

## Reversible Pore Block of Connexin Channels by Cyclodextrins\*

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**Cyclodextrins (CDs), a series of hollow cyclic glucosaccharides, can reversibly block molecular permeation through channels formed by connexin-32 and/or connexin-26 reconstituted into liposomes. The character of the block changes as a function of the size of the CD relative to the connexin pore diameter, suggesting that the block occurs via entry of the CD into the pore lumen and occlusion of the permeability pathway. The block occurs only when the CD is applied to the side of the pore that is normally cytoplasmic and not from the side that is normally extracellular. The block is potentiated when organic analytes are sequestered in the hydrophobic interior of the CDs. CDs may be useful as molecular tools with which to explore the structure of the connexin pore and to alter molecular movement through connexin channels.**

Connexin channels, which compose gap junctions, mediate direct intercellular movement of cytoplasmic signaling molecules. There are ~20 isoforms of connexin protein (1), each of which forms channels with distinct permeability properties. Molecular permeability through connexin channels varies dramatically among connexins and can be surprisingly selective (2–11). Every functional deletion of a connexin isoform produces a distinct pathology, and genetic replacement of one connexin by another (“knock-in”) fails to fully compensate (1, 12–14). The pathologies that arise from altered connexin channel function must arise from abnormal molecular movement through connexin channels, whether in magnitude, regulation, or molecular specificity.

Although the key biological function of each type of connexin channel is defined by which molecules permeate (and do not permeate) the pore, little is known about the structural locus of the molecular selectivity, the connexin pore itself. Specifically, the mechanisms of selective molecular permeability through connexin channels are not understood. In the absence of a high resolution structure, such understanding must come from molecular probes of the pore. For many channels, prior to the recent progress in crystallographic analysis, mechanisms of channel selectivity and permeation were elucidated by studies using hydrophilic molecular reagents that enter and/or occlude the conducting pathway (*cf.* Refs. 15–17). For connexin channels, such molecular tools would be useful for identifying key amino acids and protein regions that line the pore and define

its molecular selectivity; the absence of such tools has hindered progress in this area. This report identifies cyclodextrins as a class of pore blockers for connexin channels.

Cyclodextrins (CDs)<sup>1</sup> are cyclized glucose oligomers consisting of six or more glucose units. Their most stable molecular configuration is that of a toroid, wider at one end than the other, approximating a truncated cone with a hole down the center (Fig. 1). Hydroxyl groups on the external surface confer aqueous solubility, and linking glucosidic oxygen atoms lining the inner cavity give it a hydrophobic character, allowing CDs to sequester “guest” molecules (18). The unmodified naturally occurring CDs used in this study are uncharged.

CDs have been shown to reversibly enter and modify the pore properties of  $\alpha$ -hemolysin ( $\alpha$ HL) (19) and CymA bacterial porin (20). When lodged in the  $\alpha$ HL pore, CDs reduce the ionic conductance. The degree of conductance block and the stability of the CD within the  $\alpha$ HL pore were potentiated when the cavity of the CD was occupied by small guest molecules. In this context, CDs have been referred to as “molecular adapters” (21). In contrast, CDs appear to fully block the ionic conductance of CymA pores (20, 22).

Connexin channels are homo- or hetero-oligomers of isoforms of connexin protein (23–27). Connexin channels have two functional and structural forms. The basic unit is a hexamer, called a “hemichannel” or “connexon.” The hemichannel is the single membrane form of connexin channels, which can be found in the plasma membrane of many cells and has been implicated in a variety of cellular processes (28, 29). Gap junction channels are end-to-end dimers of hemichannels. The intercellular location of gap junction channels presents challenges for detailed biophysical investigation; hemichannels are more amenable for study. By and large, the properties of junctional channels follow those of the component hemichannels (10, 30–36).

This paper reports that CDs can reversibly block molecular permeability through connexin hemichannels formed by connexin-32 (Cx32) and/or connexin-26 (Cx26). The correlation between the block and the relative sizes of the CDs and the connexin pores indicates that the block occurs by direct occlusion of the pore by the CDs. Preliminary reports of this work have appeared in abstract form (37, 38).

### EXPERIMENTAL PROCEDURES

**Materials**—Lipids (egg phosphatidylcholine, bovine brain phosphatidylserine, and lissamine rhodamine B-labeled egg phosphatidylethanolamine) were purchased from Avanti Polar Lipids Inc. (Alabaster, IL); *n*-octyl  $\beta$ -D-glucoside was purchased from Calbiochem or Glycon Biochemicals (Luckenwalde, Germany). Bio-Gel (A-0.5m, 100–200 mesh, exclusion limit 500 kDa) was from Bio-Rad. Cyclodextrins were from Sigma. All other reagents were from Sigma unless stated.

**Purification of Homomeric Cx32 and Heteromeric Cx26/Cx32 Hemichannels from Rodent Liver**—Connexin was immunopurified from

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<sup>1</sup> The abbreviations used are: CDs, cyclodextrins;  $\alpha$ HL,  $\alpha$ -hemolysin; Cx, connexin; TSF, transport-specific fractionation; HA, hemagglutinin; 3H2NA, 3-hydroxy-2-naphtholic acid; 2AA, 2-amino-adamantanamine.

*n*-octyl  $\beta$ -D-glucoside-solubilized crude plasma membranes from rat or mouse liver using a monoclonal antibody specific for Cx32, as described and characterized (39). Previous biochemical and functional studies have shown that hemichannels purified in this manner from rat liver are predominantly homomeric Cx32 and from mouse liver are heteromeric Cx26/Cx32. The heteromeric channels are functionally heterogeneous, presumably because of heterogeneities of isoform stoichiometry and/or arrangement (25, 40). The primary sequences of rat and mouse Cx32 are identical. The sequences for Cx26 have conservative substitutions at two positions, neither of which is in a membrane-spanning domain (ratnmouse, Y68H and I215V).

**Reconstitution of Purified Connexin into Unilamellar Liposomes**—Liposome formation and connexin incorporation into unilamellar liposomes followed the protocol described previously (39, 41). Liposomes were formed by gel filtration of a 1 mg/ml mixture of phosphatidylcholine, phosphatidylserine, and rhodamine-labeled phosphatidylethanolamine at a molar ratio of 2:1:0.03 in urea buffer (see below) and affinity-purified connexin in 80 mM *n*-octyl  $\beta$ -D-glucoside. The protein/lipid/detergent mixture was applied to a column of Bio-Gel beads pretreated with sonicated liposomes. Connexin-containing liposomes were collected in the void volume. The protein/lipid ratio corresponded to an amount of connexin equivalent to less than one hemichannel per liposome so that some liposomes did not contain functional channels, which were used as internal controls in the permeability assay (TSF, described below).

**Transport-specific Fractionation (TSF)**—Molecular permeation of connexin channels was assessed by TSF of the liposomes. TSF has been described and characterized previously (39, 41, 42). TSF separates liposomes into distinct populations within a density gradient by exploiting the permeability of an open connexin channel to two molecular solutes, urea and sucrose. The liposomes are formed in, and entrap, urea-containing solution and are centrifuged through linear iso-osmotic TSF density gradients formed from 459 mM urea (500 mOsm/kg, density ( $\rho$ ) 1.0055 g/ml) and 400 mM sucrose (500 mOsm/kg,  $\rho$  1.0511 g/ml). During centrifugation, urea and sucrose exchange through open connexin channels, equilibrating the aqueous solution density inside and outside the liposomes, making the phospholipid membrane the determinant of liposome density. The gradient is constructed so that the solution density equivalent to the density of phospholipid is near the bottom, so liposomes with and without functional channels band in the lower and upper regions of the gradients, respectively. The positions of liposomes are monitored via rhodamine-labeled lipid fluorescence ( $\lambda_{\text{ex}}$  570 nm,  $\lambda_{\text{em}}$  590 nm) making it possible to visualize, collect, and quantitate the liposomes. The typical positions of the upper and lower bands are centered at densities of  $\sim$ 1.02 and 1.04 g/ml, respectively. Liposomes that contain channels permeable to one solute but not the other undergo osmotically driven shrinkage that results in a band of intermediate density of  $\sim$ 1.03 g/ml (43, 44). Because addition of CDs to the TSF gradients could affect the densities of the solutions, the bands were assigned as upper, intermediate, and lower on the basis of density determination by refractometry. Urea TSF buffer contained 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 3 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, and 459 mM urea at pH 7.6. Sucrose TSF buffer was identical to the urea buffer except that an osmotically equivalent concentration of sucrose (400 mM) was substituted for the urea.

Equilibration of the TSF solutes across the liposome membrane through an open hemichannel is very rapid (milliseconds for these 900-Å liposomes (45)), so any significant channel permeability to the solutes results in sufficient solute exchange to cause a liposome to move to a lower position determined by solute permeability. TSF is therefore an all-or-none assay of per liposome channel permeability. Stated another way, only complete block of TSF solute permeability through the connexin channel will cause a liposome containing a functional channel to remain at the upper position. A change in liposome distribution between the upper and either of the two lower band positions, relative to control, is a measure of a change in the fraction of channels that are permeable to the gradient solutes. Such a change can be due to pore block or to a reduction to zero of channel open probability.

**Purification of Tagged Homomeric Cx26 and Cx32 Hemichannels from Transfected HeLa Cells**—Homomeric Cx26 and homomeric Cx32 hemichannels were purified from HeLa cells transfected with an inducible vector. The connexin coding sequence was followed by a sequence coding for a thrombin-cleavable C-terminal mass tag consisting of an HA epitope followed by a 6xHN sequence (46). Connexin was immunopurified by using a monoclonal anti-HA antibody. The tag added  $\sim$ 3.2 kDa to the coding protein.

**Thrombin Cleavage of Tagged Connexin**—To 200  $\mu$ l of purified protein or pre-formed connexin proteoliposomes (pH > 7), 5.0 units of

thrombin (restriction grade thrombin as supplied; Novagen, Madison, WI) were added. The reaction was allowed to proceed on ice for 24 h and stopped by addition of 0.75 mM diisopropyl fluorophosphate (final concentration) to inactivate the thrombin.

**Connexin Recovery from Detergent Solutions and from Liposomes**—Connexin was recovered from detergent/lipid solutions according to Ref. 47 and recovered from liposomes according to Ref. 48.

**Reduction of Intraliposomal Cytochrome *c* by Extraliposomal Ascorbate**—Bovine heart cytochrome *c* was entrapped into liposomes at a concentration of 5 mg/ml during reconstitution. To ensure that cytochrome *c* was available without dilution during the reconstitution, 2 ml of 5 mg/ml cytochrome *c* was loaded onto the gel filtration column prior to connexin/lipid/detergent. Osmolarity was maintained with NaCl rather than urea buffer. Patent hemichannels reconstituted into liposome membranes were identified by the reduction of intraliposomal cytochrome *c* by an external solution of 150 mM sodium ascorbate (49). Reduction of cytochrome *c* was measured by absorbance at 417 nm at 25 °C.

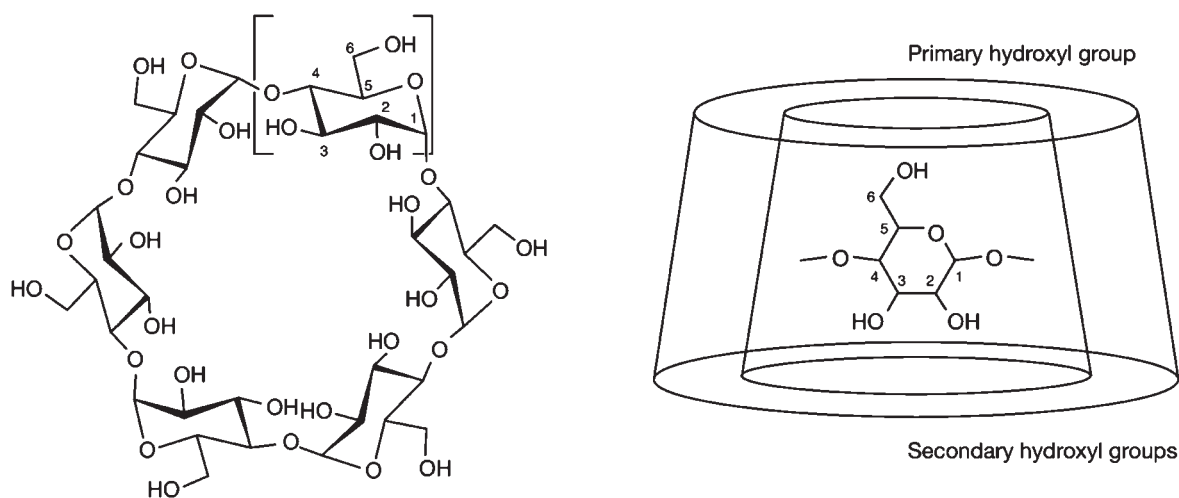
**Competitive Guest Complexation**—Fluorescence emission intensity of 3-hydroxy-2-naphtholic acid (3H2NA; 20  $\mu$ M) was measured at  $\lambda_{\text{ex}}$  350 nm and peak  $\lambda_{\text{em}}$  521 nm (50) in the presence and absence of CDs and/or TSF buffer components at pH 7.4 and 25 °C using a Cary Eclipse fluorescence spectrometer (Varian Inc., Palo Alto, CA). 3H2NA concentration and final volumes were kept constant. Measurements were made in double-distilled water with TSF buffer components added as indicated.

## RESULTS

Homomeric Cx32 channels and heteromeric Cx26/Cx32 channels were obtained from rat and mouse liver, respectively, as described and characterized previously (25, 39). Homomeric Cx26 channels and additional homomeric Cx32 channels were obtained from HeLa cells expressing HA-6xHN-tagged connexin (46). The relative limiting pore widths of the homomeric and heteromeric channels were characterized in previous work using uncharged fluorescent carbohydrate probes (25).<sup>2</sup> The data showed that homomeric Cx32 channels have a wider limiting diameter for permeation by uncharged molecules than do homomeric Cx26 or heteromeric Cx26/Cx32 channels. This size distinction is supported by work in cellular systems (5, 6). The heteromeric Cx26/Cx32 channel population was shown to be heterogeneous with regard to molecular selectivity (25) and sensitivity to modulatory ligand (40), indicating that it is composed of channels that vary in the stoichiometry and/or arrangement of Cx26 and Cx32 isoforms.

Hemichannels were reconstituted into unilamellar phospholipid liposomes. Molecular permeability of the reconstituted channels was assessed by TSF of the liposomes. TSF has been well characterized (42, 43, 51) and effectively used in studies of connexin channel permeability and modulation (25, 39, 40, 52–57). TSF separates liposomes into distinct populations within a density gradient on the basis of the permeability of an open connexin channel to two solutes of different densities (typically urea and sucrose; see “Experimental Procedures”). The salient feature is that liposomes that contain channels permeable to both gradient solutes come to an equilibrium position deep in the gradient, whereas liposomes that contain channels not permeable to the gradient solutes (or that contain no open channels) band near the top of the gradient. Liposomes that contain channels permeable to one solute but not the other undergo osmotically driven shrinkage that results in a band of intermediate density (43, 44). TSF is of particular utility in the study of connexin channels because of the constraints of studying connexin channels *in situ* and their apparent refractoriness to functional reconstitution in planar bilayers (30). In addition, TSF focuses on molecular permeability, not ionic conductance, which is the key functional property of connexin channels in most tissues.

<sup>2</sup> D. Locke, T. Stein, C. Davies, J. Morris, A. L. Harris, P. Monaghan, and B. Gusterson, submitted for publication.



Cyclodextrin (CD)	n	Da	Cyclodextrin diameter		Annular diameter		Depth
			Primary	Secondary	Primary	Secondary	
Alpha	$\alpha$ CD	6	12.7 Å	14.0 Å	4.7 Å	5.2 Å	8.0 Å
Beta	$\beta$ CD	7	14.6 Å	15.5 Å	6.0 Å	6.4 Å	8.2 Å
Gamma	$\gamma$ CD	8	17.6 Å	17.5 Å	7.5 Å	8.3 Å	8.2 Å

FIG. 1. **Schematic illustrations of CD structure.** *Left*, structure of cyclodextrin, a cyclic  $\alpha[1\rightarrow4]_n$  glucose sugar. One glucose subunit is enclosed in *brackets*. *Right*, CD is typically represented as a truncated cone. *Table*, dimensions of CDs, where  $n$  is the number of glucose units. The annular diameter from the primary (narrow) end of the cone and the secondary (wider) end of the cone are determined from Corey-Pauling-Koltun models, as is the annular depth from the primary to secondary hydroxyl groups (18, 70, 104).

The three CDs used in these studies are as follows:  $\alpha$ CD (6 glucose units),  $\beta$ CD (7 glucose units), and  $\gamma$ CD (8 glucose units). Their approximate dimensions are given in Fig. 1. The diameters of the internal cavities and the external widths of the CDs increase with the number of glucose subunits. The external widths approach and exceed the minimal pore widths of connexin channels inferred from tracer studies (58). Unless stated otherwise, CDs were applied by including them in the TSF gradients.  $\beta$ CD is known to affect membranes by sequestering cholesterol (which fits well in the internal cavity) (59), but the liposomes used in these studies contained no cholesterol.

In this report TSF is used to assess permeability to gradient solutes: the fraction of the liposomes in the lower band(s) is the fraction permeable to one or both solutes. This fraction, normalized to controls (*e.g.* without CD), is depicted as the fraction of channels that are unblocked.

**Cyclodextrin Block of Homomeric Cx32 Channels**—The two smaller CDs ( $\alpha$ CD and  $\beta$ CD) show complete block of homomeric Cx32 channels at CD concentrations of  $\sim 6$  and  $\sim 15$  mM, respectively (Fig. 2, *upper panel, left and middle graphs*). Above these concentrations, permeability to urea and sucrose was blocked; there was no lower TSF band. An intermediate band (indicating permeability to urea but not to sucrose) did not occur at any concentration.

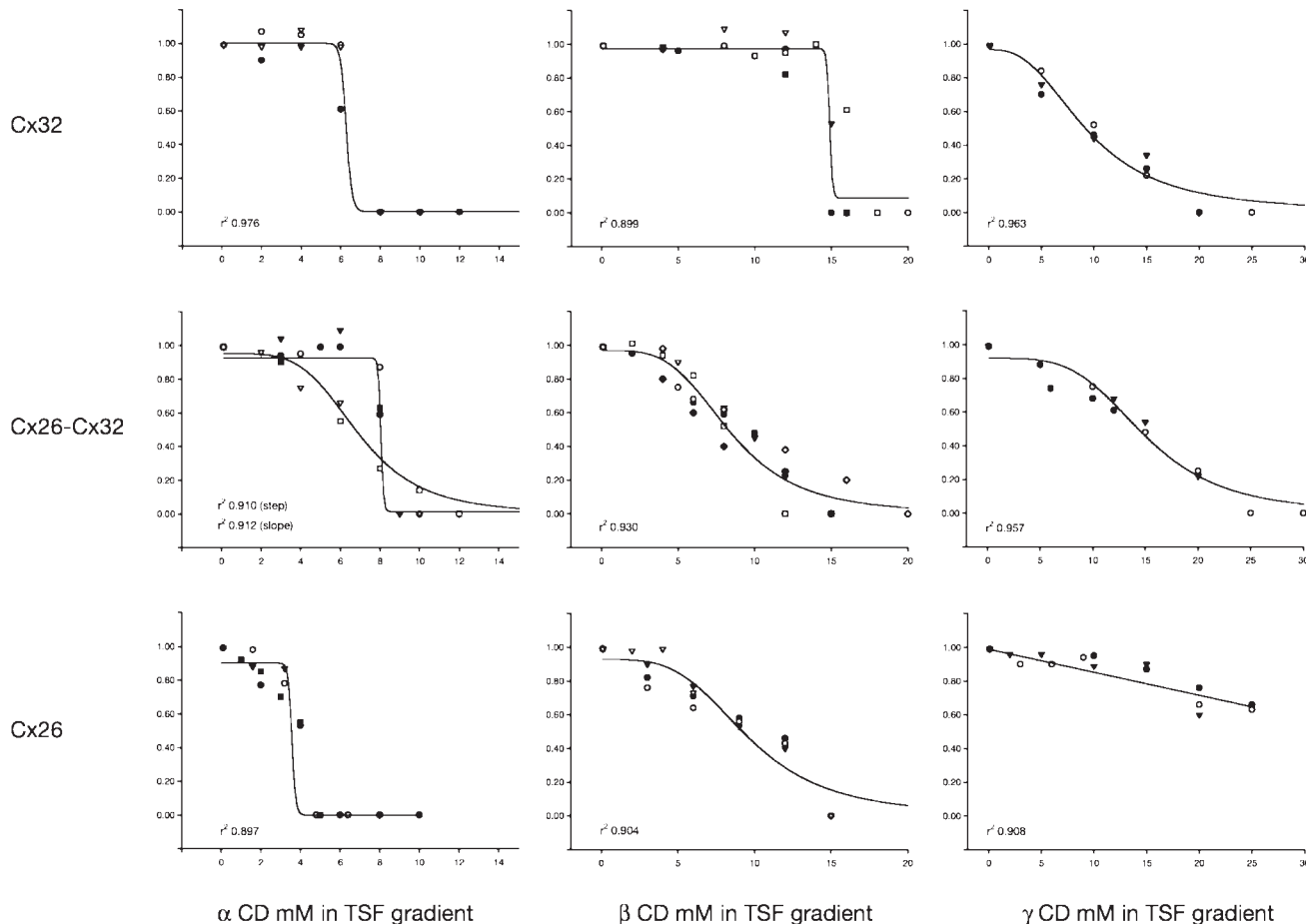
The step-change nature of the block-concentration relation for these CDs is expected for simple block of a homogeneous channel population. Because solute equilibration through a connexin channel across a liposome membrane is rapid, the solute permeability of a given liposome/channel reported by TSF is essentially all-or-none, either a liposome is detectably permeable to the gradient solutes or it is not. Only complete block of the TSF solutes will cause a liposome containing a functional channel to remain at the upper position. A step in the block-concentration relation occurs at the concentration at

which channel occupancy by the blocker is essentially complete in the population of channels. TSF does not give classical Michaelis-Menten binding parameters (*e.g.* a  $K_m$ ), because it fractionates liposomes according to whether the channels they contain have permeability/open probability greater than a very low threshold. Consequently, the concentration of a compound that produces block detectable in TSF is much greater than that needed to see an effect in biochemical studies and corresponds to the concentration needed for blocker occupancy very close to 1.0.

These data therefore indicate simple block of permeability to urea and sucrose by  $\alpha$ CD and  $\beta$ CD. The effects of the largest CD,  $\gamma$ CD, on Cx32 channels are more complex and the mechanism less clear. The  $\gamma$ CD data differ in two ways. The first is that the block-concentration relation has a different shape; block develops in a graded fashion with increasing CD concentration (Fig. 2, *upper panel, right graph*). For a homogeneous channel population, this shape of curve is inconsistent with simple block. The other difference is that in the concentration range that shows graded block ( $\sim 5$ – $15$  mM), the only lower TSF band was at the intermediate density position. This indicates permeability of the connexin channels to urea but not to sucrose; in this concentration range there was no band of liposomes corresponding to the density position indicating permeability to both gradient solutes. In other words, in the concentration range where an increasing fraction of the channels was blocked to both solutes, the other channels were blocked to sucrose but not to urea. These data show interaction of the  $\gamma$ CD with the pore, but that it is of a different character than that of the smaller CDs. It seems reasonable to attribute this difference to its larger size relative to the Cx32 pore.

A graded TSF activity-concentration relation occurs when there is heterogeneity of ligand-channel interactions. This can occur if the channel population is structurally heterogeneous. For a structurally homogeneous channel population such as

Fraction unblocked



**FIG. 2. Block of connexin channels as a function of CD concentration in TSF experiments.** For each curve, the fraction of unblocked channels is plotted as a function of CD concentration. Data are normalized to the fraction of the channels that are unblocked in the absence of CD. The curves are four-parameter logistic functions fit to the data points. *Left to right*, data for  $\alpha$ CD,  $\beta$ CD, and  $\gamma$ CD. *Upper panel*, homomeric Cx32. *Middle panel*, heteromeric Cx26/Cx32. *Lower panel*, homomeric Cx26. With increasing size of CD relative to connexin pore (from *upper left to lower right*), the block concentration relations change from approximate step functions to graded relations of decreasing slope. For each graph, each set of symbols is for a different preparation of connexin channels.

this one, graded effects occur if ligand interactions are themselves heterogeneous (*i.e.* multiple binding sites with different affinities (40)), or if the TSF measurement is not at steady state (*i.e.* the on-rate of the interaction or the time constant of solute equilibration in the presence of the CD is comparable with the time of the TSF centrifugation).

To test whether the CD-induced block was at steady state, the liposomes were incubated for several hours in a concentration of  $\gamma$ CD that in the standard 3-h TSF caused partial block, prior to TSF in gradients containing  $\gamma$ CD. The extent of inhibition was essentially unchanged from that without preincubation (inhibition in 10 mM  $\gamma$ CD in a standard 3-h TSF 1.00 (normalized), with preincubation  $0.93 \pm 0.02$ ). The duration of the TSF centrifugation was also increased, without effect (inhibition in 10 mM  $\gamma$ CD in a 6-h TSF  $0.95 \pm 0.08$  (normalized to 3 h TSF)). Overnight preincubation in  $\alpha$ CD or  $\beta$ CD prior to TSF also did not alter the extent of block (inhibition 1.00 (normalized), preincubation in 5 mM  $\alpha$ CD  $1.01 \pm 0.08$ , preincubation in 10 mM  $\beta$ CD  $0.91 \pm 0.03$ ).

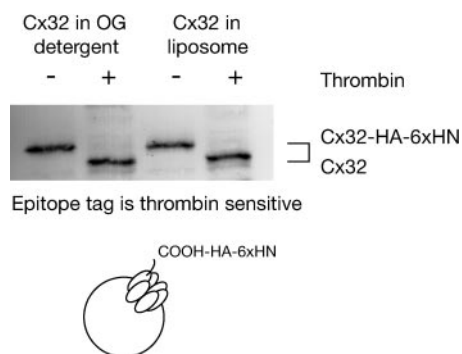
The lack of effect of these manipulations on the degree of inhibition shows that the interaction of the CDs with connexin during TSF is at steady state. Therefore, the graded block seen with  $\gamma$ CD is most readily explained by heterogeneous interactions of  $\gamma$ CD with the pore. Multiple modes of interaction that have different effective “affinities” produce a graded slope of effect, which is really a mixture of step changes, each reflecting

a different effective affinity. This mechanism is responsible for the graded effects of protonated aminosulfonates on heterogeneous mixtures of Cx26/Cx32 channels (40).

One possible reason for heterogeneity is that if a CD is too large relative to the pore diameter, it may lodge in the pore imperfectly and/or sideways in multiple configurations. The block to only the larger of the two gradient solutes (sucrose) is consistent both with imperfect fit of the CD in the pore (leak around the CD and the wall of the pore, allowing urea to get by) or with permeability of urea but not sucrose through the central pore of  $\gamma$ CD if the cavity is aligned with the axis of the pore. Either hypothesis can account for the partial permeability, but the imperfect fit idea accounts for both differences. One can speculate that the increasing block to urea that develops with CD concentration arises from mechanisms such as stacking of multiple CDs in the pore or a mass action-driven occupancy of a conformation that excludes urea from the central pore.

No matter what the specific mechanism, it appears that the different character of the  $\gamma$ CD effects is due to the larger size of  $\gamma$ CD relative to the Cx32 pore; the two smaller CDs have a single, dominant mode of interaction that blocks permeability to both TSF solutes. In this view, the effects of  $\gamma$ CD show what TSF data look like when a CD is too large for the pore. This is supported by the data below on channels that are narrower (*i.e.* that contain Cx26).

*Cyclodextrin Block of Cx32 Is Reversible*—If block by CD was



**FIG. 3. Orientation of connexin channels in the liposomes.** Cx32 channels with a C-terminal thrombin-cleavable mass tag (3.2 kDa) were exposed to thrombin when reconstituted into liposomes and when solubilized in detergent. Thrombin was applied only to the outside of the liposomes. The Western blot immunostained for Cx32 shows essentially complete cleavage of the mass tag of the reconstituted connexin under both conditions. This result indicates that the channels preferentially reconstitute into liposomes with the C-terminal domain exposed to the outside.

irreversible, one would expect that CD concentrations close to those that produce block in a 3-h TSF would eventually produce block with extensive preincubation (recall that the TSF step occurs only when essentially all the channels are blocked). As described above in the tests for steady state block, this was not the case, even with overnight preincubation. As a direct test of reversibility, upper band liposomes containing channels fully blocked by 10 mM  $\alpha$ CD, 20 mM  $\beta$ CD, or 30 mM  $\gamma$ CD were collected from TSF tubes and recentrifuged in TSF gradients containing no CD. The activity of the re-spun liposomes was unchanged from those re-spun from TSF gradients not containing CD (data not shown); the CD-induced block is fully reversible.

**Cyclodextrin Block Is from the Cytoplasmic, Not Extracellular, End of the Connexin Pore**—In the studies described thus far, CDs were applied only to the extraliposomal solution in the TSF gradient. To determine whether CD could affect the channels from the inside of the liposomes, blocking concentrations of CDs were trapped within the liposomes during connexin reconstitution. These liposomes were centrifuged through TSF gradients that did not contain CD. There was no pore block (untreated control 1.00 (normalized), with 10 mM  $\alpha$ CD trapped inside liposomes  $0.96 \pm 0.03$ ). Thus the CDs act only from the extraliposomal side of the channel.

The topology of the reconstituted connexin channels was determined by a protease protection assay. Cx32 with a thrombin-cleavable C-terminal HA-6xHN mass tag (~3.2 kDa) was reconstituted into liposomes. The liposomes were exposed to thrombin, followed by recovery of the connexin and analysis by Western blot. Thrombin cleavage of detergent-solubilized connexin was used as a positive control for complete enzymatic cleavage of the tag, and for nonspecific proteolysis. Fig. 3 shows that essentially all of the mass tag was sensitive to thrombin proteolysis, both in the liposomes and when detergent solubilized. The same results were obtained for mass-tagged Cx26 (not shown).

These results show that the connexin channels reconstitute into the liposome membranes with the C-terminal domain exposed to the outside. In the plasma membrane, the C-terminal domain of connexin is cytoplasmic (60–62). Therefore, the reconstituted channels are oriented cytoplasmic side outward. Intriguingly, this corresponds to the native topology one would expect for connexin channels in Golgi/endoplasmic reticulum prior to plasma membrane insertion, and to that of *in vitro* translated connexin inserted co-translationally (Cx32 and

Cx26) or post-translationally (Cx26) into microsomal membranes (49, 63), as well as Cx43 channels reconstituted into liposomes (57). Because CDs act only from the extraliposomal side, these studies show that they interact with connexin pores only from the “intracellular” aspect of the channel and do not have any detectable (by TSF) inhibitory effects on connexin channel permeability from the “extracellular” side.

**Cyclodextrin Block of Homomeric Cx26 Channels**—The actions of  $\alpha$ CD and  $\beta$ CD on homomeric Cx26 channels purified from transfected HeLa cells were assessed. For  $\alpha$ CD (Fig. 2, bottom panel, left graph), the block-concentration relations show a very steeply graded block between 2 and 4.5 mM CD, followed by an abrupt step to full block at about 4.5 mM.

Block by  $\beta$ CD (Fig. 2, bottom panel, center graph) is much more gradual, similar to the block of Cx32 channels by  $\gamma$ CD. As for the Cx32 data, in the regions of graded effect the lower band was at the intermediate density corresponding to permeability to urea but not sucrose. The largest CD,  $\gamma$ CD (Fig. 2, bottom panel, right graph), was without substantial effect even at the highest concentrations, suggesting that it does not significantly occlude the Cx26 pore. Thus the pattern seen in the Cx32 data, in which with increasing size of CD the block changes from steep to gradual, is confirmed and extended in the Cx26 data.

The main difference between the Cx26 and the Cx32 data is that transition from steep to gradual block occurs for smaller CDs in the Cx26 data, between  $\alpha$ CD and  $\beta$ CD for Cx26 and between  $\beta$ CD and  $\gamma$ CD for Cx32. This difference is understandable in view of the previous work showing that Cx26-containing channels are narrower than Cx32 channels (5, 6, 25).<sup>2</sup> The interaction of  $\alpha$ CD with Cx26 pores appears to have some heterogeneity (expected for some size mismatch) but is dominated by an interaction that blocks permeation, imparting a near-step quality to the relation. The interaction of  $\beta$ CD with Cx26 is like that of  $\gamma$ CD with Cx32, and the  $\gamma$ CD data suggest that this CD is too large to enter the Cx26 pore.

**Cyclodextrin Block of Heteromeric Cx26/Cx32 Channels**—The block-concentration relations of the CDs were determined for several preparations of heteromeric Cx26/Cx32 channels purified from mouse liver. The relations of each preparation corresponded to one of the types obtained previously for the homomeric channels. For  $\alpha$ CD (Fig. 2, middle panel, left graph), some preparations showed a step change (as for  $\alpha$ CD and  $\beta$ CD with Cx32) and some a gradual change (as for  $\beta$ CD with Cx26 and for  $\gamma$ CD with Cx32). The variations from preparation to preparation are attributable to variable isoform stoichiometries and/or arrangements of heteromeric channels in these populations of native channels. It is interesting to note that the preparations showing graded block mimicked the relations of homomeric Cx26 with  $\beta$ CD. Empirically, this indicates that these preparations may have more restrictive pores than homomeric Cx26 channels (*i.e.* even the smallest CD,  $\alpha$ CD, cannot fit well). One may speculate, without evidence, that this is perhaps due an irregularly shaped pore lumen because of their heteromeric composition. Nevertheless, this indication that heteromeric channels have a more restrictive pore than the corresponding homomeric channels could indicate that they have distinct molecular selectivity as well (25).<sup>2</sup> For the larger CDs,  $\beta$ CD and  $\gamma$ CD (Fig. 2, middle panel, center and right graphs), the relations were exclusively graded.

Even so, the same overall pattern was seen as for the homomeric channels, a tendency toward step changes for smaller CDs in narrower connexin channels and graded activity changes for larger CDs. As previously, in the regions of graded effect the lower band was at the intermediate density corresponding to connexin channel permeability to urea but not sucrose.

With the exception of the few preparations with graded relations with  $\alpha$ CD, the data are consistent with the idea that the heteromeric channels have a limiting pore diameter that is between that of homomeric Cx32 and homomeric Cx26 channels, with  $\alpha$ CD able to cleanly block some of the heteromeric channels (*i.e.* better than for  $\alpha$ CD with Cx26), and  $\gamma$ CD able to interact to some degree with the pores (unlike  $\gamma$ CD with Cx26).

**Pattern of Block**—The scenario that emerges is that as the size of the CD increases relative to the pore, the interactions change from simple pore block, to interactions indicative of less good fit, to no fit at all (moving from *upper left* to *lower right* in Fig. 2).

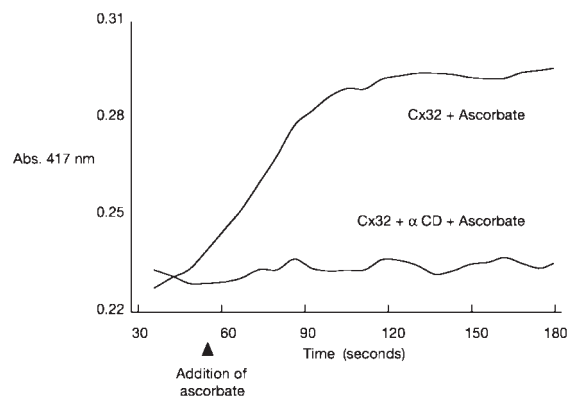
A step change in the block-concentration relation is expected if the interaction of a modulatory agent is the same for all the channels in the population. For the data presented, the simplest mechanistic explanation is that the step changes reflect simple pore block of the channels, at least for urea and sucrose, in which the CD resides in the pore as a cork. This type of interaction occurs for Cx32 with  $\alpha$ CD and  $\beta$ CD and for some Cx26/Cx32 preparations. It is also the dominant interaction for Cx26 with  $\alpha$ CD. There is simple and complete block by the two smaller CDs of the widest pore (Cx32) and by the smallest CD only of the narrower pores (Cx26 and Cx26/Cx32).

For each size of connexin channel, the larger CDs produce a more complex, graded block. They appear to interact with each channel in a less well defined manner, likely due to their larger size. For all instances of graded block, the remaining unblocked channels are impermeable to sucrose but permeable to urea, suggesting partial occlusion of the pore by the CD. Although CDs are usually described as rigid molecules, there is evidence that they have some flexibility and change their shape with binding (64) and as a function of hydrogen-bonding within the internal cavity (65). Such flexibility may contribute to heterogeneous interactions with a pore and to eventual full block by poorly fitting CDs at higher concentrations where more than one CD may lodge in the pore.

The correlation between the block and the relative sizes of the pore and the CDs strongly suggest that the pore is the site of CD action. Specifically, the data are consistent with the idea that the step changes in permeability are produced by entry and tight fit of the CD(s) into the connexin pore(s), and the gradual changes in activity seen with larger CD(s) reflect a size mismatch between the pore(s) and the CD(s), *i.e.* they are too large to enter the pore and/or interact with it in the same way. The apparent degree of mismatch correlates with Cx26 content.

**CD-induced Block Is Not Specific for the Urea or the Sucrose in the TSF Gradient**—TSF gradients are linear gradients between 459 mM urea at the top and 400 mM sucrose at the bottom. This urea concentration is well below the concentration required to denature or unfold proteins, but it was a concern that the effects of urea as a “water structure breaker,” or in diminishing the hydrophobic effect or adsorption to hydrophilic residues (66–68), might play a role in the block. To test this possibility, TSF gradients were run in which the urea was osmotically replaced by glycerol (438 mM). The density of glycerol is comparable with that of urea, so the density range of the TSF gradient was largely unaffected. Glycerol substitution had little effect on Cx32 channel permeability, in the absence or presence of  $\alpha$ CD (urea/sucrose without CD 1.00 (normalized), glycerol/sucrose without CD  $0.96 \pm 0.04$ , 5 mM  $\alpha$ CD in urea/sucrose 1.00 (normalized), and 5 mM  $\alpha$ CD in glycerol/sucrose gradients  $1.01 \pm 0.02$ ).

In addition, several ethylene glycols (484 mM ethylene glycol, 430 mM triethylene glycol, 420 mM PEG200) were also osmotically substituted for urea. Because the relation between osmolarity and density of aqueous solutions of these compounds



**FIG. 4. Block of ascorbate permeation through connexin channels by CD.** Graph shows reduction of cytochrome *c* ( $A_{417\text{nm}}$ ) entrapped in Cx32-containing liposomes by extraliposomal application of ascorbate in 150 mM NaCl. When ascorbate was added the cytochrome *c* was reduced (*upper line*). When ascorbate was added in the presence of 10 mM  $\alpha$ CD (*lower line*), reduction of cytochrome *c* was fully blocked. These data indicate that ascorbate can permeate Cx32 channels, and that this permeability is blocked by the same concentration of  $\alpha$ CD that showed block in the TSF experiments, in the absence of the TSF solutes.

differed from that of urea and glycerol, adjustments to the osmotic and density properties of the TSF and reconstitution solutions were required, but the results were the same as for glycerol; there was no effect of these substitutions on channel permeability with or without co-application of CD. These results show that CD block of connexin pores does not depend on chemistry specific to urea.

An additional experiment was performed to assess CD-induced block in the absence of TSF gradient solutes. Cytochrome *c*, which is too large (12.4 kDa) to permeate connexin channels, was loaded into Cx32 liposomes during formation. Osmotic balance was obtained with NaCl rather than urea or sucrose. The liposomes were then incubated with and without a blocking concentration (10 mM) of  $\alpha$ CD. Ascorbate (150 mM) was then added to the extraliposomal solutions, and reduction of the entrapped cytochrome *c* monitored photometrically ( $A_{417\text{nm}}$ ) (49) (Fig. 4). Following addition of ascorbate, in the absence of CD, the cytochrome *c* was rapidly reduced by ascorbate flux into the liposomes through connexin channels. With addition of ascorbate in the presence of CD, there was no reduction of ascorbate. Liposomes containing no Cx32 showed no cytochrome *c* reduction with the addition of ascorbate (data not shown). Also, reduction of cytochrome *c* in solution (not in liposomes) by ascorbate was not affected by the presence of CD. This study shows that ascorbate is permeable through Cx32 channels and that  $\alpha$ CD blocks permeability to ascorbate, in the absence of urea and sucrose.

**Occupancy of the CD Cavity Potentiates Block**—CDs form stable inclusion complexes with various molecules (“guests”) that interact with the internal cavity. Many factors contribute to the stability of these inclusion complexes, but because the formation of most complexes is exothermic, lowering the solution temperature favors complex formation and stability (69, 70). For the  $\alpha$ HL pore, the stability of CD within the  $\alpha$ HL pore was potentiated when the cavity of the CD was occupied by small organic guest molecules (19).

To characterize further the nature of CD interaction with connexin, permeability studies were carried out in the presence of CDs and some of their known water-soluble and hydrophobic guest molecules (19, 71, 72). For these studies, the CDs were applied at concentrations that had no effect on Cx32 channels when applied alone (2 mM  $\alpha$ CD and 5 mM  $\beta$ CD) at 4 °C. The guest molecules were naproxen for  $\alpha$ CD, and 2-amino-adamantanamine (2AA) and cinnamic acid for  $\beta$ CD, all applied at 10

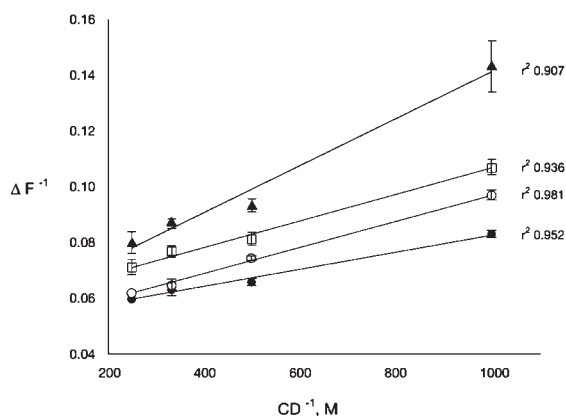


FIG. 5. Benesi-Hildebrand plots showing effect of TSF components on occupancy of the CD. The fluorescence of 3H2NA increases when complexed with  $\beta$ CD (50). The 3H2NA fluorescence ( $\lambda_{\text{ex}}$  350 nm,  $\lambda_{\text{em}}$  521 nm) was measured over a range of  $\beta$ CD concentrations in the presence of TSF buffer components and graphed as double-reciprocal plots. The slopes of the relations give the  $\beta$ CD-3H2NA binding constants (75) (see text for values). The slopes are as follows: filled triangles, control; open circles, with 459 mM urea; open squares, with 400 mM sucrose (open squares); filled circles, with urea and sucrose. The data show that maximal TSF concentrations of urea or sucrose cause a modest disruption of the  $\beta$ CD-3H2NA complex and that the effects are synergistic when both are present.

mM. By themselves these guest compounds had no effect on channel activity (naproxen could not be tested due to insolubility). The water-soluble guests 2AA and cinnamic acid were added to the TSF solutions, and the water-insoluble guest naproxen was dehydrated into the CD annulus prior to TSF by repeated lyophilization (71).

In each case, with co-application of CD plus guest there was substantial pore block at 4 °C as follows: 35  $\pm$  4% for  $\alpha$ CD-naproxen, 31  $\pm$  5% for  $\beta$ CD-2AA, and 49  $\pm$  0% for  $\beta$ CD-cinnamic acid. Taking advantage of the instability of the CD-guest complexes at higher temperature, permeability in the presence of the  $\beta$ CD-2AA was assessed at 37 °C. The block of channel permeability was eliminated at the higher temperature, consistent with release of guest.

These data show that the presence of guest causes block at CD concentrations that do not cause block on their own. As mentioned, guest occupancy of CDs enhances the affinity of CDs for the  $\alpha$ HL pore (19). The data reported here suggest that a similar effect occurs for connexin channels. The mechanism for guest-induced stability is unknown but likely involves distortion of the CD molecule because of guest occupancy (73). No matter what the mechanism, at a minimum the guest data indicate that the central cavity of the CD, and thus the CD, is located within the connexin pore.

The partial rather than complete block caused by CD-guest is likely because of incomplete loading of the water-insoluble guest naproxen and the relatively low stability constants for the other two (74). Under these conditions, guest occupancy cannot be assumed to be 1.0.

**Effect of Buffer Components on CD Occupancy**—Because occupancy of the central CD cavity influences block, we investigated the influence of TSF buffer components on CD inclusion properties by using the fluorescence of guest molecule (3-hydroxy-2-naphtholic acid; 3H2NA). The fluorescence of 3H2NA increases when complexed with  $\beta$ CD, with which it forms 1:1 inclusion complexes at 20  $\mu$ M (50). The 3H2NA fluorescence ( $\lambda_{\text{ex}}$  350 nm,  $\lambda_{\text{em}}$  521 nm) was measured over a range of  $\beta$ CD concentrations in the presence of various combinations of TSF buffer components and graphed as double-reciprocal Benesi-Hildebrand plots (75) (Fig. 5). The slopes of the relations give the CD-3H2NA binding constants. The data show that the

maximal TSF concentrations of urea and sucrose each cause a modest disruption of the  $\beta$ CD-3H2NA complex and that the effects are synergistic when both are present: control  $K_m$ , 33.1  $\pm$  2.9  $\mu$ M, with urea 47.0  $\pm$  3.2  $\mu$ M, with sucrose 44.0  $\pm$  0.9  $\mu$ M, and with urea plus sucrose 90.2  $\pm$  4.7  $\mu$ M. In contrast, the other TSF components (10 mM KCl and 10 mM HEPES) were without significant effect, either alone or in combination with the other solutes. We found that 3H2NA did not form inclusion complexes with the other two CDs (data not shown), so analogous experiments were not carried out for them. However, urea and sucrose can be expected to have effects similar in character, if not degree, on their complexation properties.

These data show that urea and/or sucrose can disrupt the  $\beta$ CD-3H2NA complex to some extent. The possible relevance of effects on CD cavity occupancy by urea and sucrose is discussed below.

## DISCUSSION

The data show that CDs can reversibly block molecular permeation through connexin channels. The block-concentration relations change as a function of the relative sizes of the connexin pores and the CDs, indicating that the CDs act within the pore. The smaller CDs produce a readily interpretable block of Cx26 and Cx32 channels. The nature of the interaction of CDs that are large relative to the pores is different, complex, and unclear.

The interactions of  $\alpha$ CD and  $\beta$ CD with Cx32 channels can be characterized as simple binding within the pore, in which binding results in block to sucrose and urea. The step-change nature of the block-concentration relations and the absence of an intermediate band indicate that there is a single dominant mode of interaction that reaches saturation at  $\sim$ 6 and  $\sim$ 15 mM for  $\alpha$ CD and  $\beta$ CD, respectively. The interaction of  $\alpha$ CD with Cx26 channels is similar, but the initial sharply graded effect likely indicates minor, secondary mode(s) of interaction. The CDs block permeation of ascorbate, and complexation of guest molecules in the CD cavity enhances the block of connexin channels, as it does for the  $\alpha$ HL pore.

For each type of connexin channel, with increasing size of CD the block becomes graded with concentration and has a component of block to sucrose but not urea, indicating a different set of interactions. These effects are difficult to interpret but are clearly consequences of the increased CD size. One can speculate that permeability to urea but not sucrose occurs when the CD is improperly configured within the connexin pore for complete or consistent block, with urea leaking between the CD and the pore wall.

The fact that CDs block only when applied from the cytoplasmic (extraliposomal, in these studies) end of the pore precludes an effect due to binding or entry at the extracellular end. Thus we would not expect CDs to act on open hemichannels in nonjunctional plasma membrane.

The site of the narrowing of the Cx26-containing pores is unknown, but given that Cx26 pores are more size-restrictive than Cx32 pores (25)<sup>2</sup> and yet have a greater ionic conductance (76, 77), it is presumed that the narrowing in Cx26 is over a short axial distance. One does not know where the narrowest part of the pore is for either channel formed by these connexins, although the 7-Å resolution structure of channels formed by a truncated Cx43 indicates it is toward the extracellular end of the pore for that connexin (78). The CD-limiting site may be at the narrowest part of pore or it may be separate, at a position more cytoplasmic. For this latter possibility, the unique pattern seen for Cx26 and  $\alpha$ CD (graded slope followed by a step) could correspond to poor fit at the initial constriction, followed by mass action-driven occupancy near the permeant-restricting

narrowing deeper in the pore, allowed by flexibility of the  $\alpha$ CD (64, 65).

However, the simplest explanation is that the barrier to CDs and to the uncharged sugar probes that characterize pore diameter (25) is the same structure. Our data cannot distinguish whether this barrier is at the cytoplasmic mouth of the pore or within the pore lumen.

**Possible Role of Sucrose in Block of Urea Permeation**—From size considerations, urea (diameter  $\sim 3.6$  Å (79)) ought to permeate the hydrophobic internal cavities of all the CDs tested. However, the data show block to both urea and sucrose. There are two possible explanations. One is that urea is unable to pass through the CD inner cavities when the CD is in the connexin pore, and the other is that a CD-sucrose inclusion complex forms that blocks urea permeability through the CD central cavity.

In support of the first possibility, it has been reported that all three CDs completely block ionic conductance through the CymA pore (22). If ionic conductance is blocked, one expects a block to small molecules such as urea, glycerol, and sucrose.

In apparent contradiction, other studies show that when the CDs are lodged in the  $\alpha$ HL pore, a substantial ionic conductance remains, which can be reduced by occupancy of a guest molecule in the CD cavity (19, 21). This suggests either that the remaining conductance is through the central cavity or that guest occupancy alters CD structure so that it makes a tighter fit with the walls of the pore.

The difference in the two studies may imply that the host pore (*i.e.* CymA or  $\alpha$ HL) can affect the conformation of an internally bound CD so as to alter the size of the central cavity and what can pass through it. There are no data on this point or directly showing molecular flux through the central cavity of CD in any system, with the exception of water (80).

If when the CDs are in the connexin pores the central cavity allows permeation by urea, is it possible that in TSF gradients sucrose forms an inclusion complex within the CD cavity and thereby blocks urea permeability? Typically, a guest molecule displaces bound water from the internal cavity and becomes “solvated” via interactions with the internal surface of the cavity (70). The Einstein-Stokes size of hydrated sucrose (diameter  $\sim 10.4$  Å (79)) is too large to fit in the cavity of even  $\gamma$ CD, the largest tested. With dehydration it could occupy the cavity of  $\beta$ CD, as it is approximately the same dimension and is weakly hydrophobic. In fact, formation of 1:1 and 2:1 complexes between sucrose and  $\beta$ CD has been demonstrated (81). In addition, we demonstrated that sucrose can displace the fluorescent reporter 3H2NA from the  $\beta$ CD cavity to some degree. Our 3H2NA data also suggest that formation of a sucrose inclusion complex could be aided by the presence of urea.

The experimental data on this point apply only to  $\beta$ CD. Even fully dehydrated sucrose is too large to fit completely into the cavity of  $\alpha$ CD. There are no data on formation of sucrose inclusion complexes with  $\alpha$ CD, but there is evidence that the glucosyl and fructosyl portions of sucrose individually fit well in  $\alpha$ CD (74, 82), which could lead to occlusion of the  $\alpha$ CD pore by the sucrose molecule.

Thus, if the CD-connexin complex allows a patent cavity in the CD, it is possible that the formation of a sucrose-CD inclusion complex produces the observed block to urea permeation. This is an intriguing possibility, and if correct, CD-induced block to small permeants such as urea, as opposed to somewhat larger ones such as ascorbate, may depend on formation of inclusion complexes with molecules such as sucrose. It will be interesting to test in cellular studies whether cytoplasmic components can play this role.

To test whether urea block was secondary to occupancy of the

CD cavity, we sought to identify a suitable solute to replace sucrose in the TSF gradients. We could not identify a compound with the required characteristics (small, uncharged, permeable, and with appropriate osmolarity-density relation). The gradient medium hypaque (sodium diatrizoate; 635 Da) was a potential candidate for use with acetate rather than urea (both gradient solutes must be either uncharged or charged to avoid development of voltages across the liposome membrane because of combined Donnan and osmotic effects). However, we found that hypaque (weakly hydrophobic) displaced 3H2NA from the  $\beta$ CD cavity as well as did sucrose (and the acetate had no effect), so it was unsuitable to use with acetate to determine whether the urea block was because of CD cavity occlusion.

Thus there is evidence supporting two explanations as to why CDs block permeability to urea: it may be impermeable, or it may be blocked by formation of an inclusion complex involving sucrose. If the latter explanation, it may be that interactions between the TSF solutes and CDs allow detection of CD residency in the connexin pore more dramatically than would otherwise be the case.

No matter what the explanation regarding urea permeability, CDs can block molecular permeation through connexin pores. This was independently demonstrated by the block to ascorbate permeation by CDs in the absence of TSF solutes.

**Mechanism of CD-Connexin Interaction**—For studies of connexin channel structure function, it will be important to identify the site(s) where CDs interact with the lumen of the connexin pore. Unfortunately, the existing data on CD-protein interactions are not informative on this point. There are crystal structures of CDs bound to several proteins, including cyclodextrin glycosyltransferases (64, 83),  $\alpha$ - and  $\beta$ -amylase (84–86), and maltose-binding protein (87–89). The identifiable mechanisms of binding are similar. Typically, the external apolar surface composed of the primary hydroxyls has a stacking interaction with aromatic side chains of the protein. There is a relatively low amount of hydrogen bonding and some direct van der Waals contacts. However this information is not informative in localizing a binding site. Not surprisingly, there are several aromatic amino acids in the connexin domains suspected to be pore-lining (90–94).

Several bacterial proteins bind or transport CDs (CymE, CymA, and CycB) (20, 22, 95, 96). Their affinities for CDs range from 30  $\mu$ M to 3 mM depending on the protein and the CD. Unfortunately, a binding site has not been identified.

Gu *et al.* (97) attempted to identify a cyclodextrin-binding site in  $\alpha$ HL by mutagenesis at a position in the pore thought to be at the narrowest point. Replacement of a methionine with 19 other residues identified 6 that decreased the dissociation rate of the CD 3 to 4 orders of magnitude. Unfortunately, the properties of these amino acids (Val, His, Tyr, Asp, Asn, and Phe) do not suggest a common mechanism by which they affect the CD off-rate in  $\alpha$ HL pores.

It is clear, however, the CDs must dehydrate substantially to enter the pore. The “diameters” of the CDs with all associated water molecules is  $\sim 56.6$  Å for  $\alpha$ CD, 73.0 Å for  $\beta$ CD, and 85.8 Å for  $\gamma$ CD (98); such dimensions would preclude entry into connexin channels.

**Connexin Pore Blockers**—Until recently, no pore blockers of connexin channels had been identified. Voltage-dependent block of Cx40 and Cx43 junctional channels by several tetraalkylammonium ions has been demonstrated (99), as has block of Cx40 junctional channels by the polyanion spermine (100).

For the tetra-alkylammoniums, complete block could not be achieved even at high voltages for the highest concentration tested (10 mM). Their usefulness is limited by their poor permeability to the blocking site. The block by spermine seems to

be specific for Cx40, because it does not block Cx43 channels (100) and readily permeates Cx38 channels (101). In addition, its voltage dependence was such that very high concentrations were required for complete block near zero membrane voltage (the  $K_m$  at +4 mV was 25 mM). Despite these limitations, these agents may be useful for the study of specific connexins (102).

For cellular studies, it would be useful to have open pore blockers that give complete block of connexins at reasonable concentrations at 0 mV. For biophysical studies, it would be useful for blockers to have reasonably rigid, well defined structures amenable to chemical modification. This report identifies CDs as a class of blockers that satisfies most of these criteria. Because the block is a function of pore diameter, it is connexin isoform-specific to the degree that the pores formed by the different connexin isoforms have different limiting diameters.

Because of the interest in CDs as drug delivery agents, a large number of modified CDs have been synthesized and characterized (18, 103). Most of the modifications are substitutions at the primary and secondary hydroxyl groups at the upper and lower ends of the torus and offer structural variants that differ in size, hydrogen bonding capability, external hydrophobicity, and charge. Preliminary studies (38) using CDs derivatized with sulfate or hydroxypropyl moieties suggest enhanced interaction with connexin channels. These modified CDs, as well as the unmodified forms studied here, have substantial promise as molecular tools for the study of the structure-function of the connexin pore, and potentially for modulation of connexin channel permeability.

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