# Inhibition of anion transport across the red cell membrane by dinitrophenylation of a specific lysine residue at the $H_2$DIDS binding site of the band 3 protein

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The inhibition of anion transport by dinitrophenylation of the red cell membrane is brought about by the modification of a single lysine residue located on the 17-kDa segment of the band 3 protein. This residue is identical with Lys a, which is also capable of reacting with one of the two isothiocyanate groups of 4,4'-diisothiocyano dihydro-stilbene-2,2'-disulfonate ($H_2$DIDS). The rate of reaction between Lys a and 1-fluoro-2,4-dinitrobenzene is reduced when a second lysine residue on the 35-kDa segment of the band 3 protein becomes dinitrophenylated. This latter residue is not identical with Lys b which is known to be present on the 35-kDa segment and involved in the cross-linking of this segment with the 17-kDa segment by $H_2$DIDS.

*Band 3 protein* *Anion transport* *1-Fluoro 2,4-dinitrobenzene* *$H_2$DIDS* *Erythrocyte*

## 1. INTRODUCTION

Exposure of the red cell membrane to 1-fluoro-2,4-dinitrobenzene ($N_2$ph-F) causes inhibition of anion transport [1–3]. Concomitantly, the amino lipids [2] and most peptides that can be detected on SDS polyacrylamide gel electropherograms of the isolated red cell membrane become dinitrophenylated [4,5].

The site of action of $N_2$ph-F could be determined by differential labelling, using $^{14}$C-labelled $N_2$ph-F ($^{14}$C-$N_2$ph-F) and non-radioactive, selective inhibitors of anion transport like SITS and other stilbene disulfonates. When these latter compounds are present during treatment with $^{14}$C-$N_2$ph-F, lipids and membrane proteins still become labelled as in red cells that had not been pretreated. The only exception is the band 3 protein whose labelling is greatly reduced [4,5]. Conversely, dinitrophenylation prior to the addition of stilbene disulfonates prevents their covalent binding to the band 3 protein [3,6]. Thus, the binding to the band 3 protein of $N_2$ph-F and the stilbene disulfonates are mutually exclusive, suggesting that band 3 is the site of action [4,5].

Binding of $N_2$ph-F to band 3 is maximally reduced when $H_2$DIDS or its covalently binding analogues are applied at concentrations that produce complete inhibition of anion transport; i.e., when 1 mol of these inhibitors is bound per mol of band 3 [6,7]. The stoichiometry of the replacement of $N_2$ph-F by the stilbenes is 1:1, suggesting that covalent binding of stilbene isothiocyanates and $N_2$ph-F involves a common amino acid residue [8,9].

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*Abbreviations:* $N_2$ph-F, 1-fluoro-2,4-dinitrobenzene; $H_2$DIDS, 4,4'-diisothiocyano dihydrostilbene-2,2'-disulfonate; DNDS, 4,4'-dinitro stilbene-2,2'-disulfonate; SITS, 4-isothiocyano 4'-acetamido stilbene-2,2'-disulfonate; PCMBS, *p*-chloro mercuribenzene sulfonate; EDTA, ethylene diamine tetra acetic acid; SDS, sodium dodecyl sulfate



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$N_2$ph-F is capable of reacting with many different amino acid residues, including SH, tyrosine and histidine groups [10]. Most notable targets are lysine residues, of which there are 27–28 per band 3 molecule [11]. Nevertheless, the decrease of $^{14}$C-$N_2$ph-F binding capacity after the binding of a single molecule of a stilbene isothiocyanate per band 3 molecule can be easily measured [4,5]. This indicates that under the conditions employed, the amino acid residues involved in the inhibition by $N_2$ph-F are much more reactive than many others that bear no relation to anion transport. The experiments presented here substantiate this and lead to the identification of two lysine residues which are responsible for the effects produced by dinitrophenylation on anion transport.

## 2. METHODS AND RESULTS

Resealed red cell ghosts [12] were incubated at a range of $N_2$ph-F concentrations at 37°C for 30 min. They were then washed to remove excess $N_2$ph-F and its breakdown products. Subsequently, each batch of dinitrophenylated ghosts was subdivided into two. One of the two batches was used to measure sulfate equilibrium exchange, the other to measure the capacity for $^3H_2$DIDS-binding, by exposure to a large excess of $^3H_2$DIDS (25 $\mu$M) for a long time (90 min) at slightly alkaline pH (7.4). Fig.1 shows the effects of dinitrophenylation on transport (a) and on the capacity of band 3 to bind $^3H_2$DIDS (b,c). A nearly linear relation exists between the two effects, suggesting a causal relationship (d).

Fig.2 shows that the relationship between the binding of $N_2$ph-F to the band 3 protein and the $N_2$ph-F concentration in the medium is biphasic. Two $N_2$ph-residues are bound much more easily than the others. At a $N_2$ph-F concentration of 0.175 mM, where the transport is inhibited by 80%, 2.5 $N_2$ph-residues are bound per band 3 molecule. After dinitrophenylation under these conditions, ion-exchange chromatography (Beckman M 71 resin, 12 × 0.9 cm column, buffers as in [17] at 54°C) of acid hydrolysates (6 N HCl, 110°C, 24 and 48 h hydrolysis in vacuum-sealed tubes) of the isolated band 3 protein lead to the recovery of 99% of the radioactivity; 80% of the radioactively labelled amino acid was $\epsilon$-$^{14}$C-labelled $N_2$ph-lysine ($\epsilon$-$^{14}$C-$N_2$ph-lysine), 20% were decomposition products besides the $N_2$ph-lysine. After more exhaustive dinitrophenylation causing 95% inhibition of transport, neither the recovery of $^{14}$C nor the percentage of decomposition products changed while up to about 6 $N_2$ph-residues were bound per band 3 molecule. These observations suggest that the inhibition is due to the modification of lysine residues on the band 3 protein, while the modification of other residues is of little, if any significance.

The inhibition of anion transport seen after exposure to $N_2$ph-F for a given length of time is reduced when that exposure is carried out in the presence of a reversibly binding analogue of $H_2$DIDS; e.g., the dinitro derivative DNDS. This is due to the fact that the analogue occupies, for much of the time, a site which controls access to the specific $N_2$ph-F binding site(s) involved in the inhibition of anion transport. At sufficiently high concentrations of the analogue, the modification by $N_2$ph-F of the specific $N_2$ph-F binding site(s) can be reduced up to the point where $N_2$ph-F, applied for 30 min at 37°C (pH 7.4), no longer produces measurable changes of the capacity of band 3 to transport $SO_4^{2-}$ (table 1). Thus, any extra $N_2$ph-F-binding that takes place on band 3 outside the binding site for $H_2$DIDS or its reversibly binding analogues has only a secondary influence on the anion transport system. We conclude, therefore, in accordance with previously expressed views, that the inhibition of anion transport by $N_2$ph-F is essentially due to dinitrophenylation of lysine residues that are located on the band 3 protein in the region that acts as the binding site for $H_2$DIDS and its analogues [8,9].

The $H_2$DIDS molecule carries two isothiocyanate groups. They are capable of reacting with two lysine residues (previously designated a and b) thereby cross-linking two adjacent segments of the peptide chain [13]. These segments can be identified by treatment with external chymotrypsin, which splits the band 3 molecule into two fragments of 60 kDa and 35 kDa, without loss of transport activity. From the 60-kDa peptide a further fragment of 42 kDa can be removed by internal trypsin, again without loss of the capacity to transport anions. The remaining 17-kDa fragment is found to be cross-linked by $H_2$DIDS with the 35-kDa fragment, yielding the so-called 55-kDa fragment [13] (fig.3).

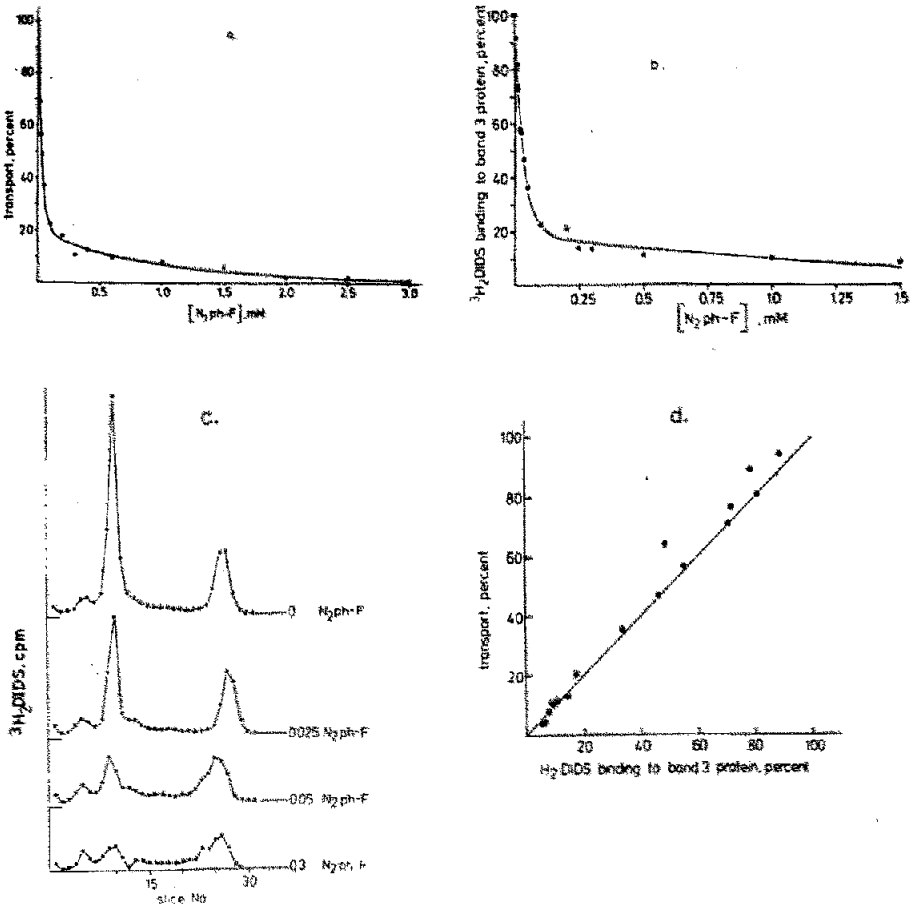


Fig.1. Capacity to transport sulfate (a) and to bind $^3H_2$DIDS to the band 3 protein (b), after dinitrophenylation of resealed red cell ghosts at the $N_2$ph-F concentrations indicated. (c) shows examples of $^3H_2$-DIDS labelling patterns of SDS polyacrylamide gel electropherograms (prepared on 5% gels) on which the determinations of $H_2$DIDS binding capacity are based. The peaks at slices 9–10 represent band 3, the peaks at the front represent lipids. The ordinates indicate the radioactivity in cpm. The origins of the ordinates for the various gels are marked by the short horizontal lines at slices 1–3. In (d) the ordinate values of fig.1a,b for equal $N_2$ph-F concentrations are plotted against one another, indicating the linear relation between the surviving capacities to perform $SO_4^{2-}$ transport and to bind $^3H_2$DIDS. The straight line indicates identity. Dinitrophenylation and subsequent flux measurements were carried out in 5 mM $Na_2SO_4$, 20 mM Na-phosphate (pH 7.4), 130 mM NaCl inside and outside resealed ghosts. For gel electrophoresis, the resealed ghosts were lysed and washed in 0.1% saponin, dissolved in 10 mM KCl, 5 mM EDTA, pH 7.4; $^3H_2$DIDS binding to the band 3 protein was determined as in [6]. The plotted curves represent non-linear computer fits of the sums of two exponential to the data. 100% refers to the flux or $^3H_2$DIDS binding in control cells that had not been exposed to $N_2$ph-F.

To localize the lysine residues that are responsible for the inhibition of anion transport by $N_2$ph-F, it is only necessary to consider the 17-kDa and 35-kDa fragments, which both play a role in anion transport [13–15]. The former contains about 5, the latter about 15 lysine residues [11].

The experiment represented in fig.4a shows that the inhibition of anion transport by $N_2$ph-F is associated with the dinitrophenylation of both the 35-kDa and the 17-kDa fragment of the band 3 protein. This experiment was executed with resealed ghosts that had been made from chymotryp-

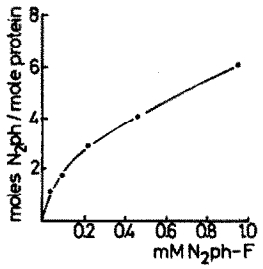


Fig.2. $N_2$ph-F binding to the band 3 protein as a function of $N_2$ph-F concentration in the medium. Red cell ghosts were labelled for 30 min at pH 7.4 in the medium described in the legend to fig.1. They were then prepared for and subjected to SDS–PAGE as indicated there. Ordinate: mol $N_2$ph-residues/mol band 3 protein. Abscissa: concentration of $N_2$ph-F in the medium during dinitrophenylation.

sinized red cells (1 mg/ml chymotrypsin, 60 min, 37°C) and whose anion transport had been inhibited by exposure to 175 $\mu$M $N_2$ph-F at pH 7.0 and 37°C for 30 min. After dinitrophenylation, the ghosts were lysed by saponin (see legend to

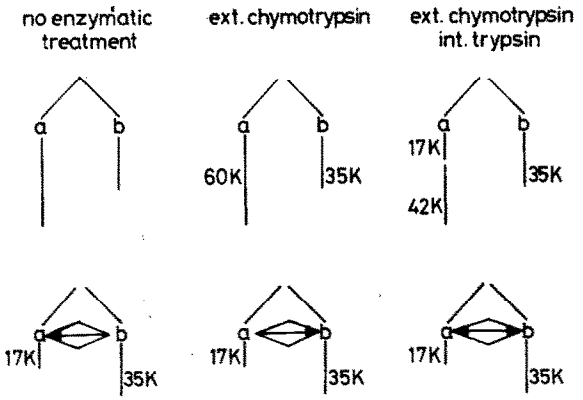


Fig.3. Upper row: Schematic representation of the fragments of the band 3 protein molecule obtained after exposure to external chymotrypsin, internal trypsin and reaction with $H_2$DIDS as in [13]; a and b refer to Lys a and Lys b, respectively. Lower row: $H_2$DIDS binding to Lys a and Lys b in chymotrypsinized red cells, and the formation of the cross-link. The figures represent the fragments that can be expected to occur on gel electropherograms after trypsinization of the isolated membranes at the end of the experiment. The $H_2$DIDS molecule is represented by the parallelogram. The establishment of covalent bonds is indicated by the black zones. The top headings refer only to the upper row.

Table 1

Rate constants for anion equilibrium exchange ($^{o}k_s$) as measure after dinitrophenylation of resealed red cell ghosts in the absence or presence of DNDS

| DNDS ($\mu$M) | $N_2$ph-F ($\mu$M) | $^{o}k_s$ ($10^2$ min$^{-1}$) |
|---|---|---|
| 0 | 0 | 5.9 |
| 125 | 0 | 5.2 |
| 250 | 0 | 5.1 |
| 0 | 175 | 1.7 |
| 125 | 175 | 5.0 |
| 250 | 175 | 5.4 |

Resealed ghosts were prepared as in [12] from chymotrypsinized (1 mg/ml, 1 h, 37°C) red cells. They were subsequently exposed to $N_2$ph-F (pH 7.4, 37°C, 30 min) in the presence of DNDS at the concentrations indicated. After removal of DNDS and excess $N_2$ph-F by washing, measurement of sulfate equilibrium exchange at pH 7.4, 37°C (1 mM $Na_2SO_4$, 130 mM NaCl, 20 mM EDTA). Ghosts from chymotrypsinized red cells had been used to make the experimental conditions similar to those used in the experiments represented in fig.4. The results are indistinguishable from those observed in ghosts made from untreated red cells

fig.1), exposed to trypsin (100 $\mu$g/ml for 30 min in a medium composed of 20 mM EDTA, 130 mM NaCl, 1 mM $Na_2SO_4$, pH 7.0), and then stripped by PCMBS [16]. This treatment not only removes the 42-kDa fragment of the band 3 molecule but also all other major membrane proteins that are susceptible to dinitrophenylation. Thus the label on the 35-kDa and 17-kDa fragments of band 3 is clearly seen, without much interference by dinitrophenylation of the other membrane proteins. One notices that the 35-kDa fragment is slightly less labelled than the 17-kDa fragment.

When $^{14}$C-$N_2$ph-F and $^{3}H_2$DIDS are added simultaneously to resealed ghosts, then the $^{3}H_2$DIDS binds much faster to the band 3 protein than the $^{14}$C-$N_2$ph-F. Almost instantaneously, the $^{3}H_2$DIDS becomes non-covalently attached. Thereafter, the covalent reaction of the isothiocyanate groups occurs. At pH 7.0 this reaction is almost exclusively confined to the formation of the thiourea bond with a lysine residue which is located on the 17-kDa fragment of band 3 (Lys a) (fig.3). The binding of $^{14}$C-$N_2$ph-F to this frag-

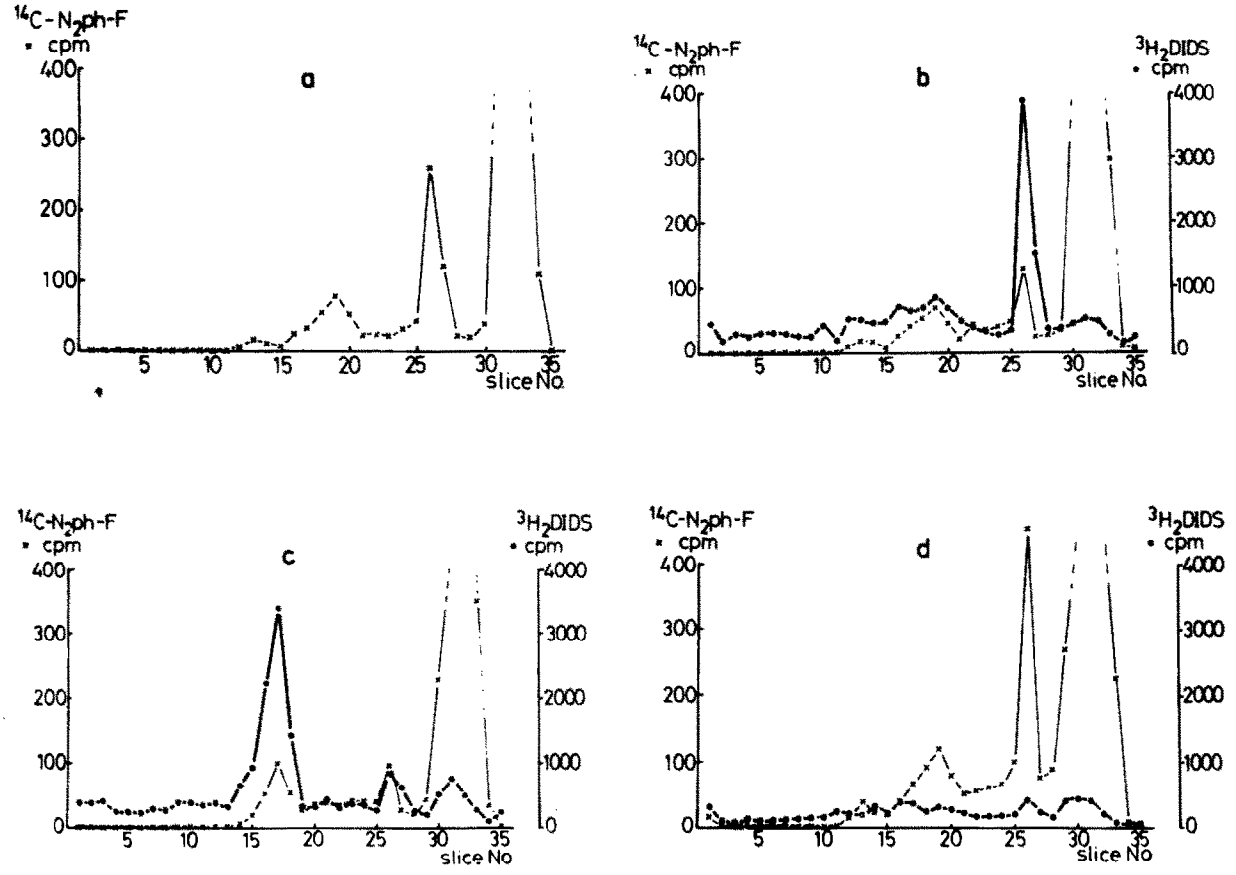


Fig.4. Labelling with $^{14}$C-N$_2$ph-F and $^{3}$H$_2$DIDS of resealed ghosts made from chymotrypsinized red cells. Gradient gel electrophoresis (4–30% polyacrylamide) after haemolysis of the ghosts by saponin, exposure to trypsin and stripping by PCMBS. (a) Labelling profile after exposure to $^{14}$C-N$_2$ph-F alone; (b) labelling profile of N$_2$ph-F and $^{3}$H$_2$DIDS, after simultaneous exposure to both agents; (c) same as (b) but after removal of excess $^{14}$C-N$_2$ph-F and $^{3}$H$_2$DIDS exposure to pH 9.5 to cross-link 35-kDa and 17-kDa fragments; (d) same conditions as in (a) but after dinitrophenylation with $^{14}$C-N$_2$ph-F a large excess of non-radioactive N$_2$ph-F was added and dinitrophenylation continued for another 30 min. Thereafter, exposure to $^{3}$H$_2$DIDS. The 35-kDa and 17-kDa fragments are located at slices 19 and 26, respectively. The so-called 55-kDa fragment (the product of the cross-linking of the 17-kDa and 35-kDa fragments) is located at slice 17. The big peak around slice 30 represents lipid-labelling. For explanations, see text and fig.3.

ment is correspondingly reduced while binding to the 35-kDa fragment is little, possibly not at all, affected (fig.4b). Since each band 3 molecule binds only one H$_2$DIDS molecule [5–7,13], and since the binding of this single H$_2$DIDS molecule reduces N$_2$ph-F-binding to the 17-kDa fragment to a low value, we conclude that the $^{14}$C-N$_2$ph-F-labelling of the 17-kDa fragment represents little more than 1 mol of N$_2$ph-F/mol of band 3 protein. This is compatible with the demonstration (see above) that of the 2.5 N$_2$ph-residues bound at 80% inhibition, only one resides on the 17-kDa fragment.

Quantitative evaluation of the counts on the gels yields a molar ratio of the labelling of the 17-kDa and 35-kDa fragments of 1.4 ($n$ = 3). Since each 17-kDa fragment binds about 1 N$_2$ph-residue, the 35-kDa segment also binds about 1 N$_2$ph-residue. For our purposes, it was necessary to explore whether or not the label resides predominantly on Lys b (see fig.3), a residue that is known to participate in the control of anion transport [15]. To do this, resealed ghosts made from chymotryp-

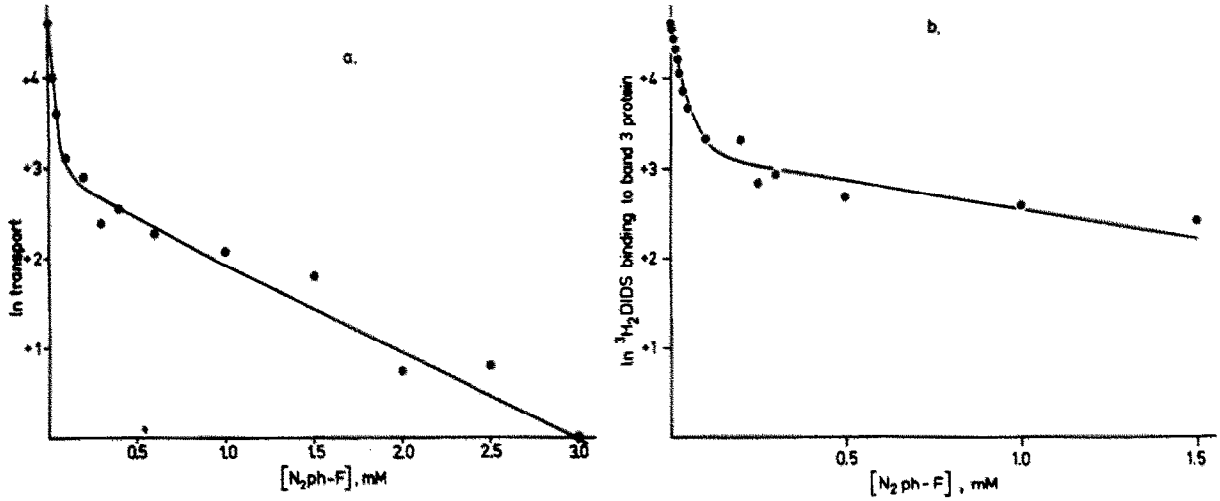


Fig.5. Semilog plots of (a) sulfate transport and (b) capacity to bind $^{3}H_2$DIDS, both as a function of $N_2$ph-F concentration. Time of exposure to $N_2$ph-F, 30 min; temperature, 37°C. Data taken from fig.1.

sinized red cells were first exposed to $^{3}H_2$DIDS and $^{14}$C-$N_2$ph-F, at pH 7.0 for 30 min. At the end of the reaction time, excess $^{3}H_2$DIDS and $^{14}$C-$N_2$ph-F were removed and the ghosts incubated at pH 9.5 for 90 min. Under these conditions, the $H_2$DIDS molecules that became attached to Lys a on the 17-kDa fragment cross-linked with Lys b on the 35-kDa fragment (fig.3, lower row), provided the latter is not dinitrophenylated. Fig.4c shows that a 55-kDa fragment appears and hence that the cross-link between the 35-kDa and 17-kDa fragments is indeed established. We conclude, therefore, that modification of Lys b is not involved in the inhibition of anion transport by $N_2$ph-F. Instead, the inhibition is due to the dinitrophenylation of Lys a.

Closer inspection of fig.4c reveals certain details that are in accordance with this conclusion but need further explanations. The small amounts of $^{14}$C-$N_2$ph-F and $^{3}H_2$DIDS that remain at the original location of the 17-kDa fragment indicate that a small fraction of that fragment did not cross-link. This was to be expected. The initially non-covalently bound $H_2$DIDS molecule has the option to react first, either with Lys a or Lys b. Although the probability for the reaction first with Lys b is much lower than with Lys a, it is still measurable [16]. When the $^{3}H_2$DIDS reacts first with Lys b, then Lys a becomes dinitrophenylated and no cross-link can be formed. The $^{14}$C-$N_2$ph-F bound to the 17-kDa fragment remains at the original location on the gel. Conversely, there exists a certain probability that Lys b on the 35-kDa fragment becomes dinitrophenylated even though its reactivity is much smaller than that of Lys a. If this happens, $^{3}H_2$DIDS bound to Lys a cannot establish a cross-link. This accounts for the residual $^{3}H_2$DIDS on the 17-kDa location in the experiment of fig.4c. Finally, one notices at the location of the 55-kDa fragment not only most of the $^{3}H_2$DIDS that was originally bound to the 17-kDa fragment but also most of the $^{14}$C-$N_2$ph-F that was originally associated with the 35-kDa fragment. This shows that the $N_2$ph-F binding site on the 35-kDa fragment is distinct from the lysine residue b, which is involved in the cross-linking reaction with the $^{3}H_2$DIDS.

In the final experiment of this series, resealed ghosts made from chymotrypsinised red cells were incubated with $^{14}$C-$N_2$ph-F (175 $\mu$M, pH 7.0, 30 min) to achieve labelling of the 35-kDa and 17-kDa fragments. Subsequently, they were further dinitrophenylated with a large excess of non-radioactive $N_2$ph-F. When the ghosts are then exposed to $^{3}H_2$DIDS, the binding to both the 17-kDa and the 35-kDa fragment is prevented and no cross-links are formed. All lysine residues, including Lys b, that could serve as potential reactants with the isothiocyanate group of the $H_2$DIDS molecule are now occupied by $N_2$ph-F residues (fig.4d).

Since the inhibition of anion transport can be attributed to the dinitrophenylation of one single amino acid residue on the 17-kDa segment, and since essentially all of the $^{14}$C-$N_2$ph-F bound to that segment at nearly complete inhibition can be accounted for as $\alpha$-$N_2$ph-lysine residues, we believe that the inhibition is due to the modification of Lys a. To support this conclusion, we studied the effects of $H_2$DIDS on the formation of $\epsilon$-$^{14}$C-$N_2$ph-lysine on the 17-kDa fragment by hydrolysis of the 17-kDa peptide and subsequent amino acid analysis. Prior to isolation of the peptide, resealed ghosts had been treated either first with $^{14}$C-$N_2$ph-F (175 $\mu$M, pH 7.0, 30 min) or first with $H_2$DIDS (20 $\mu$M, pH 7.0, 30 min). Subsequently, the dinitrophenylated batch was treated with $H_2$DIDS and the $H_2$DIDS-treated batch with $^{14}$C-$N_2$ph-F. After haemolysis by saponin and exposure to trypsin, SDS polyacrylamide gel electropherograms (14% polyacrylamide) were run. The 17-kDa band was cut out, eluted, subjected to acid hydrolysis and the number of $\epsilon$-$^{14}$C-$N_2$ph-lysine residues were determined by ion-exchange chromatography. The recovery of the radioactivity was close to 100%.

Treatment with $H_2$DIDS caused about 80% reduction of the dinitrophenylation of $\epsilon$-$^{14}$C-lysine residues as determined by acid hydrolysis and amino acid analysis. Thus, the reduction of $N_2$ph-F binding to the 17-kDa fragment by treatment with $H_2$DIDS is indeed directly correlated to the reduction of the dinitrophenylation of lysine residues. Since the stoichiometry of the reaction of $H_2$DIDS and the band 3 molecules is 1:1, it follows that the binding of $N_2$ph-F to the 17-kDa fragment is largely confined to a single lysine residue. This residue is identical with the lysine residue a that is involved in the covalent reaction with one of the isothiocyanate groups of $H_2$DIDS, or is allosterically linked to it.

Inhibition of anion transport by dinitrophenylation is complete when all Lys a residues on the band 3 molecule are blocked and hence the capacity of band 3 to bind $^3H_2$DIDS covalently is reduced to zero (fig.1). Thus, titration with $^3H_2$DIDS enables one to determine the degree of dinitrophenylation of Lys a, regardless of the dinitrophenylation of additional amino acid residues on the band 3 protein [9]. This result does not preclude that the dinitrophenylation of the $\epsilon$-amino group of the easily modifiable lysine residue on the 35-kDa fragment affects the reactivity of the $\epsilon$-amino group of Lys a on the 17-kDa fragment, and vice versa.

Interactions between the lysine residues on the 2 fragments of band 3 can indeed be recognized if one analyzes the kinetics of the modification by $N_2$ph-F of $H_2$DIDS binding capacity and anion transport in more detail. The independent reaction with a single amino acid residue would follow first-order kinetics. One should expect, therefore, that the relationship between the modification of Lys a (and hence of the capacity of band 3 to bind $^3H_2$DIDS covalently) and the $N_2$ph-F concentration in the medium could be represented by a single exponential. Evaluation of the data in fig.1 by a suitable curve-fitting procedure or replotting them on a semilog scale (fig.5) shows, however, that the rate of reaction with Lys a decreases with increasing $N_2$ph-F concentration more than predicted by a single exponential. At least two exponentials are required to describe the clearly biphasic behaviour observed. At low concentrations of the $N_2$ph-F, a high probability exists for a $N_2$ph-F molecule to react with Lys a on a band 3 molecule in which the lysine residue on the 35-kDa fragment is not yet modified. This accounts for the rapid increase of inhibition when the $N_2$ph-F concentration is raised at the lower end of the concentration range covered. At the upper end of the $N_2$ph-F concentration range, the probability increases for a reaction with Lys a on band 3 molecules in which the $\epsilon$-lysine residue on the 35-kDa fragment has already been dinitrophenylated. Under these conditions, the susceptibility of Lys a on the 17-kDa fragment is reduced: a second exponential with a lower time constant is required to describe the deviations from the first exponential pertaining to the low concentration range. Fig.5 and a more detailed analysis of the kinetic aspects that will be presented elsewhere shows that the parameter values needed to fit the relationship between inhibition of transport and $N_2$ph-F concentration are slightly different from those needed to fit the relationship between modification of Lys a and $N_2$ph-F concentration. This gives rise to the slight deviation of the slope of the straight line relationship in fig.1, from the identity line plotted there, and indicates a slight activation of anion transport that is associated with the dinitrophenylation of the lysine residue on the 35-kDa fragment of band 3. It

should be recalled that this residue is not identical with Lys b, the lysine residue involved in the cross-link formation between the 35-kDa and 17-kDa fragment.

The relative reaction rates of $N_2$ph-F with the inhibitory (Lys a) and the activating lysine residue on the 17-kDa and 35-kDa fragment, respectively, can be varied by variations of pH and ion composition of the medium. This allows one to choose experimental conditions in which the two effects are more conspicuous than in the experiments described here. However, a presentation of this material would be outside the scope of this paper.

In summary, our data indicate that the inhibition of anion transport by dinitrophenylation of the red cell membrane is associated with the modification of 2 lysine residues in the band 3 protein. One of them resides in the 17-kDa segment, the other in the 35-kDa segment of that protein. The former is identical with Lys a (or allosterically linked to it), the lysine residue involved in the covalent attachment of one of the isothiocyanate groups of $H_2$DIDS. The latter is distinct from Lys b, to which the other isothiocyanate group of $H_2$DIDS becomes attached when the 17-kDa and 35-kDa segments are cross-linked. The two lysine residues that are susceptible to dinitrophenylation are allosterically coupled. Modification of the lysine residue in the 35-kDa segment reduces the rate of dinitrophenylation of the lysine residue in the 17-kDa segment (Lys a). The modification of the latter is responsible for the inhibition of transport. It will be shown elsewhere that the modification of the former not only reduces the susceptibility of Lys a to dinitrophenylation but also induces a slight enhancement of anion transport.

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