CHARACTERIZATION OF PHENYLISOTHIOCYANATE AS A HYDROPHOBIC MEMBRANE LABEL

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1. Introduction

Various labeling techniques have been widely employed to classify those portions of membrane proteins exposed to the aqueous environment [1–3]. With the recent introduction of hydrophobic azides, the lipophilic membrane-integrated domains became specifically accessible for chemical modification [4,5]. However, a marked disadvantage of the rapid, photo-induced reactivity of azides is that it is not exclusively directed towards selected functional groups of proteins [6]. Lipid components of the membrane system are quite highly labeled [7]. For specific modification of the hydrophobic moieties of membrane constituents group specific reagents are necessary.

Successful modification in the apolar phase requires that the reagent be hydrophobic and partitions in favor of the apolar phase. Furthermore, the functional groups of the membrane protein must be in a reactive state. No less important, the reaction and reaction products formed are required to be non-ionogenic and non-charged, respectively. Water should not be involved in the reaction, neither as reactant nor as product.

PITC is able to fulfill these requirements. It reacts with amino groups solely in the unprotonated form [8]. The reactivity of the protein functional groups can therefore be controlled by the pH in the aqueous phase. At neutral pH, the exposed protein amino groups are expected to be protonated. The buried, bulk pH-independent amino functions, however, can be in the reactive state. This implies selectivity of the reaction for non-aqueous systems.

Abbreviations: PITC, phenylisothiocyanate; SDS, sodium dodecylsulfate

2. Methods and materials

Sarcoplasmic reticulum membranes were prepared from rabbit skeletal muscle [9]. Sheep and human erythrocyte membranes were isolated by the method in [10]. Spectrin depletion was performed as in [11]. Hemoglobin content was analysed by $A_{540\ nm}$. To quantify the degree of lysis, total hemolysate hemoglobin was defined as 100%.

2.1. *[^{14}C]PITC-labeling (general procedure)*

[^{14}C]Phenylisothiocyanate in ethyl alcohol (1.25 μCi/μl) was added to the membrane suspension to final 0.2 mM, final alcohol was 0.1%. The membrane preparations were suspended in 10 mM sodium phosphate buffer, pH 7.3 (6 mg protein/ml), incubated in covered glass tubes and stirred during the time of reaction. In all analyzed systems, aliquots taken from the reaction mixture immediately after the addition of PITC served as control. Unless otherwise mentioned, separation or purification procedures were performed in 10 mM sodium phosphate buffer, pH 7.3 or 9.0.

2.2. *Incorporation into membrane systems*

The degree of incorporation into different membrane systems was determined in the pelleted membrane fraction, which was washed and recovered by repeated centrifugation (30 min, 100 000 × g, 4°C) of a 50-fold diluted aliquot (200 μl). Labeling of washed human erythrocytes was performed with 250 nmol [^{14}C]PITC/0.5 ml packed erythrocytes. Following 60 min incubation at 37°C, pH 7.3 or 9.0, the labeled cells were repeatedly washed (20 min, 1500 × g, 4°C), lysed and the membranes isolated.

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2.3. *Labeling of sarcoplasmic reticulum membrane proteins*

Incorporation of [^{14}C]PITC into sarcoplasmic reticulum membrane proteins was terminated by repeated (3 times) acetone precipitation. The final precipitate, solubilized in 2% SDS was analyzed for protein, radioactivity and by gel electrophoresis.

2.4. *Labeling of soluble proteins and aminophospholipids*

Upon incubation of bovine serum albumin and ovalbumin (5 mg/ml) with [^{14}C]PITC (200 nmol) aliquots of the reaction mixture were separated on Sephadex G-50 (0.3 cm diam. × 20 cm) and analyzed for protein and radioactivity. Multilayer liposomes were prepared from phosphatidyl serine and phosphatidyl ethanolamine [12] in 100 mM sodium phosphate buffer and combined with equimolar amounts of [^{14}C]-PITC (14.7 μCi/mmol).

Protein was determined in the presence of 0.1% SDS by the Lowry method [13]. SDS gel electrophoresis was carried out in accordance with Weber and Osborn using gradient slab gels (3.75–10% acrylamide). The electrophoresis was performed in 0.1% SDS. Sodium phosphate buffer, 50 mM, pH 7.3. Gels were stained with Coomassie brilliant blue and destained with 7% acetic acid. For autoradiography the destained gels were dried and exposed (10 days) to a Kodirex X-ray film (Kodak).

All chemicals were of the highest purity commercially available. Phenyl [^{14}C]isothiocyanate (12.6 mCi/mmol) was purchased from Amersham Radiochemicals, phosphatidyl serine (bovine spinal cord) and phosphatidyl ethanolamine (egg yolk) from Lipid Products.

3. Results

The solubility of PITC in aqueous systems is restricted. Unless detergents are present, homogeneous solutions in aqueous buffer systems are difficult to obtain. In a hexane/water system, the reagent partitions to 97% into the apolar phase. During incubation, the specific incorporation in the pelleted membrane increases 2–8-fold compared with the initial value (table 1). This corresponds to a partition coefficient of 99% in favor of the membrane phase. That radioactivity was present in the recovered membrane preparation, even after short periods of incubation with PITC, is most probably due to the reaction going on during the washing process.

Table 1
Incorporation of [^{14}C]phenylisothiocyanate into sarcoplasmic reticulum and erythrocyte membranes

Membrane system	Incubation		Incorporation (cpm × 10^{-5}/ mg protein)
	Time (min)	Temp. (°C)	
Sarcoplasmic reticulum membranes	0	22	1.8
	240	22	2.6
Human erythrocyte membranes	0	22	0.7
	240	22	2.4
Sheep erythrocyte membranes	0	37	0.4
	60	37	3.2

Isolated membranes were incubated with [^{14}C]PITC (0.2 mM) under conditions in section 2. At time points mentioned, aliquots were sedimented by repeated centrifugation (30 min, 100 000 × g, 4°C). The final pellet was analyzed for protein and radioactivity

The incorporation of [^{14}C]PITC in sheep erythrocyte ghost membranes is shown in fig.1. The electrophoretic peptide pattern did not alter under the conditions used. [^{14}C]PITC binds preferentially to band 3, whereas incorporation into spectrin and actin is extremely poor or not detectable. The radioactivity in the front of the gel coincides with free [^{14}C]PITC and modified aminophospholipids. As detected by autoradiography, partial incorporation of [^{14}C]PITC into hemolysate hemoglobin did occur. This is most probably due to the unusually low pK_a value (6.72) of the amino-terminal valine in the α-chain [14].

Membrane modification of whole washed human erythrocytes with PITC was also performed. At pH 7.3, the isolated membrane fraction showed a ^{14}C-distribution identical to that reported for ghost membranes (fig.1). The addition of [^{14}C]PITC did not induce erythrocyte lysis ($\leqslant$ 3%). Spectrin depletion of [^{14}C]PITC-modified erythrocyte membranes induced a 2-fold increase in specific activity (cpm/mg membrane protein) (table 2), indicating a predominant labeling of membrane-integrated proteins. Increasing the reaction pH from 7.3–9.0 doubled the label incorporation, where more extensive labeling of aminophospholipids occurred (see table 3).

A more detailed study of the association of PITC

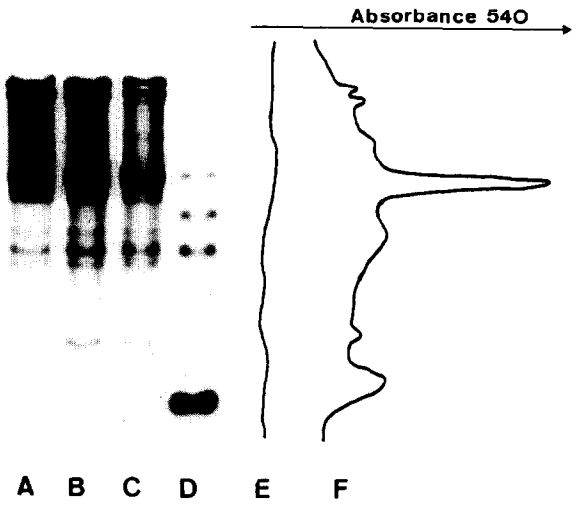


Fig.1. Incorporation pattern of protein and radioactivity in [^{14}C]PITC-labeled sheep erythrocyte membranes: Gradient SDS–gel electrophoresis of sheep erythrocyte ghost membranes (50 μg). (A) Untreated membranes; (B) membranes washed immediately after addition of [^{14}C]PITC; (C) PITC-labeled membranes (60 min, 37°C, pH 7.3); (D) molecular weight marker: phosphorylase *a* (94 000), bovine serum albumin (68 000), ovalbumin (45 000), lysozyme (14 300), insulin (5700); (E,F) densitometric scan (540 nm) of the film image of sample B and C, respectively.

with an integrated membrane protein was carried out with sarcoplasmic reticulum membranes. The membranes were incubated with the label for a determined length of time, excess unreacted PITC was then removed by repeated (3 times) acetone precipitation. The association of up to 15% of totally applied ^{14}C radioactivity in the acetone-precipitated pellet is therefore due to covalent protein modification. In fig.2a the time and temperature dependence of [^{14}C]PITC-labeling is shown. The autoradiogram (fig.2b) demonstrates the association of the label with the Ca^{2+}-dependent ATPase protein. No radioactivity was detected in the lipid region of the gel, indicating complete removal of possible reactive lipid species (see table 3).

The reactivity of [^{14}C]PITC with soluble proteins and aminophospholipids at pH 7.3 and 9.0, respectively, was tested. The results are summarized in table 3. Whereas the functional groups of ovalbumin are not reactive under either condition tested, bovine serum albumin appeared to bind 0.75 mol PITC/mol covalently since protein-associated radioactivity was detected in polyacrylamide gels. Interestingly, in a multi-layer liposomal system phosphatidyl serine bound PITC as well at pH 7.3 as at pH 9.0. In contrast, modification of phosphatidyl ethanolamine occurred at pH 9.0, but not at pH 7.3.

4. Discussion

The coupling reaction of PITC with proteins in the aqueous phase is restricted due to the solubility properties of the reagent and its spontaneous hydrolysis in aqueous systems [8]. Reactivity of the aqueous-exposed reactive groups is additionally reduced at neutral pH since PITC reacts with amino groups in their non-protonated form only.

Aqueous-soluble reagents such as cycloheptaamylose–fluorescamine complex and the diazonium salt of sulfanilic acid have been used at neutral pH to modify surface-exposed membrane protein moieties of erythrocyte [1,15] and sarcoplasmic reticulum membranes [16]. With these reagents, protein-related

Table 2
Spectrin depletion of [^{14}C]PITC-labeled erythrocyte membranes

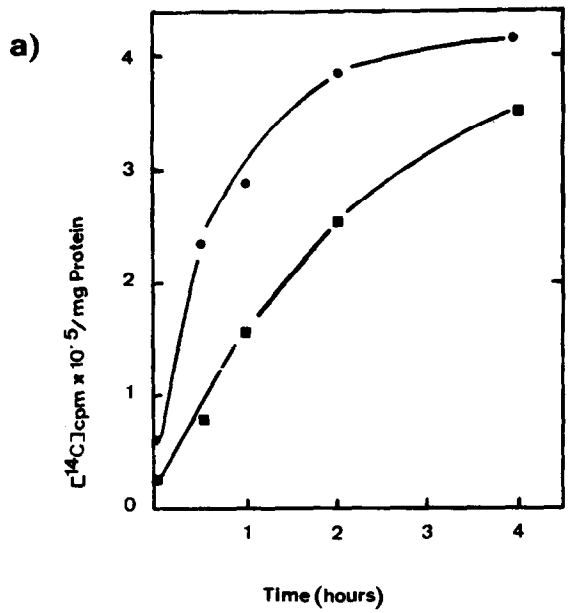
Human erythrocytes	pH	Recovery (%)		Incorporation (cpm × 10^{-3}/ mg protein)
		^{14}C cpm	Protein	
Erythrocyte membranes	7.3	100	100	56.5
Spectrin-depleted membranes	7.3	71.4	37.0	109.0
Erythrocyte membranes	9.0	100	100	101.6
Spectrin-depleted membranes	9.0	67.0	41.1	165.6

Human erythrocytes were labeled in 0.5 mM [^{14}C]PITC. Labeling, membrane isolation and spectrin depletion were performed as in section 2

Table 3
Reactivity of [^{14}C]phenylisothiocyanate in aqueous systems, at pH 7.3 and pH 9.0, respectively

	Incorporation ([^{14}C]PITC mol/mol)[a]	
	pH 7.3	pH 9.0
Ovalbumin	0.04 ± 0.01	0.08 ± 0.03
Bovine serum albumin	0.71 ± 0.06	0.75 ± 0.14
Phosphatidyl serine	+	+
Phosphatidyl ethanolamine	–	+

[a] All the data listed refer to an incubation for 60 min at 37°C. For phosphatidyl serine and phosphatidyl ethanolamine equimolar amounts of reactant were used



modification occurred, implicating the presence and availability of protein amino groups. PITC in contrast reacted almost exclusively with erythrocyte band 3 and the sarcoplasmic ATPase. The preference of the label for the non-aqueous phase is demonstrated by the increased specific activity following spectrin depletion of erythrocyte membranes (table 2).

The primary interaction of PITC with bovine serum albumin is most probably of a hydrophobic nature, followed by covalent modification. In fact, this protein has been repeatedly used to eliminate excess hydrophobic label in membrane modification studies [4,17].

Neither one of the soluble proteins tested, ovalbumin nor bovine serum albumin, showed a pH-dependent increase of PITC incorporation, demonstrating the poor reactivity of PITC in the aqueous phase. The modification of liposomal aminophospholipids is in agreement with the results in [12]. Phosphatidyl serine does not change its electrophoretic mobility between pH 5 and pH 11, being therefore in a reactive state. Significant electrophoretic changes occur, however, for phosphatidyl ethanolamine in the pH range tested.

Fig.2. (a) Incorporation of [^{14}C]PITC into sarcoplasmic reticulum membrane proteins: Incorporation was performed at 22°C (■) and 37°C (●) as described in section 2. (b) SDS–gel electrophoretic and corresponding autoradiographic pattern of: (A) Molecular weight standards (as in fig.1); (B) sarcoplasmic reticulum membranes (50 μg) immediately precipitated after the addition of [^{14}C]PITC; (C) incubation for 30 min, 37°C, pH 7.3; (D) incubation for 60 min, 37°C, pH 7.3; (B′, C′, D′) autoradiography of B, C, D, respectively.

PITC as a membrane label is specific for the non-aqueous domains, i.e., the region insensitive to the bulk pH. In a facile procedure erythrocyte membranes and the sarcoplasmic ATPase are modified to such an extent that topological studies become feasible. It is hoped that the probe and method described here may be of general use in membrane-related protein chemistry leading to structural and functional information.

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