4-*N*,*N*-DIMETHYLAMINO-AZOBENZENE-4′-ISOTHIOCYANATE: A CHROMOPHORIC, HYDROPHOBIC REAGENT FOR PROBING MEMBRANE-BURIED SEGMENTS OF INTRINSIC PROTEINS

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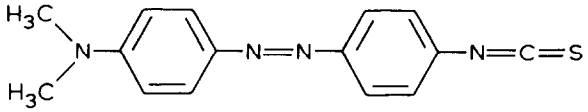
1. Introduction

Hydrophobic labeling techniques have been employed frequently to investigate the disposition of integral membrane proteins from within the lipid bilayer [1–3]. The advantageous use of nitrene- or carbene-generating reagents is restricted though, due to the relatively unselective interaction of these probes with membrane proteins and lipids. Hydrophobic arylisothiocyanates (phenylisothiocyanate, *p*-azidophenylisothiocyanate) have been introduced as apolar membrane reagents which provide the requested group-specificity for hydrophobically located nucleophiles [4,5]. The detection of phenylisothiocyanate-modified protein segments is, however, confined to the use of costly radioactive isotopes. 4-*N*,*N*-dimethylamino-azobenzene-4′-isothiocyanate (DABITC):



an intensively colored reagent [6], also possesses the requested characteristics of a hydrophobic, group-specific label. Additionally, protonation of this probe causes bathochromic and hyperchromic effects in the absorption. By adjusting the aqueous media to the appropriate pH, penetration of the label into the lipid bilayer can be spectroscopically followed. Here, the utility of DABITC as a label of membrane proteins is reported. The investigations have been done on the human anion-transport protein whose disposition in the membrane has been described [7–9].

2. Materials and methods

DABITC was purchased from Pierce Chemical Co. (IL). All other reagents were of the highest quality commercially available. Erythrocyte membranes were prepared as in [10]. Thermolytic and peptide digestion of labeled erythrocyte ghosts and isolation of the membrane-integrated segments of band 3, the 17 000 (GThl) and 10 000 M_r peptides (P5, GTh2) was done as in [11]. SDS gel electrophoresis was done as in [12]; protein was determined as in [13].

2.1. *Spectroscopic characterization of DABITC and its butylamine derivative (*N-*(4*-N,N-*dimethyl-amino-azobenzene-4′-)*-N′-*butylthiourea: DABUT)*

DABUT was prepared by combining 0.2 mmol DABITC with a 10-fold excess of butylamine in 25 ml ethanol. The mixture was stirred for 1 h at 25°C. The reaction product was purified by recrystallization from ethanol–water. The homogeneity of DABUT was confirmed by thin-layer chromatography on silica gel plates (solvent system: ether, R_F 0.73). The product was further characterized by NMR and mass spectroscopy. UV–VIS spectra were recorded with a Uvikon 810 spectrophotometer. The reagents were dissolved in 1% SDS containing either 10 mM phosphate buffer (pH 7.3), 0.1 M or 1 M acetic acid.

2.2. *Labeling of erythrocyte ghosts with DABITC*

Erythrocyte ghost membranes (4 mg protein/ml) were incubated with 5 mM DABITC at 37°C for 1 h in 10 mM sodium phosphate buffer (pH 7.3) (stirred suspension). DABITC was added as a sonicated suspension in ethanol (final conc. ethanol: 2%, v/v). After incubation the ghost membranes were sedi-

mented by centrifugation at 18 000 × g, 20 min and washed 3 times with 10 mM sodium phosphate buffer (pH 7.3).

2.3. *Fragmentation of erythrocyte band 3 and isolation of the membrane-buried segments GTh1, GTh2 and P5*

DABITC-labeled erythrocyte ghosts were digested by either thermolysin or pepsin. The above fragments were isolated by column chromatography as in [11]. Non-covalently bound DABITC was removed by extensive dialysis of the isolated peptides against water (24 h) and methanol (12 h) in spectrapor 6 dialysis tubes (excluding M_r 2000). Label incorporation into the purified, electrophoretically homogeneous proteolytic fragments was spectroscopically determined.

3. Results and discussion

3.1. *UV–VIS spectroscopy*

The ϵ-amino group of lysine located in a hydrophobic environment is the most likely candidate for covalent interaction with arylisothiocyanates [14]. Butylamine, providing the molecular structure of the lysine sidechain, has been chosen as a representative nucleophile to simulate the interaction of DABITC with membrane-buried lysine residues. As shown in fig.1 DABITC and the butylamine derivative have distinct spectroscopic characteristics. The absorption maxima are significantly different in neutral as compared to acidified media. Protonation of both DABITC and DABUT results in batho- and hyperchromic effects. The high molecular extinction coefficient of DABUT (λ_{554} = 34 000 $M^{-1} . cm^{-1} . l^{-1}$) allows binding studies in the μM range (table 1).

3.2. *Partition of DABITC into the lipid bilayer*

Differences in the absorption characteristics were not observed when labeled membranes were spectroscopically analyzed in either neutral or acidified media (fig.2a,b). The partitioning of the hydrophobic label into the lipid bilayer rendered it non-accessible to protonation from the aqueous phase. Upon solubilization of labeled erythrocyte membranes with 1% SDS in presence of 1 M acetic acid, the absorption maximum shifted to 536 nm. Simultaneously, a shoulder appeared at 554 nm (fig.2c) which coincided with the absorption maximum of DABUT (fig.1b). Covalent protein modification is therewith indicated.

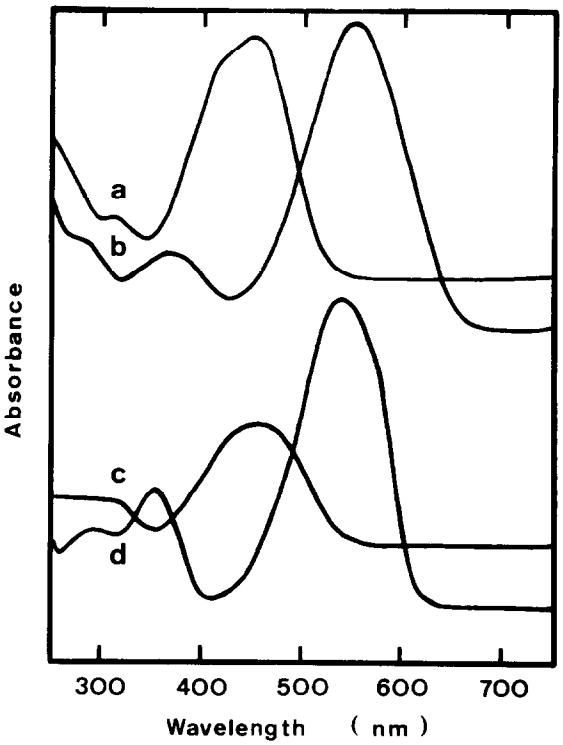


Fig.1. pH-dependent absorbance of DABITC and its butylamine derivative (DABUT): (a) DABUT in 10 mM sodium phosphate buffer (pH 7.3), 1% SDS; (b) DABUT in 1 M acetic acid, 1% SDS; (c) DABITC in 10 mM sodium phosphate buffer (pH 7.3), 1% SDS; (d) DABITC in 1 M acetic acid, 1% SDS.

3.3. *Incorporation of DABITC into the 10 000* M_r *segment (P5, GTh2) of band 3*

Erythrocyte ghosts were labeled with 5 mM DABITC. The labeled membrane proteins were then digested with thermolysin or pepsin, respectively. Upon solubilization (10% SDS, 5% β-mercaptoethanol,

Table 1
Spectroscopic data of DABITC and its butylamine derivative (DABUT)

Solvent containing 1% SDS	pH	DABITC		DABUT	
		λ_{max} (nm)	log ϵ	λ_{max} (nm)	log ϵ
10 mM phosphate buffer (pH 7.3)	7.3	460	4.18	452	4.43
0.1 M acetic acid	2.85	536	4.56	554	4.53
1.0 M acetic acid	2.35	536	4.56	554	4.53

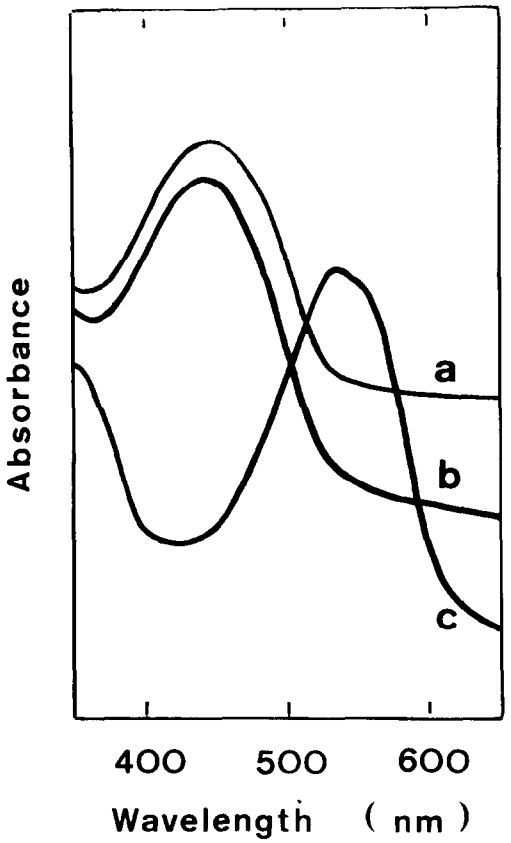


Fig.2. Spectroscopic analysis of DABITC labeled erythrocyte ghost membranes. Erythrocyte ghosts were labeled with 5 mM DABITC for 1 h at 37°C in 10 mM sodium phosphate buffer (pH 7.3). After washing by repetitive centrifugation the labeled membranes were suspended in 10 mM phosphate buffer pH 7.3 (a) or 1 M acetic acid (b) and absorption spectra recorded. Spectrum (c) shows the absorption of labeled ghosts in 1 M acetic acid after addition of 1% SDS.

1 mM EDTA, 50 mM Tris–HCl buffer (pH 8.0)) the membrane-bound fragments were isolated by gel filtration chromatography (Sephadex G-75 or G-50). An elution profile of the thermolytic membrane-bound fragments is shown in fig.3. The thermolytic peptide GTh2 (nomenclature from [11], app. M_r 10 000) is the predominantly modified fragment of band 3, as indicated by its 420 nm absorbance (1% SDS, pH 8.0). In an analogous experiment, preferential labeling of the peptic fragment P5, an overlapping fragment of GTh2, was found (not shown). An insignificant amount of label is recovered in the GTh1 fragment, as judged by column chromatography (fig.3) and SDS gel electrophoresis. The covalent

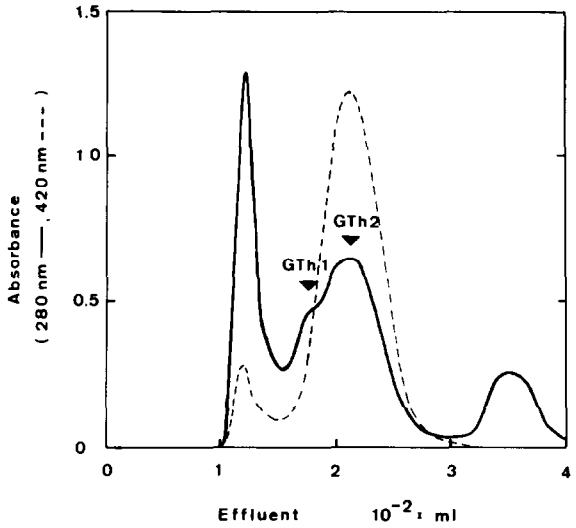


Fig.3. Isolation of thermolytic, membrane-bound fragments of DABITC-modified band 3 by gel filtration. The fractionation of peptides was performed on a 2 × 110 cm Sephadex G-75 column (elution buffer: 1 mM EDTA, 1% SDS (pH 8.0)); A_{280} (——) and A_{420} (– – –) was recorded.

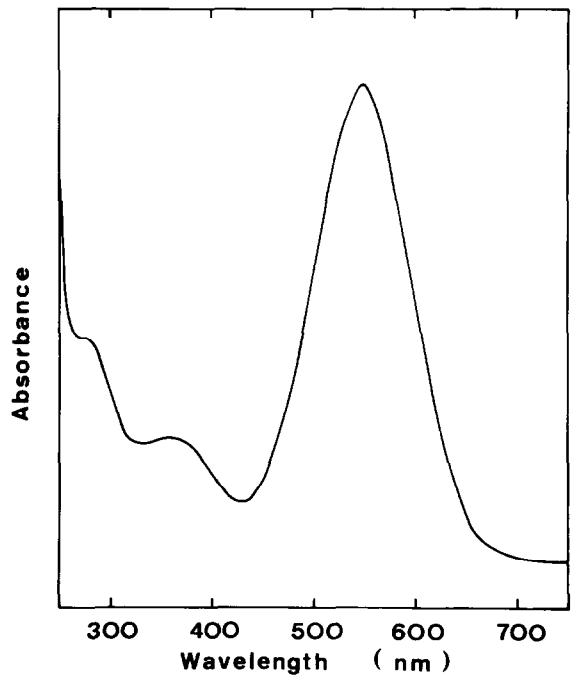


Fig.4. Covalent DABITC modification of GTh2, a membrane-integrated segment of band 3. The purified, membrane integrated 10 000 M_r thermolytic peptide GTh2 was dissolved in 1 M acetic acid containing 1% SDS. The absorption spectrum (λ_{max} = 554 nm) demonstrates covalent attachement of DABITC to the peptide.

binding of DABITC to the protein is demonstrated in fig.4. Upon DABITC modification of ghost membranes the isolated peptide GTh2 absorbs maximally at 554 nm, when analyzed under conditions described for DABUT. By using the latter derivative as reference a binding ratio of 2.3 mol DABITC/10 000 M_r peptide GTh2 was determined (2.2 mol DABITC/P5). With respect to label distribution in the fragments GTh1, GTh2 and P5, the results reported for DABITC confirm the binding studies with phenyl-[^{14}C]isothiocyanate [11].

4. Conclusions

Regions of integral membrane proteins which are in contact with the hydrophobic domain of the membrane are of obvious importance for the protein function. To explore the nature of these regions and their location in the intact polypeptide, group-specific labeling techniques have been applied. Here, we report the advantageous use of DABITC as a chromophoric, group-specific, hydrophobic label. The results indicate that covalent attachment of the label to membrane proteins occurs. Spectroscopic analysis of labeled erythrocyte ghost membranes and proteolysis experiments document that the label is directed toward those membrane-integrated protein segments, which were, as reported earlier, modified by phenylisothiocyanate. DABITC offers therefore a high potential in the elucidation of the molecular anatomy of integral membrane proteins.

Acknowledgements

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