J. Membrane Biol. 116, 87–91 (1990)

The Journal of
Membrane Biology

Inhibition of Chloride Binding to the Anion Transport Site by Diethylpyrocarbonate Modification of Band 3

Naotaka Hamasaki†, Kenji Izuhara†, Kenshi Okubo†, Yoko Kanazawa‡, Akira Omachi§, and Robert A. Kleps‖

†Department of Clinical Chemistry and Laboratory Medicine, Fukuoka University School of Medicine, and ‡Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan; §Department of Physiology and Biophysics, and ‖Nuclear Magnetic Resonance Laboratory of the Research Resonance Center, University of Illinois at Chicago, Illinois

Summary. The line widths of $^{35}Cl^-$ nuclear magnetic resonances were used to measure chloride binding by Band 3. Since this procedure relates directly to binding, the data obtained may be interpreted more unequivocally than affinities derived from kinetic data which could be related to either translocation or binding. Chloride binding to the active sites in Band 3 was assessed from that portion of the total line width which was sensitive to 4,4′-dinitrostilbene-2,2′-disulfonic acid. These sites appeared to be completely inhibited by treatment of erythrocyte membranes with diethylpyrocarbonate. This result is consistent with our previous observation that this reagent inhibits anion transport in resealed erythrocyte ghosts (Izuhara, Okubo & Hamasaki, 1989, *Biochemistry* **28**:4725–4728). Hydroxylamine could not reverse the diethylpyrocarbonate inhibition of chloride binding to Band 3. The pH-dependence of diethylpyrocarbonate reactivity suggests that the modified residues may be those of histidine.

Key Words anion transport · $^{35}Cl^-$ NMR · Band 3 · DEPC · hydroxylamine · erythrocyte membrane

Introduction

In the present study, we used a ^{35}Cl nuclear magnetic resonance (NMR) technique (Shami et al., 1977; Forsen & Lindman, 1981; Falke, Pace & Chan, 1984*a,b*; Falke, Kanes & Chan, 1985; Falke & Chan, 1986*a–c*) to investigate chloride binding by Band 3. The NMR method provides a direct measure of anion binding, whereas binding inferred from affinities calculated from transport rates is subject to both anion binding to the carrier and translocation of the anion-carrier complex.

We suggested previously that histidine residue(s) in Band 3 may participate in anion transport along with lysine and arginine residues (Matsuyama, Kawano & Hamasaki, 1986) and demonstrated that diethylpyrocarbonate (DEPC), a reputedly histidine-reactive reagent, inhibits inorganic phosphate exchange across the human erythrocyte membrane (Izuhara, Okubo & Hamasaki, 1989). The exchange rates were decreased only when the functional amino acids at the cytosolic surface of resealed ghosts were modified by this reagent. The intracellular modification of these residues inhibited the extracellular binding of [^{3}H]dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid([^{3}H]H_2-DIDS) to Band 3 and, conversely, extracellular 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) protected intracellular DEPC-susceptible residues from DEPC modification (Izuhara et al., 1989). Thus, the DEPC-susceptible residue(s) is hidden from the cytosolic surface when Band 3 is in the *outward* form and appears in the cytosolic compartment only when Band 3 is in the *inward* conformation (Hamasaki, Izuhara & Okubo, 1989). It appears, therefore, that the conformational changes which occur in Band 3 take place across the entire breadth of the erythrocyte membrane and are relevant to anion transport.

In the present study, the ^{35}Cl NMR technique was employed to investigate the chloride binding sites of Band 3 following treatment of this protein with DEPC. The results indicate that chloride binding to Band 3 is inhibited by this reagent and that this inhibition is likely to be involved in the inhibition of phosphate transport by this reagent (Izuhara et al., 1989), though the chloride transport activity under the same conditions remains to be done to further clarification of the relationship between the chloride transport activity and the binding to the transport site of Band 3.

Materials and Methods

Preparation of Red Cell Membranes

Freshly outdated human blood was used in these studies. Erythrocytes which had been stored in citrate/phosphate/dextrose solution were washed three times with phosphate-buffered saline at

0°C. The washed red cells were lysed with 30 volumes of 5 mM phosphate at pH 8 (5P8) at 0°C for 30 min. The membranes were pelleted by centrifugation for 10 min at 27,300 × *g* and 0–4°C and were washed with ice-cold 5P8 until the membranes became white. To avoid resealing of the membranes during the preparative procedures, all lysing and washing solutions were precooled in ice. The membranes were kept in ice for no longer than a week before use in the NMR experiment.

Treatment of the Erythrocyte Membrane with DEPC and Hydroxylamine

DEPC was freshly prepared as a 600 mM solution in ethanol. The cell membranes were incubated with 6 mM DEPC in ice for 30 min. For additional treatment with hydroxylamine, DEPC-treated membranes were mixed with an equal volume of 1 M hydroxylamine and incubated at 37°C for 0–180 min. The membranes were washed twice with a large volume of 5P8.

Preparation of NMR Samples

Each NMR sample contained membranes suspended in a solution containing 62.5 mM NaCl and 20 mM sodium phosphate at pH 8.0 or 6.0 to make the membrane protein concentration 1.9 mg/ml. In a preliminary study, different chloride concentrations were tested and a NaCl concentration of 62.5 mM was found to be a good working solution to obtain DNDS-sensitive line widths. The membrane concentration in the NMR samples was varied from 0 to 2.0 mg protein/ml. The pH of the NMR samples was measured with a glass electrode before and after the NMR measurements. To determine the anion transport-specific chloride binding sites (Falke & Chan, 1986*a*), the line width of each sample was determined before and after addition of 20 μl of 40 mM DNDS.

^{35}Cl NMR Spectroscopy

Spectra were obtained using a Nicolet 200 MHz spectrometer (^{35}Cl resonance frequency was 19.6 MHz). Samples were contained in 12-mm tubes and the following parameters were selected for the acquisition of spectra. The spectral width was 2000 Hz and contained 256 data points. Two thousand acquisitions were accumulated without spinning over a period of 4.8 min at 22°C. Line broadening of 10 Hz was used to increase the signal/noise ratio and the free induction decays were zero-filled twice. The line widths of the ^{35}Cl peaks at half-height were calculated with the aid of Nicolet's curve-fitting routine.

$[^3H]H_2$-DIDS Binding to Band 3 in the Membranes

Resealed ghosts were prepared in a pH 7.4 medium and incubated with and without 5 mM DEPC for 30 min at pH 7.4 and 0°C. Aliquots of the DEPC-pretreated ghost preparation were incubated with either 1 M hydroxylamine or 5P8 (in the control sample) for 30 or 180 min at 37°C. The incubated ghosts were washed and resuspended in 5P8, and used for the $[^3H]H_2$-DIDS binding experiments as described previously (Izuhara et al., 1989).

Analytical Procedures

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for protein analysis was carried out according to the method of Laemmli (1970).

Materials

DEPC and DNDS were obtained from Aldrich Chemical, WI. $[^3H]H_2$-DIDS was purchased from HSC Research Development, Toronto, Canada. Other reagents were of analytical grade.

Results

Effect of DEPC on the Line Width of the ^{35}Cl NMR

Figure 1 shows a plot of the line widths of $^{35}Cl^-$ NMR derived from solutions containing 62.5 mM NaCl, 20 mM sodium phosphate and 0–2 mg protein/ml of solution as a function of membrane protein concentration. The line broadening which resulted was due to the increase in concentration of the membranes in the NMR samples, consistent with the results of Falke et al. (1986*a*). The slope of the line describing the line broadening was 7.30 Hz per mg protein/ml and the line widths were reduced by adding DNDS to a final concentration of 0.2 mM in these same NMR samples. The slope of the DNDS-reduced line width curve was 2.70 Hz per mg protein/ml. The DNDS-sensitive line width was therefore 4.6 Hz per mg protein/ml, and this difference reflects chloride binding to anion transport sites of Band 3 as shown by Falke et al. (1986*a*). Treatment of native membranes with DEPC completely abolished the DNDS-sensitive line broadening. The slope of the line broadening was also decreased from 7.30 to 2.70 Hz per mg protein/ml when membranes were treated with DEPC at pH 7.8 (Fig. 1). This indicates that chloride binding to the anion transport sites of Band 3 was completely inhibited by this procedure. This inhibitory effect of DEPC was diminished by decreasing the pH of the solution to 6.3 (Fig. 1) as we observed previously in anion transport studies (Izuhara et al., 1989). The control values in the absence of DEPC at this pH were similar to the values observed at pH 7.8 and are not shown.

Hydroxylamine Treatment

Hydroxylamine has been used to test whether DEPC-modified residues are histidine groups,

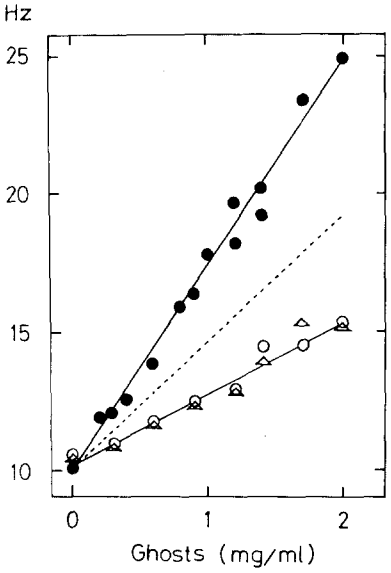


Fig. 1. DEPC and DNDS inhibit the line broadening of the $^{35}Cl^-$ NMR from human erythrocyte membranes. The line width of the $^{35}Cl^-$ NMR from unsealed ghosts was plotted against protein concentration of the ghosts. Native erythrocyte membranes with (○) or without (●) 0.2 mM DNDS and with 6 mM DEPC at pH 7.8 (△) were examined with the $^{35}Cl^-$ NMR line broadening procedure described in Materials and Methods. The dotted line indicates the line broadening of samples containing membranes treated with 6 mM DEPC at pH 6.3

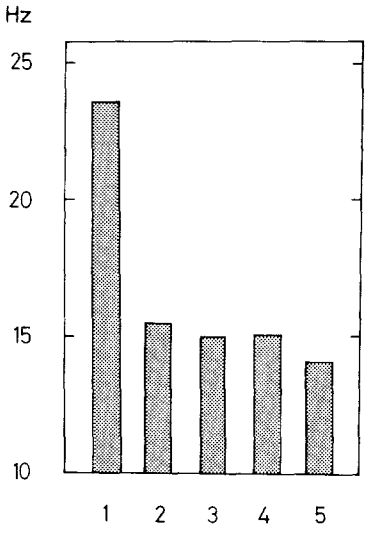
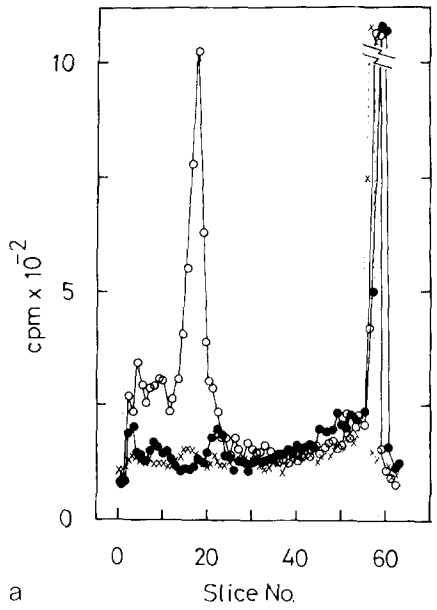


Fig. 2. Effect of hydroxylamine on the line widths of the $^{35}Cl^-$ NMR from DEPC-pretreated membranes: column *1*, the linewidth of native erythrocyte membranes (1.9 mg/ml); column *2*, the line width of membranes (1.9 mg/ml) incubated with 0.2 mM DNDS; column *3*, the line width of membranes (1.9 mg/ml) pretreated with 6 mM DEPC at pH 7.8; column 4, the line width of DEPC-pretreated membranes (1.9 mg/ml) after treatment with 0.5 M hydroxylamine at 37°C for 30 min; column *5*, the line width of DEPC-pretreated membranes (1.9 mg/ml) after treatment with 0.5 M hydroxylamine at 37°C for 180 min. Each value indicates the average of two different experiments

which would have been indicated by reversal of the DEPC inhibition (Miles, 1977). In this study, we also used this reagent for the same purpose. Erythrocyte membranes were treated with 0.5 M hydroxylamine at 37°C for 0–180 min. As shown in Fig. 1, the DNDS-sensitive line broadening was abolished by treating ghosts with DEPC, indicating that the chloride binding site for anion transport was blocked by DEPC. This reduction in line broadening was not reversed by 0.5 M hydroxylamine (Fig. 2). Essentially the same results were obtained by treating the ghosts with 0.2 or 1 M hydroxylamine (*data not shown*). Hydroxylamine alone up to 1 M had no effect on the DNDS-sensitive line broadening of native ghosts for 3 hr at 37°C (*data not shown*). The irreversible $[^3H]H_2$-DIDS binding site of Band 3 was intact when ghosts were incubated with 1 M hydroxylamine at 37°C for 30 min, but was completely destroyed by 3 hr of incubation (Fig. 3*a*), a procedure which would have minimally degraded the Band 3 protein (Fig. 3*b*). Prolonged incubation (19 hr) of ghosts with hydroxylamine degraded Band 3 (Fig. 3*b*) as well as membrane proteins such as spectrin (Bands 1 and 2).

Discussion

In the present communication, we used ^{35}Cl NMR spectroscopy to examine how DEPC modifies the anion transport system in the erythrocyte membrane. In anion transport, three general steps are actually involved: binding to a carrier, translocation of the anion-carrier complex, and release of the anion from the carrier. The NMR method employed in this study provides a more direct measure of Cl^- binding to and release from the carrier than the calculation of affinities from transport rates since translocation can also be involved in the determination of a given transport rate. In fact, translocation rather than binding appears to be the rate-limiting step in normal chloride transport (Passow, 1986). In the present study, DEPC inhibited anion binding and in our previous study (Izuhara et al., 1989) anion transport was decreased by this reagent. In this case, therefore, it is clear that the binding step is definitely involved in the inhibition of transport, although the modification might sterically hinder the anion binding. It is also clear that binding studies with ^{35}Cl NMR provide unique information which



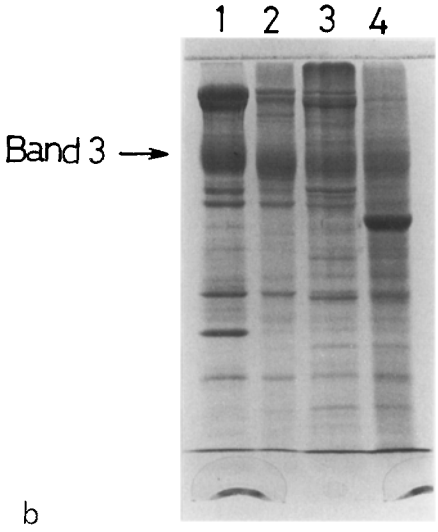


Fig. 3. (*a*) Influence of hydroxylamine on $[^3H]H_2$-DIDS binding to Band 3 in erythrocyte membranes with and without prior DEPC treatment. $[^3H]H_2$-DIDS was preferentially incorporated into Band 3 (slice # 14–20) and lipid fraction (slice # 53–57) of erythrocyte membranes which had been preincubated with 1 M hydroxylamine for 30 min in 5P8 at 37°C (○), but the radioactive incorporation into Band 3 (slice # 14–20) was completely inhibited by incubating the membranes with 1 M hydroxylamine for 180 min (×). Incorporation of $[^3H]H_2$-DIDS into Band 3 (slice # 14–20) into DEPC-pretreated membranes was not reversed by incubating the membranes with 1 M hydroxylamine for 30 min in 5P8 at 37°C (●) (*b*) SDS-PAGE analysis of erythrocyte membranes treated with 1 M hydroxylamine for 0 min (lane *1*), 30 min (lane *2*), 3 hr (lane *3*) and 19 hr (lane *4*) in 5P8 at 37°C. The gels were stained with Coomassie brilliant blue

aid in the greater understanding of not only the overall transport process but the mechanism of inhibition of a particular inhibitor as well.

DEPC modifies not only histidine but also lysine, tryptophan and cysteine residues, although histidine is considered to be the most susceptible of these amino acids (Miles, 1977). It has been known that the carboethoxy-histidine groups of some proteins are removed by hydroxylamine (Bornstein & Balian, 1977; Miles, 1977). Therefore, we used this reagent to test whether the DEPC inhibition of chloride binding to Band 3 could be restored by hydroxylamine. We could not demonstrate that this reagent was able to reverse the DEPC effect on chloride binding (Fig. 2). The changing reactivity of DEPC to pH (Fig. 1), however, strongly suggests that the modified residues may indeed be those of histidine. Further investigation would be desirable in order to secure additional evidence in support of this view.

Hydroxylamine alone inhibited the irreversible binding of $[^3H]H_2$-DIDS to Band 3 (Fig. 3*a*) before the anion transport protein was significantly degraded by this reagent (Fig. 3*b*). On the other hand, hydroxylamine did not inhibit chloride binding to Band 3 as indicated by the absence of an effect on the line broadening of ghosts incubated for 3 hr at 37°C. These results appear to be in accord with the previous observation that the minimal structure of Band 3 containing anion binding sites is relatively stable as demonstrated by the ^{35}Cl NMR technique, even after extensive digestion of Band 3 with proteolytic enzymes (Falke & Chan, 1986*c*). However, these results seem paradoxical since binding of chloride and of $[^3H]H_2$-DIDS to Band 3 are both reflections of anion transport activity. This apparent discrepancy may be due to the difference in size between the stilbene compound and chloride, since a minor alteration in membrane structure could sterically affect binding of the larger ion before that of the smaller ion. It is interesting that hydroxylamine cleaves Asn-Gly peptide bonds (Bronstein & Balian, 1977). According to the deduced amino acid sequence of human Band 3 (Tanner, Martin & High, 1988), there are three Asn-Gly sites within the molecule at 91–92, 365–366 and 482–483. The hydroxylamine-cleavage site could therefore be a crucial locus for the binding of the stilbene compound to Band 3.

We gratefully acknowledge the Nuclear Magnetic Resonance Laboratory of the Research Resonance Center, University of Illinois at Chicago, which provided the equipment and assistance necessary to conduct the NMR experiments. This study was supported in part by grants from the Ministry of Education, Science and Culture of Japan.

References

Bornstein, P., Balian, G. 1977. Cleavage at Asn-Gly bonds with hydroxylamine. *In:* Methods in Enzymology. C.H.W. Hirs and S.N. Timasheff, editors, Vol. 47, pp. 132–145. Academic, New York—San Francisco—London

Falke, J.J., Chan, S.I. 1986*a*. Molecular mechanisms of Band 3 inhibitors. I. Transport site inhibitors. *Biochemistry* **25:**7888–7894

Falke, J.J., Chan, S.I. 1986*b*. Molecular mechanisms of Band 3 inhibitors. 2. Channel blockers. *Biochemistry* **25:**7895–7898

Falke, J.J., Chan, S.I. 1986*c*. Molecular mechanisms of Band 3 inhibitors. 3. Translocation inhibitors. *Biochemistry* **25:**7899–7906

Falke, J.J., Kanes, K.J., Chan, S.I. 1985. The minimal structure containing the Band 3 anion transport site. *J. Biol. Chem.* **260:**13294–13303

Falke, J.J., Pace, R.J. Chan, S.I. 1984*a*. Chloride binding to the anion transport binding sites of Band 3. *J. Biol. Chem.* **259:**6472–6480

Falke, J.J., Pace, R.J., Chan, S.I. 1984*b*. Direct observation of the transmembrane recruitment of Band 3 transport sites by competitive inhibitors. *J. Biol. Chem.* **259:**6481–6491

Forsen, S., Lindman, B. 1981. Ion binding in biological systems as studied by NMR spectroscopy. *In:* Methods of Biochemical Analysis D. Glick, editor. Vol. 27, pp. 289–486. John Wiley & Sons, New York—Chichester—Brisbane—Toronto

Hamasaki, N., Izuhara, K., Okubo, K. 1989. Involvement of an intracellular histidine residue of Band 3 in the conformational changes taking place during anion transport. *In:* Anion Transport Protein of the Red Blood Cell Membrane. N. Hamasaki and M.L. Jennings, editors. pp. 47–58. Elsevier, Amsterdam

Izuhara, K., Okubo, K., Hamasaki, N. 1989. Conformational change of Band 3 protein induced by diethylpyrocarbonate modification in human erythrocyte ghosts. *Biochemistry* **28:**4725–4728

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227:**680–685

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Matsuyama, H., Kawano, Y., Hamasaki, N. 1986. Involvement of a histidine residue in inorganic phosphate and phosphoenolpyruvate transport across the human erythrocyte membrane. *J. Biochem.* **99:**495–501

Miles, E.W. 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. *In:* Methods in Enzymology. C.H.W. Hirs and S.N. Timasheff, editors. Vol. 47, pp. 431–442. Academic, New York—San Francisco—London

Passow, H. 1986. Molecular aspects of Band 3 protein-mediated anion transport across the red blood cell membrane. *In:* Reviews of Physiology Biochemistry and Pharmacology. E. Habermann, E. Helmreich, H. Holzer, R. Jung, R.J. Linden, P.A. Miescher, J. Piiper, H. Rasmussen, W. Singer, M.U. Trendelenburg, K. Ullrich, and M.W. Vogt, editors. Vol. 103, 61-223. Springer-Verlag, Berlin—Heidelberg—New York—Tokyo

Shami, Y., Carver, J., Ship, S., Rothstein, A. 1977. 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. *Biochem. Biophys. Res. Commun.* **76:**429–436

Tanner, M.J.A., Martin, P.G., High, S. 1988. The complete amino acid sequence of the human erythrocyte membrane anion-transport protein deduced from the cDNA. *Biochem. J.* **256:**703–712

Received 31 October 1989; revised 12 January 1990