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INHIBITION OF ANION TRANSPORT IN HUMAN RED BLOOD CELLS BY 5,5′-DITHIOBIS(2-NITROBENZOIC ACID)

REINHART A.F. REITHMEIER

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

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The uptake of [^{32}P]phosphate into human red blood cells was inhibited (K_i = 0.6 mM) by the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). 2-Nitro-5-thiobenzoic acid (NTB), the reduced form of DTNB, was a less potent inhibitor (K_i = 7 mM). The inhibition of anion transport by DTNB could be reversed by washing DTNB-treated cells with isotonic buffer, or by incubating DTNB-treated cells with 2-mercaptoethanol, which converted DTNB to NTB. DTNB competitively inhibited the binding of 4-[^{14}C]-benzamido-4′-aminostilbene-2,2′-disulfonate, a potent inhibitor of anion transport (K_i = 1–2 μM), to band 3 protein in cells and ghost membranes. These results suggest that the stilbene-disulfonate binding site in band 3 protein can readily accommodate the organic anion DTNB, and that inhibition by DTNB was not due to reaction with an essential sulfhydryl group.

Introduction

The band 3 protein of human erythrocyte membranes catalyzes the exchange of anions across the plasma membrane, a process necessary for respiration [1]. Stilbenedisulfonates are potent inhibitors of anion transport and have been shown to bind to a site on the band 3 polypeptide [1–8]. Band 3 protein contains five sulfhydryl groups that can be modified with *N*-ethylmaleimide without affecting anion transport [9]. A sixth sulfhydryl, located in a membrane-spanning portion of band 3 protein [10] can be modified with PCMBS [11]. Modification of red cells with PCMBS did not alter the rate of sulfate efflux from human erythrocytes [12]. In this paper, I report that another sulfhydryl reagent, DTNB, inhibits transport by binding reversibly to the stilbenedisulfonate site in band 3 protein.

Abbreviations: BADS, 4-benzamido-4′-aminostilbene-2,2′-disulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoic acid; PCMBS, *p*-chloromercuribenzenesulfonate.

Materials and Methods

Materials. Carrier-free [^{32}P]phosphate was obtained from New England Nuclear. DTNB was purchased from Pierce Chemical Co. [^{14}C]BADS (1300 cpm/nmol when counted in 10 ml Aquasol) was synthesized using [^{14}C]benzoyl chloride (Amersham, 11.5 mCi/mM) according to Kotaki et al. [13]. Red cell ghosts were prepared by the method of Dodge et al. [14] from outdated human blood, kindly provided by the Canadian Red Cross.

Transport [15]. Red blood cells were washed three times in 5 mM sodium phosphate, pH 7.4, 150 mM NaCl and twice in 205.3 mM sucrose, 28.5 mM sodium citrate, pH 7.4 at 4°C. Cells were suspended at a 50% hematocrit in sucrose-citrate buffer and various concentrations of DTNB were added. After incubation for 15 min at 30°C, [^{32}P]phosphate was added to a final concentration

of 10 mM. The uptake of radioactivity was measured at 30°C by centrifuging aliquots (200 μl) of the cell suspension in a microfuge and counting the radioactivity remaining in the supernant. No change in the pH of the media occurred during the transport assay.

[^{14}C]BADS binding [8]. Cells, at a 25% hematocrit in 205.3 mM sucrose, 28.5 mM sodium citrate, pH 7.4 were incubated at room temperature with various concentrations of [^{14}C]BADS after addition of various amounts of DTNB. The total concentration of BADS was corrected for the volume occupied by the cells. After a 15 min incubation, the suspension was centrifuged for 15 s in a microfuge and an aliquot of the supernatant was counted to determine the concentration of free BADS. BADS binding to ghost membranes was measured as follows. Ghosts at a protein concentration of 1 mg/ml in 28.5 mM sodium citrate, pH 7.4 were incubated at room temperature for 15 min. Aliquots of the suspension were counted to determine the total concentration of BADS. The free concentration of BADS was determined by counting an alquot of the supernatants obtained after removing the membranes by centrifugation at 100 000 × *g* for 15 min. Binding data are plotted according to Scatchard [16].

Assays. Protein was determined according to Lowry et al. [17] except that all samples contained 1% sodium dodecyl sulfate.

Results and Dicussion

Fig. 1 shows that DTNB at a concentration of 5 mM inhibited the uptake of [^{32}P]phosphate into human red blood cells. This inhibition was completely reversed by washing the DTNB-treated cells with isotonic buffer. Inhibition by DTNB was greatly reduced by direct addition of 1% 2-mercaptoethanol to DTNB-treated cells. 2-Mercaptoethanol alone had no effect on the uptake of inorganic phosphate. DTNB, when reduced to NTB by 2-mercaptoethanol [18] was a less potent inhibitor of anion transport. Cleavage of the DTNB in situ to 10 mM NTB reduced the inhibition of transport to the same value as direct addition NTB to 10 mM concentration. The fact that the inhibitory effect of DTNB could be completely reversed by washing the cells and that

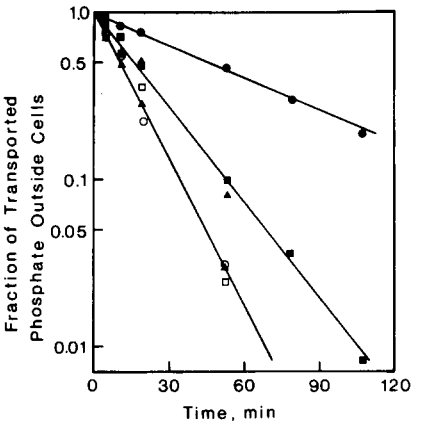

Fig. 1. Inhibition of [^{32}P]phosphate uptake into red blood cells by DTNB. (○) control cells; (●) cells in the presence of 5 mM DTNB. Cells pretreated with 5 mM DTNB were washed once with 20 vol. of sucrose-citrate buffer and resuspended in the same buffer to the original volume: (□) cells in the presence of 1% 2-mercaptoethanol; (■) cells in the presence of 5 mM DTNB and 1% 2-mercaptoethanol; (▲) cells in the presence of 10 mM NTB.

cleavage of DTNB by 2-mercaptoethanol reduced the inhibition but did not eliminate it, suggest that DTNB had not reacted covalently with an essential sulfhydryl in band 3 protein.

The effect of various concentrations of DTNB and NTB on anion transport is shown in Fig. 2. Half-maximal inhibition was obtained with 0.6

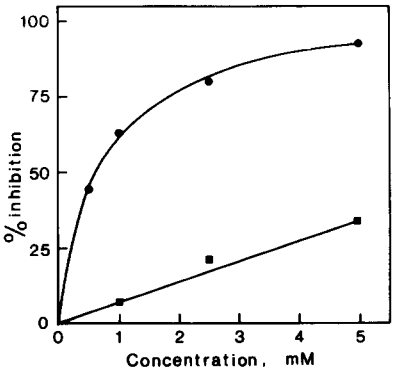

Fig. 2. Concentration dependence of the inhibition of transport as a function of the concentration of DTNB (●) and NTB (■). Cells at a 50% hematocrit were incubated at 30°C with the indicated concentrations of reagents for 15 min. [^{32}P]phosphate was added and the transport rates were detemined. The percent inhibition was calculated from the half-times of transport:

% inhibition = $(1 - t_{1/2}\ \text{control}/t_{1/2}\ \text{reagent}) \times 100\%$.

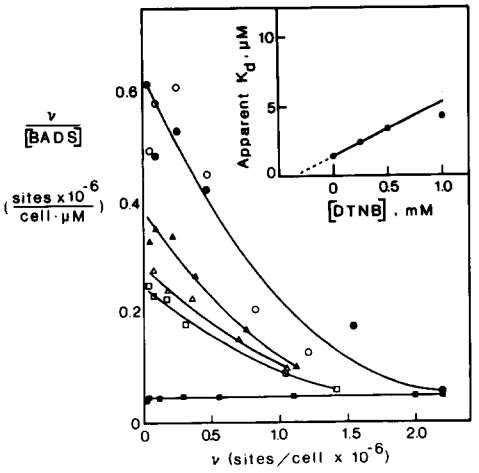

Fig. 3. Scatchard plot of the effect of DTNB on the binding of [^{14}C]BADS to cells. Cells at a 25% hematocrit in 205.3 mM sucrose, 28.5 mM sodium citrate, pH 7.4, were incubated with 0 mM (●), 0.25 mM (▲), 0.5 mM (△), 1.0 mM (□), 10 mM (■) DTNB. (○) cells pretreated for 15 min with 1 mM DTNB were washed with 20 volumes of sucrose-citrate buffer and resuspended to the original buffer in the same buffer. After 15 min at 25°C, the cells were removed by centrifugation and an aliquot of the supernatant was counted to determine the concentration of free BADS. The total concentration was determined by counting aliquots of the stock [^{14}C]BADS solution and correcting for the volume occupied by the cells. Inset: Effect of DTNB on the apparent BADS-binding constant. Binding constants were determined assuming $0.82 \cdot 10^6$ BADS-binding sites per cell, which was calculated from the BADS-binding data obtained in the absence of DTNB after subtraction of the non-saturable BADS-binding component. The intercept on the x-axis is the DTNB binding constant ($K_d = 0.35$ mM). The intercept on the y-axis at 0 DTNB is the binding constant for BADS ($K_d = 1.4$ μM).

mM DTNB while NTB at a similar concentration only inhibited transport by less than 5%. NTB was a less potent inhibitor with an estimated $K_i = 7$ mM. This suggests that the inhibitor site has a higher affinity for DTNB than for NTB. A similar sensitivity of phosphate uptake to DTNB was observed when [^{32}P]phosphate was added immediately after addition of DTNB.

The site of action of DTNB was examined by studying the effect of DTNB in the reversible binding of [^{14}C]BADS to erythrocytes and ghost membranes. Stilbenedisulfonates, such as BADS, inhibit anion transport in human red blood cells by binding to a single site on the band 3 protein [7,8,19–21]. [^{14}C]BADS inhibits anion transport ($K_i = 1$–2 μM) [19,20] by binding to band 3 protein with an affinity of 1–2 μM at physiological ionic strength, at a stoichiometry of one BADS per band 3 molecule (Lieberman and Reithmeier, unpublished data). This stilbenedisulfonate was used since it can be readily synthesized in radioactive form. Fig. 3 shows the effect of various concentrations of DTNB on the binding of [^{14}C]BADS to red blood cells. Scatchard plots of BADS binding to cells in the absence of DTNB were curved (Fig. 3, Lieberman and Reithmeier, unpublished results). The low-affinity portion of the curve which predominated at high BADS concentrations was due to non-specific (i.e., nonsaturable) binding of BADS to cells, perhaps due to binding to lipid and

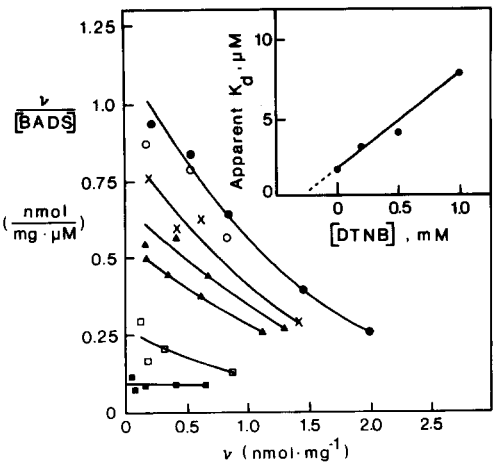

Fig. 4. Scatchard plot of the effect of DTNB on [^{14}C]BADS binding to ghost membranes. Membranes at a protein concentration of 1 mg/ml were incubated in 28.5 mM sodium citrate, pH 7.4 with 0 (●), 0.1 mM (×), 0.25 mM (▲), 0.5 mM (△), 1.0 mM (□), 10 mM (■) DTNB. (○) membranes pretreated for 15 min at 25°C with 1 mM DTNB were washed with 20 volumes of citrate buffer and resuspended to the original volume in the same buffer. After 15 min at 25°C, various concentrations of [^{14}]BADS were added. Aliquots of the suspension were counted to determine the total concentration of [^{14}C]BADS. After an additional 15 min at 25°C, the membranes were removed by centrifugation at 100000 × g for 15 min at 25°C. Aliquots of the supernatant were counted to determine the free concentration of [^{14}C]BADS. Inset: Effect of DTNB on the apparent BADS-binding constant. Binding constants were determined assuming BADS binding of 2.05 nmol per mg membrane protein (equivalent to $0.82 \cdot 10^6$ sites per cell). This value was determined in the absence of DTNB after subtraction of the non-saturable BADS-binding component. The intercept on the x-axis is the DTNB binding constant ($K_d = 0.35$ mM). The intercept on the y-axis at 0 DTNB is the binding constant for BADS ($K_d = 1.8$ mM).

is unrelated to the inhibition of transport. Equilibrium binding data indicated that there are two classes of 4,4′-dibenzamidostilbene-2,2′-disulfonate binding sites on red cell ghosts [7]. No evidence of negative cooperativity in BADS binding to band 3 protein was observed. Subtraction of the non-specific binding component revealed that there were approximately 10^6 high-affinity BADS-binding sites per cell, equivalent to the number of band 3 molecules. The binding constant ($K_d = 1.4$ μM) is equivalent to the affinity measured by inhibition of anion transport by BADS ($K_i = 1-2$ μM) (Lieberman and Reithmeier, unpublished results). As seen in Fig. 3, DTNB competitively inhibited the binding of BADS to red blood cells. In addition, washing DTNB-treated cells with isotonic buffer resulted in recovery of the high-affinity BADS-binding sites. The inset of Fig. 3 shows the change in apparent binding constant (K_d) of BADS as a function of DTNB concentration. The binding constant of BADS in the absence of DTNB was 1.4 μM. Extrapolation of the line revealed that the binding of DTNB to band 3 protein in cells was 0.35 mM, in good agreement with the affinity determined by inhibition of phosphate uptake (K_i = 0.6 mM, Fig 2).

Although the reversibility of the inhibition of transport and BADS binding by DTNB in cells suggested that DTNB is not reacting covalently with band 3 protein, it is possible that glutathione present inside the erythrocyte was cleaving the mixed disulfide. This was shown not to be the case, since the inhibition of BADS binding to band 3 protein in ghosts by DTNB (Fig. 4) was readily reversed by washing membranes with citrate buffer. DTNB also competitively inhibited the binding of BADS to ghost membranes. The binding constant for BADS was 1.8 μM and the binding constant for DTNB was 0.3 mM in agreement with the results obtained with intact cells.

The results in this paper show that DTNB inhibited anion transport in red blood cells by binding to the stilbenedisulfonate-binding site in band 3 protein. This is not surprising since DTNB has a structural resemblance to stilbenedisulfonates such as 4,4′-dinitrostilbene-2,2′-disulfonate. Indeed, reduction of the double bond has little effect on the potency of the stilbene [1]. Cleavage of DTNB to NTB reduced the potency of the inhibitor by 10–20-fold. A similar difference in potency was observed between stilbenedisulfonates and benzenesulfonates [19]. The fact that the inhibition of anion transport by DTNB can be readily reversed by washing cells or by direct addition of 2-mercaptoethanol makes DTNB a useful probe in the study of band 3 protein and anion transport.

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