The antigenic structure and topography of bacteriorhodopsin in purple membranes as determined by interaction with monoclonal antibodies

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The spatial organization and the antigenic structure of the bacteriorhodopsin molecule in the purple membrane were studied by immunochemical techniques. Five monoclonal antibodies directed against exposed parts of the protein molecule in the membrane were prepared and characterized. Antigenic determinants were localized in the bacteriorhodopsin polypeptide chain by analysis of the interaction between monoclonal antibodies and protein fragments. The structure of antigenic determinants was revealed by the interaction of monoclonal antibodies with (i) isolated bacteriorhodopsin fragments further modified by sequential Edman degradation and (ii) derivatives of bacteriorhodopsin obtained biosynthetically or by selective chemical modification. Five antigenic determinants were localized in the following parts of bacteriorhodopsin: <Glu^{1}-Met^{20} involving one of the 3 amino acid residues of the N-terminal part; Gly^{33}-Met^{56} involving Asp^{36} and/or Asp^{38} and Phe^{42}; Phe^{156}-Met^{163} involving Phe^{156}; Glu^{194}-Leu^{207} involving Glu^{194}; Pro^{200}-Leu^{207}.

Bacteriorhodopsin *Monoclonal antibody* *Membrane* *Antigenic structure* *Protein* *Enzyme-linked immunoadsorbent assay*

1. INTRODUCTION

Bacteriorhodopsin (BR) is a retinal-containing chromoprotein from purple membranes (PM) of *Halobacterium halobium* (reviews [1,2]). Its complete amino acid sequence [3,4] and general scheme of arrangement of the polypeptide chain in the membrane [5] have been established. Several models [2,6,7] have been proposed to describe the fine structure of the BR molecule in membranes, i.e., to define which regions of the polypeptide chain are intramembranous and which are exposed on the cell surface. These models are based on the identification of exposed amino acid residues using partial proteolytic cleavage of the polypeptide chain [8–10] and selective chemical or enzymatic modifications [11,13].

Monoclonal antibodies (mAb) represent a powerful new tool for the investigation of the structure and function of membrane proteins [14,15]. We have prepared a set of mAbs directed against the BR molecule, and used them to study the BR structure and topography in the PM [16,17]. The results obtained permit us to refine the chromoprotein topography and identify amino acid residues essential for interaction with particular mAbs.

2. MATERIALS AND METHODS

BR was isolated according to [18]. Fluorinated BR analogs were prepared as described in [19]. Chymotrypsin cleavage of the polypeptide chain into fragments 1–71 and 72–248 (C-2 and C-1) and subsequent separation of the fragments as well as delipidation were carried out as in [20]. Chromatography on Sephadex LH-60 was performed in nonaqueous solvents [18]. To cleave the polypeptide chain at bond Gly^{155}–Phe^{156} [18], $NaBH_4$ was added to a suspension of PM

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(1.5 mg/ml) in 0.05 M NH_4HCO_3, pH 10.0, up to a final concentration of 3% and stirred for 24 h at 4°C in the dark. After centrifugation (4 times) in H_2O, the membranes were treated with papain to remove C-terminal fragment 232–248 [8]. Peptide 156–231 was isolated by Sephadex LH-60 chromatography in 98% formic acid-absolute ethanol (3:7). Peptide 156–231 was cleaved with BNPS-skatole as in [21], then fragments 156–182, 183–189 and 190–231 were separated on Sephadex LH-20 and LH-60. Homogeneity of isolated BR fragments was confirmed by amino acid and N-terminal analyses. Localization of the regions of the polypeptide chain cleaved with $NaBH_4$ or BNPS-skatole was supported by the Edman degradation of peptides 156–231 and 190–231, respectively.

Papain cleavage of BR in PM was carried out according to [8]. To modify carboxyl groups [22], the PM (1 mg/ml) were dissolved in 5 ml of 0.2 M sodium phosphate buffer, pH 4.75, containing 2 M ethylenediamine, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to a final concentration of 0.4 M. The pH value was carefully controlled as carbodiimide was added over a 1 h interval and the reaction allowed to proceed for a further 5 h. After the reaction was completed, the membranes were spun down in water. The limited acid hydrolysis of BR fragment 1–71 was performed as follows: peptide 1–71 (8 nmol) was dissolved in 0.06 N HCl, containing 40% CH_3COOH and hydrolysed for 22 h at 105°C under vacuum. The acid was then evaporated off. The reaction products were analyzed by polyacrylamide gel electrophoresis in the presence of SDS [23].

Preparation of hybridomas, cloning and screening of positive clones were carried out as in [24,25]. To obtain preparative amounts of mAbs, hybridomas were grown in mice of the corresponding strain or in a culture by means of rollers. mAbs were isolated and purified either using ammonium fractionation followed by ion-exchange chromatography, or by affinity chromatography on protein A-Sepharose. The purified mAbs were characterized by polyacrylamide gel electrophoresis [23] and by isoelectric focusing [26].

The enzyme-linked immunoadsorbent assay (ELISA) was employed for screening hybridomas and for studying the antigenic structure of BR. Affinity purified rabbit antibodies to mouse immunoglobulins, conjugated with horseradish peroxidase were applied as the developing reagent. The isotype of heavy chains and types of light chains were determined using conjugates of goat antibodies to isotypes of mouse immunoglobulins. 50-μl aliquots of the PM suspension (1 mg protein per ml) were placed in each well of a 96-well plate, incubated for 1 h and then washed thoroughly with phosphate buffer containing 0.05% Tween 20. The supernatant, ascites fluid or purified mAbs were added, incubated overnight at 4°C and washed out; finally, substrate (5-aminosalicylic acid or *o*-phenylenediamine) was added and the reaction allowed to proceed. When using the delipidated protein or peptides as antigen, the procedure was changed slightly. The delipidated protein was sonicated in distilled water at a protein concentration of 1 mg/ml and 50 μl of this suspension was added to the wells. Incubation was carried out for 1 h and further steps were as described above. Cyanogen bromide fragments dissolved in formic acid were placed into the wells containing 20 μl of the acid, dried by vacuum, treated with PBS and tested as described above.

3. RESULTS AND DISCUSSION

We have recently prepared and characterized 60 hybridomas producing antibodies to bacteriorhodopsin [16,17]. Five of them secreting mAbs against exposed regions of the polypeptide chain were chosen for further study. The properties of these mAbs are listed in table 1.

To define epitopes of BR, recognized by these mAbs, the interaction of mAbs with fragments of BR was studied using ELISA. The fragments were obtained by cyanogen bromide, BNPS-skatole or proteolytic cleavage of BR and taken together covered the whole length of the polypeptide chain (table 2). The results are summarized in table 3. mAb ID_2G_1 interacts with the fragments B1, C2 and BrCN-2; consequently, the epitope they recognize must be situated within the BrCN-2 fragment (residues 1–20). mAb H_5E_5 interacts with B1, C2 and BrCN-1 fragments; so, the corresponding epitope must be located within the BrCN-1 fragment (residues 33–56). mAb $A_{14}H_3$ interacts only with fragments B2 and (BrCN-7 + BrCN-11), which have overlapping region Phe^{156}–Met^{163}; the

Table 1

Monoclonal antibodies against bacteriorhodopsin

No.	Code of hybridoma	Antigen used for immunization	Strain of mice	Myeloma cells	Type of mAb	K_a (M^{-1})
1	ID_2G_1	C_2^a	F_1 (B/c·Bl/6)	Ag8.653·63	γ_1 k	2×10^8
2	H_5E_5	BR	BALB/c	Ag8.653·63	γ_1 k	5×10^8
3	$A_{14}H_3$	BR	BALB/c	Ag8.653·63	γ_1 k	1.5×10^8
4	ID_2R_1	BNPS-skatol	F_1(B/c·Bl/6)	Ag8.653·63	γ_{2a}k	6×10^8
5	H_5F_3	BR	BALB/c	Ag8.653·63	γ_1 k	9×10^7

[a] See table 2

Each K_a was calculated on the basis of a Scatchard plot

Table 2

Fragments of bacteriorhodopsin used for the study of antigenic specificity of monoclonal antibodies

No.	Designation of fragment	Region of the BR polypeptide chain
1	B_1	$<Glu^1$–Gly^{155}
2	B_2	Phe^{156}–Gly^{231}
3	C_2	$<Glu^1$–Phe^{71}
4	BrCN-1	Gly^{33}–Met^{56}
5	BrCN-2	$<Glu^1$–Met^{20}
6	BrCN-3	Gly^{21}–Met^{32}
7	BrCN-6	Val^{210}–Ser^{248}
8	(BrCN-7 + BrCN-11)	Ile^{119}–Met^{163}
9	BrCN-10	Gly^{72}–Met^{118}
10	BrCN-9	Arg^{164}–Met^{209}
11	BNPS-skatol	Leu^{190}–Gly^{231}

corresponding epitope is most likely located within this region. The two other antibodies H_5F_3 and ID_2R_1 interact with fragments BrCN-9 and BNPS-1, overlapping in the region Leu^{190}–Met^{209}; therefore, corresponding epitopes are located within this region. The results of this study are shown schematically in fig.1.

To define more precisely the structure of the epitopes, derivatives of BR or its fragments were obtained and their interactions with mAbs were investigated.

Treatment of PM with ethylenediamine in the presence of water-soluble carbodiimide drastically decreased binding of mAb H_5E_5 without any influence on interaction of PM with mAbs ID_2G_1, $A_{14}H_3$ and H_5F_3 (table 4). Only carboxylic groups (Asp or Glu) should be modified under these con-

Table 3

Interaction of monoclonal antibodies with fragments of bacteriorhodopsin

No.	mAb	Binding of monoclonal antibodies to different antigens												Region of BR polypeptide chain containing antigenic determinant
		PM	BO	B_1	C_2	BrCN-1	BrCN-2	B_2	BrCN-10	BrCN-7 BrCN-11	BrCN-9	BrCN-6	BNPS-skatol	
1	ID_2G_1	+	+	+	+	−	+	−	−	−	−	−	−	$<Glu^1$–Met^{20}
2	H_5E_5	+	+	+	+	−	−	−	−	−	−	−	−	Gly^{33}–Met^{56}
3	$A_{14}H_3$	+	+	−	−	−	−	+	−	+	−	−	−	Phe^{156}–Met^{163}
4	ID_2R_1	+	+	−	−	−	−	+	−	−	+	−	+	Leu^{190}–Met^{209}
5	H_5F_3	+	+	−	−	−	−	+	−	−	+	−	+	Leu^{190}–Met^{209}

The interaction of mAbs with the purple membrane (PM), bacterioopsin (BO) and fragments of BR (see table 2) was tested by ELISA; (+) positive interaction, (−) negative interaction

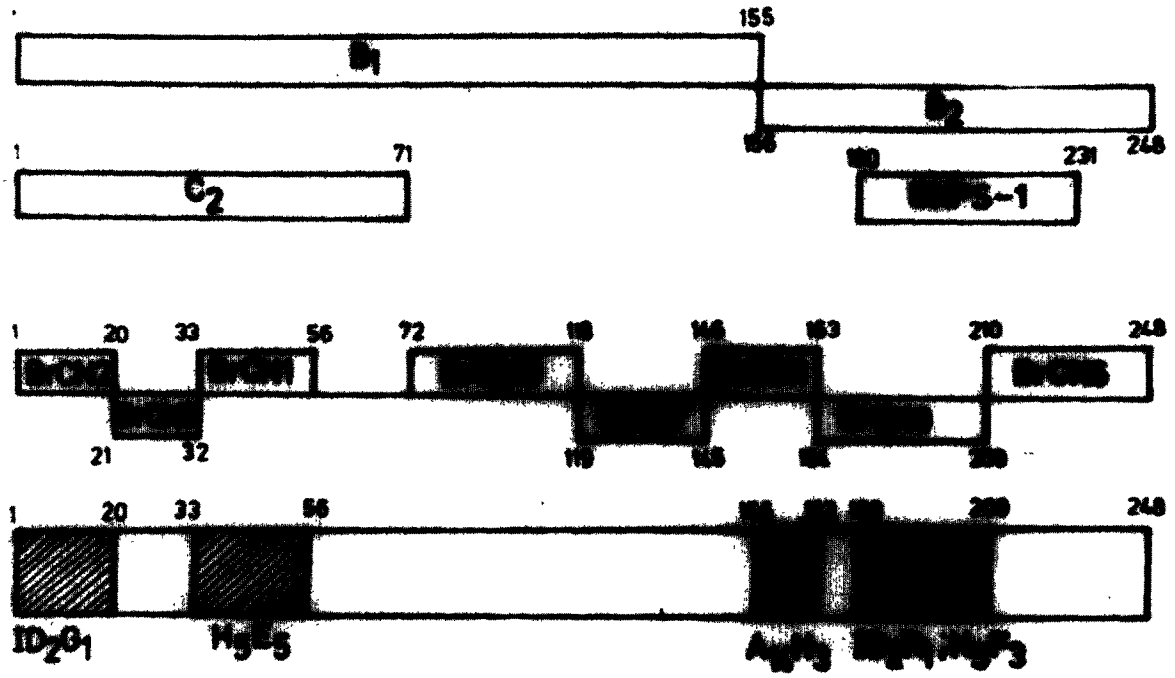

Fig.1. Localization of antigenic determinants of bacteriorhodopsin by means of overlapping fragments. The fragments are listed in table 2; ID_2G_1, H_5E_5, $A_{14}H_3$, ID_2R_1 and H_5F_3 are monoclonal antibodies (table 1); (▨) regions of polypeptide chain within which antigenic determinants are located.

Table 4

Influence of selective chemical, biosynthetic and proteolytic modifications of bacteriorhodopsin on the binding of monoclonal antibodies

No.	Modification of BR	Interaction of BR derivatives with monoclonal antibodies					Essential residue constituting antigenic determinant
		ID_2G_1	H_5E_5	$A_{14}H_3$	ID_2R_1	H_5F_3	
1	Cleavage of as residues 1–3 with papain	+	–	–	–	–	(<Glu^1; Ala^2; Gln^3)
2	Modification of as residues Asp and Glu with ethylenediamine	–	+	–	+	–	Asp^{36}/Asp^{38} Glu^{194}/Glu^{204}
3	Biosynthetic modification of bacteriorhodopsin by replacement of Phe with F-Phe	–	+	+	–	–	Phe^{42}; Phe^{156}
4	Biosynthetic modification of bacteriorhodopsin by replacement of Trp with F-Trp	–	–	–	–	–	–

(+) Modification caused the inhibition of the interaction of mAb with the BR derivatives; (–) no inhibition

ditions. Within region 33–56 where the epitope for mAb H_5E_5 is located, the only carboxylic groups are those of Asp^{36} and Asp^{38}. Consequently, one or both of these residues must be the constituent part of the antigenic determinant. To prove this suggestion, fragment C2 (1–71) was quantitatively cleaved by partial acid hydrolysis into peptides with an apparent molecular mass of 3 kDa. The N-terminal analysis showed the splitting of bond Asp^{36}–Pro^{37}. The mixture of these peptides interacted with mAb H_5E_5 much less effectively than original fragment 1–71, while the interaction with mAb ID_2G_1 was unaffected by acid hydrolysis of the C2 fragment. The observed inhibition of binding of mAb ID_2R_1 after modification of acidic groups in BR implicates glutamic acid residues 194 and 204 in the binding site, since mAb ID_2R_1 is directed against region 190–209.

mAb ID_2G_1 interaction with PM was completely abrogated after treatment of membranes with papain (as in [8]), resulting in cleavage of regions 1–3, 66–72 and 232–248 from the protein molecule (table 4). Since, as shown above, the epitope for mAb ID_2G_1 is located within the region 1–20, this result means that at leasts one of 3 removed residues, $<Glu^1$, Ala^2 or Gln^3, is a part of the epitope.

Region Leu^{190}–Met^{209} includes epitopes for mAbs H_5F_3 and ID_2R_1. To identify the composition of these epitopes the interaction of mAbs with truncated peptides was studied. Such peptides were obtained by the stepwise Edman degradation of the BNPS-1 fragment (190–231), beginning with Leu^{190} and up to Pro^{200}. Binding of mAb ID_2R_1 was drastically decreased after removal of Glu^{194} (fig.2a) but removal of Leu^{190}–Pro^{200} residues did

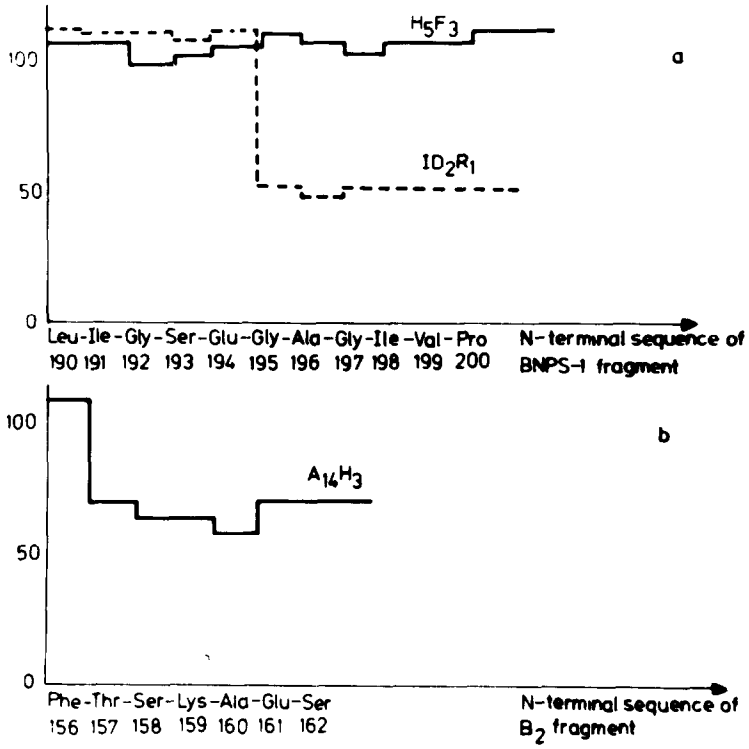

Fig.2. Influence of the truncation of bacteriorhodopsin fragments on the interaction with monoclonal antibodies. Truncation was carried out by sequential Edman degradation of the fragments of bacteriorhodopsin. (A) Interaction of mAbs H_5F_3 and ID_2R_1 with the truncated fragments of BNPS-1 (Leu^{190}–Gly^{231}); (B) interaction of mAb $A_{14}H_3$ with truncated fragments of B_2 (Phe^{156}–Gly^{231}). The vertical direction shows binding of mAb with shortened fragments after removal of certain residues as measured by ELISA; the horizontal direction shows the amino acid sequence of fragments.

not affect the interaction of the shortened peptides with mAb H_5F_3. So the antigenic determinant for mAb ID_2R_1 includes Glu^{194}, while the epitope for mAb H_5F_3 is situated nearer to the C-terminal part of the molecule, within the region Pro^{200}–Met^{209}.

A similar approach was used for identification of the residues constituting the epitope recognized by mAb $A_{14}H_3$. In the course of the Edman degradation of the B2 fragment, the inhibition of binding of the mAb was observed immediately after the removal of residue Phe^{156} (fig.2). Thus, residue Phe^{156} is the essential one in the antigenic determinant recognized by mAb $A_{14}H_3$.

Biosynthetically fluorinated analogs of BR, similar to the natural chromoprotein in the structural and functional properties [19], were used for further identification of the structure of antigenic determinants. Replacement of 56% of Phe residues with 3-F- or 4-F-Phe caused reduction of binding of mAb $A_{14}H_3$ and H_5E_5 to PM by 57%, whereas the interaction with the 3 other mAbs remained the same (table 4). Therefore, Phe residues appear to be essential in the antigenic determinants for these two mAbs. In the case of mAb $A_{14}H_3$, this result correlates with the data shown above, which also places Phe^{156} in the epitope for mAb $A_{14}H_3$. In the case of mAb H_5E_5 residue Phe^{42} located in the vicinity of residues Asp^{36}/Asp^{38} (discussed above) may be included in the epitope. These data also imply that residue Phe^{208} is not included in the antigenic determinant of mAb H_5F_3.

Table 5

Location and composition of antigenic determinants of bacteriorhodopsin

No.	Region of polypeptide containing antigenic determinant	Essential amino acid residues	Code of mAb
1	1– 20	$<Glu^1$–Gln^3	ID_2G_1
2	33– 56	Asp^{36}/Asp^{38}, Phe^{42}	H_5E_5
3	83–118	–	[a]
4	156–163	Phe^{156}	$A_{14}H_3$
5	194–207	Glu^{194}	ID_2R_1
6	200–207	–	H_5F_3
7	232–248	Ala^{246}–Ser^{248}	[a]

[a] Data from Kimura et al. [27]

Hence, this determinant appears to be located within region 200–207, rather than in 200–209.

PM with the 95% substitution of 5-F-Trp for Trp residues bind all mAbs with the same efficiency as the native PM. This means that tryptophan residues either are not involved in any epitope studied, or are not essential for the interaction of mAbs with PM. If the first assumption is correct, the epitope for mAb ID_2G_1 does not include the Trp^{10} residue and appears to be located within region 1–9. It should be mentioned that similar results were obtained by modification of Lys residues, which also did not influence the interaction of mAbs H_5E_5 and $A_{14}H_3$ with PM (not shown).

The data on the location of epitopes of BR and their composition are summarized in table 5. Together with data obtained earlier by Kimura et al. [27], our findings revealed the presence of 8 sequential antigenic determinants in the BR molecule. These determinants are located in the central, N- and C-terminal regions of the molecule. It is of note that most epitopes, studied by us and Kimura et al. [27], contain Asp or Glu residues. Apparently, negatively charged amino acid residues of BR are essential for interaction with antibodies.

As mentioned above, all mAbs studied can bind to PM. Consequently, the corresponding epitopes must be exposed on the membrane surface. The location of N-terminal fragment 1–3 and region 162–163 as antigenic determinants correlates with previous data concerning exposure of amino acid residues [1,3], whereas the localization of residues Asp^{36}/Asp^{38}, Phe^{42}, Glu^{194} and region 200–207 (or part of it) on the surface of the membrane is shown here for the first time.

Thus the presence of exposed regions within sequences 4–65 and 156–231 predicted earlier have received experimental support. Together with the previous data about exposed regions within sequences 66–72 and 231–248 [8], this means that each sequence 4–65 and 156–231 contains two transmembrane segments. The topography of BR according to these findings is shown in fig.3.

Of special interest is the exposure of residue Glu^{194} and the region belonging to sequence 200–207, since this allows the identification of the boundaries of the C-terminal transmembrane segment. The topography of this region is of

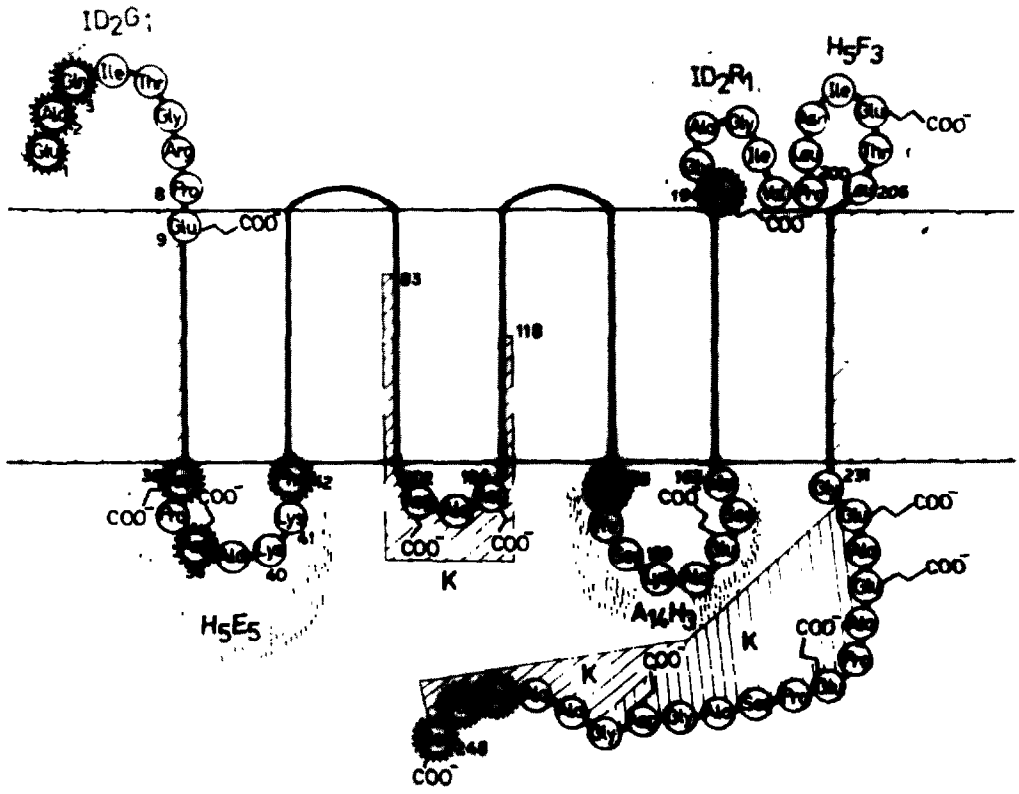

Fig.3. Location of antigenic determinants and topography of bacteriorhodopsin as revealed by mAbs ID_2G_1, H_5E_5, $A_{14}H_3$, H_5F_3, ID_2R_1 – monoclonal antibodies; (Asp), essential amino acid residues; K, antigenic sites determined by Kimura et al. [27].

significance as Lys^{216} is the retinal attachment site. The spatial disposition of retinal is undoubtedly very important in vectorial proton transfer across the membrane. Neutron diffraction [28], fluorescence [29,30] and the analysis of photoinduced cross-linking [7] do not allow the unequivocal positioning of retinal in relation to the outer and inner membrane surface. In particular, photoinduced cross-links were identified between the photoactivable aromatic analog of retinal and residue Glu^{194}, which was assumed to be inside the membrane according to the recently proposed model [7]. Our data, in contrast, indicate the exposure of the Glu^{194} residue on the surface, and in this respect support the earlier models [3]. Evidently, further investigations are necessary in order to establish the topography of the polypeptide chain and chromophore of BR. Here the application of monoclonal antibodies can undoubtedly be of significance.

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