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Labelling of erythrocyte spectrin in situ with phenylisothiocyanate

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The labelling of erythrocyte spectrin in situ with the hydrophobic reagent phenylisothiocyanate (Sigrist, H. and Zahler, P. (1978) FEBS Lett. 95, 116–120) is studied. Spectrin isolated from erythrocytes which have been incubated with phenylisothiocyanate is covalently modified by the probe. The modification in the spectrin molecule is stable under an excess of nucleophile in alkaline conditions. The labelling is very little or not affected by preincubation of erythrocytes of membranes with the polar, structural analogue of phenylisothiocyanate, *p*-sulfophenylisothiocyanate. When erythrocyte ghosts are subjected to labelling, a substantial increase in the degree of spectrin modification is observed. Subunits of labelled spectrin separated electrophoretically show similar amounts of attached label.

Introduction

Hydrophobic arylisothiocyanates are group-specific reagents wich are assumed to react with proteins from within the hydrophobic phase of the membrane (reviewed by Sigrist and Zahler [1]). Sigrist et al. [2–4] introduced radioactive phenylisothiocyanate for labelling a variety of membrane systems; e.g. bacteriorhodopsin of the purple membrane, and band 3 protein of the erythrocyte membrane. They found that phenylisothiocyanate binds covalently preferentially to membrane-integrated segments of those proteins.

The covalent interaction of integral membrane proteins with a wide variety of hydrophobic probes (for a review, see Ref. 5) has been well documented. The data on the reactivity of membrane-extrinsic proteins with such probes are rather poor and inconsistent [6–8].

In our experiments we have found that in washed erythrocytes spectrin, the main extrinsic protein of the erythrocyte membrane, was labelled with hydrophobic arylisothiocyanates. In this report a more detailed study on the labelling of spectrin in whole cells and erythrocyte ghosts employing [^{14}C]phenylisothiocyanate is presented.

Materials and Methods

Human blood was supplied by Wrocław Blood Bank and used within 2 days after expiry date. [U-^{14}C]Phenylisothiocyanate (58 or 114 GBq per mol) was obtained from Isocommerz (Leipzig, G.D.R.). Nonradioactive *p*-sulfophenylisothiocyanate was synthesized according to the method of Braunitzer et al. [9]. Erythrocytes and erythrocyte ghosts were isolated by the method of Dodge et al. [10], using sodium phosphate buffers (pH 7.3).

Labelling with [^{14}C]phenylisothiocyanate was performed as described by Sigrist et al. [2]. To the packed suspension washed with isoosmotic (310

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Abbreviations: SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; imosM, ideal milliosmomolar.

imosM) sodium phosphate buffer (pH 7.3) or packed erythrocyte ghosts (3 mg protein/ml) the appropriate volume of undiluted phenylisothiocyanate was added. The suspensions were then incubated at 37°C with gentle shaking for 120 min. Cells were washed at least three times with isoosmotic phosphate buffer (pH 7.3) and membranes were isolated. If isolated ghosts were subjected to labelling, washing was carried out using 20 imosM phosphate buffer (pH 7.3). Ghosts either labelled and washed or isolated from labelled erythrocytes were subjected to low-ionic-strength extraction with 1 mM EDTA in 0.3 mM phosphate buffer (pH 7.2) containing 50 μg/ml of PMSF for 30 min at 37°C. To the extract dithiothreitol (final concentration 10 mM) was added to prevent the secondary interaction of free phenylisothiocyanate with proteins and the extract was concentrated by reduced-pressure dialysis. Then dithiothreitol and Tris were added to a final concentration of 100 mM and the extract was chromatographed on a Sepharose CL-4B column (1.6 × 50 cm) equilibrated with 5 mM sodium phosphate buffer (pH 7.2), containing 50 mM NaCl, 4 mM 2-mercaptoethanol and 1 mM EDTA. Eluted peaks were dialysed overnight against 5 mM Tris-HCl buffer (pH 7.8), containing 0.1 mM EDTA, 20 μg ml of PMSF, and then concentrated by reduced-pressure dialysis.

When the effect of *p*-sulfophenylisothiocyanate was to be tested, washed erythrocytes or erythrocyte ghosts were incubated for 60 min with 10 mM (final concentration) of this compound at 37°C with gentle shaking, washed with 310 imosM (erythrocytes) or 20 imosM (ghosts) sodium phosphate buffer (pH 7.3), and then labelled for 120 min with [^{14}C]phenylisothiocyanate at specified concentrations.

When isolated spectrin was to be labelled, the dimeric spectrin solution (obtained by chromatography of the low-ionic-strength extract of the erythrocyte ghosts on a Sepharose 4B column) was made up to 0.1 M NaCl and 0.05 M sodium phosphate buffer (pH 7.3). The effect of *p*-sulfophenylisothiocyanate was tested by adding 0.1 ml of 100 mM solution of this compound in 0.05 M phoshate buffer (pH 7.3) to 0.9 ml of the sample. Both sets of samples (with and without *p*-sulfophenylisothiocyanate) were incubated for 30 min at 37°C. Then to all samples an appropriate volume of 950 mM ethanolic solution of [^{14}C]phenylisothiocyanate was added and the samples were left for a further 60 min at 37°C. The reaction was stopped by the addition of dithiothreitol and Tris to final concentrations of 100 mM, and labelled spectrin was isolated using a Sephadex G-25 (1 × 30 cm) column.

Protein concentration was determined according to the method of Lowry et al. [11]. Radioactivity measurements were carried out in a liquid scintillation counter using a scintillation cocktail prepared according to Fricke [12]. The radioactivity of labelled, isolated spectrin was measured after precipitation with 10% trichloroacetic acid, followed by chloroform/methanol (2 : 1, v/v) extraction, to ensure removal of noncovalently bound hydrophobic compounds. SDS-polyacrylamide gel electrophoresis was performed according to Fairbanks et al. [13], except that the concentration of SDS was 0.1% and the concentration of *N*,*N*′-methylenebisacrylamide was 0.07%. Gel (5.6%) containing a lowered amount of bisacrylamide was more easily soluble with H_2O_2. For radioactivity measurements gels with 3.5-cm-wide slots were prepared. Gels were stained with 0.25% Coomassie brilliant blue R-250 in 30% methanol and 10% acetic acid. The stained gels were cut into 2-mm slices (to prepare radioactivity patterns), or bands 1 and 2 were cut out (to determine radioactivity in subunits). The gel slices were solubilized with 1 ml 30% H_2O_2 containing 50 μl of 1 M NaOH for 15 min in a boiling water bath. The resulting solution was measured for its phenylisothiocyanate radioactivity. Strips of each gel (5 mm wide) were cut out along the migration direction and scanned at 600 nm.

Results

If the membranes of labelled erythrocytes are isolated, and extracted with low-ionic strength solution, and chromatographed on Sepharose 4B, the profiles seen in Fig. 1A are obtained. The protein profile is similar to those obtained previously by others [14,15]. The last peak corresponds to low-molecular-weight substances, which are introduced during sample processing. Apart from the dimer (c) and the so-called macro-molecular-

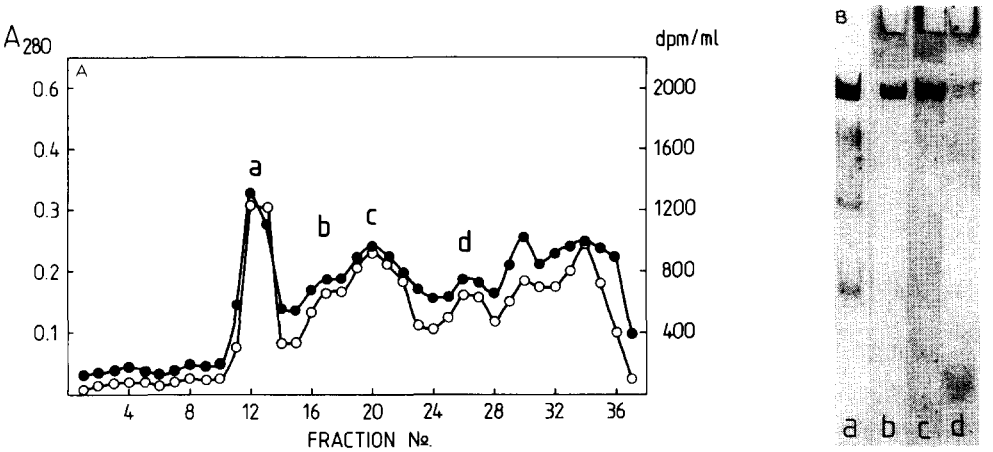

Fig. 1. (A) Protein and radioactivity distribution obtained by gel filtration of low-ionic-strength extract of erythrocyte ghosts isolated from phenylisothiocyanate-labelled erythrocytes. Washed erythrocytes were incubated with 0.5 mM [^{14}C]phenylisothiocyanate at 37°C for 120 min. After washing, ghosts were isolated and extracted with 1 mM EDTA in 0.3 mM phosphate buffer (pH 7.2) containing 50 μg/ml PMSF at 37°C for 30 min. Concentrated low-ionic-strength extract was treated with 100 mM dithiothreitol and 100 mM Tris (final concentrations) and chromatographed on a Sepharose CL-4B column (1.6×50 cm) equilibrated with 5 mM phosphate buffer (pH 7.2) containing 50 mM NaCl, 1 mM EDTA and 4 mM 2-mercaptoethanol. O——O, absorbance at 280 nm; ●——●, radioactivity. (B) SDS-polyacrylamide (5.6%) gel electrophoretograms stained with Coomassie blue of the material eluted from the column. (a) Macromolecular complex; (b) spectrin tetramer; (c) spectrin dimer; (d) actin and hemoglobin.

weight complex (a), tetrameric spectrin (b) is found in all preparations extracted from labelled erythrocyte ghosts. The proportion of tetramer is subject to change. The protein and radioactivity tracing patterns of SDS-polyacrylamide gel electrophoresed low-ionic strength extract of the membranes isolated from labelled, washed cells are shown in Fig. 2. Both the column chromatography and SDS-gel electrophoresis show that spectrin is labelled upon incubation of whole erythrocytes with [^{14}C]phenylisothiocyanate.

In order to follow the dependence of the degree of labelling of spectrin on the concentration of the probe in the incubation mixture, different samples of washed erythrocytes were incubated with increasing concentrations of phenylisothiocyanate. Low-ionic strength extracts of isolated membranes from labelled erythrocytes were subjected to Sepharose 4B column chromatography. The degrees of labelling of spectrin purified from erythrocytes as a function of the probe concentration are presented in Fig. 3. The reaction of phenylisothiocyanate with spectrin in whole erythrocytes might be considered as a saturating process. The relatively slow increase of incorporation of phenylisothiocyanate into spectrin in whole cells with the increasing concentration of the probe is probably due to the presence of hemoglobin and other proteins reactive with the probe [2].

As shown in Fig. 3, in erythrocytes about 3–4

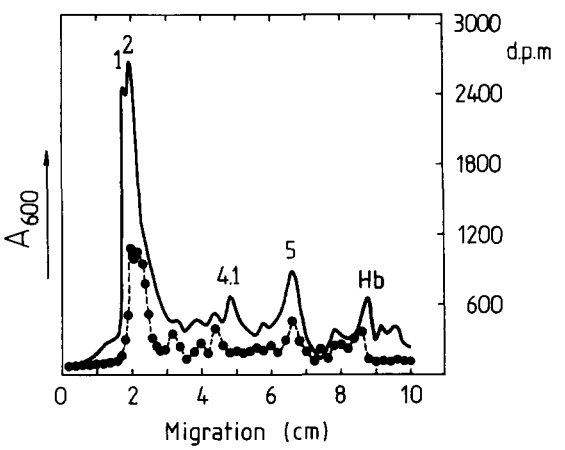

Fig. 2. SDS (0.1%)-polyacrylamide (5.6%) gel electrophoretogram of low-ionic-strength extract of erythrocyte ghosts isolated from erythrocytes labelled with 3.8 mM [^{14}C]phenylisothiocyanate. Labelling and extraction conditions as in Fig. 1. Coomassie blue-stained gel (35 mm wide) was cut into slices. Each slice was solubilized with 1.0 ml 30% H_2O_2 and 50 μl of 1 M NaOH by heating in boiling water bath for 15 min and the radioactivity of resulting solution was measured. ————, Coomassie blue staining pattern; ●— —●, radioactivity pattern.

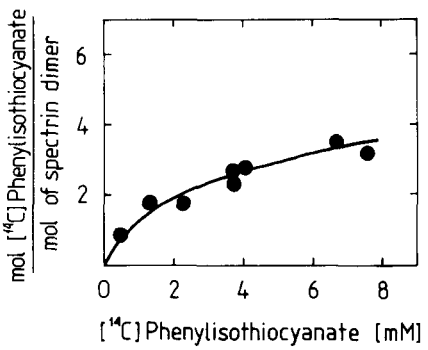

Fig. 3. The dependence of phenylisothiocyanate incorporation into spectrin (mol of phenylisothiocyanate per mol of spectrin dimer) in erythrocytes on the concentration of the probe (mM) in the incubation mixture. Samples of washed erythrocytes were incubated with the indicated [^{14}C]phenylisothiocyanate concentrations. Then spectrin was isolated as described in Fig. 1. Radioactivity was measured after 10% trichloroacetic acid precipitation and chloroform/methanol (2:1, v/v) extraction of purified spectrin.

mol of phenylisothiocyanate can be incorporated into spectrin dimer (mol. wt. 460000). When a double-reciprocal plot (not shown) was used and the correlation was tested by the least-squares method a maximal amount of 3.6 mol phenylisothiocyanate bound per spectrin dimer and a correlation coefficient of 0.97 were obtained.

When erythrocyte ghosts are labelled with phenylisothiocyanate at a concentration of 3.8 mM, the degree of labelling is 5–6 mol per spectrin dimer (Table I). This increase in reactivity might be due to the increased accessibility of the reactive regions in the spectrin molecule, or to the increase in the concentration of the probe at the internal face of the membrane. The second possibility seems to be unlikely, since in whole cells the degree of incorporation of the probe even at 7.6 mM does not exceed 3.5 mol per spectrin dimer.

It is also possible that at least a part of the phenylisothiocyanate would react in the aqueous phase [1,3]; therefore its nonradioactive, structural analogue, which is expected to react preferentially with functional groups accessible from the aqueous phase [3], was employed. Erythrocytes and erythrocyte ghosts were preincubated with *p*-sulfophenylisothiocyanate at a concentration of 10 mM, and were washed and treated with [^{14}C]phenylisothiocyanate. Results of labelling carried out with and without preincubation with *p*-sulfophenylisothiocyanate (Table I) indicate that the preincubation of the sample with hydrophilic isothiocyanate causes no inhibition of the reaction with [^{14}C]phenylisothiocyanate in the case of erythrocytes and very little (no more than 15%) in the case of erythrocyte ghosts.

In Table II the data of phenylisothiocyanate incorporation into spectrin subunits determined by gel electrophoresis, relating the peak area of Coomassie blue-stained spectrin subunits to the recovered radioactivity of excised gel slices corresponding to bands 1 and 2, are shown.

The results of such an analysis indicate that in the spectrin preparations tested both subunits are

TABLE I

EFFECT OF PREINCUBATION OF ERYTHROCYTES AND GHOSTS WITH *p*-SULFOPHENYLISOTHIOCYANATE ON THE LABELLING OF SPECTRIN IN THE MEMBRANE WITH [^{14}C]PHENYLISOTHIOCYANATE

Erythrocytes or ghosts were incubated with or without *p*-sulfophenylisothiocyanate at 37°C for 60 min, washed and incubated for 120 min with [^{14}C]phenylisothiocyanate at the same temperature. Spectrin was isolated as in Fig. 1. Radioactivity was determined after precipitation of protein with trichloroacetic acid and chloroform/methanol (2:1, v/v) extraction. Average values of duplicate experiments are shown.

Material	*p*-Sulfo-isothio-cyanate (mM)	[^{14}C]Phenyl-isothio-cyanate (mM)	Degree of labelling	
			mol phenylisothiocyanate/mol spectrin dimer	specific radioactivity (dpm/mg protein)
Erythrocytes	–	4.0	2.9	22500
Erythrocytes	10	4.0	2.9	22000
Ghosts	–	3.8	5.7	42000
Ghosts	10	3.8	5.08	37000

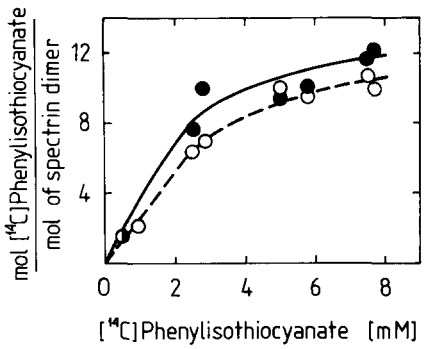

Fig. 4. Labelling of purified spectrin dimer with phenylisothiocyanate. Spectrin in 0.05 M sodium phoshate buffer (pH 7.3) containing 0.1 M NaCl was preincubated with (O— — —O) or without (●———●) 10 mM *p*-sulfophenylisothiocyanate (30 min, 37°C) and then with specified concentrations of [^{14}C]phenylisothiocyanate (60 min, 37°C). After termination of the reaction labelled spectrin was purified by passing the samples through a Sephadex G-25 (1×30 cm) column. The incorporation of the probe expressed in mol phenylisothiocyanate per spectrin dimer.

labelled to the same degree. Although data obtained this way are of rather relative value, assuming a linear correlation between protein concentration and the area of scanned, Coomasie blue-stained bands, the 'calculated' values of degree of labelling are in good agreement with the data for isolated spectrin preparations (Fig. 3 and Table I).

The data on labelling of isolated spectrin with phenylisothiocyanate in the presence or absence of *p*-sulfophenylisothiocyanate are shown in Fig. 4. The results of this experiment show the substantial increase in the reactivity of extracted spectrin in comparison with the reactivity of spectrin in whole cells and in the ghosts. With the concentrations of phenylisothiocyanate used in this experiment isolated spectrin can bind up to 10 mol of the probe after preincubation with, and up to 12 mol without, *p*-sulfophenylisothiocyanate per dimer molecule.

TABLE II

RADIOACTIVTY OF PHENYLISOTHIOCYANATE-LABELLED SPECTRIN SUBUNITS

Concentrated low-ionic strength extracts obtained from [^{14}C]phenylisothiocyanate-labelled ghosts or ghosts isolated from labelled erythrocytes were subjected to SDS-polyacrylamide gel electrophoresis in gels with 35-mm-wide slots. After staining with Coomassie blue gel slices containing bands 1 and 2 were cut out, solubilized with H_2O_2 and radioactivity was measured. A 5-mm-wide gel strip was cut out to make a gel scan at 600 nm. Values of dpm/cm^2 are related to the radioactivity per mg of protein. Under the conditions used 1 cm^2 corresponded to 36 μg of protein. The recovery of radioactivity in the described conditions was 23.6% The 'calculated' values of specific radioactivity were obtained on the basis of the above parameters (compare to 'specific radioactivity', Table I).

Material	Electrophoretic band	Specific radioactivity (dpm/cm^2)	'Calculated' specific radioactivity (dpm/mg protein)
Erythrocytes	1	91	10800
(2.5 mM phenylisothiocyanate)	2	108	12800
Erythrocytes	1	186	22000
(3.8 mM phenylisothiocyanate)	2	207	24500
Erythrocytes	1	184	21700
(4.0 mM phenylisothiocyanate)	2	195	23000
Erythrocytes	1	160	19000
(10 mM *p*-sulfophenyl-isothiocyanate, 4 mM phenylisothiocyanate)	2	162	19100
Ghosts			
(3.8 mM phenylisothiocyanate)	1	389	46000
	2	339	40000
Ghosts			
(10 mM *p*-sulfo-phenylisothiocyanate, 3.8 mM phenylisothio-cyanate)	1	342	40400
	2	321	38000

Discussion

In the present study we found that spectrin in whole erythrocytes is labelled with phenylisothiocyanate. The reaction displayed the following properties: (1) it proceeds in a saturable manner; (2) the resulting modification is insensitive to treatment with dithiothreitol and Tris (excess of nucleophile under alkaline conditions [16]); and (3) the labelling is not inhibited by preincubation of erythrocytes or ghosts with *p*-sulfophenylisothiocyanate. Histidine imidazole imino and α-amino groups partially deprotonated at neutral pH should therefore be blocked after treatment with polar isothiocyanate [1,3]. Since the attempts of Harris and Lux [17] to detect the N-terminal amino-acid residue using phenylisothiocyanate in standard conditions were unsuccessful, the conclusion was drawn that the N-terminal residues in both subunits are blocked.

The abovementioned features of the reaction would suggest the presence of hydrophobically disposed nucleophiles in the spectrin molecule in situ. This would occur in the case of penetration of the hydrophobic domain of the bilayer by small regions of spectrin molecule. Evidence for such interactions has previously been demonstrated in model systems by Mombers et al. [18–20].

Another possibility exists, that in the spectrin molecule, either in situ or extracted, highly hydrophobic domains are present. This would explain the labelling of spectrin in the solution. The reaction of bovine serum albumin with phenylisothiocyanate was similarly interpreted by Sigrist and Zahler [21]. There are reports from Gratzer's group [22,23] demonstrating the presence of hydrophobic regions in isolated spectrin, by applying high-resolution proton nuclear magnetic resonance and fluorescence techniques. However, it should be noted that isolated spectrin may undergo conformational changes, which may cause an increase in the reactivity of certain lysine ϵ-amino groups towards phenylisothiocyanate. On the other hand it is possible that the presence of millimolar concentrations of phenylisothiocyanate, which is highly hydrophobic, may introduce changes in the protein molecule environment leading to the increase in reactivity towards the probe. Similar results (with respect to the amount of label per protein molecule) were obtained with fluorescein isothiocyanate, but the reactions are inhibited by preincubation with *p*-sulfophenylisothiocyanate (Sikorski, A.F., unpublished data). The problem of diverse reactivity of spectrin in the cells and in the isolated state with different arylisothiocyanates needs further exploration. Further studies on labelling kinetics, localization and characterization of phenylisothiocyanate binding sites in the spectrin molecule are to be carried out.

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