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IDENTIFICATION OF THE Cl^- TRANSPORT SITE OF HUMAN RED BLOOD CELLS BY A KINETIC ANALYSIS OF THE INHIBITORY EFFECTS OF A CHEMICAL PROBE

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Summary

H_2DIDS, the dihydro analog of DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) can interact covalently with membrane sites, resulting in an irreversible inhibition of anion exchange. At low temperatures (0°C) and for relatively short times, however, its interaction is largely reversible, so that a kinetic analysis of the nature of its inhibitory effect on Cl^- self exchange can be performed. The effects of variations in the chloride concentration on the inhibitory potency of H_2DIDS are consistent with the concept that Cl^- and H_2DIDS compete for the transport site of the anion exchange system. The value of K_i for H_2DIDS is 0.046 μM, indicating that H_2DIDS has a higher affinity for the transport system than any other inhibitor so far examined. If, as seems probable, the covalent labelling of H_2DIDS occurs at the same site as the reversible binding, H_2DIDS can be used as a covalent label for the transport site. The specific localization of H_2DIDS in the band-3 protein thus indicates that this protein participates directly in anion exchange.

Introduction

Anion permeability in human red blood cells can be effectively blocked by DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) or by its dihydro derivative, H_2DIDS [1—5]. These two compounds bind almost exclusively to band 3 (nomenclature of Fairbanks et al. [6]), suggesting that this protein is involved in the transport process. Because these compounds form covalent, irreversible bonds, the nature of their inhibitory effects cannot be assessed by the usual

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; H_2DIDS, dihydro analog of DIDS; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

methods involving kinetic analysis. Thus, the precise role of the DIDS binding sites in anion transport remains obscure.

Recently it has been demonstrated by NMR techniques that DIDS reduces the capacity of band 3 to bind Cl^-, suggesting that the probe may react with Cl^- binding sites of this protein [7] and that the inhibition may be directly related to displacement of Cl^- from the transport system. Such evidence is not sufficient to identify the DIDS binding site, however, since the transport system contains at least two different Cl^- binding sites [8]. One is a transport site and the other a modifier site whose occupancy by anions results in a non-competitive inhibition of transport.

Previous evidence [1,4,5] suggests that the covalent reaction of disulfonic stilbenes is preceded by reversible combination at the same binding sites. This reversible interaction causes the same amount of inhibition as the irreversible binding. This study takes advantage of the fact that chloride fluxes can be measured under conditions (0°C and short times of exposure) where the irreversible binding of H_2DIDS is minimal [3,5]. The effects of variations in chloride concentration on the reversible inhibition of Cl^- exchange by H_2DIDS are consistent with the concept that H_2DIDS competes with Cl^- for binding to the chloride transport site.

Materials and Methods

Human blood cells were washed and loaded with different concentrations of chloride using the nystatin technique [9] as described by Dalmark [10], except that 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer was added to the final wash solutions. The wash solutions, which were also used for the determination of chloride fluxes, contained 1 mM NaCl, 27 mM sucrose, 5 mM HEPES, and 19—600 mM KCl as indicated. The pH was 7.2 at 0°C.

For measurement of chloride fluxes, cells were incubated at 50% hematocrit for 5 min at 0—4°C in a medium of the same composition as the final wash solution, except containing ^{36}Cl. The cell suspensions were transferred to small polyethylene tubes and centrifuged for 1 min in a Beckman Microfuge. After removal of the supernatant, the bottom of the tube was cut off with a scalpel and a syringe containing about 5 ml of the efflux medium (at 0°C) was attached to the top of the tube. At zero time, the cells were flushed into the efflux medium by depressing the syringe. The final hematocrit during the efflux was 0.03%. Samples of the supernatant were taken at various times by the method of Dalmark and Wieth [11]. Duplicate samples of the suspension were also taken. The logarithm of $(P_\infty - P_t)/P_\infty$ (where P_∞ represents the counts per min of ^{36}Cl per ml of suspension and P_t represents the counts per min of ^{36}Cl per ml of supernatant at time t), was plotted against time.

The rate constant for the efflux, k, was determined from the slope of the line which best fitted the data, as determined by the method of least squares. The fractional inhibition, i, was calculated from the observed values of k with and without H_2DIDS [12], which was either present in the medium at zero time or was added at a later time as indicated. All fluxes were carried out at 0°C and pH 7.2.

For experiments with bicarbonate, cells were prepared with nystatin as described above in a medium containing 550 mM KCl. Half of the cells were then incubated for 30 min at 0—4°C in a similar medium containing 400 mM HCO_3^- (in place of an equivalent amount of chloride) which had been bubbled with 95% CO_2. The cells were loaded with ^{36}Cl and fluxes were measured as described above, except that the efflux medium was gassed with CO_2 for the bicarbonate fluxes. After addition of cells to the medium, five samples were taken over a period of 60—90 s to establish a control flux value, then H_2DIDS was added and five more samples were taken. Similar experiments were run with the other half of the cells in chloride medium, and the effects of H_2DIDS in the two circumstances were compared.

H_2DIDS, synthesized by the procedure of Lepke et al. [5], was supplied by Dr. S. Ship of this laboratory. All other chemicals were reagent grade.

Results and Discussion

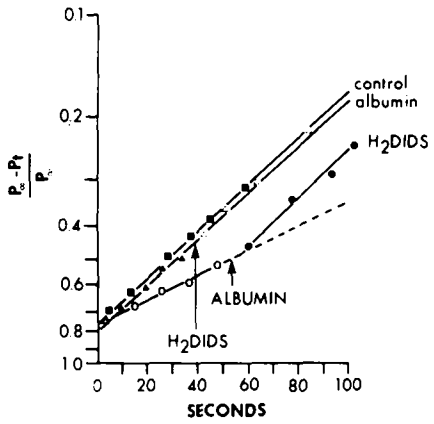
The reversibility of the inhibitory effect of H_2DIDS is illustrated in Fig. 1. Because albumin readily binds disulfonic stilbenes [1], it would be expected that after addition of a large excess of albumin, almost all of the H_2DIDS reversibly bound to the cells, as well as the free H_2DIDS in solution, would be sequestered by albumin. As expected, albumin added prior to H_2DIDS completely prevented inhibition of chloride transport. When albumin was added 53 s after the cells had been exposed to H_2DIDS, the inhibitory effect was completely reversed within a few seconds *. Albumin itself had no significant effect on chloride exchange. Thus, under the conditions of the present experiments, the inhibitory effects of H_2DIDS were completely reversible.

In the experiment of Fig. 1, inhibition by H_2DIDS was fully evident at the time of the first sample (about 2 s) and did not increase with time of exposure to the agent. In nine similar experiments, the points for times less than 4 s did not deviate significantly ($P > 0.1$) from the line. The reversal was similarly rapid. Thus, the measured inhibitions can be considered to reflect an equilibrium between free H_2DIDS and H_2DIDS bound reversibly to the inhibitory sites.

From Fig. 2 it can be seen that inhibition asymptotically approaches 100% with increasing H_2DIDS concentration. Thus no significant H_2DIDS insensitive Cl^- flux is evident. Inhibition was half-maximal at 0.3 μM. This value is much lower than that observed in previous studies [1,3,5,13], where much higher cell concentrations were used so that most of the reagent was bound by the cells and the degree of inhibition was dependent on the ratio of the amount of reagent to the number of cells [13]. Under the conditions of the present experiments, a 4-fold variation in the cell concentration (0.015—0.06% hematocrit) did not significantly affect the inhibition. Thus these data reflect the apparent affinity of H_2DIDS for the inhibitory site, rather than depletion of reagent by the cells.

Modulation of the Cl^- concentration by the nystatin technique [9], such

* Hemoglobin at a final concentration of 2 mg/ml at 0°C was ineffective in reversing the effects of H_2DIDS. Thus its binding affinity for the probe must be low. Furthermore, small amounts of hemolysis should not influence the inhibitory effects.



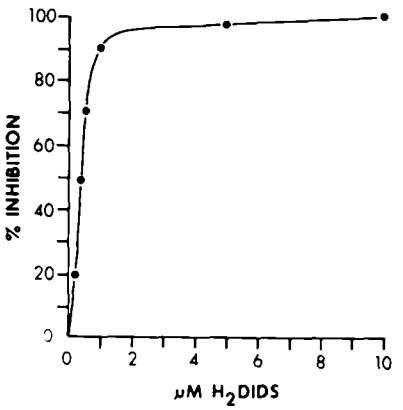


Fig. 1. Reversal of H_2DIDS inhibition of Cl^- self-exchange. Fluxes were carried out as described in Materials and Methods in a medium containing 300 mM Cl^-. The composition of the medium prior to the start of the flux is indicated to the right of each line. Additions to the medium during the course of the flux are indicated by arrows. ■, control; ▲, 0.05% bovine serum albumin added before start of flux; △, 0.75 μM H_2DIDS added after 40 s; ○, 0.5 μM H_2DIDS present in the medium; ●, albumin (final concentration 0.05%) added after 53 s.

Fig. 2. Effect of H_2DIDS on Cl^- self-exchange. Fluxes were measured at 0°C in a medium containing 150 mM Cl^-. Inhibition was calculated by comparison with fluxes measured in the same medium without H_2DIDS. Each point represents the average of dubplicate fluxes determined from at least four time points.

that the inside and outside chloride concentrations were nearly equal and the membrane potential was close to zero, had a pronounced effect on the inhibition of chloride exchange by H_2DIDS (Fig. 3). For example, at 0.5 μM H_2DIDS the inhibitions for 150 300 and 600 mM Cl^- were 64, 42 and 28%, respectively. This finding suggests an interaction of the inhibitor with a Cl^- binding site. The presence of two chloride binding sites in the anion-exchange system, however, one the transport site and the other a modifier site at which halides cause a non-competitive inhibition of transport [8], makes it more difficult to determine the mechanism of action of the inhibitor in this case.

In such cases where there is substrate inhibition, many of the classical methods for plotting kinetic data fail to yield straight lines or are difficult to interpret [14—16]. Straight lines can be obtained, however, by plotting $1/i$ (where i is the fractional inhibition) versus $1/I$ (where I is the concentration of the inhibitor, H_2DIDS). In this double reciprocal plot (Fig. 4), the slope of the line represents the concentration of inhibitor required to cause 50% inhibition, the apparent K_i. For each chloride concentration, the data fit straight lines, indicating that H_2DIDS acts at a single site within the concentration range studied. The increase in apparent K_i with increasing Cl^- concentration suggests that Cl^- and H_2DIDS compete for a common binding site.

The site can be identified in terms of its affinity for chloride by use of the Hunter-Downs plot, in which $I(1-i)/i$, a measure of the apparent K_i, is plotted against the chloride concentration. As discussed elsewhere (Knauf, P., Ship, S., Breuer, W., McCulloch, L. and Rothstein, A., unpublished), for models in which the inhibitor acts at the substrate or modifier site, this plot yields a

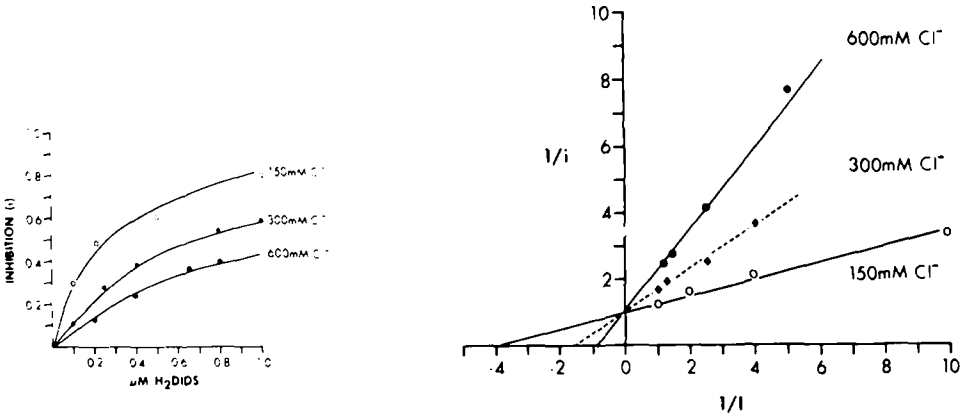


Fig. 3. Effect of Cl^- concentration on the inhibition of Cl^- self-exchange by H_2DIDS. Fluxes were measured at 0°C and inhibition was calculated as described in Fig. 2, except that the medium contained different Cl^- concentrations as indicated.

Fig. 4. Double reciprocal plot of fractional inhibition versus inhibitor concentration. The data are taken from Fig. 3 (points with 10% or less inhibition were not used). The lines were determined by the method of least squares.

straight line in which the x-intercept is equal to the negative of the dissociation constant for chloride at the site where it competes with H_2DIDS. The experimental data (Fig. 5) can be fitted by the method of least squares to a straight line with an x-intercept of −62 mM (90% confidence interval −104 to −26 mM) *. This value corresponds very closely with the reported affinity of chloride for the transport site, K_s, which is estimated at 65 [18] or 67 [8] mM. It is significantly different ($P < 0.001$) from the value of −335 mM [8] which would be predicted on the basis that H_2DIDS binds to the modifier site. The simplest interpretation consistent with the data, therefore, is that H_2DIDS competes with Cl^- for the transport site.

The y-intercept of the line in Fig. 5 is 0.092 μM (90% confidence interval 0.042—0.143), the apparent K_i at zero chloride concentration. For a mobile carrier system, only half of the transport sites are exposed to H_2DIDS at the external side of the membrane [19]. Thus, the true K_i, which represents the dissociation constant for the interaction of H_2DIDS with the transport site, is actually half of the y-intercept value (Knauf, P. et al., unpublished), or 0.046 μM. Thus H_2DIDS has a higher affinity for the anion exchange system that any other inhibitor which has so far been examined.

It can be noted from Fig. 5 that a clear distinction of the nature of the inhibition (competitive or non-competitive) and a quantitative assessment of the values for the constants is facilitated by the use of high substrate concentra-

* Statistical analysis was performed as follows: The confidence interval for the y-intercept was calculated by standard techniques [17] based on the assumption that the error in y was random. The confidence interval for the x-intercept was determined by finding the points at which the lines describing the upper and lower bounds of the confidence interval for y intersect the x-axis. The 99.9% confidence interval extended from −167 to 11 mM. Thus, the x-intercept was significantly different at the $P < 0.001$ level from the value of −335 mM expected if H_2DIDS were acting at the modifier site.

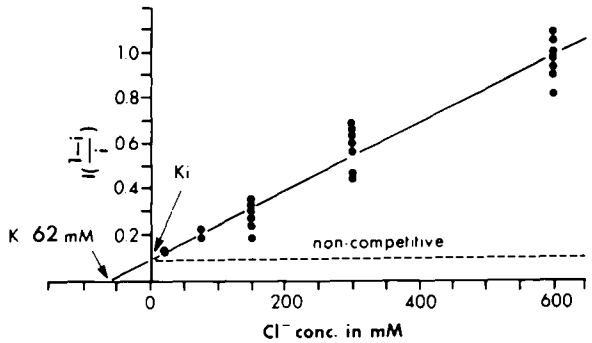


Fig. 5. Hunter and Downs plot. The data were obtained from experiments in which either the Cl^- concentration or the H_2DIDS concentration was varied, with the other parameter held constant. Fluxes and inhibitions were determined as described in Fig. 2. The line which best fit the data was determined by the method of least squares. The broken line represents the theoretical line in the case where H_2DIDS and Cl^- do not compete.

tions, since the substrate affinity is rather low (K_s = 65—67 mM). At low substrate concentrations the effects on the degree of inhibition would not be adequate for kinetic analysis unless the data were unusually precise.

Dalmark [8] has shown that the transport and modifier sites differ in their relative affinities for chloride and bicarbonate. The transport site has a higher affinity for bicarbonate than for chloride, while the reverse is true for the modifier site. Therefore attempts were made to measure the effects of substitution of bicarbonate for chloride on the inhibition by H_2DIDS. Difficulties in maintaining constant pH during the flux measurements preclude a quantitative treatment of the results, but in each of three experiments the inhibition was reduced (7—20%) by substitution of bicarbonate for chloride. These results are consistent with the concept that H_2DIDS binds to the transport site.

The experiments described above were all done under conditions in which the binding of H_2DIDS is reversible. At higher temperatures and after longer times of exposure, the H_2DIDS becomes covalently bound [3—5]. All of the available data are consistent with the hypothesis that the site of covalent binding and the site of reversible binding are the same [4,5]. It seems, therefore, that labelled H_2DIDS can serve as a marker for the transport site. Its specific localization in band 3 [1,2], with a linear relationship between binding and inhibition [3,5], thus indicates that the band 3 protein is directly involved in anion transport. Ultimately, as band 3 is dissected and sequenced, labelled H_2DIDS can be used to identify the anion transport site within the protein structure.

Acknowledgement

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References

1 Cabantchik, Z.I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311—330
2 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membrane Biol. 15, 207—226

3 Ship, S., Shami, Y., Breuer, W. and Rothstein, A. (1977) J. Membrane Biol. 33, 311—323
4 Passow, H., Fasold, H., Zaki, L., Schuhmann, B. and Lepke, S. (1974/75) in Biomembranes, Structure and Function (Gardos, G. and Szasz, I., eds.), pp. 197—214, Proc. 9th FEBS meeting, Budapest
5 Lepke, S., Fasold, H., Pring, M. and Passow, H. (1976) J. Membrane Biol. 29, 147—177
6 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
7 Shami, Y., Carver, J., Ship, S. and Rothstein, A. (1977) Biochem. Biophys. Res. Commun. 76, 429—436
8 Dalmark, M. (1976) J. Gen. Physiol. 67, 223—234
9 Cass, A. and Dalmark, M. (1973) Nat. New Biol. 244, 47—49
10 Dalmark, M. (1975) J. Physiol. Lond. 250, 65—84
11 Dalmark, M. and Wieth, J.O. (1972) J. Physiol. Lond. 224, 583—610
12 Knauf, P.A. and Rothstein, A. (1971) J. Gen. Physiol. 58, 190—210
13 Halestrap, A. (1976) Biochem. J. 156, 193—207
14 Webb, J. (1963) Enzyme and Metabolic Inhibitors, Vol. 1, pp. 111—139, Academic Press, New York
15 Segel, I.H. (1975) Enzyme Kinetics, pp. 818—826, A. Wiley Interscience Publ., New York
16 Cleland, W.W. (1967) Annu. Rev. Biochem. 36, 77—112
17 Schefler, W.C. (1969) Statistics for Biological Sciences pp. 150—157, Addison-Wesley Publ. Co. Reading, Mass.
18 Brazy, P. and Gunn, R. (1976) J. Gen. Physiol. 68, 583—599
19 Dalmark, M. (1975) J. Physiol. Lond. 250, 39—64