HETEROBIFUNCTIONAL CROSSLINKING OF BACTERIORHODOPSIN BY AZIDOPHENYLISOTHIOCYANATE

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1. Introduction

Phenylisothiocyanate has been described for group-specific modification of membrane proteins in the apolar membrane phase [1]. The method has been applied in various membrane systems [1–4]. Upon modification of purple membranes a single unique nucleophilic group of bateriorhodopsin (lysine 215) is labeled by phenylisothiocyanate [5]. The phenylisothiocyanate binding site is not accessible for the aqueously soluble analog *p*-sulfophenylisothiocyanate. Therefore, the phenylisothiocyanate modification is concluded to occur in the hydrophobic membrane domain.

The newly described probe *p*-azidophenylisothiocyanate combines the group specificity of phenylisothiocyanate and the advantages of photoinduced reactivity of arylazides. Since both reagents react with proteins from within the lipid bilayer, the new probe possesses the requirements necessary for hydrophobic, heterobifunctional crosslinking. The reagent interacts in a first step with deprotonated proteinaceous nucleophiles. The crosslink is then formed by light activation of the hetero-function (arylazide).

In this study the investigation of molecular characteristics of *p*-azidophenylisothiocyanate, the modification of bateriorhodopsin by the bifunctional probe, and the flash-induced crosslink-formation are described. The versatility of the group-specific, heterobifunctional probe is discussed.

2. Materials and methods

2.1. *Materials*

Halobacterium halobium (strain R_1M_1) was grown and the purple membrane isolated according to [6]. Phenyl-[^{14}C]isothiocyanate (12.5 Ci/mol) was obtained from Amersham, England. *p*-Azidophenylisothiocyanate was synthesized from the corresponding arylamine (manuscript in preparation). Photolysis was achieved by flashes from an electronic flash unit of a camera [7]. All handling of azidophenylisothiocyanate or modified bacteriorhodopsin was carried out in subdued light. Absorbance spectra were recorded on a Perkin Elmer 554 spectrophotometer.

2.2. *Azidophenylisothiocyanate labeling of bacteriorhodopsin*

Purple membrane containing bacteriorhodopsin (0.2 mM) in 25 mM sodium phosphate buffer, pH 7.0 was combined with specified amounts of azidophenylisothiocyanate (ethanolic solution). Equal amounts of ethanol (5–10% final concentration) were added to control samples. Labeling was performed by incubation during 90 min at 37°C in stirred suspensions. Purple membranes were then exhaustively dialyzed against bidistilled water in repeatedly replaced dialysis bags. The modified membranes were spectroscopically analyzed in a mixture of 60% glycerol in 25 mM sodium buffer, pH 7.0.

2.3. *Competitive binding*

For competitive binding studies purple membranes (0.2 mM bacteriorhodopsin) were preincubated with (or without) a 50-fold molar excess azidophenylisothiocyanate during 90 min at 37°C in 25 mM sodium phosphate buffer, pH 7.0. Phenyl-[^{14}C]isothiocyanate was then added to the pretreated membranes without separation of excess crosslinker and incubated further during 90 min at 37°C. The samples were then exhaustively dialyzed against bidistilled water. After

exposure to 10 flashes from the electronic unit the protein was repetitively treated (three times) with ice-cold acetone/ammonia 5 : 1 (v/v). The final pellet was solubilized in 2% SDS and analyzed for protein [8,9] and radioactivity [10].

In an additional series of experiments purple membranes (0.2 mM bacteriorhodopsin) were first modified with either non-radioactive phenylisothiocyanate (10 mM) or *p*-sulfophenylisothiocyanate (10 mM). The samples were then dialyzed against 10 mM sodium phosphate buffer, pH 7.0 and modified in a second incubation with a 50-fold molar excess phenyl-[^{14}C]isothiocyanate with respect to prelabeled bacteriorhodopsin (0.1 mM). Radioactivity incorporation was determined in the precipitated protein as described above.

2.4. *Crosslinking*

Azidophenylisothiocyanate-labeled purple membranes were photolyzed by 10 flashes and polymer formation was analyzed by 10% acrylamide SDS gel electrophoresis [11]. Before electrophoresis the samples were solubilized in 2% SDS, freeze-thawed and treated during 10 min at 100°C in the presence of 2.5% mercaptoethanol. 0.1 mg protein was applied per gel. Alternatively, for quantitative evaluation of crosslinked products azidophenylisothiocyanate-labeled membranes were additionally modified by phenyl-[^{14}C]isothiocyanate before light activation and SDS gel electrophoresis. The relative amount of the polymer formed was calculated by triangulation of the area in the densitometric trace of stained gel. In addition Coomassie blue-stained gels were cut into 1 mm slices, the slices were dissolved in H_2O_2/ammonia by overnight incubation at 80°C and the relative amount of radioactivity present in the oligomers was determined.

3. Results

3.1. *Photolysis of azidophenylisothiocyanate*

Ethanolic solutions of the compound were prepared for either addition to purple membranes or for photolysis experiments. Significant changes in the spectral characteristics of azidophenylisothiocyanate are observed when the reagent is exposed to light flashes from the electronic flash unit (fig.1). The exposure of the sample to 8 consecutive flashes results in complete activation of the light-sensitive compound, as expressed in the disappearance of the 290 nm absorbance and concomitant formation of colored products.

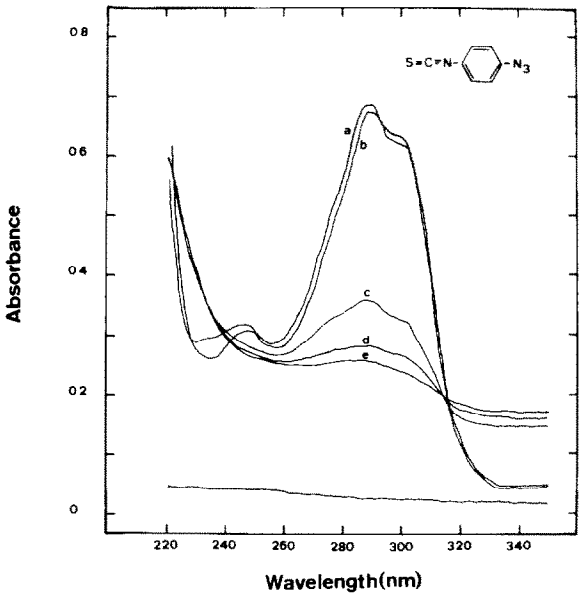

Fig.1. Photolysis of *p*-azidophenylisothiocyanate. Absorption spectra of azidophenylisothiocyanate (0.03 mM in ethanol) were recorded before (a) and after exposure of the sample to the indicated number of light flashes: (b) 1 flash, (c) 4, (d) 6, (e) 8 flashes.

3.2. *Azidophenylisothiocyanate-modified bacteriorhodopsin*

As compared to a control (ethanol-treated) sample (fig.2B) the absorption characteristics of azidophenylisothiocyanate-modified and exhaustively dialyzed purple membrane (fig.2A) differ in two obvious spectral properties: Azidophenylisothiocyanate modification results in the formation of a shoulder at 310 nm. This spectral change is best observed by second derivative spectroscopy. Secondly, the ratio E_{280}/E_{570} is 2.5 for the azidophenylisothiocyanate-modified protein as compared to 1.8 for the control. The increase indicates the addition of an arylic compound to the protein moiety. No changes are observed for the control at 310 nm, the spectrum of which is identical with native purple membranes [6]. It is both interesting and important to observe that the 570 nm absorption characteristic of the crosslinker-modified membrane is not altered. Since the absorption at 570 nm sensitively reports protein denaturation [12], it is concluded that the chromo-

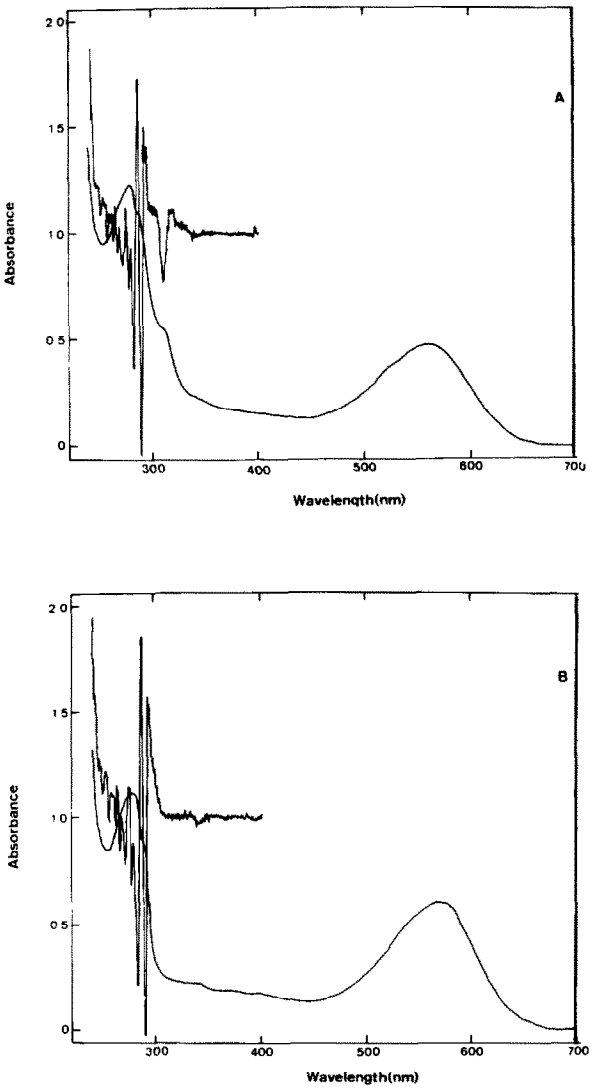

Fig.2. Effect of azidophenylisothiocyanate-modification on the spectrum of purple membrane. Spectra of azidophenylisothiocyanate-labeled and exhaustively dialyzed membranes (A), and a control sample (B) are recorded. The protein concentration is 0.19 and 0.25 mg/ml respectively. The upper line represents the second derivative spectra of the corresponding samples.

phore–protein interaction is not disturbed by the chemical modification, a necessary requirement for valid crosslinking studies.

3.3. *Competitive binding*

The apolar probe phenylisothiocyanate binds to a single unique site of bacteriorhodopsin [5]. Competitive binding studies demonstrate that the hydrophobic crosslinking reagent azidophenylisothiocyanate does compete for the phenylisothiocyanate binding site. Competition for this site is comparably effected by non-radioactive phenylisothiocyanate (table 1). The somewhat different physico-chemical properties of azidophenylisothiocyanate (m.p. 62°C) and phenylisothiocyanate (m.p. −21°C) are most likely responsible for the observed differences in the respective reagent binding capacity. In contrast to the mentioned hydrophobic probes the aqueously soluble *p*-sulfophenylisothiocyanate does not interact with the phenylisothiocyanate binding site. The modification by apolar arylisothiocyanates is therefore concluded to occur in a hydrophobic membrane domain.

3.4. *Crosslinking*

The extent of flash-induced crosslinking of azido-

Table 1
Competitive binding of arylisothiocyanates to bacteriorhodopsin

Preincubation (10 mM reagent)	Phenyl-[^{14}C]isothiocyanate incorporation		Competition for phenyl-[^{14}C]isothiocyanate binding (%)
	(cpm · 10^{-3}/mg protein)	(%)	
None	388	100	0
Phenylisothiocyanate	153	39	61
Azidophenylisothiocyanate	186	48	52
Sulfophenylisothiocyanate	386	99	1

Purple membranes (0.2 mM bacteriorhodopsin) were preincubated with a 50-fold molar excess of arylisothiocyanate followed by a modification of the dialyzed membranes (0.1 mM bacteriorhodopsin) with phenyl-[^{14}C]isothiocyanate (5 mM). Upon dialysis radioactivity incorporation was determined in the acetone/ammonia treated protein

phenylisothiocyanate-modified bacteriorhodopsin is dependent upon time and label concentration. Whereas dimers of bacteriorhodopsin are the predominant products formed when purple membranes were modified with a 100-fold excess of crosslinker, trimers and high MW aggregates are additionally detected when samples were modified with a 1000-fold molar excess of label (fig.3). A comparable protein pattern as in fig.3C is obtained upon incubation of purple membranes for prolonged time (16 h, 37°C) using a 100-fold molar excess of azidophenylisothiocyanate (table 2). Apparent MW of 19 000 (monomer), 35 000 (dimer) and 52 000 (trimer) have been determined for the crosslinked products using the indicated proteins as MW standard.

Quantitative analysis of recovered monomers and polymeric forms of bacterio-opsin by densitometry of the Coomassie blue-stained gels or by measurement of the incorporated radioactivity gave essentially identical results (table 2). In the latter experiment azidophenylisothiocyanate-modified purple membranes were labeled in a second incubation with phenyl-[^{14}C]isothiocyanate. Then the samples were exposed to light (10 flashes). Upon protein precipitation and SDS gel electrophoretic separation the relative amount of radioactivity present in the oligomeric forms was determined. The predominance of non-crosslinked bacterio-opsin is compatible with the restricted extent of covalent nucleophile interaction. Possible occurring intramolecular crosslinking can not be excluded to date.

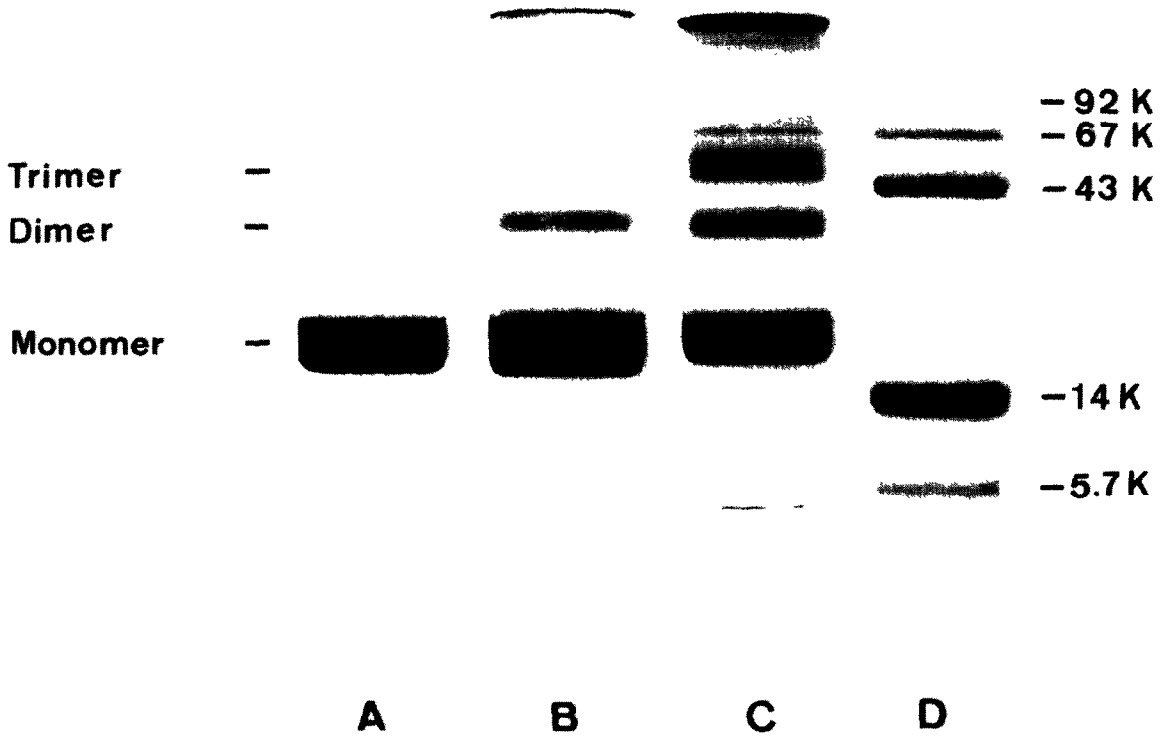

Fig.3. SDS polyacrylamide gel electrophoretic analysis of azidophenylisothiocyanate crosslinked purple membranes. 10% acrylamide gel electrophoresis of crosslinked membranes was performed in a Tris–acetate system, pH 7.4. Coomassie blue-stained protein pattern of control (A), and azidophenylisothiocyanate-modified and photoactivated membranes labeled with a 100-fold excess (B), or with a 1000-fold molar excess crosslinker (C). All samples were identically treated with 10 flashes of light before gel electrophoresis. (D) MW markers: phosphorylase *a*, bovine serum albumin, ovalbumin, lysozyme, insulin.

Table 2
Oligomer distribution in azidophenylisothiocyanate-crosslinked purple membranes

	Monomer (%)	Dimer (%)	Trimer (%)	Top of gel (%)
Crosslinked purple membranes	61 (77)	13 (13)	5 (4)	19 (6)
Control	89 (91)	3 (3)	0 (0)	7 (8)

Purple membranes (0.2 mM bacteriorhodopsin) were labeled with or without (control) a 100-fold molar excess crosslinker during 16 h at 37°C and additionally treated (90 min, 37°C) with phenyl-[^{14}C]isothiocyanate. After removal of excess arylisothiocyanate and flash-induced crosslinking the relative amount of radioactivity present in the electrophoretically separated protein bands was determined. The percentage of recovered oligomers by densitometric analysis of the Coomassie blue stained gels are given in the parentheses

4. Discussion

The heterobifunctional crosslinker azidophenylisothiocyanate is capable of forming a crosslink over the range of 10 Å. Projection of this dimension into the scattering density map of the purple membrane [13] confirms that, on a theoretical basis, an azidophenylisothiocyanate-modified protein can reach adjacent protein molecules. The data reported in this study provide evidence for hydrophobic heterobifunctional crosslinking of bacteriorhodopsin. Azidophenylisothiocyanate occupies a single hydrophobically located site in bacteriorhodopsin. The group-specific interaction of azidophenylisothiocyanate (aryl-NCS – deprotonated nucleophile) with membrane proteins is advantageous for controlled crosslink formation in a topologically defined membrane domain. The probe is expected to facilitate the investigation of hydrophobically interacting sites between proteins and membrane lipids.

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