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Fluorescence studies on the interaction of a synthetic signal peptide and its analog with liposomes

Qingda Wang, Dafu Cui, Qishui Lin *

State Key Laboratory of Molecular Biology, Shanghai Institute of Biochemistry, Academia Sinica, 320 Yue-yang Road, 200031 Shanghai, China

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Abstract

The N-terminal signal sequence of glucitol permease of *Escherichia coli* (Gut22: MIETITHGAEWFIGLFQKGGEC) and its analog (Gut22Ana: MIETITPGAVWFIGLFQKGGEC) were synthesized. The analog had a Pro residue substituted for the His at the 7th position of Gut22 and a Val residue substituted for the Glu at the 10th position. Previous studies indicated that due to its structural rigidity, the interaction of Gut22Ana with lipid bilayer was much weaker than that of Gut22 (Wang, Q.D., Cui, D.F. and Lin, Q.S. (1996) Science in China (Series C) 39, 395–405). To further probe the location of the tryptophan residues of the peptides in lipid bilayer, the membrane penetration depth of the tryptophan residue of Gut22 was measured using spin-labeled phospholipids, and fluorescence quenching of the peptides by iodide and acrylamide in the presence and absence of phosphatidylserine/phosphatidylcholine liposomes were also studied. Fluorescent labeling of the peptides enabled the study of their association with membrane by fluorospectrophotometry. In the presence of liposomes, the peptides were protected from reaction with chymotrypsin, indicating that the peptide incorporated into the membrane. However, dithionite, which acts external to the membrane, reacted with the peptide, showing that the peptides did not translocate across lipid bilayer spontaneously.

Keywords: Signal sequence; Glucitol permease; Analog; Liposome; Lipid–peptide interaction

1. Introduction

The phosphoenolpyruvate-dependent phosphotransferase system (PTS) is an important system in bacteria catalyzing sugar transport. Enzymes II of the PTS are embedded in the plasma membrane of bacteria. The N-terminal sequences of enzymes II of the PTS are similar to mitochondrial signal sequences which can potentially form amphiphilic α helices. The PTS signal sequences have their distinct characteristics: they are not cleaved following their insertion into the membrane; they exhibit higher than normal frequencies of charged residues, with most of them positively charged, but also some of them negatively charged; Ser and Thr residues are common in mitochondrial signal sequences, but rarely occur in PTS signal sequences.

Signal sequences could not only bind membrane

Abbreviations: 5-doxyl PC, 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine; 10-doxyl PC, 1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; IANBD, *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PC, phosphatidylcholine; PS, phosphatidylserine; PTS, phosphoenolpyruvate-dependent phosphotransferase system; SUV, small unilamellar vesicle; TFA, trifluoroacetic acid

* Corresponding author. Fax: +86 (21) 6433 8357; e-mail: qslin@sunm.shcnc.ac.cn

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receptor, but also interact with lipid membrane directly. It helps to elucidate the process of membrane binding, insertion and translocation of proteins to study the interaction of synthetic signal peptides and their analogs with liposomes as model membranes.

Two peptides were synthesized by the solid phase method, one corresponding to the N-terminal signal sequence of glucitol permease of *Escherichia coli* (Gut22), and the other (Gut22Ana) was an analog of Gut22, in which the His at the 7th position was substituted by Pro and the Glu at the 10th position was substituted by Val. Previous studies showed that Gut22 could insert into lipid bilayers spontaneously and drastic conformational changes were induced, while Gut22Ana could only bind lipid bilayers weakly [1]. The interaction of the peptides with model membranes were further studied by techniques based on tryptophan fluorescence. Fluorescence quenching of the peptides by aqueous and membrane-incorporated quenchers in the absence and presence of phosphatidylserine/phosphatidylcholine liposomes were studied. To make clear whether the peptides could translocate into the liposomes, the protection of 7-nitrobenz-2-oxa-1,3-diazole(NBD)-labeled peptides from reaction with chymotrypsin and dithionite by liposomes was also studied.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC), phosphatidylserine (PS) and chymotrypsin were from Sigma; 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine (5-doxyl-PC) and 1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine (10-doxyl-PC) were from Avanti Polar Lipids; acetonitrile was from BDH; trifluoroacetic acid (TFA) was from Merck; *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD) was from Molecular Probes; Triton X-100 was from ROTH; dithiothreitol (DTT) was from Serva; all other reagents were of analytical grade and made in China.

2.2. Peptides

Gut22 (MIETITHGAEWFIGLFQKGGEC) and Gut22Ana (MIETITPGAVWFIGLFQKGGEC) were both synthesized on an ABI 430A peptide synthesizer by the solid phase procedure and purified by high performance liquid chromatography. Their purity was more than 95% respectively. Amino acid analysis of the two peptides clearly showed the correct composition.

2.3. Preparation of vesicles

Small unilamellar vesicles (SUVs) with a potential negative inside were prepared as follows: liposomes were first prepared in a pH 7.4, 10 mM PIPES buffer containing 150 mM KCl using the same procedure described in Ref. [1] (K^+-containing liposomes), and then diluted 20-fold with a pH 7.4, 10 mM PIPES buffer containing 150 mM NaCl (Na^+ buffer) and treated with 1/400 (molar ratio to lipids) valinomycin overnight; a K^+ diffusion potential negative inside was thus created. For comparison, K^+-containing liposomes were also diluted with K^+ buffer and treated with valinomycin. In this case there would be no valinomycin-induced potential.

2.4. Fluorescence quenching experiments

Fluorescence quenching experiments were performed on a Hitachi F-4010 spectrofluorometer using 295 nm excitation wavelength in order to minimize absorptive screening by the quenchers used. Aliquots (8 μl) of freshly prepared sodium iodide (4 M) or acrylamide (4 M) stock solutions were added to a constantly stirred 1.6-ml peptide solution. After each addition of the quencher, the emission intensity at 340 nm was measured. Stock solution of sodium iodide contained 1 mM sodium thiosulfate ($Na_2S_2O_3$) to prevent I_3^- formation. For acrylamide quenching, correction for inner filter effects ($\epsilon_{295} = 0.25$ $M^{-1}\,cm^{-1}$, for acrylamide) were made [2]. The fluorescence quenching data were analyzed according to the Stern–Volmer equation for the collisional quenching [3]:

$$F_0/F = 1 + K_{sv} \cdot [Q] \quad (1)$$

where, F_0 and F are fluorescence intensities in the absence and presence of quencher, respectively, K_{sv} is Stern–Volmer constant for the collisional quenching process, [Q] is quencher concentration. The above equation predicts a linear plot of F_0/F versus [Q] for

a homogeneous emitting solution. The slope of this plot yields the value of K_{sv}.

2.5. Measurement of penetration depth of peptide's tryptophan residue in the membrane

SUVs for *n*-doxyl PC quenching experiments contained PS/spin-labeled PC/PC (1:1:3). Fluorescence emission spectra of the two peptides in the presence of PS/PC (1:4), PS/5-doxyl-PC/PC (1:1:3) or PS/10-doxyl-PC/PC (1:1:3) vesicles were recorded from 300 nm to 400 nm and fluorescence intensities at 345 nm were read. Excitation wavelength was 280 nm, peptide concentration was 5 μM and lipid concentration was 160 μM. The data were analyzed according to the parallax method by Chattopadhyay and London [4]. The distance between the bilayer center and the fluorophore, Z_{CF}, was calculated from Eq. (2),

$$Z_{CF} = -\frac{1}{2L_{m-n}}\left[\frac{1}{\pi C}\ln\left(\frac{F_1}{F_2}\right) + L_{m-n}^2\right] + L_{c-m} \quad (2)$$

where C is the two-dimensional concentration of the quenchers in the membranes (0.002 molecules/Å^2, assuming that the cross section of a lipid molecule is 70 Å^2). F_1, F_2 are the fluorescence intensities in the presence of *m*- and *n*-doxyl PC, respectively. L_{c-m} is the length between the bilayer center and the doxyl group of *m*-doxyl PC (12.15 and 7.65 Å for $m = 5$ and 10, respectively). The vertical length between the *m*- and *n*-doxyl groups, L_{m-n}, was obtained from these L_{c-m} values. This quantitative estimation is possible due to the ability of spin-labeled phospholipids to quench the intrinsic tryptophan fluorescence of the peptides over a short range.

2.6. Translocation assay with NBD-labeled peptides

30 μl 3 mM Gut22 or Gut22Ana solution was incubated with 160 nmol DTT at 37°C in nitrogen for 1 h to reduce potentially oxidized cysteine residues. Subsequently a 3-fold excess of IANBD (1.2 μmol, in acetonitrile) over the total of thiol groups was added and the coupling reaction was allowed to proceed for 1 h under nitrogen at 37°C.

Unlabeled and labeled peptides were purified on a Vydac C18 reverse-phase column (bead size 5 μm, 200 × 4.6 mm) with 41% to 51% gradient of solution B (0.09% TFA in acetonitrile) versus solution A (0.1% TFA in water). Detection wavelength was 490 nm. NBD concentrations were measured spectrophotometrically at 494 nm. The absorption coefficient of IANBD in water was determined to be $27.3 \cdot 10^3$ $M^{-1}\,cm^{-1}$ and was assumed not to change upon coupling to the peptide. Peptide concentrations were determined spectrophotometrically at 280 nm ($\epsilon_{280} = 4.41 \cdot 10^3$ $M^{-1}\,m^{-1}$, for both Gut22 and Gut22Ana). Since NBD also contributed to the absorption at 280nm ($\epsilon_{NBD,280} = 1.66 \cdot 10^3$ $M^{-1}\,cm^{-1}$), it was subtracted from the actual reading at 280 nm of the labeled peptides.

PS/PC vesicles (50 μM), chymotrypsin (0.1 mg/ml) and dithionite (10 mM) were added sequentially to NBD-Gut22 or NBD-Gut22Ana solution (10 μM), and time courses of NBD fluorescence were recorded. Excitation wavelength was 485 nm, emission wavelength was 540 nm and slits were both 5 nm.

3. Results

3.1. Fluorescence quenching of peptides' tryptophan residue by iodide or acrylamide

Iodide and acrylamide were used as aqueous-phase quenchers of the tryptophan fluorescence. Iodide is considered to access only the surface tryptophans, whereas acrylamide would have good access to all but the most highly buried tryptophan residues [5]. The Stern–Volmer plots of the quenching of tryptophan fluorescence by iodide and acrylamide are shown in Fig. 1. In the presence of PS/PC vesicles, compared with buffer, tryptophans in both peptides became less accessible to the quenchers, suggesting shielding by the lipid bilayer. However, the extent of shielding from the quenchers was different for Gut22 and Gut22Ana. In buffer, the tryptophan residue in Gut22 was a little more exposed to the quenchers than that of Gut22Ana. Upon addition of liposomes, however, it became less exposed than that of Gut22Ana. Thus, tryptophan in Gut22 experienced more shielding in the lipid bilayer. The Stern–Volmer quenching constants for a bimolecular collisional quenching process were calculated from the apparent

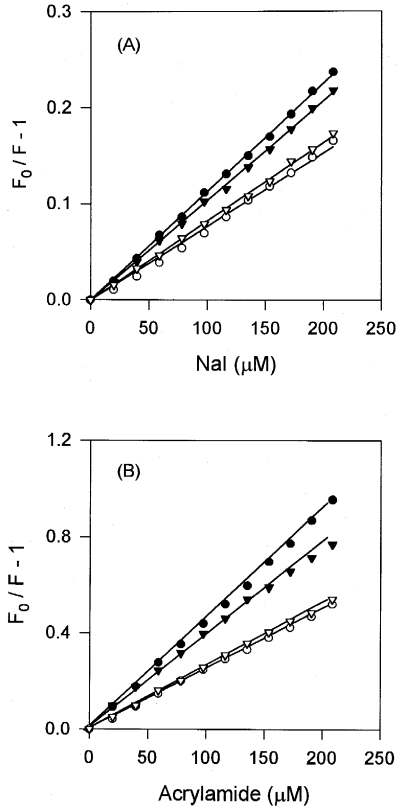

Fig. 1. Stern–Volmer plots showing the tryptophan fluorescence quenching of Gut22 and Gut22Ana by iodide (A) or acrylamide (B). Peptide concentration was 6 μM, lipid concentration was 110 μM. Excitation was at 295 nm. (○) Gut22 in buffer; (●) Gut22 with PS/PC vesicles; (▽) Gut22Ana in buffer; (▼) Gut22Ana with PS/PC vesicles.

slopes of the plots of $F_0/F - 1$ versus [Q] (slopes were calculated by linear regression analysis using the least square method) and are given in Table 1.

Table 1
Fluorescence quenching parameters of Gut22 and Gut22Ana in the absence or presence of PS/PC vesicles

Peptide	K_{sv} (M^{-1})			
	Iodide quenching		Acrylamide quenching	
	in buffer	with PS/PC vesicles	in buffer	with PS/PC vesicles
Gut22	1.13	0.77	4.53	2.49
Gut22Ana	1.03	0.82	3.85	2.61

Peptide concentration was 6 μM, lipid concentration was 110 μM. K_{sv} is Stern–Volmer quenching constant determined from the slopes of the lines for the plots of $F_0/F - 1$ versus [Q]; slopes were determined by linear regression analysis of the fluorescence quenching data using the least squared method.

3.2. Measurement of penetration depth of peptide's tryptophan residue in the membrane

To measure accurately the penetration depth of the peptides' tryptophan residues in lipid bilayer, fluorescence quenching experiments were carried out with spin-labeled PCs. The fluorescence emission spectra of the two peptides in the presence of PS/PC, PS/5-doxyl-PC/PC or PS/10-doxyl-PC/PC vesicles are shown in Fig. 2. Intrinsic fluorescence of Gut22 was almost equally efficiently quenched by 5-doxyl PC and 10-doxyl PC. According to Eq. (2), the penetration depth of the tryptophan residue of Gut22 was calculated from the fluorescence intensities at 345 nm, and it was 9.8 Å from the bilayer center. Since the thickness of one leaflet of a lipid bilayer is more than 20 Å, it is unlikely that there was

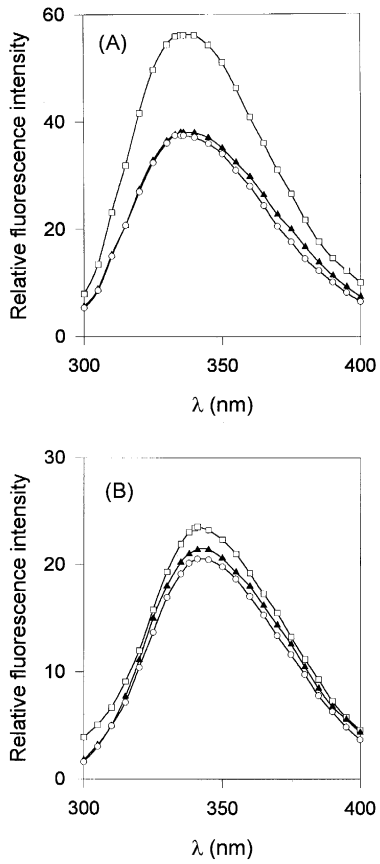

Fig. 2. Fluorescence emission spectra of Gut22 (A) and Gut22Ana (B) in the presence of PS/PC (1:4) (□), PS/5-doxyl-PC/PC (1:1:3) (▲) or PS/10-doxyl-PC/PC (1:1:3) (○) vesicles. Excitation was at 280 nm. Peptide concentration was 5 μM, lipid concentration was 160 μM.

quenching from the *trans* leaflet [4]. In comparison, as shown in Fig. 2B, the intrinsic fluorescence of Gut22Ana was so little quenched by the doxyl groups that it was impossible to calculate the penetration depth of its tryptophan residue [4].

3.3. Translocation assay of NBD-labeled peptides

Either Gut22 or Gut22Ana had a single cysteine residue at carboxyl terminal, to which the thiol-reactive fluorophore IANBD was coupled. The labeled peptides, NBD-Gut22 (Fig. 3) and NBD-Gut22Ana, were purified by reverse-phase HPLC. The degree of labeling, defined as the concentration of the label divided by the peptide concentration after separating from all free labels, was 93% and 94% for NBD-Gut22 and NBD-Gut22Ana, respectively.The intrinsic fluorescence spectra of the labeled peptides in the absence and presence of PS/PC vesicles were similar to those of unlabeled peptides (result not shown). This result supports the notion that the NBD label does not drastically alter the property of the peptides.

PS/PC vesicles (with or without a membrane potential), chymotrypsin and sodium dithionite were added sequentially to NBD-Gut22 solution and time courses of the NBD fluorescence were recorded (Fig. 4). Addition of PS/PC vesicles to NBD-Gut22 solution resulted in a large enhancement of the NBD fluorescence, indicating the entering of NBD groups into hydrophobic environment of the lipid bilayer. This reflected the rapid binding of the peptides to lipid membrane. Treatment of the suspensions with chymotrypsin resulted in a decrease of the fluorescence, as chymotrypsin could digest those peptides remaining outside the vesicles and the fluorescent fragment generated by proteolysis would not bind to the vesicles. However, the fluorescence did not decrease too much, indicating that there was a certain amount of protection of liposome on the peptide from protease digestion. This suggested that the peptide was either irreversibly associated with the vesicle membrane or translocated across the lipid bilayer.

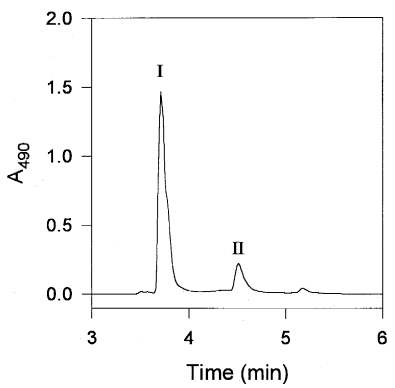

Fig. 3. Purification of NBD-Gut22 by HPLC. Elution profile of the labeling reaction solution of Gut22 with DTT and IANBD was shown. The sample was run on a reverse phase Vydac C18 column with 41% to 51% gradient of solution B (0.09% TFA in acetonitrile) versus solution A (0.1% TFA in water). Flow rate was 1 ml/min. Peak I was DTT-NBD and Peak II was NBD-Gut22.

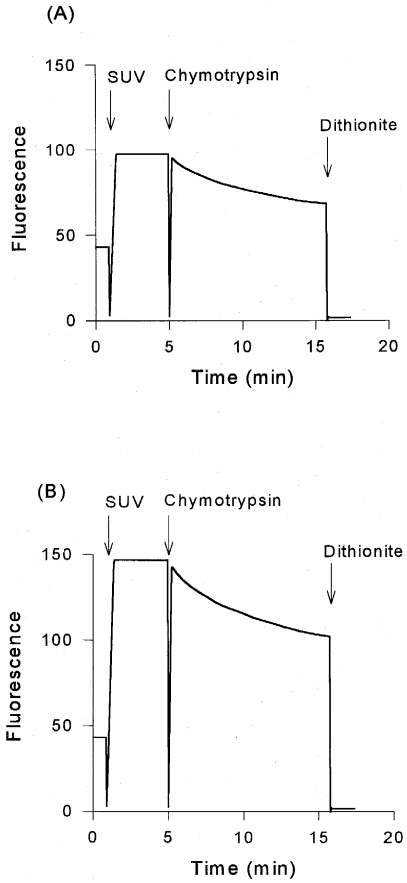

Fig. 4. Influence of chymotrypsin and dithionite on the fluorescence of NBD-Gut22 upon addition of PS/PC vesicles with (B) or without (A) a membrane potential. 50 μM K^+-containing PS/PC SUVs diluted in K^+-buffer (A) or Na^+-buffer (B) and treated with valinomycin, 0.1 mg/ml chymotrypsin, and 1 mM dithionite were added sequentially to 8 μM NBD-Gut22 in K^+-buffer (A) or Na^+-buffer (B), and time courses of the NBD fluorescence were recorded respectively. Excitation was at 485 nm and emission was at 540 nm.

Further treatment of the suspensions with sodium dithionite resulted in the disappearance of the fluorescence. Dithionite could reduce all NBD groups both remaining outside the vesicles and embedded in the outer leaflet of lipid vesicles, and the reduced form of an NBD group was not fluorescent [6], but translocated peptides would be resistant to the treatment of added dithionite and remain highly fluorescent inside the vesicles [7] on the premise that dithionite would not leak into the internal compartment of the liposomes, which was verified to be the case. When dithionite was added 10 min after the addition of Gut22 to the IANBD-contained liposomes, the NBD fluorescence nearly did not decrease (results not shown). Though Gut22 could induce vesicle leakage, the leakage process reached a slow phase shortly after the addition of the peptide [1], suggesting that the membranes of the vesicles with bound peptide were compromised such that they would still be the barrier for molecules like dithionite. The result that the addition of dithionite immediately extinguished the NBD fluorescence indicated that NBD-Gut22 could not translocate into phospholipid vesicles.

The presence of a membrane potential increased the extent of fluorescence enhancement of NBD-Gut22 upon addition of liposomes (Fig. 4B). This result further supported the conclusion that a membrane potential negative inside favored the binding of Gut22 with lipid membrane [1]. However, the peptide remained unable to translocate into liposomes.

It should be pointed out that the NBD fluorescence of the fluorescently-labeled peptide was only 2–4-fold enhanced while binding to lipid bilayers. The enhancement was not very prominent, indicating that the NBD-labeled cysteine residue at C-terminal of the peptide did not insert deeply into the lipid membrane.

As seen from Fig. 5, the extent of fluorescence enhancement of NBD-Gut22Ana was not as large as that of NBD-Gut22, and the fluorescence decrease was faster than that of NBD-Gut22 after chymotrypsin treatment, indicating that its lipid binding ability was not as strong as NBD-Gut22. The NBD fluorescence of NBD-Gut22Ana also disappeared after the addition of dithionite, indicating that NBD-Gut22Ana could not translocate into phospholipid vesicles spontaneously, just as in the case of NBD-Gut22.

Control experiments showed that though the addition of liposomes to NBD-Gut22 solution resulted in an enhancement of NBD fluorescence, further addition of 0.1% Triton X-100 to lyse the vesicles led to a decrease of the NBD fluorescence, restoring it to the previous level immediately (results not shown).

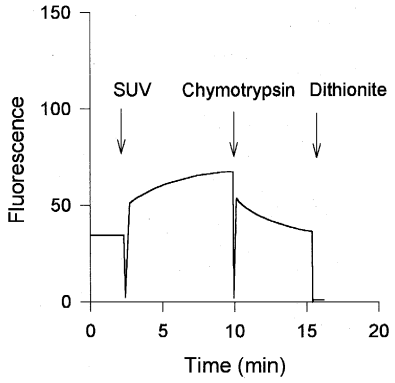

Fig. 5. Influence of chymotrypsin and dithionite on the fluorescence of NBD-Gut22Ana upon addition of PS/PC vesicles. 50 μM K^+-containing PS/PC SUVs diluted in K^+-buffer, 0.1 mg/ml chymotrypsin, and 1 mM dithionite were added sequentially to 8 μM NBD-Gut22Ana in K^+-buffer and time course of the NBD fluorescence was recorded. Excitation was at 485 nm and emission was at 540 nm.

4. Discussion

Fluorescence quenching studies (Fig. 1) indicated that the extent of shielding of the tryptophan fluorescence from the aqueous-phase quenchers iodide and acrylamide in the PS/PC complex is larger for Gut22 than for Gut22Ana. It was either due to a deeper penetration of the tryptophan residue of Gut22 into the lipid bilayer, or a higher extent of association of the peptide, or a combination of both. To clarify the two possibilities, an experiment under conditions in which all peptides are bound to the membranes would be very helpful. However, such an experiment is difficult to carry out, since the association of the peptides with lipid bilayer is a reversible process and it is thus difficult to maintain all peptides in lipid-binding state. In any case, the quenching experiments indicated a stronger interaction of Gut22 than Gut22Ana with lipid bilayer. In accordance, the extent of the liposomal protection of NBD-labeled peptides from protease digestion, and that of the fluorescence enhancement of the NBD-labeled peptides upon

addition of liposomes (Figs. 4 and 5), also indicated that Gut22 had a stronger lipid affinity than Gut22Ana. This was in accordance with the previous conclusion [1].

The signal peptide/lipid interaction was further characterized by quenching experiments using PCs that were doxyl-labeled at different positions along the acyl chain (Fig. 2). These lipids act as collisional quenchers of tryptophan fluorescence and can be used to obtain information about the extent and relative depth of penetration of the peptide into the hydrophobic core of the bilayer. The calculated penetration depth of Gut22's tryptophan residue at the 11th position was 9.8 Å from the bilayer center, which placed the tryptophan residue a little below the polar/hydrocarbon 'boundary'. This tended to support the proposal that Gut22 was buried in the bilayer with the long axis of its amphiphilic helix lying parallel to the membrane surface, though the experiments did not allow a clear-cut assessment of the peptide's membrane topology.

Since the fluorescence enhancement of NBD-Gut22 or NBD-Gut22Ana was not very large, it was proposed that the C-terminal of the peptides was not much deeply buried into the core of the lipid bilayer. In fact, the first 18 residues of Gut22 have the potential to form an amphiphilic helix, and are thought to constitute separate folding domain in the glucitol permease [8], because they are separated from the following polypeptide chain by two glycyl residues (Gly19 and Gly20). The C-terminal cysteine residue of Gut22 is located just after the supposed folding domain, separated by three residues including two glycyl residues. It might be conceived that the N-terminal 18-residue stretch of Gut22 was buried a little deeper in the membrane, leaving its C-terminal buried to a lesser extent.

The NBD-labeled synthetic peptide corresponding to the N-terminal presequence of the precursor of yeast cytochrome oxidase subunit IV (CoxIV-25) was shown to translocate across phospholipid bilayers in a potential-dependent manner [5]. Results here indicated that NBD-Gut22 could not translocate into liposomes, even in the presence of a membrane potential, though it could bind irreversibly with a lipid membrane. This is in accordance with the fact that the glucitol permease is an intact membrane protein of *E. coli*. CoxIV-25 have a net positive charge of 5 at physiological pH, while Gut22 is negatively charged. Whether an amphiphilic peptide can translocate across lipid membrane in a potential-dependent manner or not might be related to its charge condition.

Acknowledgements

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