Molecular Mechanisms of Band 3 Inhibitors. 2. Channel Blockers†

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ABSTRACT: Band 3 is proposed to contain substrate channels that lead from the aqueous medium to a transport site buried within the membrane, and which can be blocked by inhibitors. The inhibitors 1,2-cyclohexanedione (CHD) and dipyridamole (DP) each inhibit the transport site ^{35}Cl NMR line broadening, but neither competes with Cl^- for binding. Thus these inhibitors do not occupy the transport site; instead they slow the migration of Cl^- between the transport site and the medium. The simplest explanation for this behavior is that CHD and DP block one or more substrate channels. CHD is an arginine-specific covalent modification reagent, and its effectiveness as a channel blocker indicates that the channel contains arginine positive charges to facilitate the migration of anions through the channel. DP is a noncovalent channel blocker that binds with a stoichiometry of 1 molecule per band 3 dimer. DP binding is unaffected by CHD but is prevented by phenylglyoxal (PG), 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), or niflumic acid. Thus the DP and CHD binding sites are distinct, with DP binding sufficiently close to the transport site to interact with PG and DNDS. It is proposed that substrate channels may be a general feature of transport proteins.

Stoichiometric transport proteins such as band 3 differ significantly from transmembrane channel proteins in their mechanism of transmembrane transport. Transport proteins repetitively undertake a structurally predetermined transport cycle during which a stoichiometric quantum of ions or molecules bound to transport sites are moved across the membrane. The reproducibility of subsequent quanta and the inability to carry a substantial ionic current flow across the membrane rule out transmembrane channels in these proteins. Yet shorter channels that lead to transport sites buried within the membrane could play an important role in transport systems. Passive diffusion of substrate through such channels could carry substrate through part of its transmembrane journey, thereby shortening the transmembrane distance that must be spanned by the translocation event. In addition, the opening and closing of substrate channels could provide a mechanism to regulate the net transport of substrate across the membrane.

Previous studies are consistent with the proposal that a substrate channel exists in the band 3 system. The disulfonic stilbene inhibitor 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate (BADS)[1] is considered a transport site inhibitor by analogy with other stilbenedisulfonates [see preceding paper in this issue (Falke & Chan, 1986a)] and thus is thought to bind at or near the outward-facing transport site. Fluorescence energy transfer experiments using BADS as a fluorescence donor indicate that the BADS binding site is 34–42 Å from the intracellular compartment. Since this is less than the thickness of the bilayer, the BADS binding site must be buried within the membrane (Macara & Cantley, 1983). Moreover, proteolysis of band 3 to its transmembrane segments does not destroy Cl^- binding, as expected for a transport site that is protected from proteolysis by virtue of its intramembrane location (Falke et al., 1985). Presumably a buried transport site would require hydrophobic or even aqueous substrate channels to facilitate the diffusion of an anion into the hydrophobic region containing the transport site. If this is so, it should be possible to block these channels so that the migration of substrate between the transport site and the medium is inhibited. The present paper demonstrates that certain organo inhibitors of band 3 are channel blockers that inhibit the exchange of Cl^- between the transport site and solution.

Materials and Methods

All experimental details are described under Materials and Methods in the preceding paper in this issue (Falke & Chan, 1986a).

Results

Strategy of Channel Blocker Identification. Like transport site inhibitors, channel blockers generally inhibit the $^{35}Cl^-$ line broadening due to Cl^- binding to the transport site. However, these two types of inhibitors can be resolved by their differing dependence on $[Cl^-]$. The $^{35}Cl^-$ line broadening due to Cl^- binding to a homogeneous population of sites i is given by Lindman and Forsen (1976) and Falke et al. (1984a):

$$\delta_i = \alpha_i \frac{[X_i]_T}{K_{Di}} \frac{[Cl^-]^{-1}}{[Cl^-]^{-1} + K_{Di}^{-1}} \quad (1)$$

where α_i is a constant characteristic of the site, $[X_i]_T$ is the total concentration of the site, and K_{Di} is the Cl^- dissociation constant of the site. When the exchange of Cl^- between the site and solution is sufficiently slow, α_i is directly related to the rate at which bound Cl^- returns to the solution: $\alpha_i = k_{off}/\pi$. True channel blockers occupy a channel leading from the site to solution, thereby reducing k_{off} and k_{on} by the same factor

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[1] Abbreviations: CHD, 1,2-cyclohexanedione; DP, dipyridamole; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; pNBS, *p*-nitrobenzenesulfonate; PG, phenylglyoxal; DNFB, 2,4-dinitrofluorobenzene; NIF, niflumic acid; BADS, 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; EDTA, ethylenediaminetetraacetic acid.

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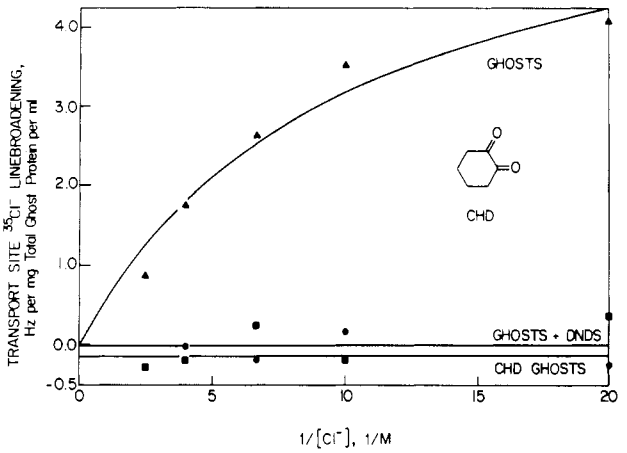

FIGURE 1: 1,2-Cyclohexanedione inhibits transport site $^{35}Cl^-$ line broadening. Shown is the line broadening due to both inward- and outward-facing transport sites on leaky ghosts modified or unmodified with 80 mM CHD and then sonicated. The solid curves are nonlinear least-squares best-fit curves for a set of inhibited (lower curve, $y = 0$) and intact [upper curve, $y = Ax/(K_D x + 1)$, $K_D = 100 \pm 20$ mM] transport sites. Each sample contained the indicated [NaCl] as well as 80 mM boric acid, 2.5 mM NaH_2PO_4, and 17% D_2O, pH to 8.0 with NH_4OH and NaOH. Sufficient citric acid (pH to 8.0 with NaOH) was added to bring the ionic strength up to that of the sample containing the highest [NaCl] = 400 mM. Spectral parameters: 8.8 MHz, 3 °C.

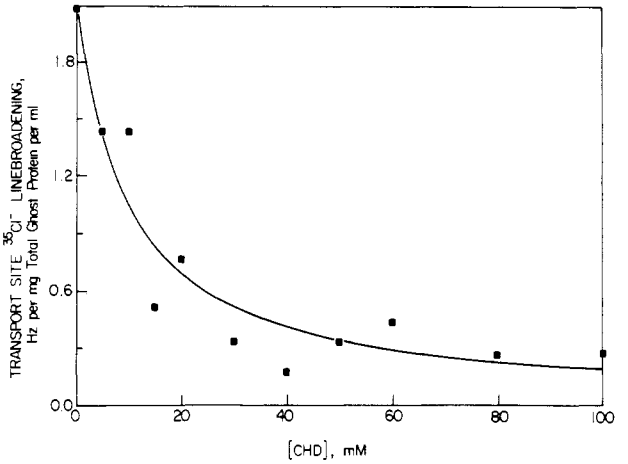

FIGURE 2: Titration of transport site $^{35}Cl^-$ line broadening with CHD. Shown is the line broadening due to both inward- and outward-facing transport sites on leaky ghosts modified with the indicated [CHD], followed by sonication. Each sample contained 250 mM NaCl, 80 mM boric acid, 2.5 mM NaH_2PO_4, and 17% D_2O, pH to 8.0 with NaOH, and also $0.8x$ mM CHD, where x is the [CHD] used in the modification reaction and indicated in the figure. Spectral parameters: 8.8 MHz, 3 °C.

while having no effect on the structure of the site, so that $[X_i]_T$ and K_{Di} remain unchanged. As a result, the binding of channel blockers is independent of $[Cl^-]$. In contrast, inhibitors of the site itself must decrease $[X_i]_T$ or increase K_{Di} so that the binding of such inhibitors is sensitive to $[Cl^-]$. Thus although channel blockers and transport site inhibitors each eliminate the transport site line broadening, they can be distinguished in experiments where $[Cl^-]$ is varied. Here we describe two channel blockers: 1,2-cyclohexanedione and dipyridamole.

1,2-Cyclohexanedione (CHD). Borate and CHD form a complex that modifies arginine residues in a covalent but reversible reaction of undetermined stoichiometry (Dietl & Tschesche, 1976; Riordan, 1979). CHD is known to inhibit anion transport by band 3 (Zaki, 1981a,b); however, it is not clear whether the inhibition stems from modification of the transport site or, alternatively, a channel.

When leaky isolated red cell membranes (or ghosts) are modified with CHD, the line braodening due to band 3 transport sites is completely eliminated, as expected for inhibition by a transport site inhibitor or a channel blocker (Figure 1). Since both inward- and outward-facing transport sites contribute to the transport site line broadening in the leaky ghost system (Falke et al., 1984b), it follows that CHD inhibits the line broadening due to both transport site conformations. The inhibitory reaction exhibits a dependence on [CHD] (Figure 2) which is similar to that previously observed in studies of transport inhibition (Zaki, 1981a), indicating that line broadening inhibition and transport inhibition are due to modification of the same arginine residue(s). These arginine residue(s) are presumed to be on band 3 since the CHD reaction inhibits H_2DIDS binding to band 3 (Zaki, 1981a). The line broadening inhibition is not due to simple denaturation of band 3 since (a) incubation of ghost membranes with 10 mM 1,3-cyclohexanedione instead of 1,2-cyclohexanedione inhibits the transport site line broadening only 17% and (b) inhibition by CHD is 46 ± 2% reversed by overnight incubation with the nucleophile NH_2OH in the absence of borate.

The mechanism of transport site inhibition by CHD is revealed by the effect of transport site substrates on the CHD reaction. The line broadening inhibition is not due to modification of a transport site residue since the inhibitory reaction is not slowed by occupation of the transport site with Cl^- [saturation of the transport site with 300 mM Cl^- changes the rate of inhibition by <15%, within experimental error of no change (Falke & Chan, 1984)]. This result, which we have shown to be repeatable, contradicts a previous transport study suggesting that increasing $[Cl^-]$ effectively protects band 3 catalyzed anion transport against inhibition by CHD (Zaki, 1981b). The explanation for this apparent discrepancy appears straightforward. In the NMR protocol only [NaCl] and [sodium citrate] were varied while the total ionic strength and the concentrations of the other buffer constituents were held constant (Falke & Chan, 1984); however, in the transport protocol [NaCl], [Na_2SO_4], [EDTA], and the total ionic strength were all varied simultaneously (Zaki, 1981b). The conclusion that transport site substrates do not affect the CHD reaction is confirmed by the observation that DNDS, like Cl^-, does not affect the reaction rate [saturation of the transport site with 200 μM DNDS slows the rate of inhibition by <15%, within experimental error of no change (Falke & Chan, 1984)]. Thus the line broadening inhibition by CHD stems from the slowing of Cl^- exchange between the transport site and solution rather than from occupation of the transport site. This slowing of exchange is not due to trapping of the transport site between the inward- and outward-facing conformations, since the site only encounters such a state when occupied by Cl^- so that the trapping would exhibit a significant dependence on $[Cl^-]$. Instead, the simplest explanation of the data is that CHD blocks a substrate channel leading to the transport site.

It appears likely that band 3 possesses both inward- and outward-facing channels, each leading to the transport machinery buried within the bilayer where it is inaccessible to proteases added to either side of the membrane (Falke et al., 1985). Thus CHD could inhibit the line broadening due to transport sites on both sides of the membrane by modifying arginine in each channel. Alternatively, CHD could react with a channel only when the transport site is exposed to that channel, so that the CHD reaction would recruit sites into the blocked conformation, thereby yielding the observed complete inhibition of line broadening due to transport sites on both sides of the membrane. The concept of simultaneous channel

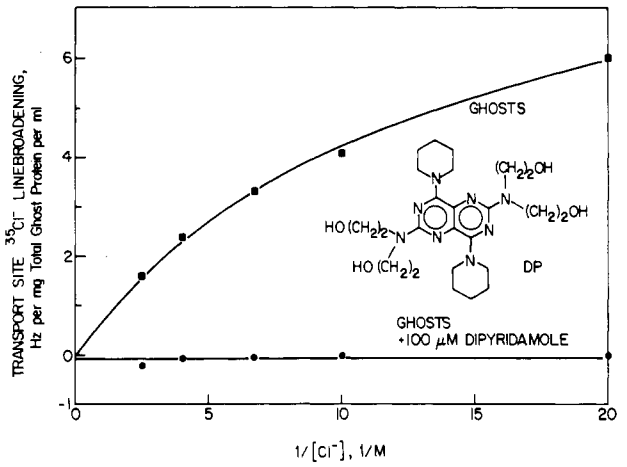

FIGURE 3: Dipyridamole inhibits transport site $^{35}Cl^-$ line broadening. Shown is the line broadening due to both inward- and outward-facing transport sites on leaky ghosts in the absence or presence of 100 μM dipyridamole. The solid curves are nonlinear least-squares best-fit curves for a set of inhibited (lower curve, $y = 0$) and intact [upper curve, $y = Ax/(K_D x + 1)$, $K_D = 80 \pm 10$ mM] transport sites. Each sample contained the indicated [NH_4Cl] as well as 2.5 mM NaH_2PO_4 and 20% D_2O, pH to 8.0 with NH_4OH and NaOH. Sufficient citric acid (pH to 8.0 with NaOH) was added to bring the ionic strength up to that of the sample containing the highest [NH_4Cl] = 400 mM. Spectral parameters: 8.8 MHz, 3 °C.

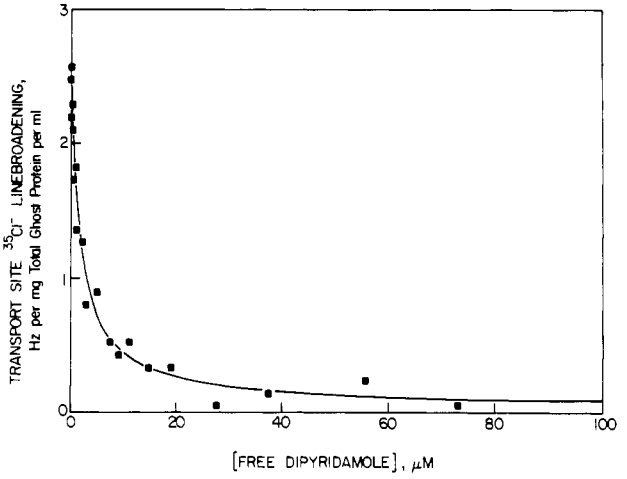

FIGURE 4: Titration of transport site $^{35}Cl^-$ line broadening with dipyridamole. Shown is the line broadening due to both inward- and outward-facing transport sites on leaky ghosts. The solid curve is a nonlinear least-squares best-fit curve for a set of homogeneous DP binding sites [$y = A[1 - x(x + K_D)^{-1}]$, $K_D = 2.0 \pm 0.1$ μM]. Samples contained 250 mM NH_4Cl, 5 mM NaH_2PO_4, and 20% D_2O, pH to 8.0 with NH_4OH and NaOH. Spectral parameters: 8.8 MHz, 3 °C.

blocking and transport site recruitment can also explain the behavior of dipyridamole.

Dipyridamole (DP). This large inhibitor lacks a negative charge and thus is not expected to bind to the transport site, yet DP is a potent inhibitor of anion transport (Deuticke & Gerlach, 1967; Knauf, 1979). DP binds noncovalently with unknown stoichiometry to a site of unknown location on red cell membranes.

The inhibitory mechanism of DP is illuminated by the observation that this inhibitor completely inhibits the line broadening due to transport sites on both sides of leaky red cell membranes (Figure 3). This line broadening inhibition is due to DP binding to the same sites that cause transport inhibition, since the observed dissociation constant for DP binding is the same in line broadening experiments ($K_D = 2.0 \pm 0.1$ μM; Figure 4) and in transport experiments ($K_D = 5$ μM; Deutiche & Gerlach, 1967). The binding of DP exhibits

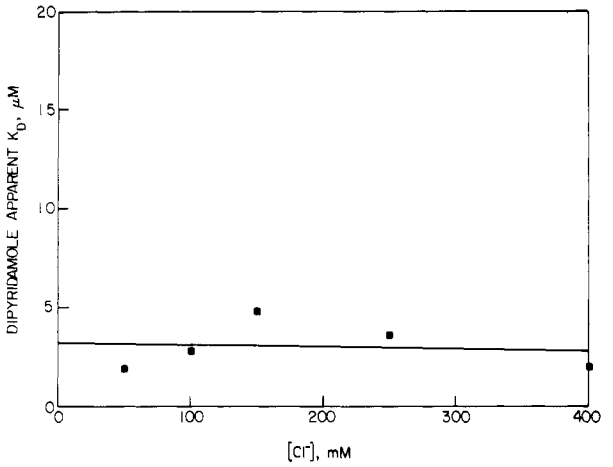

FIGURE 5: Inhibition of transport site $^{35}Cl^-$ line broadening by dipyridamole is independent of [Cl^-]. The dipyridamole apparent K_D is defined as the free DP concentration that yields half-maximal inhibition of the transport site line broadening due to leaky ghost membranes. The solid line is a linear least-squares best-fit straight line of zero slope, $K_D = 3.2 \pm 0.6$ μM. Samples are as in Figure 3. Spectral parameters: 8.8 MHz, 3 °C.

Table I: Interaction of Dipyridamole with Other Inhibitors

inhibitor[a]	covalent	DP binding sites per band 3 monomer[b]	K_D for DP (μM)
control	–	0.5 ± 0.1	1.2 ± 0.6
CHD (20 mM)	+	0.3 ± 0.2	0.7 ± 0.8
DNFB (0.2 mM)	+	1.0 ± 0.1	1.0 ± 0.3
DNFB (4 mM)	+	1.1 ± 0.1	1.0 ± 0.3
PG (10 mM)	+	0.0 ± 0.1	
DNDS (1 mM)	–	0.0 ± 0.1	

[a] For control, CHD, and PG membranes: leaky ghosts and the indicated inhibitor concentration were incubated in 80 mM boric acid, pH 8.0 with NaOH, at 37 °C for 1 h, then washed in 150 mM NH_4Cl and 5 mM NaH_2PO_4, pH to 8.0 with NH_4OH and NaOH, and finally sonicated before determination of free and bound DP as described under Materials and Methods in the preceding paper (Falke & Chan, 1986a). For DNDS membranes: leaky ghosts were treated like control membranes except DP binding was measured in the presence of DNDS. For DNFB membranes: leaky ghosts and the indicated DNFB concentration were incubated in 50 mM NaCl and 10 mM NaH_2PO_4, pH to 7.4 with NaOH, and then the membranes were washed and sonicated as above. [b] Determined from the concentration of bound DP (=total DP – free DP) and the concentration of band 3 [see Materials and Methods in the preceding paper (Falke & Chan, 1986a)].

Table II: Interaction of Dipyridamole with Niflumic Acid

inhibitor[a]	total $^{35}Cl^-$ line broadening [Hz/(mg of total ghost protein/mL)]	inhibition of transport site $^{35}Cl^-$ line broadening[b] (%)
control	6.8 ± 0.1	
1 mM DNDS	4.8 ± 0.2	100
100 μM DP	4.4 ± 0.2	120
1 mM DNDS, 100 μM DP	4.6 ± 0.1	110
100 μM NIF	6.6 ± 0.2	10
10 μM DP	5.4 ± 0.0	70
100 μM NIF, 100 μM DP	6.5 ± 0.1	15

[a] The concentrations given are total concentrations. [b] The transport site line broadening is defined as the difference between leaky ghosts ± 1 mM DNDS.

no dependence on [Cl^-] (Figure 5), indicating that DP inhibits the transport site line broadening by blocking a channel leading to the transport site.

The stoichiometry of DP binding and its interaction with other inhibitors of band 3 provide information on the location

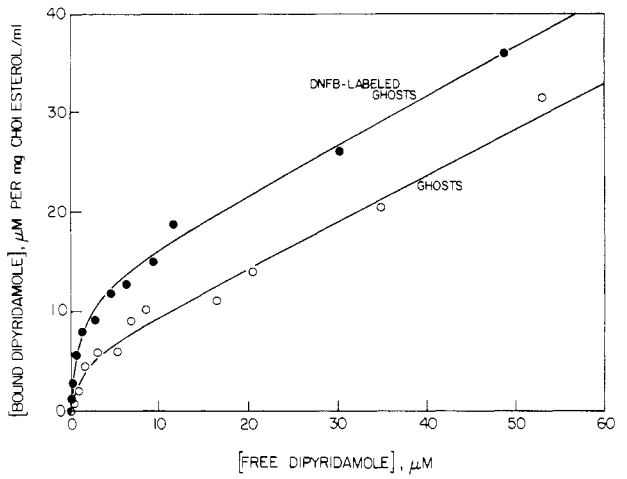

FIGURE 6: Dipyridamole binding to ghosts and DNFB-modified ghosts. Leaky ghosts and leaky ghosts modified by reaction with 4 mM DNFB were prepared and washed in 150 mM NH_4Cl, 5 mM NaH_2PO_4, pH to 8.0 with NH_4OH and NaOH. Bound and free DP was quantitated by pelleting membranes and measuring the A_{416} of free DP in the supernatant. The solid curves are nonlinear least-squares best-fit curves for a set of high-affinity sites plus a set of low-affinity sites [y = [sites]$x(x + K_D)^{-1} + cx$]. For unmodified ghosts the best-fit parameters are 5.2 ± 0.7 μM high-affinity sites/(mg of cholesterol/mL), and K_D = 1.2 ± 0.6 μM for DP binding to those sites. DNFB-modified ghosts yield 12.4 ± 0.8 μM high-affinity sites/(mg of cholesterol/mL) and K_D = 1.1 ± 0.3 μM. The concentration of band 3 is 11 μM monomers/(mg of cholesterol/mL), determined as described under Materials and Methods in the preceding paper (Falke & Chan, 1986a).

of its binding site. Unmodified leaky red cell membranes bind DP with a ratio of 1 molecule of DP per band 3 dimer (Figure 6, Table I). Niflumic acid, DNDS, and phenylglyoxal, which compete with each other for binding to extracellular sites on band 3, each prevent DP binding (Tables I and II). Surprisingly, the other channel blocker CHD has no effect on DP binding while the inhibitor, 2,4-dinitrofluorobenzene increases the stoichiometry of binding to 1 molecule of DP per band 3 monomer (Figure 6, Table I). The simplest interpretation of these results is that the large DP molecule binds to an external site on band 3, near both the transport site and the interface between the two monomers in a dimer, such that the bound DP can simultaneously block the outward-facing substrate channels on both monomers. In this picture DP binds only when the transport site faces the external solution, so that a saturating concentration of DP recruits all sites to the outward-facing conformation, where the substrate channel is blocked and the transport site line broadening is completely eliminated. CHD binds farther from the transport site, where it does not sterically interact with bound DP or DNDS; while 2,4-dinitrofluorobenzene appears to sterically uncouple the monomers so that each band 3 monomer can bind a single molecule of DP. In short, the results for CHD and DP indicate that multiple channel-blocking sites exist on band 3.

Discussion

All of the available evidence concerning band 3 inhibitors is consistent with the following model. The transport site of band 3 contains an essential arginine residue that readily reacts with the planar PG molecule. Other planar organo inhibitors such as DNDS and pNBS are also able to occupy the transport site, while nonplanar organo inhibitors such as the CHD–borate complex and DP are unable to enter the transport site. The transport site arginine is buried within the membrane and is accessed by two hydrophilic or even aqueous channels, one leading to each of the internal and external solution compartments. These channels contain positive charges that facilitate the rapid exchange of anion between the transport site and solution: the pH dependence of anion transport (Wieth & Bjerrum, 1982) and the transport site line broadening (Falke & Chan, 1985), as well as the reaction of CHD with the channels, indicate that some or all of the positive charges are provided by arginine. Due to its hydrophilic character, the CHD–borate complex can rapidly react with the charges in the hydrophilic channels, while the hydrophobic PG reacts only at the more hydrophobic transport site.

This model emphasizes the essential role played by substrate channels even in transport proteins that do not form transmembrane pores. Substrate channels could be a general feature of transport proteins, since such channels effectively shorten the transmembrane distance over which substrate must be transported. Many soluble enzymes may also contain substrate channels, since a wide variety of catalytic processes are most efficient in a low dielectric environment as in the interior of a biomolecule. Such channels could be important control elements: channels are known to exhibit substrate specificity and can be opened or closed at the appropriate time. The work presented here clearly indicates that NMR techniques can reveal important characteristics of channel structure. In the following paper in this issue (Falke & Chan, 1986b) we show that NMR techniques can also identify inhibitors that leave binding sites and substrate channels intact but prevent an enzymatic reaction that follows substrate binding, such as the transmembrane translocation of an occupied transport site.

Registry No. CHD, 765-87-7; DP, 58-32-2; DNFB, 70-34-8; PG, 1074-12-0; DNDS, 128-42-7; NIF, 4394-00-7; Cl^-, 16887-00-6; L-arginine, 74-79-3.

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