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INTERACTION OF PHENYLISOTHIOCYANATE WITH HUMAN ERYTHROCYTE BAND 3 PROTEIN

II. TOPOLOGY OF PHENYLISOTHIOCYANATE BINDING SITES AND INFLUENCE OF *p*-SULFOPHENYLISOTHIOCYANATE ON PHENYLISOTHIOCYANATE MODIFICATION

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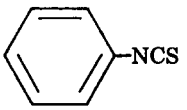
Summary

The two structurally related probes, the apolar phenylisothiocyanate and the polar, water-soluble *p*-sulfophenylisothiocyanate, were analysed for their topological interaction with human erythrocyte band 3 protein. Upon thermolytic and peptic digestion of labeled erythrocyte ghosts, the membrane-integrated segments of band 3 protein, the 17 000 and 10 000 dalton peptides, were isolated. At 2 mM initial label concentration, 90% of the hydrophobic probe phenylisothiocyanate was recovered in the 10 000 dalton peptide, the remaining amount of label being associated with the 17 000 dalton fragment. Pretreatment of the membranes with 5 mM *p*-sulfophenylisothiocyanate followed by labeling with 2 mM phenylisothiocyanate results in a consistent reduction in binding of phenylisothiocyanate by 1 mol/mol isolated band 3 protein. *p*-Sulfophenylisothiocyanate reportedly binds to the 17 000 dalton fragment (Drickamer, K. (1977), J. Biol. Chem. 252, 6909—6917). The interaction of the polar probe with the membrane protein affects binding of phenylisothiocyanate to the 10 000 dalton peptide by the equivalent of 1 mol/mol isolated peptide. The topological interrelation of the membrane-integrated segments is concluded.

Abbreviation: SDS, sodium dodecyl sulfate.

Introduction

The membrane-spanning protein — band 3 — is a major constituent of the human erythrocyte membrane. This protein is involved in transmembrane anion-exchange (Cl^-, HCO_3^-) [1]. Its functional properties can be specifically inhibited under physiological conditions by a large number of reagents [2]. Sulfonated arylisothiocyanates or analogous compounds possess the necessary requirements for effective anion transport inhibition. Both the hydrophobic probe phenylisothiocyanate (I) [3] and its polar structural analogue *p*-sulfophenylisothiocyanate (II) [4] have been utilized for chemical modification of human erythrocyte band 3 protein.



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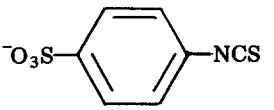

II

The apolar reagent phenylisothiocyanate partitions preferentially into the hydrophobic membrane phase [5]. The label can, therefore, interact with reactive nucleophiles present in this membrane domain. At saturating concentrations of the reagent, 4—5 mol phenylisothiocyanate were found to be bound per mol of band 3 protein [3]. The water-soluble *p*-sulfophenylisothiocyanate also binds covalently to the anion transport protein. On the basis of its physico-chemical properties, this probe would be expected to label preferentially reactive functional groups accessible from the aqueous phase. Previous studies have indicated that both reagents interact covalently with band 3 protein, resulting in comparable inhibition of its functional activity [6]. It is, therefore, of interest to investigate the topology of the phenylisothiocyanate-binding sites in the anion transport protein and the possible existence of common interacting site(s). This information would contribute to the understanding of the topology and function of erythrocyte band 3 protein.

Materials

Fresh blood was obtained from the central laboratories of the Swiss Red Cross and was stored at 4°C for no longer than 2 days. Before use, erythrocytes were washed three times with isotonic phosphate buffer, pH 7.3.

Phenyl[^{14}C]isothiocyanate and phenyl[^{35}S]isothiocyanate were obtained from Amersham, U.K. The radioactive reagents were diluted with non-radioactive phenylisothiocyanate (Pierce, sequenal grade) to final concentrations of 0.1 M in ethanol (specific activity, 6.25 Ci/mol and 23.2 Ci/mol, respectively). *p*-Sulfophenylisothiocyanate, a product of Aldrich Chemicals, was dissolved in water just before use (0.1 M). Thermolysin and pepsin were purchased from Sigma Chemicals. All other chemicals were of the highest purity commercially available.

Methods

Erythrocyte ghost membranes were prepared by the method of Dodge et al. [7]. Phenylisothiocyanate and *p*-sulfophenylisothiocyanate modification of human erythrocyte ghosts was performed as described by Sigrist et al. [3]. Protein was determined in presence of 0.1% SDS according to the method of Lowry et al. [8]. Measurement of radioactivity was carried out as described by Fox [9]. SDS-polyacrylamide gel electrophoresis was peformed according to the method of Fairbanks et al. [10] using 0.77% of the oxidative cleavable *N,N*-diallyltartardiamide instead of 0.29% *N,N*-methylene-bisacrylamide and an acrylamide concentration of 7.75% in the Tris/acetate buffer system, pH 7.4, or using the system of Laemmli [11]. Protein was visualized by Coomassie blue staining.

Differential labeling. Erythrocyte ghosts (4 mg protein/ml) were preincubated with various *p*-sulfophenylisothiocyanate concentrations (30 min, 37°C in 10 mM sodium phosphate buffer, pH 7.3, stirred suspension). After the preincubation, phenyl[^{14}C]isothiocyanate or phenyl[^{35}S]isothiocyanate was added to specified final concentrations. The mixture was then incubated for a further hour at 37°C. The labeled membranes were sedimented (30 min, 100 000 × *g*, 4°C) and washed three times with 10 mM sodium phosphate buffer, pH 7.3.

Relative phenylisothiocyanate incorporation into band 3 protein. Labeled membranes were solubilized in 1% SDS and the proteins separated by SDS-polyacrylamide gel electrophoresis. The Coomassie-blue-stained band 3 protein was cut out and solubilized in 0.8 ml 0.088 M sodium periodate. The resulting solution was measured for its relative protein content (absorption 570 nm) and phenylisothiocyanate radioactivity.

The radioactivity/absorption 570 nm ratio was utilized as a relative measure for phenylisothiocyanate binding to band 3 protein.

Isolation of band 3 protein. The isolation procedure described by Steck et al. [12] was utilized for purification of labeled band 3 protein. Phenylisothiocyanate-labeled ghosts were treated with 0.1 M NaOH. The washed extracted membranes were solubilized with 1% SDS, containing 0.05% dithiothreitol. Following separation by Sepharose 4B chromatography the fraction containing band 3 protein was concentrated by vacuum dialysis. Lipid-associated or non-covalently bound phenylisothiocyanate was removed by chloroform/methanol extraction according to Renkonen et al. [13].

Thermolysin digestion of erythrocyte ghosts and preparation of fragments Th 3, GTh 1 and GTh 2

Frozen erythrocyte ghosts were thawed by incubation at 4°C. Sodium chloride was added to a final concentration of 0.15 M, and then thermolysin to 0.2 mg/ml. Ghosts (about 2 mg protein/ml) were incubated at 0°C for 20 min and 37°C for 60 min. The digested ghosts were stripped of extrinsic proteins and polypeptides by washing with 0.1 M sodium hydroxide (90 min, 23 000 × *g*, 4°C) and then washed once with 5 mM sodium phosphate, pH 8.0, containing 5 mM 2-mercaptoethanol (90 min, 23 000 × *g*, 4°C). The ghosts were dissolved by incubating them for 10 min at 100°C in a solution

containing 50 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 10% (w/v) SDS. β-Mercaptoethanol was added to a final concentration of 5% (v/v) and the solution was loaded on to a 6 × 105 cm column of Sephadex G-75 (medium) which was developed with 1% (w/v) SDS/1 mM EDTA, pH 8.0. Fractions containing the peptides of interest were identified by polyacrylamide gel electrophoresis of samples taken at regular intervals across the column profile. After pooling the appropriate fractions the solutions containing the purified peptides were concentrated by pressure dialysis in an Amicon ultrafiltration cell with a PM10 membrane and dialysed exhaustively against distilled water (GTh 2 was dialysed in Spectrapor 6 tubing; molecular weight cut-off 1000, Spectrum Medical Industries, Los Angeles, CA). The peptides were precipitated by dialysis against methanol and distilled water successively, for 24 h each, and they were then lyophilized.

Pepsin digestion of erythrocyte ghosts and preparation of fragment P5

Pepsin digestion of erythrocyte ghosts derived from 500 ml packed human erythrocytes was done on a preparative scale as described previously [14] except that the protease digestion was allowed to continue for 60 min at 37°C. The neutralized washed membrane pellet was suspended and made up to 120 ml with 5 mM sodium phosphate buffer, pH 8.0. 5 vol. *n*-butanol/methanol (1 : 1, v/v) were added and the mixture stirred overnight at room temperature. The insoluble material was collected by centrifugation, washed once with diethyl ether and freeze-dried after the addition of a small amount of water, typically giving 130—150 mg dry weight of material. Fragment P5 was further purified by gel filtration in 1% SDS containing 1 mM EDTA on a column of Sephadex G-50 (Superfine). The sample was mixed with 5 ml 50 mM Tris-HCl buffer, pH 8.0, containing 10% SDS, 5% 2-mercaptoethanol, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, vortexed vigorously and heated in a boiling water bath for 5 min. After stirring overnight at room temperature, the insoluble material was centrifuged out and the supernatant containing the fragment P5 was applied to the column. The tubes containing the fragment P5 peak were identified by SDS-polyacrylamide gel electrophoresis and were pooled and dialysed using Spectrapor 6 membrane tubing (molecular weight cut-off 1000) against daily charges of distilled water for 2 days. After overnight dialysis against methanol, during which the peptide precipitated, the sample was dialysed against distilled water for 24 h and freeze-dried, and yielded 6—12 mg dry weight fragment P5 containing varying amounts of detergent.

Results

The effect of *p*-sulfophenylisothiocyanate on phenylisothiocyanate binding to erythrocyte band 3 protein is shown in Fig. 1. Membranes were labeled with various phenylisothiocyanate concentrations and the incorporation of the label into the electrophoretically separated band 3 protein was determined (Fig. 1A). For differential labeling studies membranes were preincubated with *p*-sulfophenylisothiocyanate. Phenylisothiocyanate was then added to a final

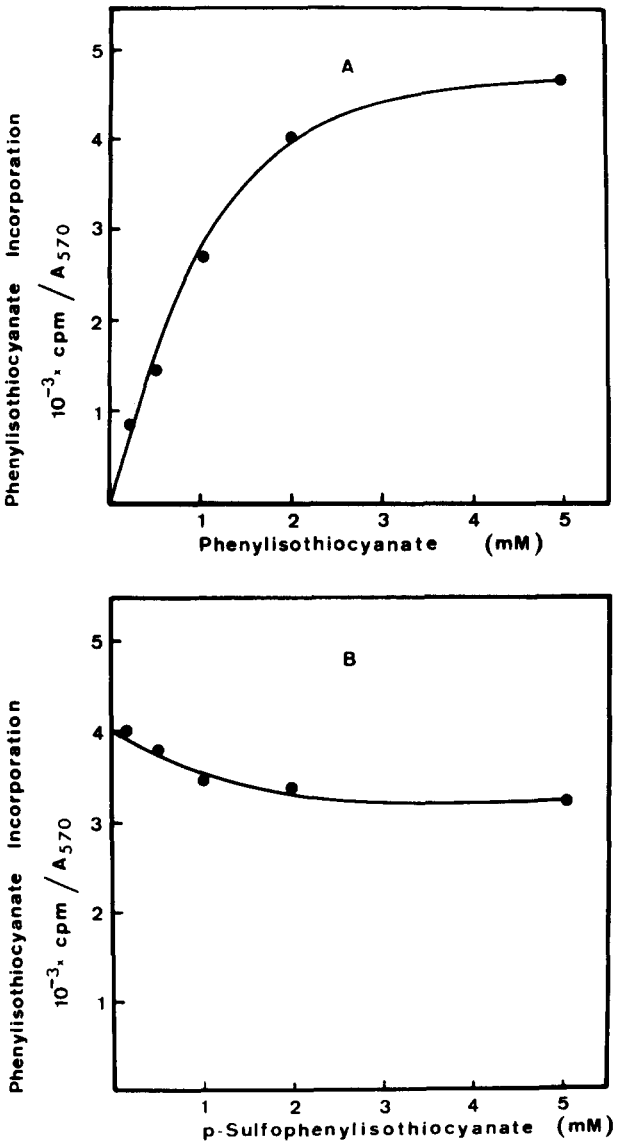

Fig. 1. Phenylisothiocyanate binding to band 3 protein and effect of *p*-sulfophenylisothiocyanate. Erythrocyte membranes were labeled with various phenylisothiocyanate concentrations at pH 7.3 for 1 h at 37°C. The membranes were washed three times and the label incorporation was determined in the electrophoretically separated band 3 protein by analysing the ratio radioactivity to Coomassie blue staining intensity (A). Prior to labeling with 2 mM phenylisothiocyanate, membranes were incubated with various *p*-sulfophenylisothiocyanate concentrations. The phenylisothiocyanate binding to band 3 protein was determined as described in Methods (B).

concentration of 2 mM. The binding of phenylisothiocyanate to band 3 protein following a pretreatment of the erythrocyte ghost membranes with various *p*-sulfophenylisothiocyanate concentrations is shown in Fig. 1B. Preincubation of erythrocyte ghosts with 5 mM *p*-sulfophenylisothiocyanate resulted in a decrease of 20—30% of the binding of phenylisothiocyanate to band 3 protein.

TABLE I

EFFECT OF *p*-SULFOPHENYLISOTHIOCYANATE ON PHENYLISOTHIOCYANATE BINDING TO BAND 3 PROTEIN

Erythrocyte ghosts were preincubated with *p*-sulfophenylisothiocyanate (30 min, 37°C, 10 mM sodium phosphate buffer, pH 7.3, stirred suspension). After the preincubation radioactive phenylisothiocyanate was added. The mixture was then incubated for a further hour at 37°C. The labeled membranes were sedimented, washed three times with 10 mM sodium phosphate buffer, pH 7.3, and band 3 protein was isolated according to Steck et al. [12]. The binding of phenylisothiocyanate to band 3 protein was determined.

p-Sulfophenylisothiocyanate preincubation (mM)	Phenylisothiocyanate (mM)	Phenylisothiocyanate bound per isolated band 3 protein (mol/mol)
5.0	1.1	1.5
0.0	1.1	2.5
5.0	1.8	2.15
0.0	1.8	3.1

Identical results were obtained when the excess *p*-sulfophenylisothiocyanate was removed before the incubation with phenylisothiocyanate (data not shown). In a further set of experiments the competitive effects of *p*-sulfophenylisothiocyanate on the binding of phenylisothiocyanate to band 3 protein was quantitatively determined (Table I). Membranes were labeled with 1.1 and 1.8 mM phenylisothiocyanate, respectively. Band 3 protein was then isolated and the binding of phenylisothiocyanate determined in the isolated protein. Pretreatment of the erythrocyte ghosts with 5 mM *p*-sulfophenylisothiocyanate resulted in a decrease of 1 mol phenylisothiocyanate bound per mol 95 000 dalton protein (Table I).

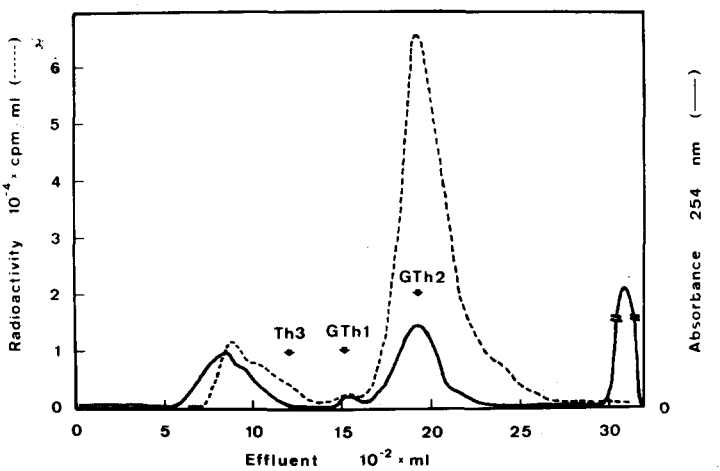

Fig. 2. Isolation of thermolytic, membrane-bound fragments of band 3 protein by Sephadex G-75 chromatography. The fractionation of peptides was performed on a 6 cm diameter × 105 cm Sephadex G-75 column (elution buffer, 1 mM EDTA/1% SDS, pH 8.0). Absorbance at 254 nm was measured (solid line). Fractions of 14 ml were collected and the radioactivity content was determined (dotted line). line).

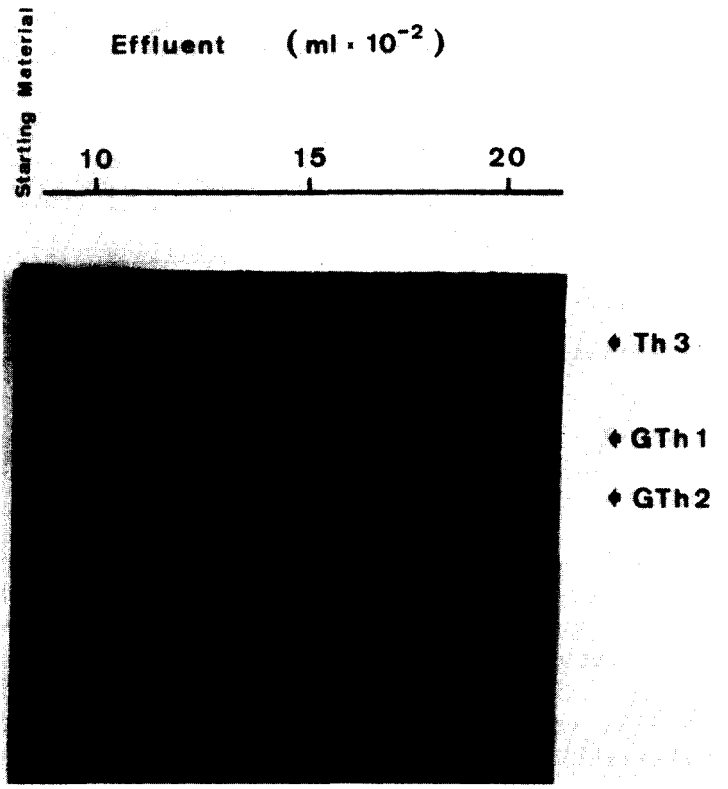

Fig. 3. Isolation of thermolytic, membrane-bound fragments of band 3 protein. Electrophoresis of samples taken at regular intervals across the profile shown in Fig. 2, carried out according to the method of Laemmli [11].

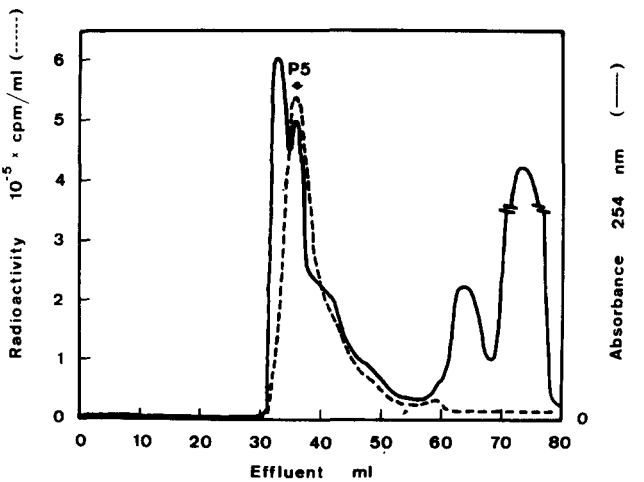

Fig. 4. Isolation of the peptic fragment P5 by Sephadex G-50 chromatography. Pepsin-digested erythrocyte membranes were treated with 5 vol. butanol/methanol (1 : 1), solubilized in 50 mM Tris-HCl buffer, 10% SDS, 5% β-mercaptoethanol, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluoride, pH 8.0, as described in Methods. The solubilized peptides were separated on a 1 cm diameter × 100 cm Sephadex G-50 (superfine) column (elution buffer, 1% SDS/1 mM EDTA, pH 8.0). 1 ml fractions were collected and analysed for radioactivity (dotted line). Absorbance at 254 nm was recorded (full line).

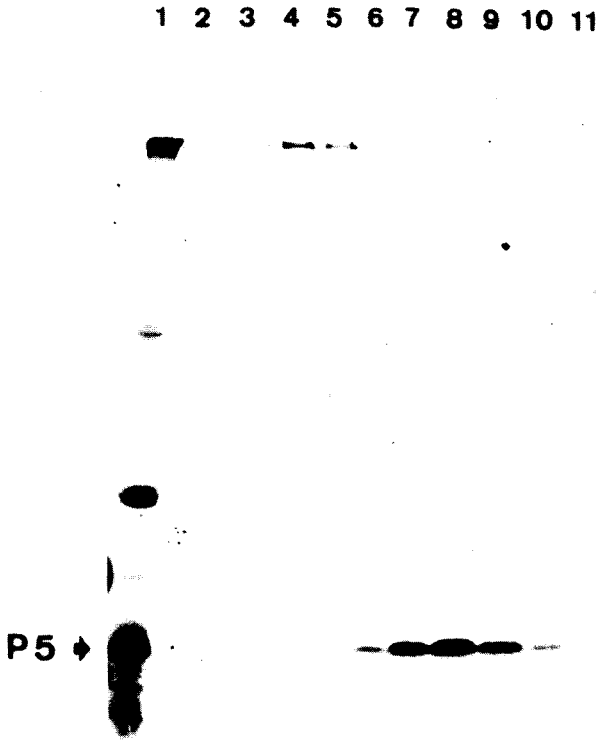

Fig. 5. SDS-polyacrylamide gel electrophoretic identification of column fractions obtained during purification of fragments P5. The butanol/methanol (1 : 1)-insoluble material from a pepsin digest was solubilized in SDS as described in Methods and applied to a column (2 × 150 cm) of Sephadex G-50 (superfine) equilibrated with 1% SDS containing 1 mM EDTA. 3.25-ml fractions were collected. 0.01 ml samples of the fractions were applied to a gel containing a gradient (12.5—25%) of acrylamide with a 5% acrylamide overlay using the system of Laemmli [11]. Track 1, sample of the total material applied to the column; tracks 2—11, fractions 37 and successive fractions to fraction 46. Track 8 (fraction 43) contains the peak fraction of P5 and is equivalent to the peak P5 fractions shown in Fig. 3.

Incorporation of phenylisothiocyanate into proteolytic fragments of band 3 protein

Erythrocyte ghosts (4 mg protein/ml) were labeled with 2 mM phenylisothiocyanate. The labeled membrane proteins were then digested with thermolysin or pepsin, respectively. Upon solubilization the membrane-bound fragments were isolated as described in Methods. Th 3, GTh 1, GTh 2 and P5 were assumed to have molecular weights of 38 000, 17 000, 10 000 and 10 000, respectively [14,15]. Although these values were derived from gel electrophoresis experiments, any inaccuracy due to the possible anomalous mobilities of membrane-associated peptides is unlikely to be sufficient to affect the conclusions of this investigation. GTh 1 is similar to the peptides ThT [15] and GTh [16], whereas GTh 2 is similar to the peptides Th 6 [15] and P5 [14]. Gel filtration elution profiles of the integral thermolytic and peptic peptides from band 3 protein and gel electrophoresis patterns of samples taken at intervals across these profiles are shown in Figs. 2 to 5. Phenyliso-

TABLE II

PHENYLISOTHIOCYANATE INCORPORATION INTO THERMOLYTIC AND PEPTIC FRAGMENTS OF BAND 3 PROTEIN

Erythrocyte membranes were preincubated with or without 5 mM *p*-sulfophenylisothiocyanate, followed by labeling with 2 mM radioactive phenylisothiocyanate. The labeled membranes were washed three times with 10 mM sodium phosphate buffer. The washed ghosts were digested with either thermolysin or pepsin. Isolation of membrane-bound protein fragments was performed as described in Methods. The number of determinations is shown in parentheses; $\bar{x}$, mean value of label incorporation into the listed fragments.

Fragment	Phenylisothiocyanate bound per mol peptide (mol)	Phenylisothiocyanate binding upon pretreatment with 5 mM *p*-sulfophenylisothiocyanate (mol/mol)
GTh 1	0.3 ± 0.1 (2)	0.3 ± 0.1 (2)
Th 3	3.0 (1)	2.1 (1)
GTh 2	3.1 (1)	2.2 (1)
P5	2.9 ± 0.2 (3)	1.9 ± 0.3 (3)
$\bar{x}$ (Th3, GTh 2, P5)	3.0 ± 0.3	2.0 ± 0.2

thiocyanate binding has been determined in these peptides (Table II).

For the peptide GTh 2 a binding ratio of 3.1 mol label per mol 10 000 dalton peptide was found. In contrast, the incorporation of phenylisothiocyanate into the 17 000 dalton peptide (GTh 1) was only 0.3 mol/mol. It has been shown earlier that the thermolytic fragment Th 6 (similar to GTh 2) overlaps with the peptic fragment P5 in the primary structure [14,15]. In support of this result a binding ratio of 2.9 mol phenylisothiocyanate per mol P5 peptide was obtained. Limited digestion by thermolysin allowed the isolation of the 38 000 dalton peptide (Th 3). In this fragment 3.0 mol phenylisothiocyanate were recovered. It was inferred from a preceding study [3] that when applied at 2 mM 3.5 mol phenylisothiocyanate are bound per mol band 3 protein. Under these labeling conditions 85 ± 8% of the total number of binding sites are recovered in the thermolytic fragments Th 3 and GTh 2 and in the peptic fragment P5. Surprisingly, only 8 ± 3% of the recovered phenylisothiocyanate was found to be localized in the 17 000 dalton fragment GTh 1.

Preincubation of erythrocyte ghosts with 5 mM *p*-sulfophenylisothiocyanate, followed by labeling with 2 mM phenylisothiocyanate and proteolytic digestion with either thermolysin or pepsin resulted in a consistent reduction in the binding of phenylisothiocyanate by 1 ± 0.15 mol per mol of Th 3 and the peptides GTh 2, P5 (Table II).

Discussion

It was reported earlier that the labeling of human erythrocyte ghosts with 2 mM phenylisothiocyanate results in the incorporation of 3.5 mol phenylisothiocyanate per mol band protein.

It is shown in this study that a total of 3.3 ± 0.4 mol phenylisothiocyanate can be recovered in the thermolytic and peptic fragments GTh 1, Th 3, GTh 2

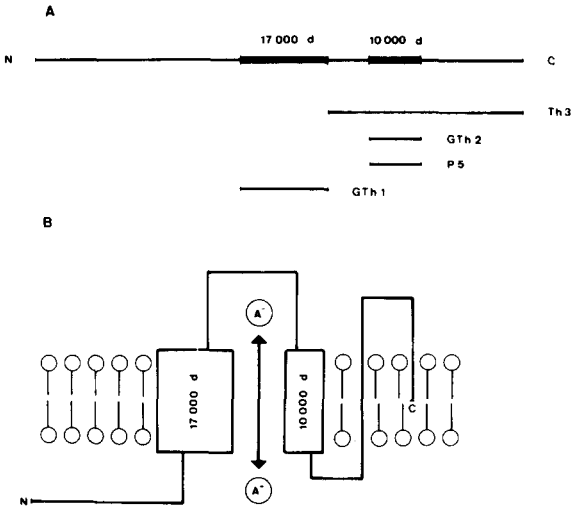

Fig. 6. Structural representation of erythrocyte band 3 protein. (A) Alignment of the thermolytic (GTh 1, Th 3, GTh 2) and pepsin P5 fragments on the primary structure of band 3 protein. The thickened portions indicate the regions which are membrane-bound and modified by arylisothiocyanates. (B) Schematic representation of the anion (A^-) transport protein in the erythrocyte membrane. N, N-terminal; C, Carboxyl terminal of the polyeptide chain; d, dalton.

and P5. The fragments GTh 2 and P5 overlap and are subfragments of Th 3 (see Fig. 6A). The three isolated fragments (Th 3, GTh 2 and P5) bind equal amounts of phenylisothiocyanate. The phenylisothiocyanate binding to band 3 is, therefore, restricted to the two membrane-bound segments, the 10 000 dalton and 17 000 dalton peptide (Fig. 6A). Under the labeling conditions used only a small amount (about 10%) of label is recovered in the fragment GTh 1. The GTh 2 fragment from the C-terminal region of the protein bears almost all the radioactivity (90%).

p-Sulfophenylisothiocyanate is reported to bind covalently to the CB-C fragment [17], which is a subfragment of the 17 000 dalton spanning section of the anion transport protein. The incorporation of phenylisothiocyanate is reduced by 1 ± 0.15 mol/mol band 3 protein upon pretreatment of the membranes with *p*-sulfophenylisothiocyanate (5 mM).

Identical results were obtained for the isolated peptides Th 3, GTh 2 and P5. However, no significant decrease in phenylisothiocyanate incorporation was found for the GTh 1 fragment. Thus, the occupation of a site in the 17 000 dalton fragment by *p*-sulfophenylisothiocyanate affects one phenylisothiocyanate binding site in the 10 000 dalton peptide.

As stated above, the probes phenylisothiocyanate and *p*-sulfophenylisothiocyanate interact with the anion transport protein in topologically distinguishable sections, but comparable inhibition has been achieved with both reagents for phosphate uptake [6]. Thus both sites are probably involved in the transport process.

Upon labeling of red blood cells with 2 mM phenylisothiocyanate, phosphate uptake is inhibited by 85% [3]. Under these conditions the 17 000 dalton seg-

ment, which is reported to be involved in anion transport [18], is only modified to the extent of 0.3 ± 0.1 mol phenylisothiocyanate. By contrast, the 10 000 dalton section is modified by 3.0 ± 0.3 mol phenylisothiocyanate. The results indicate that inhibition of phosphate transport by phenylisothiocyanate is caused by modification of the 10 000 dalton peptide. It is concluded, therefore, that the 10 000 dalton segment probably participates in anion translocation. Jennings and Passow [19] have also described the involvement of the 38 000 dalton peptide in anion transport. Moreover, the modification of the 17 000 dalton peptide by *p*-sulfophenylisothiocyanate affects the phenylisothiocyanate incorporation (reduction by one site) into the 10 000 dalton peptide. We conclude that the two fragments are therefore topologically related and synergistically involved in anion transport (Fig. 6B).

Acknowledgements

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