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INTERACTION OF PHENYLISOTHIOCYANATE WITH HUMAN ERYTHROCYTE BAND 3 PROTEIN

I. COVALENT MODIFICATION AND INHIBITION OF PHOSPHATE TRANSPORT

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Summary

The hydrophobic probe phenylisothiocyanate is utilized for chemical modification of human erythrocyte band 3 protein. The binding of phenylisothiocyanate to this protein is characterized in whole erythrocytes, erythrocyte ghost membranes and in isolated band 3 protein. The label, reactive with nucleophiles in their deprotonated form, is found in all three preparations to be covalently bound to band 3 protein. Under saturation conditions, 4—5 mol phenylisothiocyanate are covalently bound per mol protein (molecular weight 95 000). The described modification effects inhibition of phosphate entry into erythrocytes. 50% inhibition of phosphate transport is obtained following a preincubation of erythrocytes with 0.45 mM phenylisothiocyanate. Both phenylisothiocyanate binding and transport inhibition are saturating processes. The relationship of the two parameters is non-linear.

Introduction

The major trans-membrane protein of the erythrocyte membrane — band 3 (Fairbanks et al. [1] nomenclature) — is known to be involved in anion transport and has therefore been the focus of considerable research concerning both structure and function. Kinetic experiments and chemical modification studies

Abbreviation: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid.

have yielded valuable information on both of these aspects (for reviews on the subject see Refs. 2—5).

Covalent interaction of phenylisothiocyanate with erythrocyte band 3 has been presented in preliminary communications [6,7]. The hydrophobic probe interacts covalently with nucleophilic groups solely in their non-protonated form ($RS^- >> RO^- > R{-}NH_2 > OH^-$) [8]. At neutral pH, the ϵ-amino groups of lysine exposed to the aqueous phase are protonated and accordingly not reactive. In contrast, the buried bulk-pH-independent amino functions may be in a reactive state. Cysteine thiols exposed to the aqueous phase are partially deprotonated at pH 7.3 and, as a result, are reactive with the label. The reaction product formed with thiols, however, is chemically distinguishable by its reversibility under alkaline conditions in the presence of reactive nucleophiles [8].

Functionally important probes used to date for covalent chemical modification and concomitant inhibition of erythrocyte anion transport possess three common characteristics: (1) a lipophilic core (aryl), (2) an anion-site directing group ($-SO_3^-$, $-O-PO_3H^-$) and (3) an electrophilic functional group (—F, $-N_3$, —NCS) as a prerequisite for covalent binding [3]. With the exception of photolabels [9], covalently modifying inhibitors of the anion transport system are suggested to interact with proteinaceous amino groups [2,3,5,10]. Thiol modifying reagents are generally noninhibitory or their interaction site is ambiguous [2]. The possible effect of phenylisothiocyanate, which lacks an anion-site directing group, on biological function is of obvious interest.

The present study reports on the dual effect of phenylisothiocyanate: The probe interacts at physiological pH in a saturable process with band 3, both as an apolar covalent label and as a potent inhibitor of phosphate transport.

Materials and Methods

Materials

Fresh blood was obtained from the Central Laboratories of the Swiss Red Cross and stored at 4°C for no longer than two days. Before use erythrocytes were washed three times with isotonic phosphate buffer, pH 7.3. Radiochemicals were obtained from Amersham, England, with the following specifications: phenyl[^{14}C]isothiocyanate, 11.2 Ci/mol; $H_3\,^{32}PO_4$, carrier free. All other chemicals were of the highest purity commercially available.

Methods

Phenylisothiocyanate labeling of whole erythrocytes. A packed sediment of washed erythrocytes was carefully suspended in an equal volume of isotonic (310 mosM) sodium phosphate buffer, pH 7.3. The suspension was labeled with phenylisothiocyanate by incubation for 1 h at 37°C under continuous agitation. The labeled erythrocytes were washed three times by sedimentation (15 min, 1200 × *g*) with 0.3 M sodium citrate buffer, pH 6.4, at ambient temperature.

Phenylisothiocyanate labeling of human erythrocyte ghosts. Erythrocyte ghost membranes were prepared by the method of Dodge et al. [11] and labeled at a protein concentration of 4 mg/ml with various amounts of phenyl-

[^{14}C]isothiocyanate (1 h, 37°C in 10 mM sodium phosphate buffer, pH 7.3, stirred suspension). 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.

Isolation of band 3. The isolation procedure described by Steck et al. [12] was employed for purification of labeled band 3. Phenyl[^{14}C]isothiocyanate-labeled ghosts were treated with ice-cold 0.1 M NaOH. The washed extracted membranes were solubilized with 1% sodium dodecyl sulfate (SDS)/0.05% dithiothreitol (w/v). Dithiothreitol was used to prevent secondary interactions of non-bound phenylisothiocyanate which is soluble in the membrane lipid phase. Protein modification by the label during purification could thus be avoided, because phenylisothiocyanate reacts favorably with excess OH^- and is highly reactive with nucleophiles such as dithiothreitol [8]. Following separation by Sepharose-4B chromatography the fraction containing band 3 was concentrated by vacuum dialysis.

Analytical procedures. Protein was determined in presence of 0.1% SDS according to Lowry et al. [13]. Radioactivity was measured as described by Fox [14]. Extraction of lipids and lipophilic products was performed according to Renkonen [15]. For SDS-gel electrophoretic analysis the method of Fairbanks et al. [1] was followed, using 5.8% acrylamide gels in Tris-acetate buffer, pH 7.4. 10% acrylamide gels in a phosphate buffered system, pH 7.3, were used according to Weber and Osborn [16]. Protein bands were visualized by Coomassie blue staining; carbohydrate staining was performed by the periodic acid-Schiff technique of Zacharius et al. [17]. For determination of phenyl[^{14}C]isothiocyanate incorporation destained gels were cut into 1-mm slices which were then dissolved by incubation overnight at 80°C with H_2O_2 containing 0.01 vol. 25% ammonia. The resulting solution was measured for phenyl[^{14}C]isothiocyanate radioactivity.

Phosphate transport. Transport of inorganic phosphate into erythrocytes was performed as described by Ho and Guidotti [18]. Phenylisothiocyanate-labeled citrate-washed cells were incubated at 20°C with 0.3 M sodium citrate buffer, pH 6.4, containing 0.013 M [^{32}P]orthophosphate. 50% cell suspensions were used for uptake measurements. Phosphate entry into erythrocytes was measured by determining the amount of radioactivity which remained in the extracellular fluid as a function of time [18]. Percentage of inhibition was calculated by the equation

$$\% \text{ Inhibition} = \frac{1 - \text{slope inhibitor}}{\text{slope control}} \times 100$$

The respective slopes were calculated from the semilogarithmic plot of time-dependent phosphate entry according to Ho and Guidotti [18].

Results and Discussion

Binding of phenyl[^{14}C]isothiocyanate to band 3 in whole erythrocytes and erythrocyte ghost membranes

In Fig. 1 the phenyl[^{14}C]isothiocyanate incorporation into erythrocyte membranes (B) is compared with the corresponding protein (A) and glycoprotein (C) gel electrophoretic patterns. Band 3 protein is extensively labeled

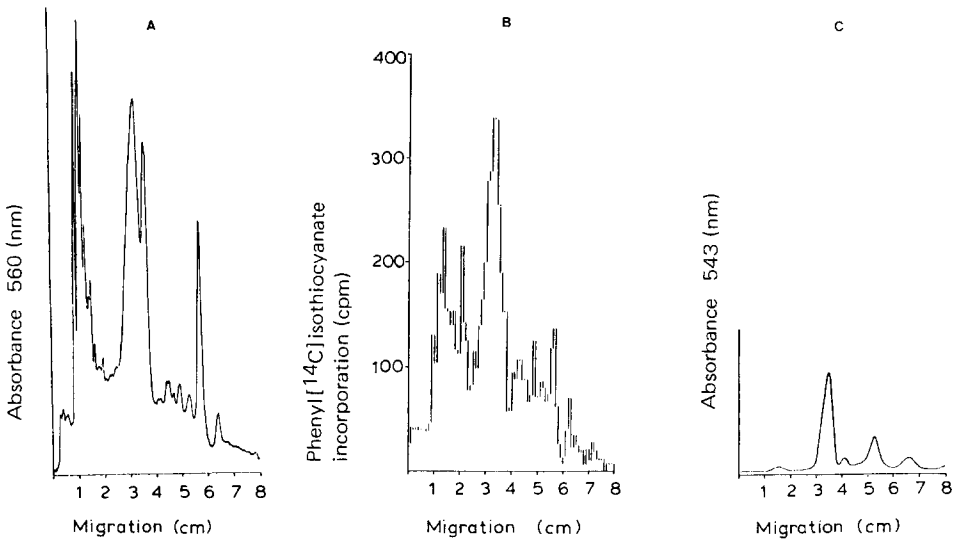

Fig. 1. Gel electrophoretic pattern of phenyl[^{14}C]isothiocyanate-labeled ghosts. Erythrocyte ghost membranes (4 mg protein/ml) were labeled with 2 mM phenyl[^{14}C]isothiocyanate by incubation at 37°C for 1 h in 10 mM sodium phosphate buffer, pH 7.3. Repeatedly-sedimented labeled membranes were analyzed by 5.8% acrylamide SDS-gel electrophoresis in a Tris-acetate system, pH 7.4. (A) Coomassie blue-stained protein pattern. (B) Phenyl[^{14}C]isothiocyanate radioactivity. (C) Glycoprotein-stained pattern.

by the radioactive probe. When erythrocyte ghost membranes are labeled with phenyl[^{14}C]isothiocyanate at a concentration of 2 mM, phenylisothiocyanate incorporation into proteins other than band 3 is observed. As reported earlier, with lower phenylisothiocyanate concentrations (0.2 mM) the almost exclusively labeled membrane protein is band 3 [6]. The binding of phenyl-[^{14}C]isothiocyanate to erythrocyte band 3 is a saturating process. Saturation can be shown by labeling either isolated erythrocyte membranes (Fig. 2A) or whole red cells (Fig. 2B). The differential increase of phenylisothiocyanate incorporation into band 3 utilizing intact cells as compared to erythrocyte ghosts is due to the presence of hemoglobin which is reactive with the membrane penetrating label [6].

Phenylisothiocyanate incorporation data presented in Fig. 2 are determined by analytical gel electrophoresis, relating the scanned Coomassie blue-stained band 3 area with the radioactivity recovered in the corresponding gel slice. Because the amount of protein present in the electrophoretically-separated band 3 is determined by absorbance at 560 nm, the values obtained contain relative rather than absolute information. Based on the linear correlation of two independent analytical methods, the relative incorporation as determined in the electrophoretically-separated band 3 (Fig. 2) and the absolute incorporation data as obtained from modified, isolated band 3 preparations (Table I), it is concluded that maximally 4—5 mol phenylisothiocyanate can be bound per band 3.

Isolated phenylisothiocyanate-labeled band 3

The electrophoretic homogeneity of the phenyl[^{14}C]isothiocyanate-labeled

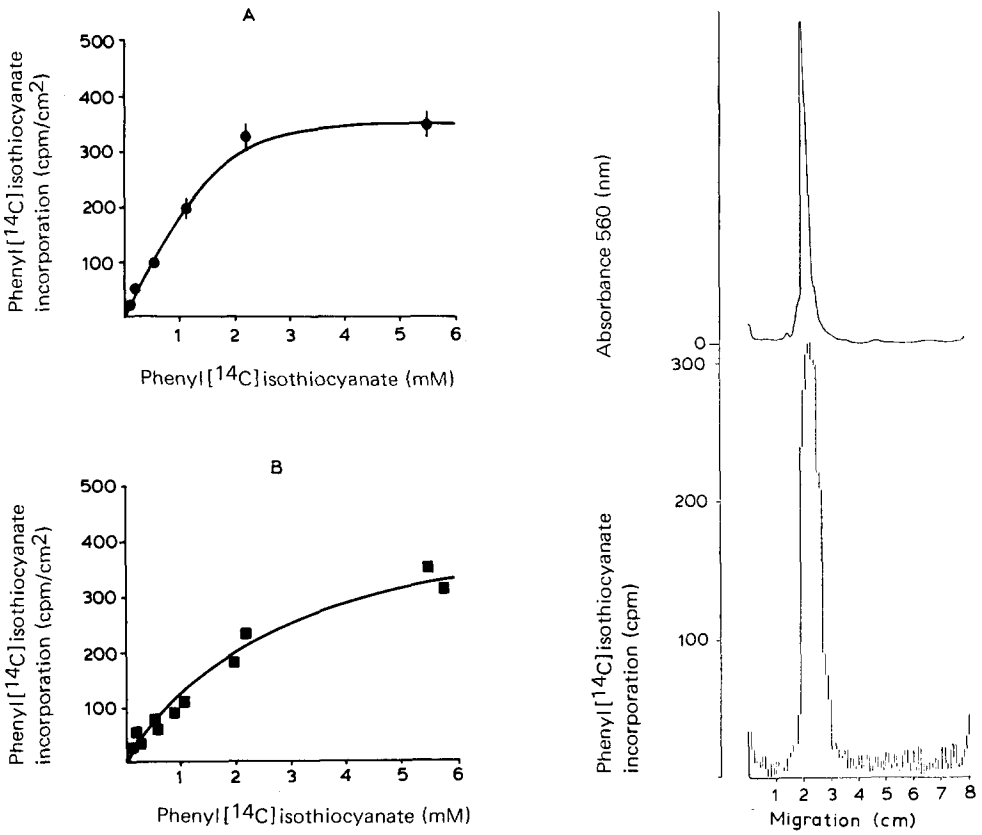

Fig. 2. Phenyl[^{14}C]isothiocyanate incorporation into erythrocyte ghosts (A) and whole red cells (B). Labeling procedures were followed as described in Methods. Band 3 associated radioactivity was determined in the dissolved acrylamide gel, following gel electrophoretic separation (10% acrylamide SDS/phosphate system, pH 7.3) of the membrane components. For Fig. 2B ghost membranes were prepared [11] from labeled erythrocytes prior to electrophoresis. Phenylisothiocyanate incorporation is defined by the ratio of radioactivity recovered in the Coomassie blue-stained band 3 and the corresponding scanned (560 nm) peak area (cpm/cm^2). Duplicate values of two independent series are shown.

Fig. 3. Phenyl[^{14}C]isothiocyanate-labeled isolated band 3. 5.8% acrylamide gel electrophoretic analysis of phenylisothiocyanate-labeled, isolated band 3 (SDS-Tris-acetate system, pH 7.4). Incorporated radioactivity is depicted below the densitometric trace of the Coomassie blue-stained gel.

isolated band 3 is shown in Fig. 3. Binding stoichiometries of phenylisothiocyanate to band 3 are listed in Table I. Label incorporation is determined in isolated band 3, following membrane modification with various phenylisothiocyanate concentrations. Because of the hydrophobic properties of the label, lipid associated or nonbound phenyl[^{14}C]isothiocyanate is determined by chloroform/methanol extraction.

Inhibition of phosphate transport

Phenylisothiocyanate irreversibly inhibits the uptake of inorganic phosphate in erythrocytes (Fig. 4A). Half-maximal inhibition is obtained upon preincubation of red cells with 0.45 mM phenylisothiocyanate (Fig. 4B). Preincubation

TABLE I

BINDING OF PHENYLISOTHIOCYANATE TO BAND 3

Erythocyte membranes were labeled with various phenylisothiocyanate concentrations. The labeled band 3 protein was then isolated by the procedure of Steck et al. [12]. Lipid-associated or nonbound phenyl-[^{14}C]isothiocyanate present in the isolated band 3 was extracted by chloroform/methanol [15]. The amount of covalently protein-bound label was determined by subtraction of the chloroform/methanol-extracted radioactivity from the total radioactivity measured in the isolated band 3 preparation.

Initial concentration of phenyl-[^{14}C]isothiocyanate in the incubation medium (mM)	Total phenyl[^{14}C]-isothiocyanate per isolated band band 3 (A) (dpm × 10^6/mg)	Chloroform/methanol extractable phenyl-[^{14}C]isothiocyanate in isolated band 3 (B) (% of total (A) dpm × 10^6/mg)	Covalent protein-associated phenyl-[^{14}C]isothiocyanate (C) (A — B, expressed as dpm × 10^6/mg)	Phenyl[^{14}C]-isothiocyanate bound per band 3 protein (C expressed in mol label per mol protein)
0.2	0.20	17.6	0.16	0.6
0.5	0.32	6.5	0.30	1.1
2.2	1.18	15.1	1.00	3.8
3.0	1.29	12.7	1.12	4.2

with 4—5 mM phenylisothiocyanate results in complete inhibition of phosphate entry. For comparative studies, the inhibitors phenylisothiocyanate and *p*-sulfophenylisothiocyanate have been applied under identical conditions using 0.5 mM inhibitor concentration. The hydrophobic phenylisothiocyanate was found to inhibit phosphate uptake to 60 ± 7% whereas the hydrophilic, anion-site directing group bearing analog induced 74 ± 11% inhibition [7]. The inhibitory effect of *p*-sulfophenylisothiocyanate by covalent interaction has been described for phosphate [18] and sulfate transport [4,19].

Correlation of phenylisothiocyanate binding to band 3 and phosphate transport inhibition

The relationship of phenylisothiocyanate modification of band 3 and phosphate transport inhibition is depicted in Fig. 5. The nonlinear curve indicates the presence of several nonequivalent phenylisothiocyanate interaction sites. The extrapolated initial inhibition (dotted line) approximates equimolar modification. Covalent chemical modification of a primary reactive site is therefore concluded to be responsible for inhibition. In contrast, the binding-inhibition relationship is linear for anion-site directed bimodal inhibitors like *p*-sulfophenylisothiocyanate [18] and DIDS [20]. Complete inhibition of anion transport is reported to occur upon binding of 1 mol DIDS per mol band 3 [20].

Discussion of possible interaction sites

The N-terminal amino acid of band 3, an obviously favored interaction site for phenylisothiocyanate, is reported to be blocked by acetylation [21]. The amino acid composition of band 3 has been described by several authors [12,18,22—24]. Among the amino acids present, cysteine, tyrosine and lysine residues are the most probable candidates for the formation of a covalent bond with the label. Possibly occurring cysteine and tyrosine modification, however,

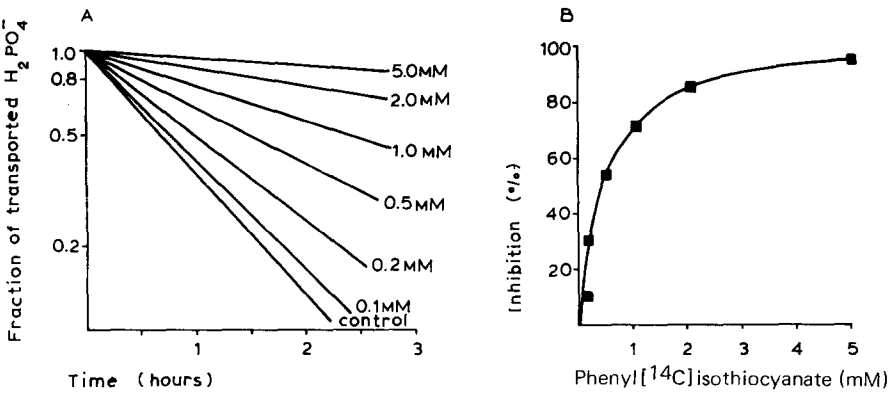

Fig. 4. Inhibition of phosphate uptake by phenylisothiocyanate in human erythrocytes. Washed erythrocytes were incubated for 1 h at 37°C in isotonic phosphate buffer, pH 7.3, containing phenylisothiocyanate. Following incubation the erythrocytes were washed three times with 0.3 M sodium citrate buffer, pH 6.4. Uptake of [^{32}P]phosphate was measured in a 50% suspension of phenylisothiocyanate-labeled, citrate-washed erythrocytes in 0.3 M sodium citrate containing 13 mM [^{32}P]phosphate at 20°C, pH 6.4. (A) Time course of phosphate transport into PITC-labeled cells. (B) Inhibition of phosphate transport as a function of PITC labeling concentration. PITC, phenylisothiocyanate.

is expected to be reversed during the membrane solubilization step. This treatment includes an incubation at pH 8 in the presence of excess dithiothreitol, a highly reactive nucleophile. Dissociation and exchange reactions are reported to occur for the S-ester of the N-monosubstituted dithiocarbamic acid derivative ($R_1NHCSSR_2$) under alkaline conditions in presence of excess nucleophile [8].

The stable covalent interaction between the ϵ-amino group of lysine and phenylisothiocyanate is the most probable modification. The chemical stability of this bond is documented by the partial resistance to acid hydrolysis (6 N HCl, 24 h, 110°C) and its stability during the Edman degradation procedure [25]. Phenylisothiocyanate modification of the lysine ϵ-amino group present in 28 copies per band 3 [12], can only occur with the deprotonated nucleo-

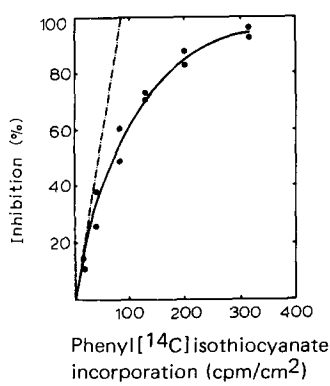

Fig. 5. Relationship between phenyl[^{14}C]isothiocyanate incorporation into band 3 and percent inhibition of phosphate transport. Phenyl[^{14}C]isothiocyanate modification and non-radioactive labeling was performed in parallel experiments using an identical sample of washed red cells. Phenyl[^{14}C]isothiocyanate incorporation and inhibition was determined as described in the legends to Figs. 2 and 4, respectively.

phile (RNH_2). Since the labeling was performed at pH 7.3, the reactive, uncharged groups are favorably existent in an apolar domain. The availability and reactivity of such proteinaceous nucleophiles in band 3 has already been demonstrated by the covalent interaction of bimodal anion transport inhibitors, including the numerous isothiocyano derivatives [2—5,18,20]. Furthermore, the covalent DIDS binding site is associated with the membrane-spanning 17 000 dalton segment of band 3 [26] and covalently bound *p*-sulfophenylisothiocyanate has been localized in a hydrophobic protein fragment [27,28].

Hydrophobic arylisothiocyanates possess the chemical characteristics for a covalent reaction with nucleophiles in an apolar environment. Presently, it cannot be stated, however, if the occupation of hydrophobic domains (see Ref. 3) alone induces the inhibition of phosphate transport. It is the subject of a further study to investigate whether common covalent interacting sites do exist for bimodal anion transport inhibitors and phenylisothiocyanate.

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