Biochimica et Biophysica Acta 856 (1986) 602–609
Elsevier

BBA 73063

Inhibition of phosphate transport in human erythrocytes by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)

James D. Craik, Kenseelan Gounden and Reinhart A.F. Reithmeier

Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7 (Canada)

(Received October 28th, 1985)

Key words: Phosphate transport; Band 3; Tyrosine modification; (Erythrocyte membrane)

Treatment of intact human erythrocytes with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) leads to inhibition of anion transport as measured by [^{32}P]phosphate exchange for intracellular chloride. Inhibition is rapid at 37°C (80% inhibition, 1.7 mM NBD-Cl, 3 min, pH 6.9) and not reversed by washing the cells with 1% bovine serum albumin in isotonic sucrose citrate buffer. Pretreatment of cells with *N*-ethylmaleimide and *p*-chloromercuribenzenesulfonic acid enhanced transport inhibition by NBD-Cl. Transport inhibition caused by brief incubations of erythrocytes with NBD-Cl could be almost completely reversed with dithiothreitol or β-mercaptoethanol. Prolonged incubation (60 min, 37°C, pH 6.4, sucrose-citrate buffer) following NBD-Cl treatment leads to partial reversal of transport inhibition. The residual inhibition is then only partially reversed by dithiothreitol treatment. Reversal of transport inhibition of dithiothreitol or β-mercaptoethanol may be prevented by incubation of the erythrocytes with sodium dithionite. Phosphate transport was readily inhibited by other tyrosine-directed reagents, tetranitromethane (55% inhibition, 1.6 mM, 3 min, 37°C, pH 8.3 in sucrose-citrate medium) and *p*-nitrobenzene sulfonyl fluoride (31% inhibition, 1.8 mM, 3 min, 37°C, pH 8.1 in sucrose-citrate medium) but not by *N*-acetylimidazole (10% inhibition, 37.5 mM, 30 min, 37°C, pH 7.5). These results suggest that NBD-Cl inhibits anion exchange by two mechanisms; a rapid inhibition reversible by sulfhydryl reagents, possibly due to modification of a tyrosine residue(s), and a slower irreversible inhibition due to modification of an essential amino group in the transporter.

Introduction

The Band 3 polypeptide, a major integral glycoprotein of the human erythrocyte membrane, mediates the rapid transmembrane exchange of anions necessary for efficient respiration [1,2]. Treatment of erythrocytes with a wide variety of chemical reagents indicates that lysine [3,4], arginine [5] and carboxylic acid [6,7] but not cysteine [8] residues are essential for anion transport. Transport inhibition by carbodiimides [6,7] could be caused in part by modification of tyrosine residues so the effect of tyrosine-selective reagents on anion exchange in intact human erythrocytes was studied. Preliminary experiments indicated that NBD-Cl was the most potent of the reagents examined.

Abbreviations: NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; O^{tyr}-NBD, *O*-tyrosyl reaction product of NBD-Cl.

Materials and Methods

Materials

[^{32}P]phosphoric acid, carrier free, from New England Nuclear; [U-^{14}C]NBD-Cl (109 mCi/mmol) from Research Products International Corp., Mount Prospect, IL. Unlabelled NBD-Cl from Pfaltz & Bauer Inc., Waterbury, CT; 4,4′-dinitrostilbene-3,3′-disulfonic acid (DNDS) and 2-

(3-trifluoromethyl)phenylamino-3-pyridine carboxylic acid (niflumic acid) from Aldrich Chemical Co., Milwaukee, WI. *N*-(4-Azido-2-nitrophenyl)-2-aminoethyl sulfonate (NAP-taurine), *p*-nitrobenzenesulfonyl fluoride from Pierce Chemical Co., Rockford, IL. *N*-Acetylimidazole, tetranitromethane, *p*-chloromercuribenzenesulfonic acid (PCMBS) and phenylmethylsulfonyl fluoride (PMSF) from Sigma Chemical Co., St. Louis. MO. Dithiothreitol from Bio-Rad Laboratories, Richmond, CA. 4-Benzamido-4′-aminostilbene-2,2′-disulfonate (BADS) was synthesized as previously described [9]. *N*-Ethylmaleimide from Fisher Scientific Co., Fair Lawn, NJ. ACS scintillation cocktail was from Amersham Corp., Arlington Heights, IL and Enhance fluorography cocktail from New England Nuclear. Human red blood cells were kindly provided by the Canadian Red Cross. All other reagents were of pure reagent grade from standard suppliers.

Methods

Preparation of erythrocytes. Human red blood cells were washed four times with 10 to 15 vols. 0.9% (w/v) NaCl to remove storage medium, contaminating white cells and platelets. If chloride-free medium was required, cells were further washed three or four times with 10 to 15 vols. buffer A (28.5 mM sodium citrate (pH 7.4)/205.3 mM sucrose).

Transport measurements. The exchange of extracellular phosphate for intracellular chloride was measured by following the loss of [^{32}P]phosphate from the assay medium at 30°C as described previously [7]. ^{32}P was determined by Cerenkov counting of 50 μl samples in 10 ml water in plastic scintillation vials using a Beckman LS7800 scintillation counter.

Inhibition of transport was estimated from the change in half time for the loss of radioactivity from the extracellular medium.

$$\text{Inhibition } \% = \left(1 - \left(T_{1/2} \text{ control}/T_{1/2} \text{ treated}\right)\right) \times 100$$

Preparation of red cell membranes. Red cell ghosts were prepared by the method of Dodge et al. [10] using buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% (v/v) dimethylsulfoxide. Ghosts were washed five times with this medium to remove haemoglobin. Alternatively erythrocytes were lysed and washed in 10 mM phosphate buffer (pH 4.4) to give pink ghosts.

Analytical techniques. Protein was determined by the method of Lowry et al. [11] except that all samples were assayed in the presence of 1% (w/v) sodium dodecyl sulfate. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [12] or by a modification of the method of Weber and Osborn [13]; tube gels were prepared by the addition of 8 ml acrylamide stock solution (25% w/v acrylamide, 0.96% bis) to 32 ml buffer (0.25 M phosphate (pH 7.4)/0.2% SDS/5% (w/v) sucrose). Polymerization was initiated by the addition of 60 μl 10% (w/v) ammonium persulfate and 30 μl TEMED. Protein samples were solubilized in sample buffer (2% SDS/10% sucrose/0.2% Bromophenol blue/0.2 M phosphate (pH 7.0)) at room temperature for 5 min before applying the sample to the tube gel. Gels were run at 8 to 10 mA/tube for 6 h. Radioactivity in gels was quantitated by slicing (2 mm slices or excision of individual bands) unstained gels. Gel slices were solubilized with 0.4 ml 30% hydrogen peroxide at 55°C for 16 h before scintillation counting in ACS scintillation cocktail. Quench correction was performed using an external standard method. Alternatively Laemmli slab gels were fixed and stained with Coomassie blue before treatment with Enhance fluorography cocktail for fluorography of the dried gel at −70°C (Kodak X-Omat X-ray film) or the gel dried without fixing for autoradiography at −70°C.

Results and Discussion

Inhibition of phosphate uptake by NBD-Cl

Treatment in intact erythrocytes with NBD-Cl (1.6 mM, 37°C, 3 min, pH 6.4 in sucrose citrate buffer at 50% haematocrit) gives 78% inhibition of phosphate transport (Fig. 1). Rapid washing of the cells twice with five volumes of ice-cold 1% bovine serum albumin in buffer A did not significantly affect transport inhibition. Transport inhibition of NBD-Cl was markedly reduced by lowering the temperature of the incubation medium. At 2°C, the inhibition of phosphate transport (3 mM NBD-Cl, buffer A, 2 min) was less than 12% while a parallel incubation at 37°C gave 87% inhibition.

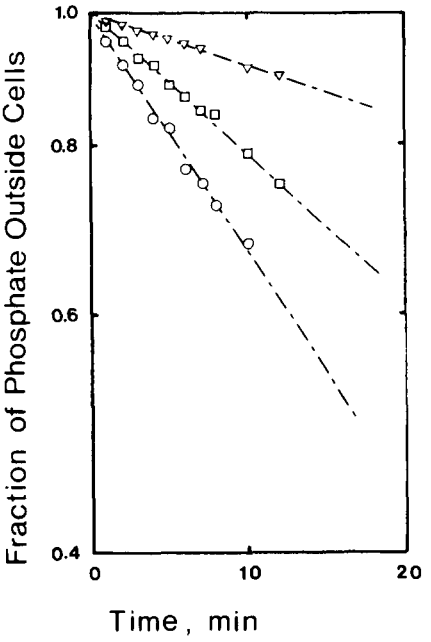

Fig. 1. Effect of addition of NBD-Cl on phosphate transport in human erythrocytes. Incubation for 3 min at 37°C. The suspension was cooled to 30°C for 1 min prior to transport assay at 50% haematocrit in sucrose-citrate buffer (pH 6.4). NBD-Cl was not removed before transport assay. ○, control; □, 0.3 mM NBD-Cl; ▽, 1.6 mM NBD-Cl.

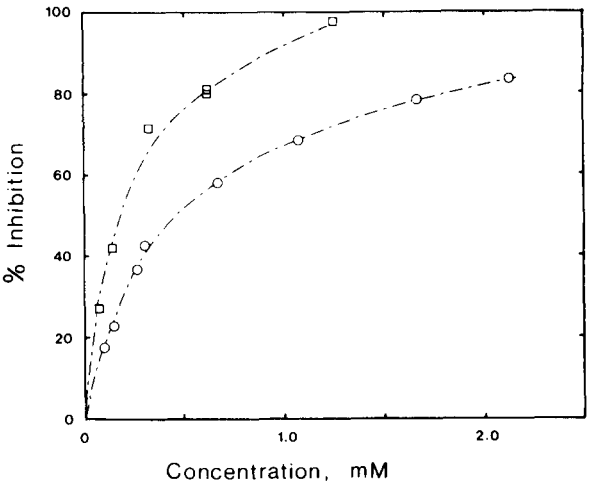

Fig. 2. Concentration dependence of the inhibition of phosphate transport by NBD-Cl. Incubation was for 3 min at 37°C, pH 6.4, 50% haematocrit, sucrose citrate medium. The cell suspension was cooled to 30°C for 1 min prior to transport assay. NBD-Cl was not removed before transport assay. ○, control red cells; □, red cells pretreated for 60 min at 37°C, pH 7.4, with 10 mM *N*-ethylmaleimide in 50 mM phosphate, 0.81% NaCl. Cells were washed five times with buffer A before NBD-Cl treatment.

No correction was made for inhibition by NBD-Cl which may remain associated with the cells through the washing procedures and react while the cells were equilibrated to 30°C for the transport assay. It is possible, therefore, that inhibition at 2°C has been overestimated. Bovine serum albumin was included to increase the efficiency of removal of NBD-Cl from erythrocytes in the washing procedure. Control experiments showed it to have only a minor effect. It is possible that NBD-Cl may partition into the membrane lipid bilayer or bind noncovalently to intracellular haemoglobin. Difficulties in ensuring complete removal of NBD-Cl by rapid washing methods prevented any accurate determination of the pH dependence of transport inhibition by this reagent. However, preliminary experiments showed inhibition following incubation at pH 5.5 was less than that following a parallel incubation at pH 6.4. Fig. 2 shows the concentration dependence of transport inhibition and the effect of pretreatment of the cells with a sulfhydryl modifying reagent, *N*-ethylmaleimide.

Transport inhibition by NBD-Cl in intact erythrocytes is extensive giving up to 80% inhibition in 3 min at 37°C, pH 6.4 and under these conditions 50% inhibition of transport is achieved with about 600 μM NBD-Cl. NBD-Cl is known to react readily with free sulfhydryl groups in polypeptides at neutral pH [14]. Pretreatment of intact red cells with *N*-ethylmaleimide under conditions which result in quantitative modification of five of the six cysteine residues in band 3 [15] markedly enhances the inhibition of transport by NBD-Cl (half maximal inhibition 200 μM NBD-Cl). Since treatment of intact human erythrocytes with *N*-ethylmaleimide enhances NBD-Cl inhibition of transport, the five sulfhydryl groups of band 3 located on the cytoplasmic side of the membrane cannot be the site of NBD-Cl inhibition. A sixth sulfhydryl located in the membrane spanning portion of the molecule [16] is unreactive to *N*-ethylmaleimide but can be modified by *p*-chloromercuribenzene sulfonate (PCMBS) [17]. Pretreatment of cells with 1 mM PCMBS, 1 mM PCMBS plus 10 mM *N*-ethylmaleimide, or 10 mM *N*-ethylmaleimide for 60 min at 37°C in phosphate-

saline buffer (pH 7.4), followed by incubation with 2.49 mM NBD-Cl for 3 min in sucrose-citrate medium at 37°C gave 83%, 81% and 74% inhibition of transport, respectively. Control cells which had not been pretreated with sulfhydryl reagents showed an inhibition of only 63% under these conditions. Inhibition by NBD-Cl is therefore not due to modification of the PCMBS sensitive sulfhydryl residue in band 3. The enhancement of inhibition following treatment with sulfhydryl reagents could be a result of an effective increase in the concentration of NBD-Cl since nonspecific reaction with intracellular sulfhydryl groups will be substantially reduced. NBD-Cl inhibition of phosphate transport is clearly not a result of a reaction with any sulfhydryl group on the band 3 polypeptide.

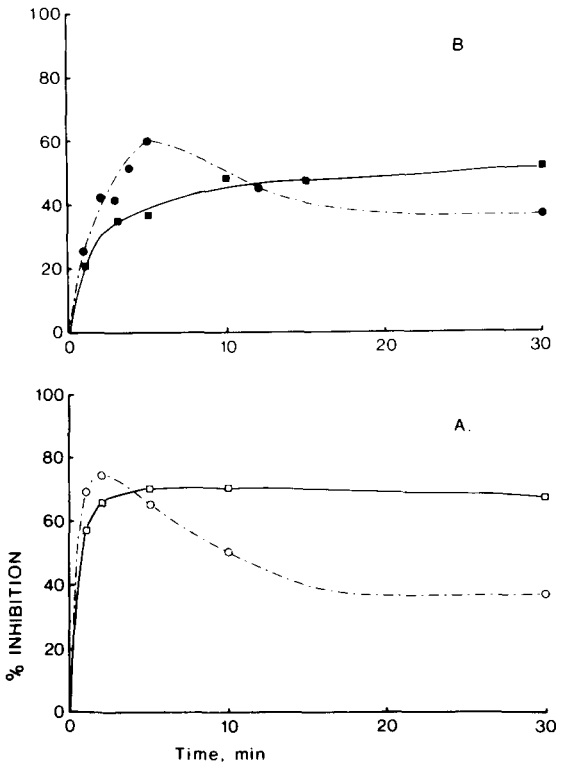

Fig. 3. Time-course of phosphate transport inhibition by NBD-Cl. (A) Control cells in buffer A at 50% haematocrit, 37°C. The cells were washed three times with 1% bovine serum albumin in ice-cold buffer A before transport assay at 30°C. ○, 3 mM NBD-Cl; □, 3 mM NBD-Cl with 6.8 mM sodium dithionite added after 1 min of incubation. (B) Cells were pretreated for 60 min at 37°C, pH 7.4 with 12 mM *N*-ethylmaleimide. Other experimental conditions were as in (A). ●, 312 μM NBD-Cl, ■, 304 μM NBD-Cl plus 9 mM sodium dithionite.

Fig. 3 shows the time-course of transport inhibition by NBD-Cl. Inhibition is rapid but partially reverses on prolonged incubation. This reversal may be prevented by the addition of sodium dithionite (maximal effect at concentrations of over 5 mM). Dithionite is a strong reducing agent which is readily transported by the band 3 anion transport system [18]. This reagent has been used to reduce an NBD-tyrosine adduct of F_1-ATPase to render it less labile during sequence determination of NBDCl-labelled peptides [19]. It has been suggested that the strongly nucleophilic nature of dithionite may promote migration of the NBD moiety and that ^{35}S from labelled dithionite can be quantitatively incorporated into the peptide during dithionite treatment of NBD-labelled protein [20]. In this study control experiments showed that the dithionite treatment itself does not significantly affect phosphate/chloride exchange transport but that it can prevent the reversal of NBD-Cl inhibition of transport resulting from prolonged incubation or treatment with dithiothreitol. Pretreatment of erythrocytes with 10 mM *N*-ethylmaleimide does not prevent these effects of dithionite.

Reversal of NBD-Cl inhibition of phosphate transport

Covalent modification of cysteine or tyrosine residues by NBD-Cl may be readily reversed by treatment with dithiothreitol or β-mercaptoethanol [21,22] while adducts formed with amino groups are not readily displaced [23]. Table I shows that after a brief treatment with NBD-Cl, transport inhibition may be almost completely reversed by dithiothreitol. Prolonged incubation of red cells following NBD-Cl treatment leads to a substantial recovery of transport activity, as shown in Fig. 3, but the remaining inhibition is no longer sensitive to dithiothreitol. In all these treatments the cells were washed free of unbound NBD-Cl before addition of the thiol reagent to prevent anomalous reaction between NBD-Cl and the low-molecular-weight thiols [24]. The results suggest that a rapid inhibition of transport following addition of NBD-Cl to intact erythrocytes at neutral pH is probably due to modification of a tyrosine re-

TABLE I

THE EFFECT OF PROLONGED INCUBATION ON THE REVERSAL OF NBD-Cl INHIBITION OF TRANSPORT BY DITHIOTHREITOL

Initial treatment [a]	Further treatment [b]	% Inhibition [c]
2.5 mM NBD-Cl, 2 min	Nil	84.7
2.5 mM NBD-Cl, 2 min	DTT [d]	4.2
2.5 mM NBD-Cl, 2 min	60 min, 37°C	32.8
2.5 mM NBD-Cl, 2 min	60 min, then DTT [d]	28.4

[a] After incubation with NBD-Cl, cells were washed with 1% bovine serum albumin (BSA) in sucrose-citrate buffer (ice-cold) then with ice-cold sucrose-citrate medium.

[b] Incubation in sucrose-citrate (pH 6.6).

[c] Inhibition expressed as a % of parallel controls from which NBD-Cl was omitted.

[d] 20 mM dithiothreitol in sucrose-citrate plus 1% BSA. Incubation for 30 min at 37°C followed by a further incubation with 10 mM dithiothreitol in the same medium for 5 min at 37°C.

sidue(s) and that after prolonged incubation a lysine residue is modified. It is also possible that after an initial reaction at a tyrosine residue the NBD moiety migrates to an adjacent amino group. The intramolecular migration of an NBD group from tyrosine to lysine has been examined in detail in purified mitochondrial F_1-ATPase [23,25,26]. Similarly, O^{tyr}-NBD-lysozyme has been shown to be an obligatory precursor for the modification of an essential amino group of lysozyme by NBD-Cl at neutral pH [27].

Effect of reversible inhibitors of anion transport on transport inhibition by NBD-Cl

4-Benzamido-4′-amino-stilbene-2,3′-disulfonate (BADS) is a reversible competitive inhibitor of anion exchange which binds with high affinity ($K_d = 1.5\ \mu M$) to the band 3 polypeptide [28]. 125 μM BADS gave no protection from NBD-Cl inhibition of phosphate transport. Similarly 235 μM 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) which also binds with high affinity to the stilbenedisulfonate-inhibitory site ($K_i = 2\ \mu M$ [29]), failed to protect phosphate transport from NBD-Cl inhibition.

NAP-taurine, a reversible noncompetitive inhibitor of anion transport (K_i 10 to 20 μM at the external face of the membrane) [30], also failed to give protection at a concentration of 114 μM. Niflumic acid, a reversible noncompetitive inhibitor of anion exchange ($K_i = 0.6\ \mu M$) whose site of action appears to interact with the stilbenedisulfonate-inhibitor site [31], did not give significant protection from NBD-Cl inhibition at a concentration of 45 μM.

It appears that NBD-Cl inhibits ion transport by interaction at a site(s) distinct from those of the inhibitors tested.

Treatment of erythrocytes with [U-^{14}C]NBD-Cl

Long incubation; red cells were pretreated with *N*-ethylmaleimide (12 mM, 37°C, 60 min, phosphate-saline buffer (pH 7.2)) prior to incubation with [U-^{14}C]NBD-Cl in buffer A. Cells were incubated for 60 min at 37°C with 300 μM NBD-Cl at 50% haematocrit while shielded from the light to minimize photolabelling. Transport measurements showed an inhibition of 40% in phosphate transport. SDS-PAGE (Laemmli methodology) showed an incorporation of radioactive label of 0.27 to 0.42 mole NBD per mole band 3 protein corresponding to a stoichiometry of about 0.7 to 1.0 for transport inhibition. [^{14}C]NBD-Cl, however, labelled most other red cell proteins, including haemoglobin, so its site of action with respect to membrane sidedness cannot be determined. Treatment of erythrocyte ghosts with trypsin according to the protocol of Jennings et al. [32] to generate the 55 kDa integral membrane domain of band 3 showed radioactive labelling of this region of the protein (Fig. 4). Treatment of intact erythrocytes with 1 mg/ml chymotrypsin for 30 min, 37°C prior to lysing the cells to prepare ghost membranes generates 59 kDa and 35 kDa fragments of the band 3 molecule [33]. Radioactive label was found to be associated with both fragments (Fig. 5). Scanning densitometry of the autoradiogram indicates that less than 26% of the band 3 label remains associated with the 59 kDa polypeptide after fragmentation of band 3.

Short incubation: Cells were pretreated as described for the long incubation and incubated with 300 μM NBD-Cl for 5 min at 37°C. Phosphate transport was assayed on a portion of the erythrocytes and the remainder were lysed in 5 mM phosphate buffer (pH 4.4). It was anticipated that lysis under mildly acidic conditions would

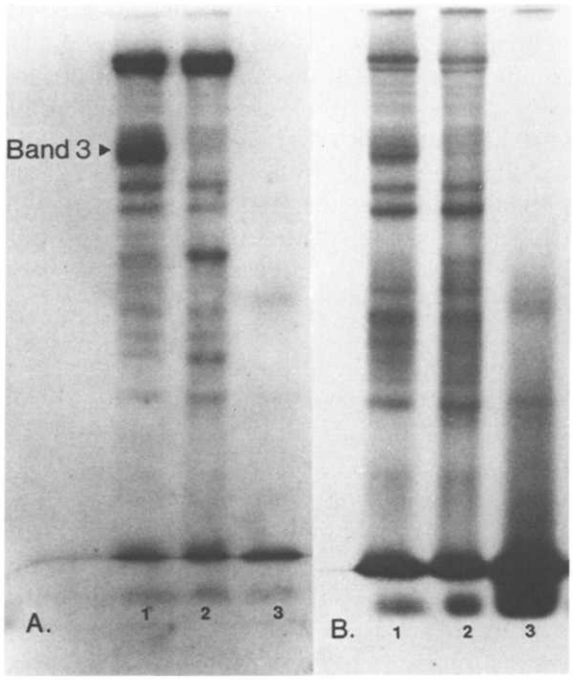

Fig. 4. Fluorography of [U-^{14}C]NBD-Cl-labelled red cell ghost membranes. Erythrocytes were pretreated with 12 mM *N*-ethylmaleimide (60 min, 37°C, pH 7.4) before incubation with 300 μM [^{14}C]NBD-Cl (60 min, 37°C, buffer A). Ghost membranes were prepared and run on SDS-PAGE (10% acrylamide, Laemmli buffer system, 40 μg protein/lane). (A) Coomassie blue-stained gel, (B) fluorogram. (1) Red cell ghost membranes; (2) ghost membranes prepared after chymotrypsin treatment (1 mg/ml, 30 min, 37°C) of intact cells; (3) red cell ghost membranes treated with trypsin using the protocol of Jennings et al. [32]. Some aggregated protein was present in the stacking gel (not shown).

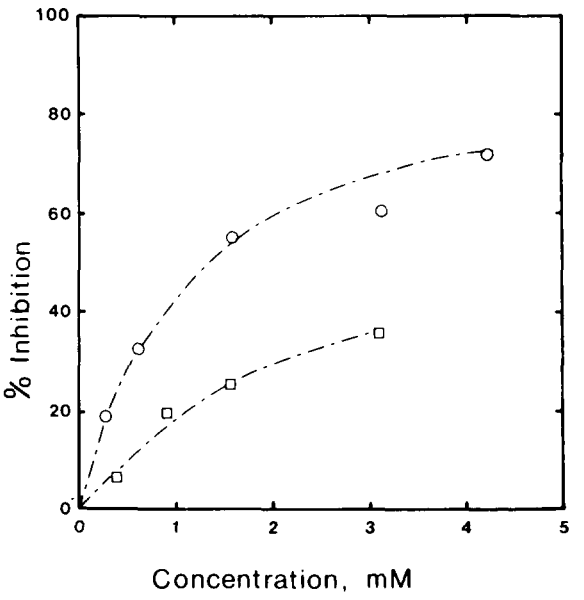

Fig. 5. Concentration dependence of inhibition of phosphate transport by tetranitromethane. Incubation for 3 min at 37°C in sucrose-citrate medium: ○, pH 8.3; ■, pH 5.5. Cells were washed three times with ice-cold 1% bovine serum albumin in buffer A before transport assay at 30°C. Cell lysis following incubation at pH 5.5 was estimated as 0.45% from haemoglobin release.

minimize the loss of NBD label. Approx. 2% of the cellular radioactive label was found associated with the washed erythrocyte ghost membranes. Most of the label was found in the cell lysate, associated with haemoglobin. The membrane proteins were solubilized in SDS sample buffer at room temperature for 5 min and separated by SDS-PAGE in phosphate medium (pH 7.4) as described in Materials and Methods. 0.043 μmole NBD per μmole band 3 were recovered by counting the gel while transport was inhibited 25%, corresponding to a stoichiometry of only 0.17 per band 3 for transport inhibition. Inclusion of 5 mM unlabelled NBD-Cl in the lysis medium did not increase the amount of radioactive label associated with the band 3 polypeptide and treatment of erythrocytes with sodium dithionite (14 mM) did not increase incorporation of NBD-Cl into erythrocyte ghosts. The NBD adduct appears unstable and does not survive the solubilization and electrophoresis procedures.

Effect of other tyrosine-selective reagents

Tetranitromethane (TNM) has been used to nitrate tyrosine residues in polypeptides [34]. In some proteins it appears to preferentially modify tyrosine residues buried in a hydrophobic environment [35]. Treatment of intact red cells with tetranitromethane at alkaline pH leads to a marked inhibition of transport (Fig. 5). Inhibition is substantially reduced at acid pH when nitration of tyrosine side chains will be largely suppressed. Part of the inhibition following incubation at pH 5.5 may result from subsequent reaction during equilibration of the cells to 30°C in sucrose-citrate medium (pH 6.9) for the transport assay.

p-Nitrobenzene sulfonyl fluoride (NBSF) is

known to be highly selective for the modification of tyrosine residues at neutral and mildly alkaline pH [36]. This reagent inhibits phosphate transport (31% inhibition, 1.8 mM NBSF, 37°C, pH 8.1, 3 min) indicating a role for tyrosine residues(s) in the anion transporter.

N-Acetylimidazole, a tyrosine methylating reagent [37] which inhibits sodium/potassium and calcium-stimulated ATPase in red cells [38], failed to give substantial irreversible inhibition of phosphate transport (10% inhibition, 37.5 mM *N*-acetylimidazole, 37°C, pH 7.5, 30 min) suggesting that this reagent does not have ready access to an essential tyrosine residue.

Conclusions

The results of this study show that anion exchange is readily inhibited by treatment of intact erythrocytes with some tyrosine-selective reagents. The temperature dependence and time-course of inhibition are consistent with covalent modification and inhibition is not readily reversed by extensive washing of the cells. Pretreatment of cells with sulfhydryl reagents clearly shows that transport inhibition by NBD-Cl is not caused by reaction of this agent with a sulfhydryl group on the band 3 polypeptide. Oxidative side reactions of tetranitromethane and NBD-Cl may also contribute to transport inhibition as a direct or indirect consequence of nonspecific chemical modification of the transport protein. The lack of protection by stilbenedisulfonates and NAP-taurine indicates that the site of action of NBD-Cl is distinct from these binding sites. The rapidity and efficiency of inhibition by NBD-Cl at slightly acidic and neutral pH where tyrosine reactivity is relatively low [27] could indicate that an essential tyrosine residue is subject to a special local environment or is in close proximity to a binding site with high affinity for NBD-Cl. The sensitivity of anion exchange towards tetranitromethane suggests that an essential tyrosine residue may be present in a hydrophobic environment at a site which does not favour stable modification by *N*-acetylimidazole. This study has not determined whether or not the different tyrosine selective reagents act at a common site. Amino acid analysis of the band 3 polypeptide [39] indicates the presence of 26 tyrosine residues per molecule of which 15 are found in the 55 kDa membrane-associated domain which mediates anion exchange [40]. The low amount of radioactive label associated with the band 3 protein following transport inhibition with radiolabelled NBD-Cl probably indicates that this reagent reacts with only a few of the tyrosine residues of the transporter under the experimental conditions used. The irreversible inhibition found on longer incubations is consistent both with the direct modification of an essential amino group or formation of an NBD-*O*-tyrosine adduct as a precursor and with nonspecific chemical modification of the transport protein. The lability of the radioactive labelling following short incubations with [^{14}C]NBD-Cl could be a result of changes in the band 3 protein during the preparation of ghost membranes. For example during membrane preparation two sulfhydryl groups in band 3 become cryptic, probably by forming a disulfide which is very resistant to subsequent reduction [15]. It is possible that this conformation change promotes the rapid displacement of NBD label from band 3. Displacement of label may also have occurred during membrane solubilisation and electrophoresis even though conditions similar to those used to identify an NBD-tyrosine adduct of (Na^+/K^+)-ATPase [22], were used. Treatment with sodium dithionite prevents rapid reversal of transport inhibition by dithiothreitol but it does not prevent a loss of label from band 3 during membrane isolation and SDS-PAGE. Although other chemical effects of the reagents examined may contribute to the inhibition of phosphate transport, the results in this paper strongly suggest that tyrosine must be added to the list [40] of residues considered essential for the anion transport function of the band 3 polypeptide.

Acknowledgements

The Medical Research Council of Canada, the Alberta Heritage Foundation for Medical Research and the Alberta Heart Foundation are thanked for generous financial support. R.A.F.R. is a Scholar of the Medical Research Council of Canada. J.D.C. holds an Alberta Foundation for

Medical Research Postdoctoral Fellowship. Dawn Oare is thanked for typing the manuscript.

References

1 Knauf, P.A. (1979) Curr. Top. Membranes Transp. 12, 249–363
2 Lowe, A.G. and Lambert, A. (1983) Biochim. Biophys. Acta 694, 353–374
3 Zaki, L., Fasold, H., Schuhmann, B. and Passow, M. (1975) J. Cell. Physiol. 86, 471–494
4 Jennings, M.L. (1982) J. Biol. Chem. 257, 7554–7559
5 Wieth, J.O., Bjerrum, P.J. and Borders, C.L. (1982) J. Gen. Physiol. 79, 283–312
6 Craik, J.D. and Reithmeier, R.A.F. (1984) Biochim. Biophys. Acta 778, 429–434
7 Craik, J.D. and Reithmeier, R.A.F. (1985) J. Biol. Chem. 260, 2404–2408
8 Knauf, P.A. and Rothstein, A. (1971) J. Gen. Physiol. 58, 190–210
9 Rao, A., Martin, P., Reithmeier, R.A.F. and Cantley, L.C. (1979) Biochemistry 18, 4505–4516
10 Dodge, J.T., Mitchell, C. and Hanahan, D.T. (1963) Arch. Biochem. Biophys. 100, 119–130
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
12 Laemmli, U.K. (1970) Nature 227, 680–685
13 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
14 Birkett, D.J., Price, N.C., Radda, G.K. and Salmon, A.G. (1970) FEBS Lett. 6, 346–348
15 Rao, A. (1979) J. Biol. Chem. 254, 3503–3511
16 Rao, A. and Reithmeier, R.A.F. (1979) J. Biol. Chem. 254, 6144–6150
17 Lukacovic, M.F., Toon, M.R. and Solomon, A.K. (1984) Biophys. Acta 772, 313–320
18 Salhany, J.M. and Rauenbuehler, P.B. (1983) J. Biol. Chem. 258, 245–249
19 Andrews, W.W., Hill, F.C. and Allison, W.S. (1984) J. Biol. Chem. 259, 8219–8225
20 Joshi, V. and Wang, J.H. (1985) Biophys. J. 47, 485a
21 Ferguson, S.J., Lloyd, W.J. and Radda, G.K. (1974) FEBS Lett. 38, 234–236
22 Cantley, L.C., Gelles, J. and Josephson, L. (1978) Biochemistry 17, 418–425
23 Ferguson, S.J., Lloyd, W.J. and Radda, G.K. (1975) Eur. J. Biochem. 54, 127–133
24 Nitta, K., Bratcher, S.C. and Kronman, M.J. (1979) Biochem. J. 177, 385–392
25 Andrews, W.W., Hill, F.C. and Allison, W.S. (1984) J. Biol. Chem. 259, 14378–14382
26 Sutton, R. and Ferguson, S.J. (1985) Eur. J. Biochem. 148, 551–554
27 Aboderin, A.A., Boedefeld, E. and Luisa, P.L. (1973) Biochim. Biophys. Acta 328, 20–30
28 Lieberman, D.M. and Reithmeier, R.A.F. (1983) Biochemistry 22, 4028–4033
29 Barzilay, M. and Cabantchik, Z.I. (1979) Membrane Biochem. 2, 255–281
30 Knauf, P.A., Ship, S., Breuer, W., McCulloch, L. and Rothstein, A. (1978) J. Gen. Physiol 72, 607–630
31 Cousin, J.L. and Motais, R. (1979) J. Membrane Biol. 46, 125–153
32 Jennings, M.L., Adams-Lackey, M. and Denny, G.H. (1984) J. Biol. Che,m. 259, 4652–4660
33 Grinstein, S., Ship, S. and Rothstein, A. (1978) Biochim. Biophys. Acta, 507, 294–304
34 Riordan, J.F. and Vallee, B.L. (1972) in Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N., eds.), Vol. 25B, pp. 515–521, Academic Press, New York
35 Myers, B. and Glazer, A.N. (1971) J. Biol. Chem. 246, 412–419
36 Liao, T.-H., Ting, R.S. and Yeung, J.E. (1982) J. Biol. Chem. 257, 5637–5644
37 Riordan, J.F. and Vallee, B.L. (1972) in Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N., eds.), Vol. 25B, pp. 500–506, Academic Press, New York
38 Masiak, S.J. and D'Angelo, G. (1975) Biochim. Biophys. Acta 382, 83–91
39 Steck, T.L., Koziarz, J.J., Singh, M.K., Reddy, G. and Kohler, H. (1978) Biochemistry 17, 1216–1222
40 Cacciola, S.O., Sigrist, H., Reist, M., Cabantchik, Z.I. and Zahler, P. (1984) J. Membrane Biol. 81, 139–147