INHIBITION OF Cl^- BINDING TO ANION TRANSPORT PROTEIN OF THE RED BLOOD CELL BY DIDS (4,4'-DIISOTHIOCYANO-2,2'-STILBENE DISULFONIC ACID) MEASURED BY [^{35}Cl]NMR

Y. Shami, J. Carver, S. Ship and A. Rothstein

Research Institute, The Hospital for Sick Children, and the Department of Cell Biology, University of Toronto (J.C.), Toronto, Ontario

Received March 22,1977

SUMMARY: The width (at half height) of the NMR spectral peak of [^{35}Cl] was increased significantly in the presence of either red blood cell ghosts or a Triton X-100 extract of the ghosts, indicating chloride binding in both preparations. The specific widening was a linear function of protein concentration of the medium, amounting to 4.2 Hz/mg of ghost protein and 6 Hz/mg of Triton X-100 extracted protein. If cells were treated with the irreversible anion transport inhibitor, DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonic acid), prior to the preparation of the ghosts, the specific widening of the NMR peak was diminished by 32% for ghost protein and 50% for the Triton X-100 extract. The effect of the inhibitor on the widening was largely limited to the pH range 6.5 - 8.5. The data suggest that the effectiveness of DIDS as an inhibitor may be related to its capacity to displace Cl^- from anion binding sites in band 3.

INTRODUCTION: Anion permeability in the human red blood cell is associated with an intrinsic protein of apparent molecular weight of 95,000 (1,2,3,4,5) usually called band 3 (nomenclature of Fairbanks et al.)(6). Although kinetic data suggest that chloride binding to membrane sites precedes its translocation (7,8,9), a demonstration of such binding to membrane proteins by conventional methods is not feasible because the apparent affinity of the transport site for Cl^- is low(K_m = 67 mM) (8,9). The difficulty can, however, be overcome by using [^{35}Cl]NMR, because the chloride relaxation times are affected by the presence of even low affinity anion binding sites in proteins from a wide variety of sources (10,11,12). Using this technique, we have measured the binding of Cl^- to red cell ghosts, to a Triton X-100 extract from the ghosts which is enriched in band 3 protein and the red cell sialoglycoproteins (13), and to purified sialoglycoprotein and phospholipids. Because of the potentially large number of non specific binding sites for chloride, we used the specific irreversible inhibitor of anion permability, (DIDS)(1,2) to distinguish the particular sites which might be involved in Cl^- transport.

ISSN 0006-291X

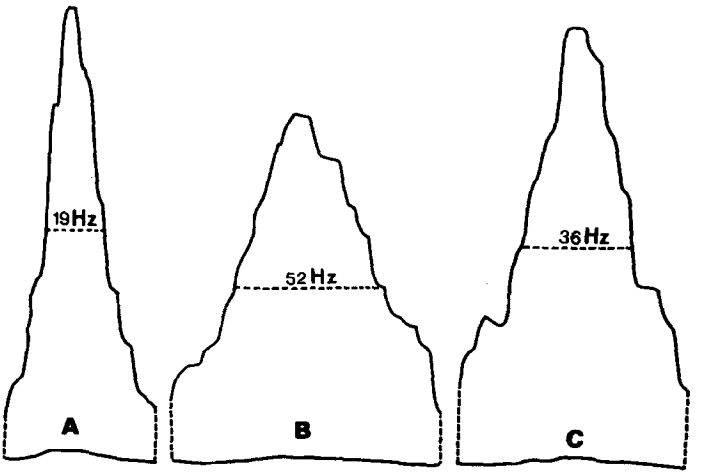

Figure 1. The line width at half height of [^{35}Cl] resonance in (A) 250 mM KCl, (B) 250 mM KCl plus Triton X-100 extract of red blood cell ghosts (5 mg protein per ml), (C) 250 mM KCl plus Triton X-100 extract of DIDS (10 μM) treated red blood cell ghosts (5 mg protein per ml).

MATERIALS AND METHODS: Human red blood cell ghosts were prepared by the method of Dodge et al.(14) and were extracted with 1% Triton X-100 (Rohm and Haas, Canada) at ionic strength, 40 mM by the procedure of Yu et al.(13). The fraction extracted by this method is enriched in band 3 and the major glycoproteins and also contains phospholipids. The extract was then concentrated eight to ten fold with a Diaflo XM100A filter to final concentration of 5.0 to 10 mg protein/ml under 15 psi N_2, the chloride concentration was adjusted by the addition of KCl, and pH was adjusted by the addition of either HCl or NaOH. To evaluate the Cl^--binding of major constituents other than band 3, the Triton extract was passed through an organomercurial sepharose column (15). Some of the sialoglycoproteins and all of the lipid components in the extract were eluted from the column but all of the band 3 was retained (15). The eluate was tested for its broadening effect on the [^{35}Cl]NMR line width. In addition, the glycoprotein components were separately purified (16) and tested. Except in the experiment of Fig. 3, the pH was 7.2. The treatment of red blood cells with 10μM DIDS (4,4-isothiocyano-2,2'-stilbene disulfonic acid) was carried out as described previously (2). Protein was determined by the method of Lowry et al.(17) and sialic acid by the method of Aminoff(18). Samples were kept at 4°C except during NMR readings which were carried out at 23°C. Pulsed Fourier transform NMR spectra of chloride were obtained on a Bruker BKR-322 variable frequency pulsed spectrometer. For a field equivalent to proton resonance at 60 MHz, [^{35}Cl] resonated at 5.88 MHz. Chloride NMR line widths were measured at half height of the spectral peak.

RESULTS: Typical [^{35}Cl]NMR spectra are shown in Fig. 1. The line width at half height for 250 mM KCl was 19 Hz ± 0.6 S.E.(Fig. 1A), a value that was used as a blank for the calculation of line width broadening. In the presence of Triton X-100 extracts of human red blood cell ghosts (5 mg protein per ml),

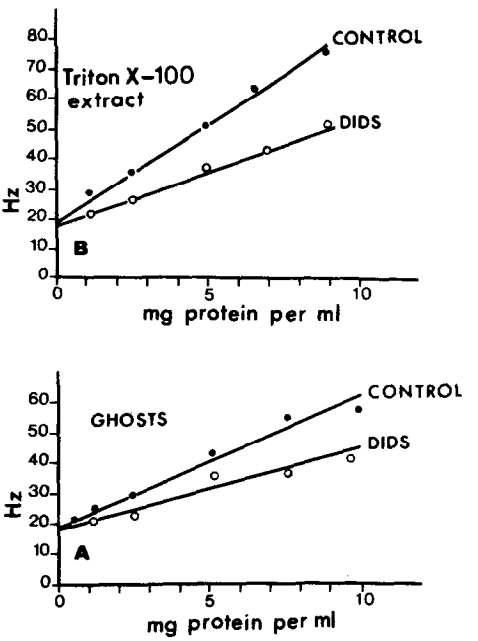

Figure 2. Variation in [^{35}Cl] (250 mM KCl) NMR line width as a function of protein concentration. (A) Ghosts (•) control; (o) from DIDS treated cells, (B) Triton X-100 extract of red blood cell ghosts (•) control; (o) from DIDS treated cells.

the line width was considerably broadened, to 52 Hz (Fig. 1B), but with similar extracts from cells treated with DIDS (also containing 5 mg protein per ml), the broadening was substantially less. The line width was only 36 Hz (Fig. 1C).

The [^{35}Cl] NMR line width increased in the presence of red cell ghosts, as well as Triton X-100 extracted proteins (Fig. 2). In either case the increase was linearly related to the amount of protein added, amounting to 4.2 Hz per mg of ghost protein and 6.0 Hz per mg of Triton X-100 extracted protein. If the cells were pretreated with DIDS, the specific widening decreased in each case. In the ghosts, the decrease was 32%, from 4.2 to 2.7 Hz per mg of protein (Fig. 2A), and in the Triton X-100 extract, the decrease was 50% from 6.0 to 3.1 Hz per mg of protein (Fig. 2B). The difference in slopes of the regression lines with and without DIDS in both cases is significant at the $p < 0.01$ level.

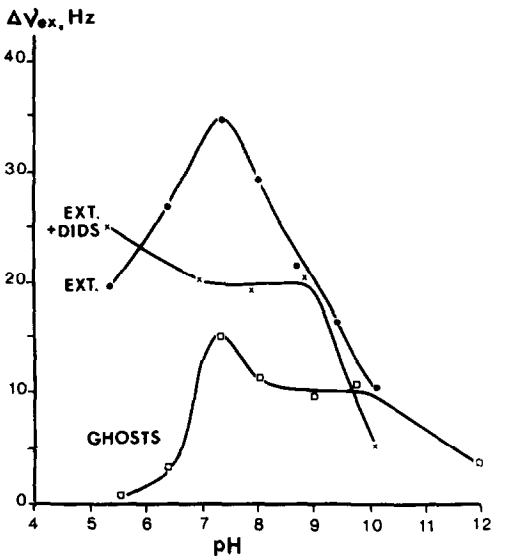

Figure 3. The excess [^{35}Cl] (250 mM KCl) NMR line width as a function of pH. (□) ghost protein; (●) Triton X-100 extract of red blood cell ghosts; (x) extract from DIDS treated cells. The protein concentration was 5 mg/ml in all cases.

The line broadening for either the ghosts or Triton X-100 extracts was maximal at pH 7.2 (Fig.3). Except at high pH values, the specific broadening per mg of protein was substantially higher for the Triton X-100 extracts than for the ghosts. The differential was especially large at low pH because the ghosts had almost no influence on the NMR spectra below pH 6. In extracts from DIDS-treated cells, the peak at pH 7.2 was eliminated, but the effects of DIDS at low or high values of pH were minimal. Exposure to ghosts or extracts to pH values ranging from 3 to 10 for up to 3 hours resulted in no irreversible effects in terms of their Cl^- binding measured by NMR.

To assess the possible effects of major components in the Triton X-100 extracts other than band 3 on the line width, several controls were carried out. Triton X-100 (1%) itself had no effect on [^{35}Cl]NMR spectra measured at pH 7.2 in the presence or absence of protein (Bovine serum albumin). The extract contains phospholipids (20 µg per mg of protein) and

protein components, largely band 3 (about 80%) and sialoglycoproteins (about 15%) (4,13). An eluate of the Triton-extract (prepared by passage through an organic mercury column - see methods) containing only the phospholipids and some sialoglycoproteins (15) did not significantly increase the [^{35}Cl] NMR line width (values were only 1 to 2 Hz above the control level). The same result was obtained using purified sialoglycoproteins in concentrations equivalent to those present in the Triton extracts. By elimination, therefore, band 3 is the only major component in the extract that could contribute to the broadening of the [^{35}Cl]NMR signal. Minor components (amounting to about 5% of the protein in the extract) might also contribute.

DISCUSSION: Binding of Cl^- to band 3 protein has been assumed to precede its translocation across the membrane, but the evidence is indirect, based largely on kinetic behavior (7,8,9) or on protection by Cl^- against inhibitors (19). The NMR technique offers an opportunity to measure Cl^- binding directly, for the widening of the [^{35}Cl]NMR absorption signal in the presence of ligand is generally interpreted as binding of Cl^- to ligand (10). Because of the difficulty of preparing the large amounts of undenatured, purified band 3 protein necessary for NMR measurements, the present study was carried out with Triton X-100 extracts of ghosts enriched in band 3 protein. Such extracts are also enriched in sialoglycoproteins and contain phospholipids in about the same lipid to protein ratio as the ghosts (13). Band 3 constitutes about 30% of the ghost protein (5), but about 80% of the protein in the extract. Apart from the sialoglycoproteins (about 15%), other proteins are present in only small quantities (4,13). The present study indicates that the phospholipids and sialoglycoproteins in the extract do not contribute significantly to the widening of the [^{35}Cl] NMR signal. Although minor components cannot be excluded as contributors to the widening, it seems likely that most of the effect is due to the binding of Cl^- to band 3. This conclusion is supported by several kinds of evidence in addition to the relative abundance of band 3: (I), the broadening per mg of ghost protein is of the same order of magni-

tude as that reported for other proteins (11,12). If it were largely due to a minor component, the broadening per mg of protein would have to be unusually large; (II), the excess of [^{35}Cl]line width is a direct measure of the transverse relaxation time of the bound Cl^-. The dominant relaxation process for such quadrupolar nuclei arises from the interaction of the quadrupole moment with the electric field gradients at the ion binding site. Modulation of this interaction can arise from both the rotation of the ion-protein complex and from exchange between bound and free ions. For Triton X-100 band 3 extract, the exchange process is most likely too slow to contribute to the relaxation times, and on this assumption a quadrupolar coupling constant can be estimated for the bound ion using the procedure of Norne et al.(12). Using a rotational correlation time of 16 nsec derived from an estimated molecular weight of 310,000 for the band 3 Triton X-100 micelle (20), a value of 2 MHz is obtained for the quadrupolar coupling constant. This value is in good agreement with values found for Cl^- binding to other metal free proteins (12); (III), DIDS is highly localized in band 3 (over 90%) with about 5% in the sialoglycoprotein, only trace amounts distributed in other proteins and none in phospholipids (2,21,22). Its substantial inhibition of the NMR peak widening supports the conclusion that DIDS-modulated Cl^- binding is located in band 3.

The line width broadening per mg of protein is somewhat higher in the Triton X-100 extract than in the ghost (4.2 compared to 6.0 Hz/mg) and the inhibitory effect of DIDS is also higher in the extract (35% compared to 50%). If band 3 contributes a disproportionate fraction of the broadening by ghost protein and almost all of the DIDS effect, then its enrichment in the extract could account for the changes. In addition, however, some alteration in Cl^- binding must accompany Triton X-100 extraction. For example, at pH 5.5 to 6.5, the line broadening is very low in ghosts, but relatively high in the extract (Fig. 3). Additional Cl^- binding sites visible in this pH range have become accessible as a consequence of detergent extraction.

The modulation of the line broadening by DIDS is restricted to a narrow pH range around the peak value of 7.2. Thus the peak (seen with both ghosts and extract) represents Cl^- binding that might be related to the inhibition of anion transport by DIDS. This conclusion is supported by the approximate correspondence of the pH peak for DIDS-modulation of Cl^- binding and that reported for Cl^- transport (23). The remaining line broadening over the whole pH range studied is probably unrelated to the inhibition of anion transport, representing non-specific Cl^- binding by band 3 and other membrane components.

The degree of line broadening cannot be used to directly determine the affinities or stoichiometry of Cl^- binding. The DIDS-binding on the other hand, is known to be in approximately a 1 to 1 ratio to band 3 protein(22,24). Thus the modulation of line broadening by DIDS can be attributed to one DIDS-binding site per band 3 monomer. We suggest that the capacity of DIDS to inhibit anion transport in the human red blood cell is related to its capacity to displace Cl^- from binding sites on band 3, the anion transport protein.

ACKNOWLEDGEMENTS: The authors would like to thank Mr. Thomas Lew for carrying out the NMR Measurements, Mr. A. Gaarn for his technical help in preparing the samples, and Dr. P. Knauf for valuable discussions. This study was supported by the Medical Research Council (Canada) by Grant # MT4665.

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. Passow, H., Fasold, H., Zaki, L., Schuhmann, B., and Lepke, S. (1974/75) in Biomembranes, Structure and Function (Gardos, G., and Szasz, I. eds.) p. 197-214 (Proceed. 9th FEBS meeting, Budapest).
4. Rothstein, A., Cabantchik, Z.I., Balshin, M., and Juliano, R. (1975) Biochem. Biophys. Res. Commun. 64, 144-150.
5. Wolosin, J.M., Ginsburg, H., and Cabantchik, Z.I. (1977) J. Biol. Chem. (in press).
6. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
7. Passow, H. (1969) in Progress in Biophysics (Butler, J.A.V., and Noble,D. eds.) 19, 425-467, Pergamon, New York.
8. Gunn, R.B., Dalmark, M., Tosteson, D.C., and Wieth, J.O. (1973) J. Gen. Physiol. 61, 185-206.
9. Dalmark, M. (1976) J. Gen. Physiol. 67, 223-234.
10. Stengle, T.R., and Baldeschwieler, J.D. (1966) Proc. Nat. Acad. Sci. USA, 55, 1020-1025.
11. Norne, J.E., Hsalmarsson, S.G., Lindman, B., and Zeppezauer, M. (1975) Biochemistry, 14, 3401-3408.
12. Norne, J.E., Lilja, H., Lindman, B., Einarsson, R., and Zeppezauer, M. (1975) Eur. J. Biochem. 59, 563-473.

13. Yu, J., Fischman, A., and Steck, T.L. (1973) J. Supramol. Struct. 1, 233-247.
14. Dodge, J.T., Mitchell, C., Hanahan, D.S. (1963) Arch. Biochem. Biophys. 110, 119-130.
15. Shami, Y., Ship, S., and Rothstein, A. (1977) Anal. Biochem. (in press).
16. Fukuda, M., and Osawa, T. (1973) J. Biol. Chem. 248, 5100-5105.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
18. Aminoff, D. (1961) Biochem. J. 81, 384-392.
19. Rothstein, A., Cabantchik, Z.I. and Knauf, P.A. (1976) Fed. Proc. 35, 3-10.
20. Clarke, S. (1975) J. Biol. Chem. 250, 5459-5469.
21. Cabantchik, Z.I., and Rothstein, A. (1974) J. Membrane Biol. 15,227-246.
22. Ship, S., Shami, Y., Breuer, W., and Rothstein, A. (1977) J. Membrane Biol. (in press).
23. Brazy, P.C., and Gunn, R.B. (1976) J. Gen. Physiol. 653, 583-599.
24. Lepke, S., Fasold, H., Pring, M., and Passow, H. (1976) J. Membrane Biol. 29, 147-177.