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Topography of surface-exposed amino acids in the membrane protein bacteriorhodopsin determined by proteolysis and micro-sequencing

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The topography of membrane-surface-exposed amino acids in the light-driven proton pump bacteriorhodopsin (BR) was studied. By limited proteolysis of purple membrane with papain or proteinase K, domains were cleaved, separated by SDS-PAGE, and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Fragments transferred were sequenced in a gas-phase sequencer. Papain cleavage sites at Gly-65, Gly-72, and Gly-231, previously only deduced from the apparent molecular weight of the digestion fragments, could be confirmed by N-terminal micro-sequencing. By proteinase K, cleavage occurred at Gln-3, Phe-71, Gly-72, Tyr-131, Tyr-133, and Ser-226, i.e., in regions previously suggested to be surface-exposed. Additionally, proteinase-K cleavage sites at Thr-121 and Leu-127 were identified, which are sites predicted to be in the α-helical membrane-spanning segment D. Our results, especially that the amino acids Gly-122 to Tyr-133 are protruding into the aqueous environment, place new constraints on the amino-acid folding of BR across the purple membrane. The validity of theoretical prediction methods of the secondary structure and polypeptide folding for membrane proteins is challenged. The results on BR show that micro-sequencing of peptides separated by SDS-PAGE and blotted to PVDF can be successfully applied to the study of membrane proteins.

Introduction

Knowledge of the structure of a protein is required to understand the mechanism of its function. For integral membrane proteins, determination of the structure to high resolution by X-ray crystallography has not usually been possible to date due to lack of suitable three-dimensional crystals. Therefore, the results obtained by a variety of biochemical and biophysical techniques have to be combined to deduce the native conformation of a protein residing in the membrane.

BR is the intensively studied transmembrane protein of the so-called purple membrane in *Halobacterium halobium* (recent reviews [1–3]). This protein consists of a single polypeptide chain of 248 amino acids (M_r 26 486) and the chromophore retinal, covalently bound to the lysine 216 via a protonated Schiff's base. Light energy absorbed by this chromoprotein is used to translocate protons across the membrane. The electrochemical gradient generated is utilized by the cell to drive energy-dependent processes, such as ATP synthesis [4]. Based on electron microscopy [5], the amino-acid sequence [6,7], and on biochemical as well as immunological methods [8,9], secondary structure models for BR have been proposed assisted by empirical secondary structure prediction methods. It is assumed that the polypeptide chain traverses the lipid bilayer via seven α-helical segments, with 17–22 amino acids of the C-terminal end protruding from the cytoplasmic side and the N-terminal sequence exposed to the outside. At present, the number of amino-acid residues in each helix is still uncertain, as well as which amino acids are protruding from the membrane surface. Even the existence of seven transmembrane α-helices has been recently challenged [10].

To provide additional experimental data for verification of existing structural models, we have examined the topography of surface-exposed amino acids by limited proteolysis with papain or proteinase K and subsequent

Abbreviations: BR, bacteriorhodopsin; PVDF, polyvinylidene difluoride; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; PMSF, phenylmethylsulfonylfluoride.

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micro-sequencing of digestion fragments. This paper describes for a membrane protein the successful application of blotting of the fragments in combination with micro-sequencing [11]. A modified structural model for BR will be presented which is compatible with the observed cleavage site at Thr-121 that has previously been assumed to be membrane-embedded.

Experimental procedures

Materials

H. halobium cells, strain ET 1001, were grown and purple membrane was isolated as described previously [12]. Papain type III and proteinase K (from *Tritirachium album*) were purchased from Sigma, SDS, *N,N*-methylene-bisacrylamide, TEMED and ammonium persulfate from Bio-Rad, and ultrapure acrylamide from BRL. The molecular weight standards were from Pharmacia. PVDF membranes (Immobilon) were obtained from Millipore.

Methods

Papain digestion. 1.3 mg BR in a suspension of purple membrane in 1 ml of 1 mM EDTA/0.06 mM 2-mercaptoethanol/5 mM cysteine hydrochloride (pH 7.0) were treated at 37 °C with cysteine-activated papain at an enzyme-to-protein ratio of 1:200 (w/w). The digestion was stopped after 1 and 2 h, respectively, by the addition of a mixture containing 50 µl of 0.1% PMSF and 10 ml of a solution of 1 M NaCl/10 mM Tris-HCl (pH 6.8) [13]. The digested purple membrane was centrifuged at 165 000 × *g* for 20 min. The resulting pellet was suspended in 10 mM Tris-HCl (pH 6.8) and centrifuged again to remove all traces of papain.

Proteinase K digestion. Proteinase K was added to the purple membrane at an enzyme-to-substrate ratio of 1:5 (w/w) and incubated for 2, 8 and 24 h at 37 °C. To terminate the reaction, each sample was stored at −20 °C until all samples had been collected and thereafter the same washing procedure as described for papain was used to inactivate and to remove proteinase K. The buffers used contained 0.2 M *N*-methylmorpholine acetate (pH 7.1) ('low-salt') or 50 mM Tris-HCl/10 mM $CaCl_2$/4 M NaCl (pH 8.0) ('high-salt'). A zero-time sample (to check for papain or proteinase K cleavage during the centrifugation and subsequent electrophoresis steps) was prepared as follows: 0.1% PMSF was added to proteinase K or activated papain, then the purple membrane/buffer solution was added.

SDS-PAGE. Aliquots (10 nmol) of untreated and proteinase-digested BR were dissolved in sample buffer containing 55 mM Tris-HCl (pH 6.8)/2% SDS/7% glycerol/4.3% 2-mercaptoethanol and boiled for 5 min. The samples were subjected to electrophoresis on a high Tris concentration SDS-PAGE with a linear gradient (10–20% acrylamide) and an 8% stacking gel [14].

Electroblotting. After PAGE, the gels were first equilibrated for 30 min in transfer buffer (25 mM Tris-HCl (pH 8.4)/0.5 mM dithiothreitol/0.02% SDS). Transfer of the fragments from SDS-PAGE on PVDF membranes (blotting) was done in an apparatus of standard design [15], using a chamber with an interelectrode distance of 10.5 cm. Electroblotting was performed for 90 min at 100 mA and thereafter for 4 h at 650 mA, at 4 °C, as described in Ref. 11. The PVDF membranes were rinsed twice for 5 min in distilled water. The blotted digestion fragments were visualized by staining with 0.1% amino black in 50% methanol for 40 s.

Sequence analysis of blotted fragments. The proteins or peptide fragments blotted onto PVDF membranes were cut out after visualization and were sequenced in a pulsed-liquid gas-phase sequencer, model 477 A (Applied Biosystems) equipped with the model 120 PTH-aa analyzer. The PVDF filter was placed above a TFA-treated glass-fiber (GF) filter to which 30 µl Bioprene were added to avoid washing-out the fragments. The amount of protein submitted to sequence analysis varied between 50 and 150 pmol of blotted material. The degradation program used for sequencing in the machine was as given in Ref. 11.

Amino-acid analysis. After sequencing of the blotted proteins, the same PVDF membranes with the digested fragments were used to analyze the remaining amounts of amino acid. 200 µl of 5.7 M HCl/0.02% 2-mercaptoethanol were added to each of the PVDF membranes and incubated for 20 h at 110 °C. The amino acids were determined after precolumn derivatization with *o*-phthaldialdehyde (OPA) by reverse-phase HPLC separation as described in Ref. 16.

Spectroscopic and diffraction methods. The functional and structural properties of the various purple membrane samples were tested by static (Shimadzu, UV-VIS spectrophotometer UV-260) and time-resolved absorbance spectroscopy by a laboratory-built laser-flash spectrophotometer, by circular dichroism (CD) measurements (Jasco J-500A spectropolarimeter), and by X-ray diffraction (Elliott GX-21 rotating anode X-ray generator).

Results

Native BR at different salt conditions

Since *H. halobium* grows at high ionic strength in its natural environment it is of interest to determine whether conformational alterations of BR in the purple membrane occur between low- and high-salt conditions. This is of importance for proteolytic studies, which are normally carried out at lower concentrations of electrolyte. It has been shown [17] that circular dichroism and absorption spectra of native purple membrane in water and increasing salt concentrations (1–4 M NaCl) are almost identical. We have performed similar experi-

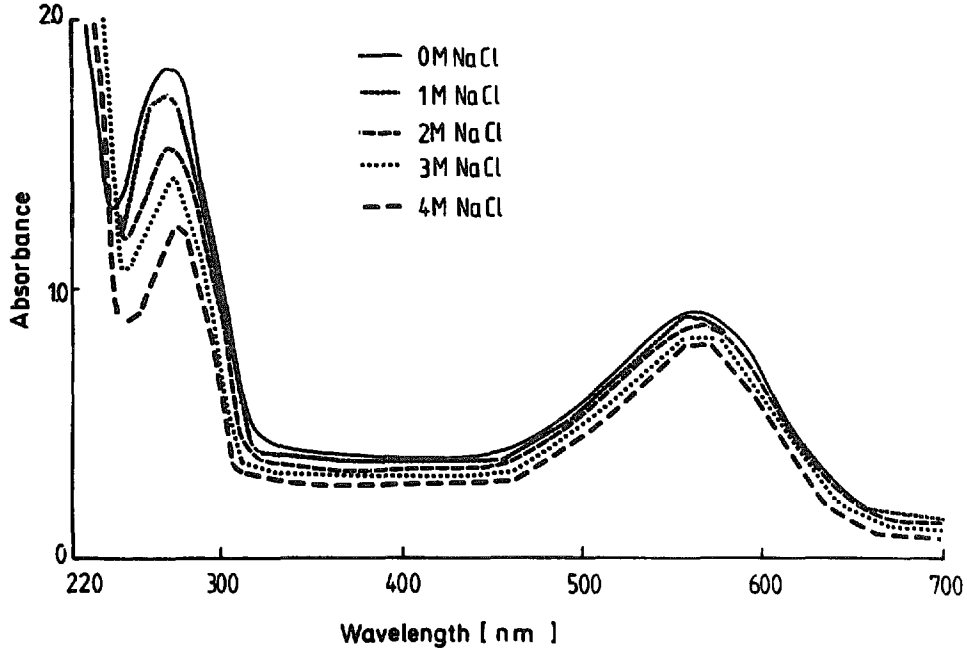

Fig. 1. Absorption spectra of purple membrane with native BR incubated at different NaCl concentrations for 20 h at 37 °C. (The same spectra were obtained after 1 h incubation.)

ments under conditions which resemble those for proteolytic studies. Purple membranes were incubated for 1 and 20 h at 37 °C in digestion buffer (50 mM Tris-HCl/10 mM $CaCl_2$ (pH 8.0)) with different concentrations of NaCl in the absence of proteinases. It was observed that purple membrane patches tend to aggregate at high-salt concentrations depending on the incubation time, which impairs circular dichroism studies. Absorption spectra (Fig. 1) demonstrate that the ionic strength does not affect the conformation of the protein in the vicinity of the retinal binding pocket since the absorption band at 568 nm remains virtually unchanged. Due to purple membrane aggregation with concomitant changes in light-scattering and absorption-flattening, however, a linear decrease of the absorption maximum of the aromatic amino acids at 280 nm occurs with increasing NaCl concentration. With respect to the proteolytic studies performed in this paper, it was necessary to clarify whether a cleavage of BR in the purple membrane as a consequence of autolysis can occur during the incubation period depending on ionic strength. Suspensions of purple membrane at NaCl concentrations between 1 and 4 M were shaken for 1 and 20 h at 37 °C. SDS-PAGE of these samples shows a single band of uncleaved BR and no indication for lower-molecular-weight digestion products (Fig. 2).

The photocycle which is connected to the pumping of protons across BR [18], was compared at zero and 4 M NaCl. This is an additional, sensitive way to look for conformational alterations influencing the function of BR. The M-decay is identical for samples without and with 4 M NaCl, only the formation of the M-state is slowed down by a factor of two in the presence of 4 M NaCl. Since the kinetics of the photocycle are not or only slightly affected, ionic-strength-dependent alterations of structural domains involved in the function of BR can be excluded.

Papain cleavage of BR

The pattern of SDS-PAGE of native and papain-treated BR is shown in Fig. 3. After incubation of purple membrane for 1 h with a low concentration of

Fig. 2. SDS-PAGE of purple membrane with native BR incubated at 37 °C in Tris buffer (50 mM Tris-HCl/10 mM $CaCl_2$ (pH 8.0)) at different NaCl concentrations. Unprimed and primed letters indicate incubation times of 1 and 20 h, respectively. The first column shows the molecular weight markers phosphorylase *b* (94000); albumin (67000); ovalbumin (43000); carbonic anhydrase (30000); trypsin inhibitor (20100); α-lactalbumin (14400). Lanes (M NaCl): a, a′, 1; b, b′, 2; c, c′, 3; d, d′, 4; o′, without NaCl. The high molecular bands in lane o′, a, and b′ represent BR aggregates.

TABLE I

Papain digestion fragments

Fragments obtained by papain digestion after different incubation periods and an enzyme-to-substrate ratio of 1:200 (see Fig. 3). s, sequenced fragments; ns, non-sequenced peptides. The M_r values and the amino-acid composition have been determined from SDS-PAGE and amino-acid analysis after acid hydrolysis as described in the text.

Incubation time (h)	M_r ($\times 10^{-3}$)	Fragments obtained		No.	Cleavage sites
		1 2 231			231 ↓ 232
1	24.7	p-Glu-Ala...Gly	ns	I	Gly ↓ Glu-Ala...
		1 2 231			231 ↓ 232
2	24.7	p-Glu-Ala...Gly	ns	I	Gly ↓ Glu-Ala...
		66 67 231			65 ↓ 66
	16.5	Leu-Thr...Gly	s	II	Gly ↓ Leu-Thr...
		73 74 231			72 ↓ 73
	15.6	Gly-Glu...Gly	s	III	Gly ↓ Gly-Glu...
		1 2 72			72 ↓ 73
	9.0	p-Glu-Ala...Gly	ns	IV	Gly ↓ Gly-Glu...
		1 2 65			65 ↓ 66
	8.2	p-Glu-Ala...Gly	ns	V	Gly ↓ Leu-Thr...

papain (enzyme-to-substrate ratio of 1:200) the native protein (M_r 26500) converted into a species with a molecular weight of 24700 (Fig. 3c). The limited proteolysis results in a fragment shortened by 17 amino acids, which is cleaved off at Gly-231 from the exposed C-terminus [19]. After 2 h digestion or longer at a 1:200 (w/w) ratio, six protein bands are observed by SDS-PAGE. The M_r values of five of these fragments (I–V), marked by arrows in Fig. 3d, are listed in Table I. The fragments II (M_r 16500) and III (M_r 15600) are produced by a cleavage of the peptide bonds between 65-Gly↓66-Leu and 72-Gly↓73-Gly. The protein band visible between III and IV could not be blotted. The disappearance of the band corresponding to native BR indicates the complete cleavage of the protein.

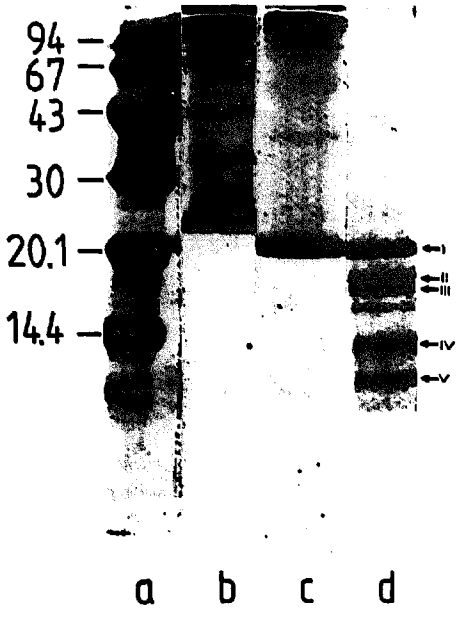

Fig. 3. SDS-PAGE of purple membrane after digestion with papain (enzyme-to-substrate ratio 1:200) for 1 and 2 h at 37°C in cysteine-activated buffer. a, molecular weight markers as in Fig. 2; b, intact BR (strong band at M_r 26500); c, BR digested with papain for 1 h (strong band at M_r 24700); d, BR digested with papain for 2 h (strong band at M_r 24700 and the five BR fragments below this band). Molecular masses ($\times 10^{-3}$) are shown on the left.

Sequencing of fragments obtained from papain digestion

As described in the Experimental procedures section the peptides were electroblotted onto Immobilon membranes (PVDF). The amido-black-stained bands were cut out and sequenced in a pulsed-liquid gas-phase sequencer. For peptide II (M_r 16500) and peptide III (M_r 15600) the following sequences were obtained:

66 231
Leu-Thr-Met-Val-Pro-Phe-···Gly (II)

73 231
Gly-Glu-Gln-Asn-Pro-Ile-···Gly (III)

The first six amino acids of these domains were sequenced. The molecular masses of both peptides were determined by SDS-PAGE and by the amino-acid composition from acid hydrolysis experiments. Sequencing of fragments I, IV and V, which all derive from the N-terminal position of the protein, was not possible due to the presence of pyroglutamic acid at the N-terminus. By amino-acid analysis of these peptides on the filter after the degradation, the remaining amounts were determined. This confirmed that enough peptides were on the blot. The small 17 amino acids containing peptide

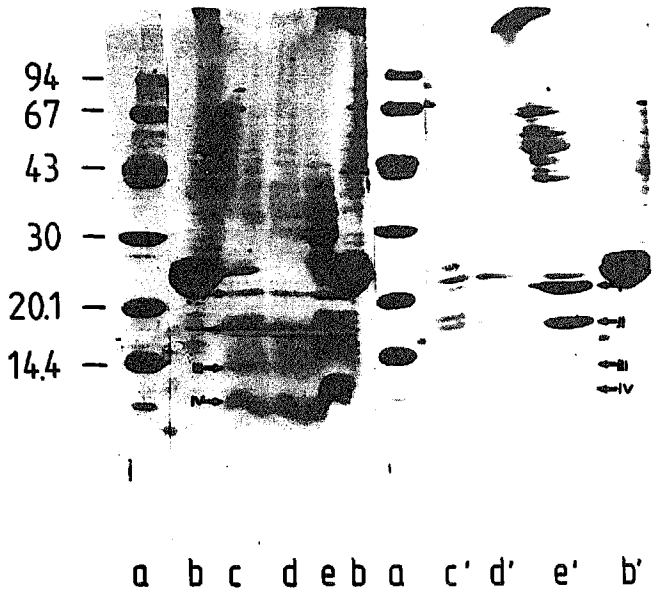

Fig. 4. SDS-PAGE of purple membrane after digestion with proteinase K (enzyme-to-substrate ratio of 1:5) at 37°C for various durations. a, molecular weight markers; b–e, 'low-salt' buffer; b'–e', 'high-salt' buffer; b, b', native BR; c, c', 2 h; d, d', 8 h; e, e', 24 h. In lanes e and e', a larger amount of sample was applied as compared to the other lanes. Molecular masses ($\times 10^{-3}$) are shown on the left.

from the C-terminal end with M_r of about 1815 migrates from the SDS-gel and was not sequenced for this reason.

The amino-acid compositions of the fragments were obtained as described in Experimental procedures. The number of amino acids found was in good agreement with M_r values determined from SDS-PAGE (data not shown).

Digestion of bacteriorhodopsin with proteinase K

The digestion of BR with proteinase K was performed at 'low-salt' (0.2 M *N*-methylmorpholine (pH 7.1)) and at 'high-salt' conditions (50 mM Tris-HCl/10 mM $CaCl_2$/4 M NaCl (pH 8.0)). The pattern of the SDS-PAGE of digested membranes after 2, 8, and 24 h in these buffers is shown in Fig. 4. Analysis (SDS-PAGE and amino-acid composition) proved that the fragments obtained under low- and high-salt conditions were identical. Since blotting at high-ionic concentrations yields low recoveries, only fragments obtained after 24 h digestion in 'high-salt' buffer were sequenced, otherwise fragments produced at low-salt conditions were analyzed. Due to the high-ionic strength, less of the sample material applied migrates into the gel, which explains the less pronounced appearance of the band pattern as compared to the 'low-salt' buffer samples.

After 2 h digestion ('low-salt') several fragments in the M_r range from 23700 to 10600 appeared (see Table II and Fig. 5). Fragment I (M_r 23700), the remainder of the peptide chain, is produced by liberation of about 22 amino acids from the C-terminus. After incubation in 'high-salt' buffer two bands are simultaneously formed with M_r of 24700 and 23700. The additional band at 24700 Da is presumably obtained by cleavage of 17 amino acids from the C-terminus. It is interesting to note that the 24700 Da molecular mass fragment is also produced by cleavage with papain (see above). Fragment II (M_r 17400) represents a mixture of two fragments formed by cleavage at 71-Phe↓72-Gly and 72-Gly↓73-Gly, the latter is also a papain cleavage site. Fragment III (M_r 13000) starts with Ile-4 and ends at Thr-121. Band IV (Fig. 4) contains four fragments. The most interesting fragment IV_1 (M_r 12000) is obtained by cleavage between Thr-121 and Gly-122, a linkage which according to all folding models of BR (except Ref. 20) is localized in the transmembrane helix D. This fragment migrates together with three other peptides (fragment IV_2–IV_4) in the SDS gel (Fig. 4). These fragments were blotted together on the PVDF membrane and sequenced. This part of the BR molecule is easily accessible to proteinase K, which hydrolyses the sequence from Gly-122 to Tyr-133 into small peptides. After incubation of BR with proteinase K for 8 h, identical sequences were found (Table II, Fig. 5). At 24 h, fragments IV_2–IV_4 are missing because of the digestion into dipeptides.

Spectroscopic measurements on proteinase-K-digested BR

To obtain information about alteration in the structure of BR upon treatment of purple membrane with proteinase K, optical absorption spectra were recorded. No differences in the spectra were observed for proteolysis in 'low'- or 'high-salt' buffers. The 568 nm absorption peak of purple membrane in the light-adapted state decreases after 2 h digestion to 90%, after 8 h to 85%, and after 24 h digestion to 75% of the native-membrane absorbance. The absorption peak shifts from 568 nm for native purple membrane to 560 nm for proteinase-K-treated membranes. The absorbance around 370 nm remained unchanged, excluding hydrolysis of the Schiff's base linkage to the retinal.

X-ray diffraction on proteinase-K-digested purple membrane

The X-ray diffraction pattern of native- and proteinase-K-incubated purple membrane in Tris buffer containing NaCl or in morpholine buffer were compared. At 100% relative humidity, oriented stacks of purple membrane were exposed to X rays for 2 h. From the diffraction pattern (data not shown) it is apparent that the BR molecules are still arranged in lattices after 2, 8, or 24 h digestion. There are no obvious differences in the lattice quality between native purple membrane samples and purple membrane incubated with proteinase K in morpholine buffer for increasing periods. The only exception was the specimen digested for 24 h. This sample has a changed colour from purple to red.

SEQUENCED FRAGMENTS

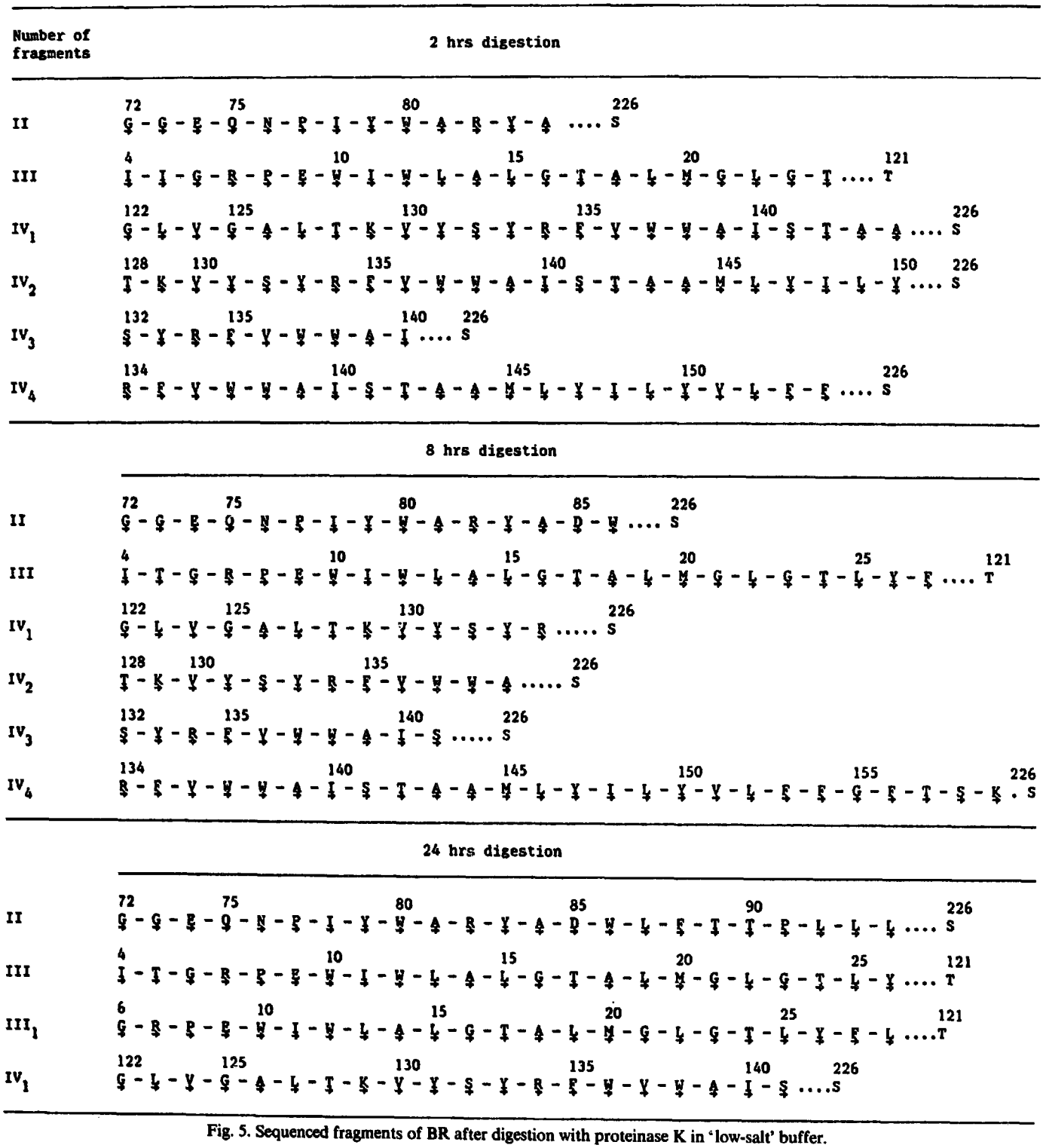

Fig. 5. Sequenced fragments of BR after digestion with proteinase K in 'low-salt' buffer.

For this sample the ratio of the peak height of the X-ray reflections to the background is decreased to about 50% compared to undigested purple membrane. However, when purple membrane samples are incubated with proteinase K in Tris buffer containing 4 M NaCl, this ratio remains the same as for undigested membranes. These differences might result from the combined action of morpholine buffer and the enzyme K.

Discussion

Knowledge of the structure of BR is important for understanding the molecular processes involved in light-induced proton translocation across this membrane protein. Since BR is a common model for a transmembrane transport protein, its structural features are of general interest and any deviation from the assumed structure will be of consequences for the structure elucidation of a great number of other membrane proteins. Often, their secondary and tertiary structures were predicted solely from the amino-acid sequence by applying empirical algorithms that rely on parameters such as hydrophobicity, transfer free energies, or turn regions in the polypeptide chain (Refs. 21–23, and references therein). Many of these empirical methods

TABLE II

Proteinase K digestion fragments

Fragments obtained by proteinase *K* digestion in 'low-salt' buffer after different digestion periods and an enzyme-to-substrate ratio of 1:5 (see Fig. 4 and 5). s, sequenced fragments; ns, non-sequenced fragments.

Incubation time (h)	M_r ($\times 10^{-3}$)	Fragments obtained		No.	Cleavage sites
		1 2 226			226 227 228
2	23.7	p-Glu-Ala...Ser	ns	I	Ser↓Arg-Ala...
		72 73 226			71 72 73
	17.4	Gly-Gly...Ser	s	II	Phe↓Gly↓Gly...
		4 5 121			3 4 5
	13.0	Ile-Thr...Thr	s	III	Gln↓Ile-Thr...
		122 123 226			121 122 123
	12.0	Gly-Leu...Ser	s	IV_1	Thr↓Gly-Leu...
		128 129 226			127 128 129
	11.5	Thr-Lys. .Ser	s	IV_2	Leu↓Thr-Lys...
		132 133 226			131 132 133
	10.9	Ser-Tyr...Ser	s	IV_3	Tyr↓Ser-Tyr...
		134 135 226			133 134 135
	10.6	Arg-Phe...Ser	s	IV_4	Tyr↓Arg-Phe...
		1 2 226			226 227 228
8	23.7	p-Glu-Ala...Ser	ns	I	Ser↓Arg-Ala...
		72 73 226			71 72 73
	17.4	Gly-Gly...Ser	s	II	Phe↓Gly↓Gly...
		4 5 121			3 4 5
	13.0	Ile-Thr...Thr	s	III	Gln↓Ile-Thr...
		122 123 226			121 122 123
	12.0	Gly-Leu...Ser	s	IV_1	Thr↓Gly-Leu...
		128 129 226			127 128 129
	11.5	Thr-Lys...Ser	s	IV_2	Leu↓Thr-Lys...
		132 133 226			131 132 133
	10.9	Ser-Tyr...Ser	s	IV_3	Tyr↓Ser-Tyr...
		134 135 226			133 134 135
	10.6	Arg-Phe...Ser	s	IV_4	Tyr↓Arg-Phe...
		1 2 226			226 227 228
24	23.7	p-Glu-Ala...Ser	ns	I	Ser↓Arg-Ala...
		72 73 226			71 72 73
	17.4	Gly-Gly...Ser	s	II	Phe↓Gly↓Gly...
		4 5 121			3 4 5 6
	13.0	Ile-Thr...Thr	s	III	[illegible]↓Ile-Thr↓Gly...
		122 123 226			121 122 123
	12.0	Gly-Leu...Ser	s	IV_1	Thr↓Gly-Leu...

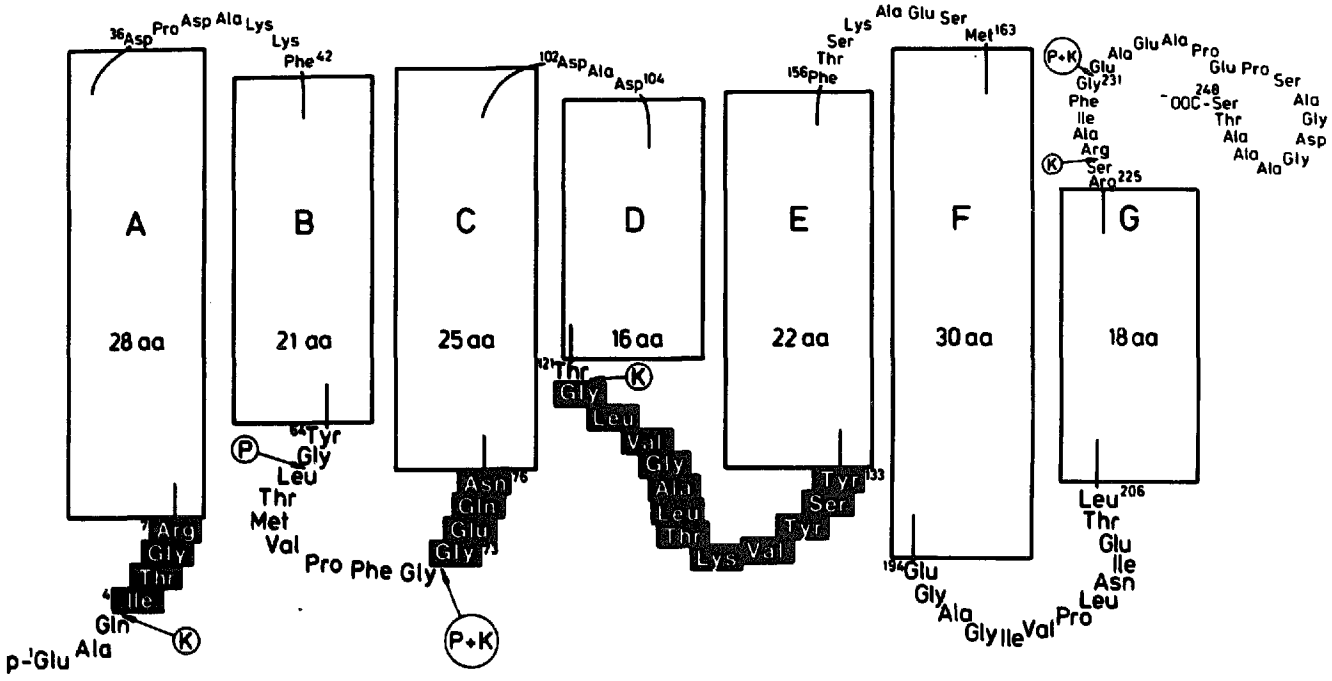

Fig. 6. Folding model of BR according to the results of our digestion studies and data from others (see Discussion). The height of the rectangular columns A to G represents the length of the amino-acid chain (aa) if arranged in an α-helical conformation. The black background represents sequenced amino acids (additional amino acids sequenced are depicted in Fig. 5). (P), papain, (K), proteinase-K cleavage sites.

are based more or less on parameters deduced from the suggested structure of BR as a reference. On the other hand, the validity of newly developed structure prediction methods is often tested on the 'known' structure of BR (e.g. Ref. 22). Furthermore, attempts have been made to identify potentially functional important amino-acid residues in membrane domains of transport proteins by using BR as one of the standards [24]. Obviously, such approaches have to result in misleading conclusions, since the structure of BR is not solved. In a recent analysis it was convincingly shown that present prediction schemes are all inappropriate for the prediction of secondary structures (less than 0.5% correlation between predicted structure and experimental result) and for membrane protein folding [21]. This is corroborated by our experimental finding that a polypeptide segment in BR previously predicted by all folding models (e.g., Refs. 8, 9, 23, 25), except (Ref. 20) to be part of the transmembrane helix D is actually membrane-surface-exposed (Fig. 6). To contribute to the BR structure, we have identified amino-acid residues exposed at the membrane surface by limited proteolysis of purple membrane with papain and proteinase K in combination with micro-sequencing of blotted fragments after separation by SDS-PAGE. Considering the number of cleavage sites and the amount of material removed from the membrane, papain and proteinase K are more effective than the enzymes trypsin, chymotrypsin, or V8.

It is important to note that the proteolytic cleavage sites between Thr-121 and Gly-122 and the following ones (Fig. 5, Table II) are generated by proteinase K both at 'low'- and 'high-salt' conditions even after only 2 h. According to the spectroscopic and X-ray diffraction data, the structure of the purple membrane is still preserved in the presence of the enzyme after this time. This excludes exposure of cleavage sites artificially produced by denaturation of BR. Inferred from the amount of material digested, all BR molecules in a purple membrane patch, and not only those located at the patch margin, contain this cleavage site. The only firm explanation for the high susceptibility to proteolysis is that, in native BR, the amino-acid segment starting with Gly-122 is freely exposed at the purple membrane surface. This contradicts the current view of the folding motif of BR which, based on empirical methods, places this peptide segment nearly in the middle of the transmembrane segment D. It is unrealistic to assume that a huge, water-soluble enzyme can cleave a peptide bond in the hydrophobic core of the membrane, especially in the case of the purple membrane, where the proteins are tightly packed with only one boundary layer of lipid. Also a large membrane-surface invagination in close contact to the transmembrane polypeptide segments as a point of attack for the proteinase can be excluded, since the surfaces of the purple membrane are fairly flat [26]. To explain our data, we present the modified

folding model of BR depicted in Fig. 6 that considers only experimental data, such as site-specific immunological probes [27, 28], limited proteolysis [19,29,30], NMR spectroscopy [31], and selective chemical or enzymatic modification [32,33]. The length of the exposed C-terminal end is deduced from the observed proteinase K and papain cleavage sites (Tables I and II) and is in accordance with a recent modification study [33]. The absence of other cleavage sites at the cytoplasmic surface under our conditions might indicate short interhelical connecting loops and/or loops situated directly at the membrane surface. The topography of these amino acids in Fig. 6 is taken from Ref. 8. The framework for the present model of BR is the three-dimensional structural analysis obtained by electron-microscopic data [5], in which seven transmembrane rods of density were derived. There remains some controversy about the type of secondary structure of these rods. The strong 1.5 Å reflection on the meridian seen in the X-ray diffraction pattern on oriented stacks of purple membrane makes it very likely that most of the seven rods contain α-helices. From the obtained sequence data of proteinase cleavage sites it is not possible to decide the type of secondary structure. According to the modified model, the membrane-spanning segment D cannot be formed by a continuous α-helix, since 16 amino acids as α-helix are not sufficient to traverse the membrane. The membrane-spanning helices of the photosynthetic reaction center are composed of at least 23 amino acids [34]. Other secondary structure elements have to participate in segment D, and their occurrence in BR is being intensively studied [35–41].

X-ray diffraction studies on papain or proteinase-K-digested specimens show that the conformation of the remaining portion of BR is unaffected by cleavage of the polypeptide chain. Changes in the lamellar spacing, which is smaller in proteinase-treated BR, are probably due to the removal of the negative charges at the C-terminus. The absence of these negative charges may also be the main reason for an increased tendency of purple membranes to aggregate. Experiments to localize the C-terminal amino acids in the structure by the Fourier difference technique were not successful. Our results therefore support previous suggestions of a random orientation of this terminal segment (Refs. 42, 43, but also Ref. 44).

Previous studies of the cleavage behavior of proteinase K on BR in the purple membrane [30] led to complete degradation of BR in the absence of a high NaCl concentration. Only in the presence of 4 M NaCl was a proteinase-K-specific cleavage site proximate to the C-terminus found. Our spectroscopic measurements (absorption- and CD-spectra, time-resolved photocycle analysis) give no evidence for any influence of the salt concentration on the conformation and function of native BR. The digestion studies with proteinase K show, in contradiction to Gerber et al. [30], that the amino-acid sequences of the fragments digested in the presence or absence of salt are identical. The only region which has an additional cleavage site in the presence of NaCl is at the carboxyl end, where equal amounts of a 17- and 22-amino-acid long C-terminal segment were cleaved off.

Dumont et al. [45] described several locations of proteolytic cleavages by proteinase K (using an enzyme-to-BR ratio 2.5 higher as compared to the present study), i.e., at both ends of the polypeptide (residues 4–7 and 231–236) and in other regions (amino acids 32–38, 66–75, 132–135 and 160–170). The peptides were separated by HPLC followed by amino-acid analysis but not be sequencing. These authors assume that peptides recovered by 'denaturating' HPLC conditions are not identical to helical parts in the native structure. By employing protein blotting and micro-sequencing of the fragments obtained we can exclude misleading results.

By sequencing some of the papain-digestion fragments we were able to confirm unequivocally cleavage sites which in previous studies were deduced only from the apparent molecular weight according to SDS-PAGE or from the total amino-acid composition [19,29].

While the three-dimensional structure of BR remains unsolved by X-ray crystallography or electron microscopy, biochemical and immunological data will have to contribute to the understanding of the polypeptide folding across the membrane. Newly developed experimental techniques such as tritium planigraphy [46] will be more helpful and reliable in supporting our present view of the structure of BR than novel empirical prediction methods.

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