ION CHANNELS WITHIN ION TRANSPORT PROTEINS

Evidence in the Band 3 System

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Many membrane proteins contain channels that allow migration of ions into the protein interior, but not all of these channels are of the transmembrane variety. The ion transport proteins that exhibit a fixed stoichiometry of ion translocation during their catalytic cycle (such as band 3) do not contain ion channels that allow passive diffusion of ions between opposite membrane surfaces. Yet these proteins may contain ion channels that lead from the surface of the protein to internal ion transport binding sites. Such channels could effectively shorten the transmembrane distance over which ions need to be transported.

Band 3 is an integral membrane protein which catalyzes the exchange of anions across human red cell membranes. Each band 3 monomer possesses a single transport site which is alternately exposed to opposite sides of the membrane. [1,2] This transport site contains essential arginine residues that provide the positive charge necessary for anion binding (1). We have found another type of essential arginine that is not associated with the transport site. This new type of essential arginine can be covalently modified with the arginine-specific reagent 1, 2-cyclohexanedione; the characteristics of the modification reaction suggest that the modified arginine residue(s) are components of an ion channel leading to the transport site.

An inhibitor of band-3–catalyzed chloride exchange that does not act at the transport site could either inhibit the translocation of bound anion, or inhibit the movement of anion into and/or out of the transport site. We have used a ^{35}Cl NMR assay to study binding of substrate chloride ion to binding sites on leaky red cell membranes, or ghosts (1, 2). This assay utilizes the fact that the linewidth of the solution chloride ^{35}Cl NMR resonance increases in the presence of chloride binding sites. Under specific conditions, the increase in linewidth, termed the linebroadening (δ), is linearly related to the concentration of chloride binding sites:

$$\delta = \sum_i \alpha_i \cdot [X_{T_i}] \cdot \frac{[Cl]^{-1}}{[Cl]^{-1} + K_{Di}^{-1}}$$

where α_i is a characteristic constant for the i'th type of site, which has a total (stoichiometric) concentration $[X_{T(i)}]$ and a chloride dissociation constant $K_{D(i)}$. The ^{35}Cl linebroadening due to leaky ghost membrane is a sum of the contributions from different types of chloride binding sites. We have identified a component of the linebroadening that stems from chloride binding to band 3 transport sites on both sides of the membrane. [1,2] This transport site linebroadening can be isolated by a subtraction procedure utilizing 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS), a competitive inhibitor of chloride binding to band 3 transport sites.[1]

RESULTS AND DISCUSSION

The arginine-specific reagents phenyglyoxal (PG) and 1,2-cyclohexanedione (CHD) have both been shown to be inhibitors of band-3–catalyzed anion exchange (1, 2). Fig. 1 indicates that CHD and PG also inhibit the ^{35}Cl NMR linebroadening of ghost membranes. The modified membranes give rise to linebroadening that is similar to that observed for unmodified membrane samples containing the band-3 inhibitor DNDS (Fig. 1). We have previously shown that DNDS inhibits the characteristic square hyperbola (Fig. 1, Eq. 1) due to band-3 transport sites[1] (K_D = 80 mM). Thus, CHD and PG both inhibit the linebroadening due to band-3 transport sites (Fig. 1). In addition, PG appears to inhibit some of the linebroadening due to low-affinity[1] ($K_D >> 0.5$ M) chloride-binding sites that remain in the presence of DNDS (Fig. 1).

The inhibition of the transport site linebroadening by PG stems from transport site destruction: PG is known to modify covalently the arginine residue(s) in the transport site (1). This essential arginine is less available for modification when the transport site is occupied with substrate chloride ion or with the inhibitor DNDS. Thus, the reaction of the transport site with PG is slowed in the presence of chloride or DNDS (Fig. 2, and reference [1]).

In the case of CHD, the inhibition of the transport site linebroadening is not a result of simple modification of the transport site, because the site is not protected from CHD by the presence of chloride or DNDS (Fig. 2). Nor is the linebroadening inhibition due to denaturation of band 3, because *a*, incubation of ghost membranes with 10 mM 1,4-cyclohexanedione (instead of 1,2-cyclohexanedione) at 37°C for 1 h inhibits the transport site linebroadening only 17%; and *b*, the inhibition by CHD is 46% ± 2% reversible (by overnight incubation at 4°C in 200 mM $NH_2OH \cdot HCl$, pH to 8.0 with NaOH, followed by incubation at 37° for 1–3 h and subsequent washing in NMR buffer). Instead,

[1]Falke, J. J., R. J. Pace, and S. I. Chan. Chloride binding to the anion transport sites of band 3. In preparation.

[2]Falke, J. J., R. J. Pace, and S. I. Chan. Direct observation of the transmembrane recruitment of band 3 transport sites. In preparation.

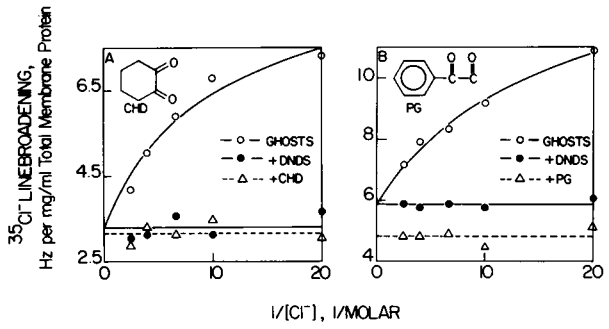

FIGURE 1 Inhibition of transport site linebroadening by DNDS, CHD, and PG. Leaky ghost membranes[1] (3 parts) were diluted with buffer (2 parts) to yield 100 mM boric acid, pH to 8.0 with NaOH, with or without 100 mM CHD (*A*) or 30 mM PG (*B*). The resulting suspensions were incubated at 37°C for 1 h. Subsequently, the membranes in *B* were washed to remove excess PG by three cycles of pelleting (48,000 g/15 min), aspiration of the supernatant, and resuspension in 83 mM boric acid, pH to 8.0 with NaOH. The washed membranes were sonicated.[2] Finally, the membrane suspensions (5 parts) were diluted with buffer (1 part) to yield *X* mM NaCl, ionic strength = (400 − *X*) mM Na citrate, pH 8.0, with 83 mM borate (*A*) or 70 mM borate (*B*), and 7% D_2O (*A*) or 17% D_2O (*B*), both at pH 8.0. Where indicated, DNDS was added to 1 mM.

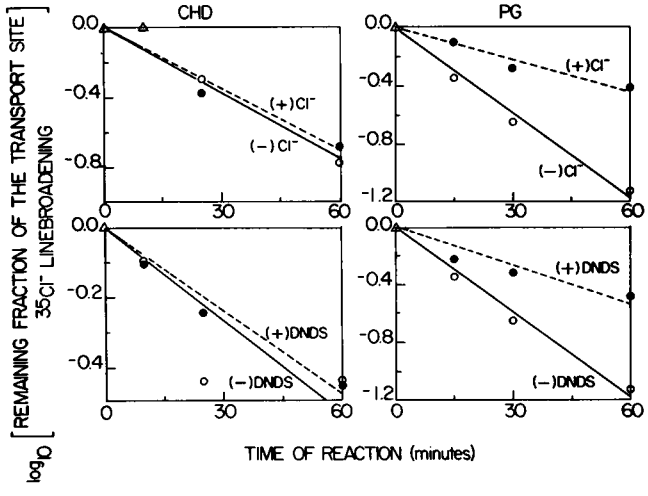

FIGURE 2 The effect of chloride and DNDS on the rate of transport site linebroadening inhibition. Leaky ghost membranes[1] (3 parts) were diluted with buffer (2 parts) to yield 81 mM boric acid, pH to 8.0 with NaOH, ± 300 mM NaCl, ± 200 μM DNDS, and 14 mM CHD (*left column*), or 15 mM PG (*right column*). The resulting suspension was incubated at 37°C for the indicated time before removal to ice. Then the membranes (except for those in the upper left graph) were washed and sonicated as above where the washing buffer also contained 250 mM NaCl and 20% D_2O. The transport site linebroadening was isolated using the DNDS-subtraction procedure.[1] The data was fit with linear least-squares best-fit straight lines.

the observed loss of transport site linebroadening results from decreased accessibility of transport sites to solution chloride ions. Because the linebroadening of both the inward- and outward-facing transport sites is inhibited by CHD (Fig. 1), the transport site is inaccessible to solution chloride at both surfaces of the modified membranes. When the exchange of chloride between transport sites and solution is sufficiently slow, the constant α_i (Eq. 1) becomes zero for the transport sites. As a result, the transport site linebroadening disappears even when the transport site remains intact.

All of the available evidence is consistent with the following model. The transport site of band 3 contains one or more essential arginine residue(s). This arginine reacts readily with the planar PG molecule. Nonplanar inhibitors are not able to interact with the transport site (5), and so CHD cannot reach the arginine(s) there. However, other essential arginines are found in ion channels of undetermined length that lead from both the inner and outer solutions to the interior of the protein where the transport machinery resides. These channel arginines provide positive charges that facilitate the diffusion of anions through the channels. The water-soluble CHD molecule can react with the channel arginines to block movement of anions between binding sites and solution. In contrast, the less water-soluble PG is less likely to be found in aqueous channels; thus, PG inhibits the transport site preferentially.

This model emphasizes the fact that channels can play an essential role even in proteins that do not form membrane pores. Substrate channels could be quite common in proteins because certain enzymatic processes are most efficient in a low dielectric environment, as is found in the interior of a protein. Moreover, substrate channels could be important control elements: channels are known to exhibit substrate specificity and can be opened or closed at appropriate times. Future structural studies should lead to an increased understanding of substrate channels in proteins.

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