phase. However, these may be covered by hydrophobic groups from the rest of the α-lactalbumin.

The relationship between the possible α-helical conformation of α-lactalbumin (80–108) fragment in the membrane and the secondary structure of the same segment before it binds to the bilayer is of importance in understanding the mechanism of penetration. However, there is no detailed X-ray structure of this protein available. The predicted α-helix stretches in the vicinity of the segment protected from tryptic digestions are 77–87, 91–99 and 105–110 according to Hanssens et al. [7] and 90–99 and 103–111 according to Lewis and Scheraga [8]. Hanssens et al. [7] also estimated the mean hydrophobic moments of these stretches and found them to be highly amphiphilic. The amphiphilic nature of this segment is in accord with two models given here. If parts of the 81–106 segment are non-helical in aqueous solution as suggested by these estimates, there should be an increase in helical content when in contact with the phospholipid vesicles. This was demonstrated by CD measurements [7]. These authors emphasized, however, that the existence of an amphiphilic α-helix segment does not automatically bring about the penetration of helix into bilayer. They noted that α-lactalbumin must be somewhat unfodled and amphiphilic helices be exposed for the protein to be able to micellize the PC vesicles. Since the penetration should precede the micellization, it is clear that the exposure of the amphiphilic α-helicis is also a prerequisite for the penetration. Our results with ovalbumin [9] and cytochrome *c* [10] also show that the penetration of a segment each of these proteins occurs only at low pH where it is known that the exposure of hydrophobic residues takes place.

Brunner et al. [3] suggested that the [^{125}I]TID labeling-sequencing method may be used for kinetic studies on the penetration into and translocation across membranes of proteins. As far as we are aware, the present investigation is the first kinetic experiment of this nature. Although the initial penetration of the middle part of 81–106 segment of α-lactalbumin into the bilayer is obvious (Table I), it is not altogether clear whether the N-terminal edge of this segment goes before the C-terminal side.

In the previous communication, the correlation between fusion and penetration by the α-lactalbumin segment was suggested. The present kinetic study further strengthens this notion by the coincidence of the penetration and fusion processes as shown in Fig. 6. From this data and Table I, it appears that fusion depends on total penetration rather than penetration of particular amino acid residues.

In any event, further studies will be needed to obtain a clearer picture of the α-lactalbumin segment topology in the bilayer. Interaction of α-lactalbumin with vesicles of phospholipids with spin labels at different carbon positions of the acyl chain should be a promising approach. The ESR method was successfully employed to clarify topologies of integral proteins [11] as well as water-soluble proteins [12]. Another logical approach may be to photoactively label the penetrating protein at different positions of the phospholipid [3]. This method was employed in demonstrating the penetration of tetanus toxin into model membrane. The elucidation of topologies of the protein segments within the bilayer is very important in understanding the molecular mechanism of protein-induced fusion of biological membranes.

The fusion of PS/PE vesicles induced by the membrane penetrating fragment is of interest. Because this small fragment is free of the rest of the protein, the determination of the topology of this segment in the membrane is of importance.

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