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Reconstitution of bacteriorhodopsin from a mixture of a proteinase V_8 fragment and two synthetic peptides

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Abstract

The peptide and retinal mixture of bacteriorhodopsin, composed of two synthetic peptides corresponding to helices F (160–197) and G (202–237) and a proteinase V_8-derived fragment V_1 (1–166), generated the characteristic features of bacteriorhodopsin with absorbance maximum at 550 nm and fluorescence quenching as in two synthetic peptides corresponded to helix A (sequence 7–31) and B (41–65), and a chymotryptic fragment (72–248). The recovery of reconstitution estimated from the absorption and the fluorescence quenching of these mixture was 16–19% and 25–32% of the native purple membrane, respectively, whereas mixtures lacking any one of the peptides exhibited no absorption recovery. Circular dichroism of each peptide fragment showed complete formation of α-helical structure in a membrane-mimetic medium of sodium dodecyl sulfate. These results indicate that the specific interactions or mutual recognitions between α-helices in lipid bilayers are essential for correct bundling of the seven helices and formation of the retinal binding pocket.

Keywords: Bacteriorhodopsin; Membrane protein; Reconstitution; Peptide fragment

1. Introduction

Specific interactions among polypeptide secondary structures are the basis of the formation of a tertiary structure of a protein [1]. From the early stage of progress of protein science, this view has been supported by the production of a functional protein from a set of complementary polypeptide chains, the classical examples are RNase S [2] and fragmented Streptococcal nuclease [3]. In general, proteins which are formed in aqueous media interact so that hydrophobic amino acid side chains are buried and their hydrophilic ones are exposed to the media. These types of proteins have been fairly well studied, particularly in the case of coiled-coils derived from packing of α-helices [4–9].

The packing principle of membrane proteins is the opposite of that of aqueous proteins, i.e. to bury hydrophilic side chains and to expose hydrophobic

Abbreviations: BO, bacterioopsin; BR, bacteriorhodopsin; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DMPC, dimyristoyl-L-α-phosphatidylcholine; Fmoc, fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLCK, N^{α}-tosyl-L-lysylchloromethyl ketone

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ones. Usually a polypeptide chain folds back and forth crossing a membrane bilayer several times to form α-helices which assemble with each other to give an α-helix bundle in the interior of a membrane. Each α-helix in a membrane bilayer is held together with polypeptide chain loops. If the mutual recognition of the α-helices was strong enough, the presence of joining loops might unnecessary for a correct pairing of the helices. Experimental evaluation of the degree of such mutual recognition of elementary secondary structures is thus needed. Although such an evaluation is fundamental to the understanding of the structure formation of membrane proteins, there is, unfortunately, no general and practical method to estimate the association state of polypeptide secondary structures. We must therefore look for a specific event to study whether a specific assembly of polypeptide secondary structures was established or not: in other words, whether mutual recognition between secondary structures occurred or not. For such a system, we chose bacteriorhodopsin as a model system.

Bacteriorhodopsin (BR) is a protein made of a single polypeptide chain (248 amino acids) and a retinal and functions as a light-driven proton pump [10,11]. Electron microscopic studies have shown that BR contains seven α-helices (A–G) connected by loop portions to form a bundle in the lipid bilayer [12–14]. The most characteristic feature of BR is its absorption maximum at 568 nm [36], which is caused by retinal in the protein. The spectrum is very sensitive to the environment of retinal. The absorption maximum is attained only when the correct BR structure is formed and, therefore, can be used to study the correct folding or packing of the polypeptide chain in membrane environments [15–17]. Using this property, Khorana's group in their historical report first showed that chymotryptic fragments (helix A–B and C–G) with retinal give complete BR structure and function [15,18,19]. Two proteinase V_8 fragments and retinal were reported later to give a correct BR structure [20]. Recently it was shown that a ternary mixture (helix A, B, and C–G) with retinal also completed the structure [21]. These results show that to form the helix bundle loop portions which connect helices seem to be unimportant and that is shown by the report that BR mutants which lack a part of loop portions correctly refold to afford BR structure [22]. Among the BR loop portions, the role of a loop connecting helix F and G attracts attention because the terminal helix G carries an essential cofactor, retinal, and the F–G loop is the only one to hold this key helix. Although the other terminal loop, loop A–B, has been shown to be dispensable for the construction of BR structure [21], nevertheless, the more critical problem of the loop F–G remained unanswered.

To clarify whether the process of mutual recognition of individual α-helix fragments is involved in establishing the correct bundle or not, studies of the reconstitution from peptide fragments have to be continued. In this paper we have described that a ternary complex (helix A–E, F, and G) with retinal can reconstitute BR structure and also confirmed the report that a connection between helix A and B is dispensable.

2. Materials and methods

2.1. Chemicals

DMPC was obtained from Avanti Polar-Lipids (Alabaster, AL, USA), CHAPS and TLCK-treated chymotrypsin from Sigma (St. Louis, MO, USA), all-*trans* retinal from Nacalai Tesque (Kyoto, Japan), *Staphylococcus aureus* proteinase V_8 from Miles Laboratories (MO, USA), amino acid derivatives used for peptide synthesis from Watanabe Chemical Industry (Hiroshima, Japan) and Peptide Institute (Osaka, Japan), Spectra/Por dialysis bags (molecular weight cut off: 3500 and 1000) from Spectrum Medical Industry (Los Angeles, USA), and poly(ethylene glycol) (average molecular weight 20 000) from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of proteolytically cleaved fragments

Purple membrane was isolated from *Halobacterium halobium* as described by Oesterhelt and Stoechenius [23]. Apomembrane and chymotryptic fragments C_1 and C_2 were prepared according to the method of Pope et al. [19]. Proteinase V_8 digestion of BR was performed according to the published procedures [20] with some modifications as follows: Lyophilized apomembrane having 25 mg protein was

suspended in 50 mM sodium phosphate buffer (pH 7.8) containing 2 mM EDTA and 0.25% SDS (weight/vol) at a concentration of 5 mg/ml. The suspension was incubated with proteinase V_8 (weight ratio of enzyme to BR, 3:100) at 37°C for 24 h. Proteolytic digestion was terminated by lyophilization.

Proteolytically cleaved fragments, C_1, C_2, V_1, and V_2, were separated by gel permeation chromatography on Sephadex LH-60 equilibrated with 88% formic acid and ethanol (3:7, v/v). Fragments obtained showed a single band by SDS-PAGE. Amino acid composition and molecular weight of each fragment were confirmed to be in good accordance with the theoretical values. Delipidated bacterioopsin was also prepared by this chromatography.

2.3. Gel-electrophoresis

SDS-PAGE was performed by a 15% polyacrylamide gel and stained by Coomassie brilliant blue R-250 [24].

2.4. Transfer of fragments to SDS solution

A solution of chymotryptic or V_8 proteinase fragment in formic acid/ethanol (3:7) was poured in a dialysis bag (Spector/Por 3) dialyzed against SDS buffer-b consisting of 0.2% SDS, 10 mM sodium phosphate, pH 8.0, and 0.025% NaN_3, until initially formed white precipitates disappeared (for 1–2 days), and further dialyzed against SDS buffer-a which had the same composition as SDS buffer-b except the pH was 6.0, for 1 day. SDS buffer-b was used first to dissolve the fragments because white precipitates was not dissolved directly in SDS buffer-a, which was used for the reconstitution system. After dialysis, fragments were concentrated to 50–150 mM by covering the dialysis bag with poly(ethylene glycol) powder (average molecular weight = 20 000). Synthetic peptides were also transferred into SDS buffer-a using Spectra/Por CE dialysis bag.

2.5. Peptide synthesis

Four peptides corresponding to amino acid sequences 7–31, 41–65, 160–197 and 202–237, representing helical regions A, B, F, and G, respectively, were synthesized on an automatic peptide synthesizer (Applied Biosystem 431A or Milligen 9050) with an Fmoc methodology [25]. The sequences of the peptides are shown as follows, where the residues assumed to the membrane-bound are underlined:

Peptide A: H-RPEWIWLALGTALMGLGTLYF-LVKGM-OH
Peptide B: H-KFYAITTLVPAIAFTMYLSMLL-GYG-OH
Peptide F: H-AESMRPEVASTFKVLRNVTVV-LWSAYPVVWLIGSEGAG-OH
Peptide G: H-NIFTLLFMVLDVSAKVGFGLI-LLRSRAIFGEAEAPE-OH

All synthetic peptides were purified by a HPLC. Peptides A and B were purified on a μBondapak C4 column with a linear gradient elution from 80% solvent A (10% w/v formic acid, 10% ethanol, 10% acetic acid in water)–20% solvent B (0.1% trifluoroacetic acid in acetonitrile) to 20% A–80% B. Peptides F and G were purified on a μBondapak C18 column with a linear gradient from solvent a (5% formic acid in water) to b (5% formic acid in ethanol). Amino acid compositions and the purity of peptides A and B thus obtained were confirmed by amino acid analysis and analytical HPLC. Crude peptide F gave several peaks (Fig. 1A) but the peak 4 fraction gave the amino acid composition identical to the theoretical value for peptide F and gave one major peak (Fig. 1A inset) upon rechromatography. Crude peptide G was purified as a single peak (Fig. 1B, peak 2) with theoretical amino acid composition. The purity of peptides F and G were also confirmed by analytical HPLC.

2.6. Reconstitution of BR-like chromophore from peptide fragments and retinal

Reconstitution of BR from peptide mixtures (BO, C_1 and C_2, V_1 and V_2, A, B and C_1 or V_1, F and G) with retinal was performed according to the method of Liao et al. [18] with some minor modifications as modified by Dr. F. Tokunaga, Osaka University (personal communication). One or two-fold molar amounts of retinal, which was taken from a stock solution of 2.7 mM in ethanol, was mixed with

peptide dissolved in SDS buffer-a. Simultaneously, an equal volume of a Chaps (1.5–4.0%) and DMPC (3.5–4.0%) solution in 100 mM sodium phosphate, pH 6.0, 0.025% NaN_3 was added to the mixture. Reconstitution was performed at 15°C in the dark. Free retinal was determined taking molar extinction at 380 nm as 43 000 cm^{-1} M^{-1} in absolute ethanol [16,26].

When retinal is incorporated in apomembrane-bound polypeptide by a Schiff base formation, the chromophore exhibits a new absorption band at 550 nm [17] or a decrease in tryptophan fluorescence due to the energy transfer from tryptophan to retinal [15,16]. The degree of reconstitution was thus determined by measuring the increase in absorbance at 550 nm or a decrease in fluorescence at 335 nm. The absorbance and fluorescence intensity of the native BR was assumed to be the 100% reconstituted stage.

2.7. Protein determination

Protein concentration was determined by an absorbance at 280 nm. Molar extinction coefficient of BO, C_1, C_2, V_1, and V_2 at 280 nm were taken as 66 000, 48 000, 18 000, 51 600, and 14 400, respectively, calculated from the contents of tryptophan and tyrosine and their extinction coefficient [27]. The concentration of reconstituted chromophore was calculated from the molar extinction coefficient (ϵ_{550}) value of 47 000 $cm^{-1}M^{-1}$ [28].

2.8. Amino acid analysis

Amino acid analysis was performed with a JASCO Model 1000 amino acid analyzer after hydrolysis in 6 M HCl at 110°C for 24 h in vacuo [29].

2.9. CD measurement

CD was measured with a JASCO-720 spectropolarimeter using quartz cells (1 mm light path) at room temperature. Spectrum of SDS buffer-a was subtracted from spectrum of peptides dissolved in SDS buffer-a and the ellipticity was expressed as the mean residue weight. Content of secondary structures was estimated by the method of Chen et al. [30].

2.10. Fluorescence measurement

Fluorescence intensities were measured using a quartz cell (5 mm light path) on a Shimadzu RF-5000 spectrofluorometer. The excitation wavelength was 285 nm and the emission wavelength was 335 nm (5 nm slit band width).

3. Results

3.1. Peptide synthesis and purification

Peptides were synthesized with an Fmoc methodology [25] and purified according to the established

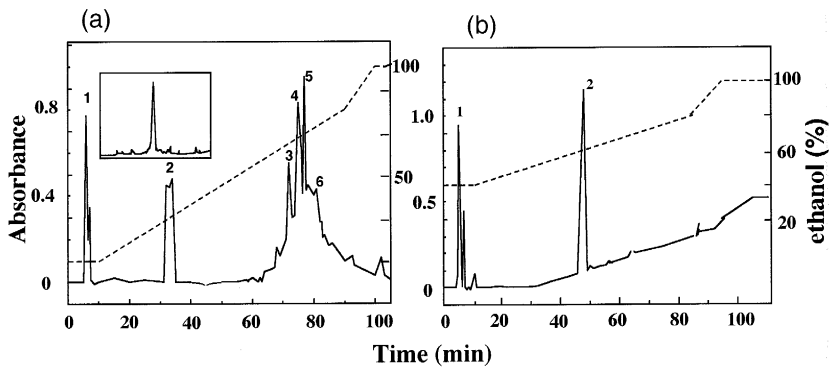

Fig. 1. HPLC of crude peptides F (a) and G (b). Peptides were dissolved in 88% formic acid and injected into a column of μBondapak C18 at a flow rate of 0.7 ml/min. A gradient elution of 5% formic acid in water to 5% formic acid in ethanol was employed as indicated by the dotted line. Peptides F and G were detected by absorbance at 280 nm and 260 nm, respectively. Inset in (a) shows a rechromatogram of peak 4.

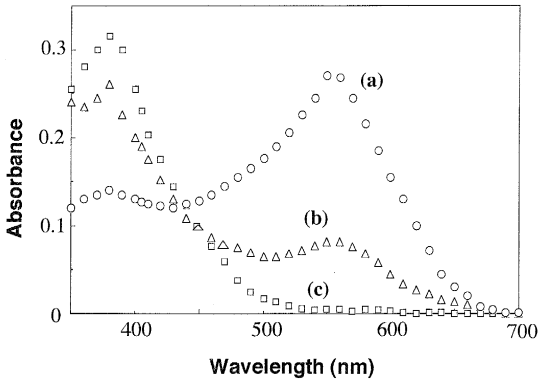

Fig. 2. The absorption spectrum of retinal with $V_1 + V_2$ (a), retinal with V_1 and peptides F + G (b), and retinal only (c). Each solution contained 10 μM retinal, 10 μM polypeptides, 0.75% CHAPS, 2.0% DMPC, 0.11% SDS, 0.025% NaN_3, and 50 mM sodium phosphate, pH 6.0.

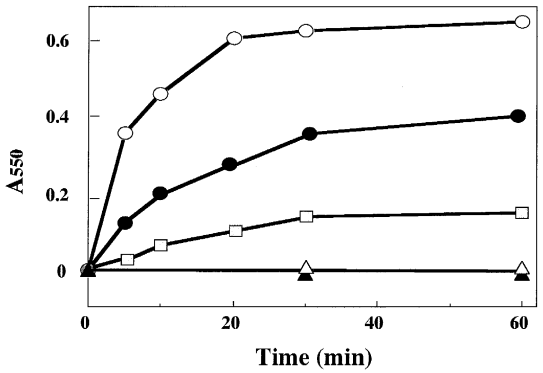

Fig. 3. Time courses of chromophore regeneration from V_8 proteolytic fragments, synthetic peptides and retinal. Peptide and retinal concentration was 15 μM and 30 μM, respectively. (○), retinal and BO; (●), retinal and $V_1 + V_2$; (□), retinal and V_1 + peptides F + G; (△), retinal and V_1; (▲), retinal and V_2.

procedure for purification of BR-derived peptide fragments [37]. The purity and amino acid compositions of peptides A, B, F and G were confirmed by analytical HPLC and amino acid analysis.

3.2. Evaluation of regeneration system

To see if the fragments could regenerate a BR-like chromophore under the present experimental conditions, V_1 and V_2, C_1 and C_2 or C_1, synthetic peptides A and B were mixed with retinal. In these experiments, solvent containing the fragments were transferred to an aqueous solution containing detergents, because the formic acid/ethanol solvent used in gel permeation chromatography is too strongly acidic for reconstitution experiments in synthetic lipids. As an example, regeneration of a BR-like chromophore is shown in Figs. 2, 3 and Figs. 4. The degree of reconstitution was thus determined by measuring the increase in absorbance at 550 nm or a decrease in fluorescence at 335 nm. The yield of these regenerations with respect to the native BR is compatible to the result of Sigrists et al. [20] as summarized in Table 1. Similar reconstitution yields were observed with the fluorescence and the absorbance data, although some discrepancies in the yields are shown with the mixture of C_1, A, B and retinal. These results show that the regeneration system employed here is pertinent to evaluate the new peptide mixture of BR.

3.3. Reconstitution from V_8 proteolytic fragments and synthetic peptides

The newly constructed mixture of V_1 fragment and synthetic peptides F and G with retinal produced the BR-like chromophore to give an absorption spectrum at 550 nm (Fig. 2). The regeneration monitored by the absorption at 550 nm reached plateau within 60 min (Fig. 3). A mixture of peptides lacking any one of peptide components showed no increase in absorption up to 12 h and gave the same absorption spectrum as that of retinal only in Fig. 2 (data not shown).

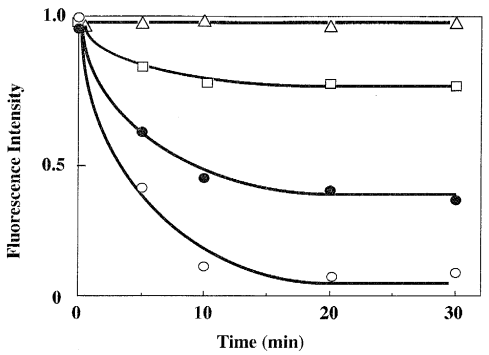

Fig. 4. Time course of decrease in tryptophan fluorescence. Experimental conditions are the same as in Fig. 2 except concentrations of retinal and polypeptides were 1.0 μM each. Excitation wavelength was 285 nm and emission wavelength was 335 nm. (○), retinal and BO; (●), retinal and $V_1 + V_2$; (□), retinal and peptides F + G + V_1; (△), retinal and V_1 fragment only.

Excess amount of retinal and synthetic peptides over the proteolytic fragment V_1 caused no additional increase in the reconstitution yield.

Quenching of tryptophan fluorescence was also observed with this mixture (Fig. 4). No fluorescence decrease was observed when the mixture lacked any fragment. The decline of the fluorescence was complete at about 10 min while regeneration monitored by the increase in absorbance was complete by about 20 min. This result may suggest a biphasic process in which the first represents some kind of assembly step necessary for regeneration. There was also a discrepancy between the reconstitution yields calculated from the fluorescence and absorbance data (Table 1). We have not yet identified the source of this discrepancy.

3.4. Effect of detergent on reconstitution

CHAPS added to the medium helped generate the BR-like chromophore, but regenerations decreased as the concentration of CHAPS increased. The highest yield was obtained when CHAPS concentration was 0.75%. Below this concentration, no chromophore formation could be detected because of the appearance of the turbidity in the mixture. These results show that the detergent is needed to keep lipids soluble in the reconstitution medium, but an excess amount of detergent disturbs the lipid assembling and prevents the chromophore forming.

3.5. Secondary structure of peptide fragments and synthetic peptides

CD spectra of the synthetic or enzymatically cleaved peptides V_1, V_2, F, and G dissolved in SDS buffer-a (see Section 2) showed that the peptide fragments form highly-helical structures (Fig. 5). Possible interfere with the CD spectra caused by the detergent in the medium can be excluded because no abnormal CD spectrum was observed with the detergent only. The concentration of SDS (0.2% w/v) present in the buffer is sufficient to form micelles;

Table 1
Reconstitution yields of BR-like chromophore determined by absorbance and fluorescence methods

Component for reconstitution	Concentration of each component (μM)	Yields of reconstitution determined by	
		Absorbance method (%)	Fluorescence method [a] (%)
Bacterioopsin	5.0	85.4	90.2
	15.0	85.1	
Chymotryptic fragments ($C_1 + C_2$)	5.0	72.0	57.6
	15.0	70.2	
Synthetic peptides (A + B) + chymotryptic fragment (C_1)	3.95	10.7	32.0
	2.9 [b]	19.0 [b]	
V_8 proteolytic fragment ($V_1 + V_2$)	15.0	65.2 (70.5) [c]	68.0
V_8 proteolytic fragments (V_1) + synthetic peptide (F + G)	15.0	16.5	25.0
V_1	15.5	< 0.5	< 1.0
V_1 + F	18.0	< 0.5	< 1.0
V_1 + G	21.1	< 0.5	< 1.0

Equimolar concentration of BO or peptide fragments (C_1 corresponding to residues 72–248; C_2, 1–71; V_1, 1–166; V_2, 167–254; A, 7–31; B, 41–65; F, 160–197; G, 202–237) and retinal were mixed and reconstitution yields were measured after 12 h incubation.
[a] Reconstitution medium contains 2.0% DMPC and 0.95% CHAPS. Peptide concentration is 1 μM each.
[b] Reconstitution medium contains 3.5% DMPC and 4.0% CHAPS.
[c] Calculated from Ref. [20].

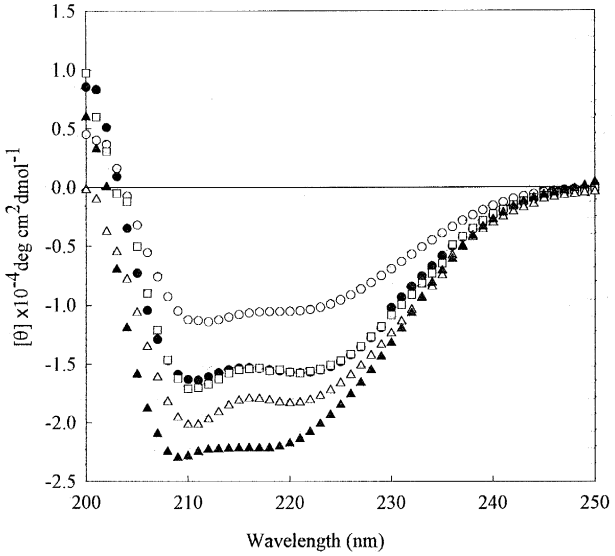

Fig. 5. Circular dichroism spectra of peptide fragments. Peptide fragments were dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 0.2% SDS and their CD spectra were measured. (○), fragment V_2 ($2.2 \cdot 10^{-5}$ M); (●), BO ($7.7 \cdot 10^{-6}$ M); (□), V_1 ($1.2 \cdot 10^{-5}$ M); (△), peptide F ($1.2 \cdot 10^{-5}$ M); (▲), peptide G ($3.0 \cdot 10^{-5}$ M). Curves were obtained by averaging over 8 scans with background subtracted.

the peptide structures can be considered to reflect those in a lipid membrane [31].

The α-helical content of each peptide fragment was estimated to be 64.0, 43.3, 60.0, and 73.3% for V_1, V_2, F, and G, respectively. These values are in good agreement with the helical contents estimated from the proposed structure of BR [14]. These results indicate that peptide fragments, V_1, V_2, F, and G spontaneously form α-helical structure in lipid membranes. The CD spectra of peptides A and B showed that these synthetic peptides also formed highly-helical structures (data not shown) and the α-helical content was estimated to be 95.0 and 92.7 for A and B, respectively. This result is in good agreement with the structure of segment B (residues 34–65) of BR in SDS micelles determined from NMR data [32].

4. Discussion

The bacteriorhodopsin chromophore is formed only when the correct bacteriorhodopsin structure is established [15–17]. The correct formation of the structure thus can be monitored by observing the absorption spectrum of the system. The method is simple and highly sensitive to a desired structure formation compared to neutron diffraction studies which investigated the position and orientation of seven transmembrane α-helices of BR [33]. The present study demonstrated that a new system containing V_1 fragments and C-terminal helices, peptides F and G, successfully reconstituted the bacteriorhodopsin chromophore. When the system lacks any one of the peptide fragments, no chromophore is generated (Fig. 4, Fig. 5 and Table 1). In particular, peptide G which has Lys 261 as the retinal-binding site has no ability to bind retinal unless the reconstitution system contains all the proposed membrane helical component of BR. These results show that peptide fragments correctly assemble to make a bundle of helices which forms the retinal-binding pocket.

The role of loop-helix interactions [34] has not yet been solved for helix bundle membrane proteins. During the course of the BR structural formation, loop-helix interactions are considered to be unnecessary because the native-like chromophore is regenerated from peptide components which lack loop portions. However, our observation of a small, but significant, difference in the reconstitution yields between the mixture of V_1, F and G and C_1, A and B suggests that loop structure plays some roles in making an efficient assembly or providing extra stability in the BR structure. The reconstitution yield for fragment C_1 with peptides A, and B was significantly higher than that for fragment V_1 with peptides F, and G (Table 1). The former system lacks a long and flexible loop connecting helices B and C (Gly63-Tyr79) comparing to the latter system which lacks a short loop connecting helices E and F (Ser158-Glu166). This result suggests that loop structures help arrange the helices in a proper position. It is also plausible that the connecting loops in the mixture of C_1, A and B help to arrange the helix G into a proper position so that the regeneration yield increases.

Each peptide fragment independently forms secondary structure in the SDS medium (Fig. 5). This indicates that an α-helical structure could be formed in the first step of the reconstitution process. Then a ternary helical complex was formed to give bacterioopsin structure, which finally gave reconstituted bacteriorhodopsin chromophore with retinal. These

speculations are supported by the fact that retinal does not bind to Lys 216-holding peptide G when a mixture of peptides lacking any one of peptide components and also there is no increase in the reconstitution rate when excess retinal is added (data not shown).

Reconstitution of bacteriorhodopsin chromophore from a peptide mixture including single helices has been reported (helices A, B, and C–G) [21]. In this paper we extended the work to show that reconstitution of the native BR chromophore is provided from a variety of peptide fragments containing extramembraneous loops. In addition, we show that there must be a special interaction between helices within the membrane. In the case of glycophorin [35], it was revealed that a synthetic peptide which corresponded to one of the α-helices in the membrane domain competitively inhibited a formation of glycophorin structure, thus showing a strong mutual recognition of α-helices in membranes.

As a conclusion, the present study shows that the α-helix of each peptide fragment is spontaneously formed in the reconstitution system, then assembles with each other, and forms a bundle by mutual recognition to make up a retinal-binding site. Each peptide has the geometry to lead correct contact with the complementary peptides and can identify should-be-neighbors once the helical structure is formed. The idea of mutual recognition is supported by the results of Henderson et al. who have identified some residues all over helical portions which take part in the non-covalent interaction among helices and between helices and retinal [27]. We believe that a mutual recognition between α-helices (or between any secondary structure) is a dominant principle in building a protein structure not only for cytoplasmic proteins but also for the membraneous domains of membrane proteins.

Substitution or deletion of an amino acid residue is an important technique to study the structure and function of BR. The approach given in the present paper, namely the use of synthetic peptides, provides a route for such studies. Especially, using synthetic peptides would provide the opportunity to incorporate non-natural amino acids, which can not be done by conventional mutagenesis and in vivo expression.

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