Localization of a Voltage Gate in Connexin46 Gap Junction Hemichannels

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ABSTRACT Cysteine replacement mutagenesis has identified positions in the first transmembrane domain of connexins as contributors to the pore lining of gap junction hemichannels (Zhou et al. 1997. *Biophys. J.* 72:1946–1953). Oocytes expressing a mutant cx46 with a cysteine in position 35 exhibited a membrane conductance sensitive to the thiol reagent maleimidobutyryl biocytin (MBB). MBB irreversibly reduced the single-channel conductance by 80%. This reactive cysteine was used to probe the localization of a voltage gate that closes cx46 gap junction hemichannels at negative potentials. MBB was applied to the closed channel either from outside (whole cell) or from inside (excised membrane patches). After washout of the thiol reagent the channels were tested at potentials at which the channels open. After extracellular application of MBB to intact oocytes, the membrane conductance was unaffected. In contrast, channels treated with intracellular MBB were blocked. Thus the cysteine in position 35 of cx46 is accessible from inside but not from the outside while the channel is closed. These results suggest that the voltage gate, which may be identical to the "loop gate" (Trexler et al. 1996. *Proc. Natl. Acad. Sci. USA.* 93:5836–5841), is located extracellular to the 35 position. The voltage gate results in regional closure of the pore rather than closure along the entire pore length.

INTRODUCTION

Gap junction channels are composed of two hemichannels, residing in apposed cell membranes. Each hemichannel is formed from six subunits, connexins (Beyer et al., 1990), which are docked to each other to form a hydrophilic path between adjacent cells. Thus the gap junction channel pore, unlike other ion channel pores, is only accessible from the inside of cells. Although the permeation pathway has been characterized in terms of permeability limits and channel conductance in complete gap junction channels (Bennett et al., 1991; Veenstra, 1996; Loewenstein, 1981), the limited access to the pore has hampered progress in identifying pore lining and gating domains.

Recently, both wild-type and mutant connexins have been discovered that form open gap junction hemichannels (Paul et al., 1991; Ebihara and Steiner, 1993; Gupta et al., 1994; Pfahnl et al., 1997). The hemichannels are gated and respond to changes in voltage or pH like complete gap junction channels (Ebihara et al., 1995). Open hemichannels provide direct access to the pore from both the extracellular and the intracellular sides. Cysteine scanning mutagenesis of cx46 gap junction hemichannels revealed two amino acid positions (I34 and L35) in the first transmembrane region (M1) that react with extracellularly applied agents, suggesting that these positions may be part of the pore lining (Zhou et al., 1997). The mutation of an amino acid in a position equivalent to position 35 in two other connexins has been shown to cause disease in humans (Kelsell et al., 1997; Tan et al., 1996). Cx32 M34T has been shown to have altered single-channel properties consistent with a pore lining function of this amino acid (Oh et al., 1997). In this study we test the accessibility of the 35 position for intra- or extracellulary applied reagents with and without activation of the voltage gate.

Channels of mammalian gap junctions, depending on their connexin composition, respond to changes in transjunctional voltage with different sensitivities. In addition, the polarity of gating is connexin specific. Gating polarity is typically determined in asymmetrical gap junction channels composed of hemichannels with different voltage sensitivities (Werner et al., 1989; Barrio et al., 1991; Verselis et al., 1994; White et al., 1994; Rubin et al., 1992; Bukauskas et al., 1995). Cx46 was found to have a positive gating polarity; i.e., in heterotypic junctions the cx46 gap junction hemichannel closed at positive potentials in the cell expressing it (White et al., 1994). On the other hand, the gating polarity of cx46 gap junction hemichannels was found to be the opposite: these channels close when the cytoplasm is negative (Paul et al., 1991). White et al. (1994) interpreted these findings as an allosteric modification of the gating polarity consequent to docking of hemichannels to each other. However, these results can also be interpreted as indicating the presence of more than one voltage gate. Indeed, Trexler et al. (1996) determined on the basis of single-channel records that the voltage sensitivity of cx46 gap junction hemichannels is much more complex. The channels close at negative intracellular potentials. However, it was also found that channel conductance (γ) was gradually reduced from negative to positive potentials. At positive potentials the channel was found to stay preferentially at a subconductance level. Paradoxically, the open probability (p_o) increased steadily from negative to positive potentials. Trexler et al. (1996) attributed this complex voltage response of cx46 gap junction hemichannels to the presence of two independent voltage gates with opposite gating po-

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larities. Because of similarities with the voltage dependence in intact gap junctions, the changes at positive potentials were referred to as the junctional gate (Vj), and the reduction of p_o at negative potentials, which closed hemichannels, was referred to as the “loop” gate.

Here, we test the two-gate hypothesis and attempt to localize a voltage gate with respect to a position in the pore segment of cx46 gap junction hemichannels. To avoid prejudice, we call the gate that closes the channels at negative potentials the p_o gate. We localize the p_o gate to be extracellular relative to the 35 position.

MATERIALS AND METHODS

Preparations of mutants, mRNA, oocytes, and whole-cell electrophysiological recording were performed as described previously (Dahl, 1992). Briefly, a cx46 clone was obtained from Dr. D. L. Paul (Paul et al., 1991) and was used to make the cx46L35C mutant as described previously (Zhou et al., 1997). Oocytes were injected with ~20 nl of in vitro transcribed connexin mRNA and incubated for 18–24 h in oocyte Ringer’s solution (OR2), with elevated Ca^{2+} concentration (5 mM) to keep the gap junction hemichannels in the closed state (Ebihara and Steiner, 1993). Whole-cell membrane currents of single oocytes were measured using a two-microelectrode voltage clamp and recorded with a chart recorder. Electrodes were pulled with a vertical puller and filled with 1 M KCl. Oocytes were transferred to a dish with regular OR2 (1 mM Ca^{2+}) and then voltage clamped at a holding potential of −10 mV, unless specified differently. The dish was perfused continuously with solution. Membrane conductance was determined using repetitive voltage steps of 5 s duration and of 5 mV amplitude, unless specified differently.

Single connexin hemichannels were studied by the patch-clamp technique (Hamill et al., 1981) with an Axopatch-1B amplifier (Axon Instruments). Recordings were filtered at 5 kHz and digitized at 10 kHz with a VR-10B digital data recorder and stored on videotape. The recordings were later transferred to a Power Macintosh (Apple) computer with an ITC-18 Computer Interface (Instrutech Corporation) and analyzed. The acquisition and analysis software was Acquire and TAC (both from Bruxton Corporation).

All recordings were made at room temperature (21–23°C). Patch pipettes were made from glass capillary tubing with filament (GC150F-15; Warner Instrument Corporation). Patch pipettes were pulled with a P-97 micropipette puller (Sutter Instrument Company), and the tips were polished with a microforge (Narishige Scientific Instruments) to a resistance of 10–20 MΩ in symmetrical solution.

After a patch was pulled and a potential hemichannel identified, the patch was moved into a microperfusion chamber. The microperfusion chamber had a diameter of 1.5 mm and was made by blowing out the sealed end of a glass pipette. The chamber was continuously perfused with solution. The basic pipette and bath solution consisted of 140 mM potassium gluconate, 10 mM KCl, 5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES), pH 7.5 (KG). Additional reagents or pH changes were made to the basic solution and are specified in the Results. The additional reagents included maleimidobutyryl-biocytin (MBB) (0.1 mM; Calbiochem) and Tris-(2-carboxyethyl)phosphine (TCEP) (1.0 mM; Molecular Probes). MBB was always freshly prepared from a stock solution in dimethyl sulfoxide (100 mM). Voltage ramps were applied by connecting to the external command of the amplifier a custom-designed voltage ramp generator.

RESULTS

Fig. 1 illustrates the strategy used in the present study to localize the voltage gate in gap junction hemichannels. We tested the accessibility of position 35 in the cysteine re-

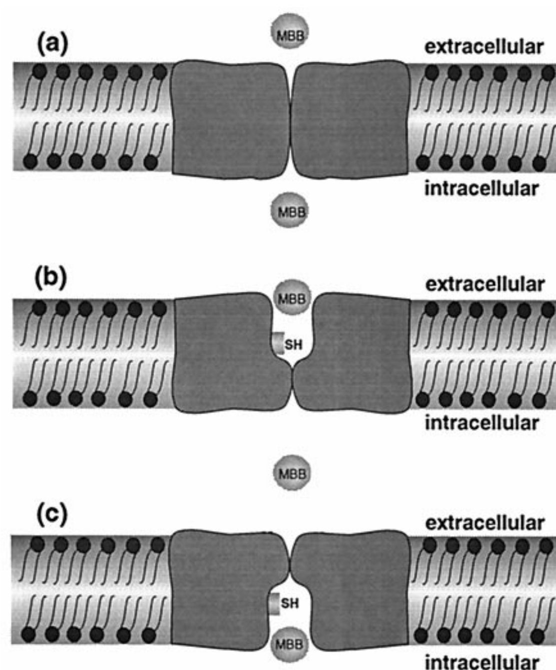

FIGURE 1 Model of gap junction hemichannel gating. Channel closure may involve collapse of a large fraction or all of the channel pore (*a*) or may be a regional phenomenon in which only part of the conduction pathway is occluded (*b* and *c*). The partial occlusion may be intracellular (*b*) or extracellular (*c*) to a reference point, in the form of a reactive cysteine within the pore (SH). Application of a thiol reagent to a closed channel would (*a*) remain without effect, (*b*) affect the channel only when applied from the extracellular side, (*c*) affect the channel only when applied from the intracellular side.

placement mutant cx46L35C to the thiol reagent MBB. Previous studies have indicated that the 35 position in cx46 and its equivalent in other connexins are part of the pore lining (Zhou et al., 1997; Oh et al., 1997). If the gate were to occlude the whole pore (Fig. 1 *a*), MBB would reach the 35 position from neither end of a closed channel. If the gate occludes only part of the pore length, however, 35C should be accessible to MBB when applied from one side but not the other while the channel is closed (Fig. 1, *b* and *c*). For example, if the gate were located on the cytoplasmic side, cytoplasmic application of MBB should not result in a reaction with 35C, whereas extracellular application should yield an altered channel (Fig. 1 *b*). The converse would be expected for a localization of the gate extracellular relative to the 35 position (Fig. 1 *c*).

Characterization of cx46L35C channels

Previously the effect of MBB had been studied with extracellular application of the thiol reagent to whole oocytes expressing the cysteine mutant cx46L35C (Zhou et al., 1997). As a first step, we therefore determined whether intracellular MBB application affected the open channel. All experiments were performed on *Xenopus* oocytes expressing wild-type cx46 or cx46L35C. For useful interpre-

tation of the thiol reaction, experiments involving intracellular application had to be performed on excised inside-out patches, preferably with single cx46L35C hemichannels.

The cx46L35C mutation per se does not seem to interfere with channel function. Both macroscopic and single-channel currents of channels formed by the cx46L35C mutant were almost identical to those of cx46 wild-type channels (Paul et al., 1991; Ebihara and Steiner, 1993; Trexler et al., 1996). Mutant and wild-type channels were similarly gated by cytoplasmic acidification, increased calcium concentration (not shown), and voltage. As reported earlier for wild-type cx46 channels (Paul et al., 1991; Ebihara and Steiner, 1993; Trexler et al., 1996), both opening and closing of the channels were slow processes requiring tens of seconds to reach steady state after a voltage jump (Fig. 2 *a*). Fig. 2 *b* shows steady-state macroscopic conductance as a function of holding potential. The channels were closed at negative potentials and started to open between −30 and −20 mV. Further depolarization increased conductance steeply. Single cx46L35C mutant channels exhibited slow openings and closings, including intermediate substates (not shown) as described by Trexler (Trexler et al., 1996). Furthermore, the voltage dependencies of mutant and wild-type channels were indistinguishable (Fig. 3, *a* and *b*, and Trexler et al., 1996). Voltage affected these channels in four ways. First, the single-channel conductance decreased when the holding potential was changed from negative to positive (Fig. 3 *a*). Second, the channel often entered a subconductance level at positive potentials; for example, in the traces shown in Fig. 3, *a* and *b*, the channels resided at a subconductance level and not at zero (between +30 and +60 mV). Third, at positive potentials transitions to the closed state were less frequent (Fig. 3 *a*). Fourth, the channels remained mainly in the closed state at negative potentials (below −40 mV). To illustrate these different points, the voltage ramps applied to the membrane patches in Fig. 3, *a* and *b*, were the opposite. For Fig. 3 *a* the patches were held at positive potentials before application of the voltage ramp. Because of the slow inactivation of these channels, channel activity was still seen to potentials as negative as −60 mV. For Fig. 3 *b* the patch was held at a negative potential (−80 mV) before application of the voltage ramp. Under this condition the channels opened at −30 mV. In the record shown (Fig. 3 *b*) the activity at potentials more negative than −30 mV was caused by a second, small-conductance, endogenous channel present in that particular patch.

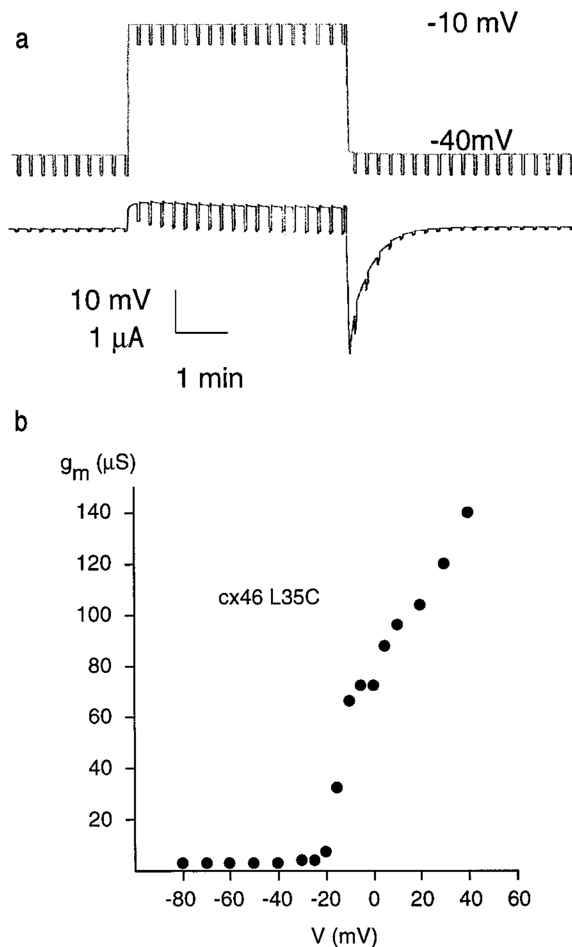

FIGURE 2 (*a*) Effect of holding potential on membrane currents induced by 5-mV test pulses in an oocyte expressing cx46. After changes in the holding potential, the currents reach steady-state levels in ~1 min. (*b*) Steady-state membrane conductance as a function of membrane potential in a single oocyte expressing cx46L35C gap junction hemichannels. Membrane conductance was tested by holding the oocyte at various potentials between −80 and +40 mV for 5 min to achieve steady state and applying 5-mV test pulses.

Macroscopic and microscopic channel records are consistent with each other despite an apparent discrepancy. While the single channel rectified and preferentially resided in a subconductance state with positive potentials, no comparable drop in macroscopic conductance was seen. Several factors probably contribute to this discrepancy. First, with the high level of membrane conductance in cx46L35C-expressing oocytes, access resistance limitation (Wilders and Jongsma, 1992) has to be considered. Second, at positive potentials other ion channels like the slow sodium channel (Baud et al., 1982) open. Finally, at positive potentials the open probability of cx46L35C increases.

Although the mutant channels are very similar to the wild-type channels, a small but significant decrease in channel conductance was noted at positive potentials for mutant channels (Fig. 3 *c*). Such a difference due to an amino acid substitution might be expected if the 35 position was part of the pore lining, as postulated. Yet other interpretations of this phenomenon are possible.

Effect of MBB on single cx46L35C channels

Bath application of the thiol reagent MBB to cx46L35C channels in excised inside-out patches held at negative potentials (−40 mV to −25 mV) substantially reduced channel conductance (Fig. 4). This reduction was irreversible, consistent with the thiol-MBB reaction. The latency of reduction of channel conductance varied, but reduction al-

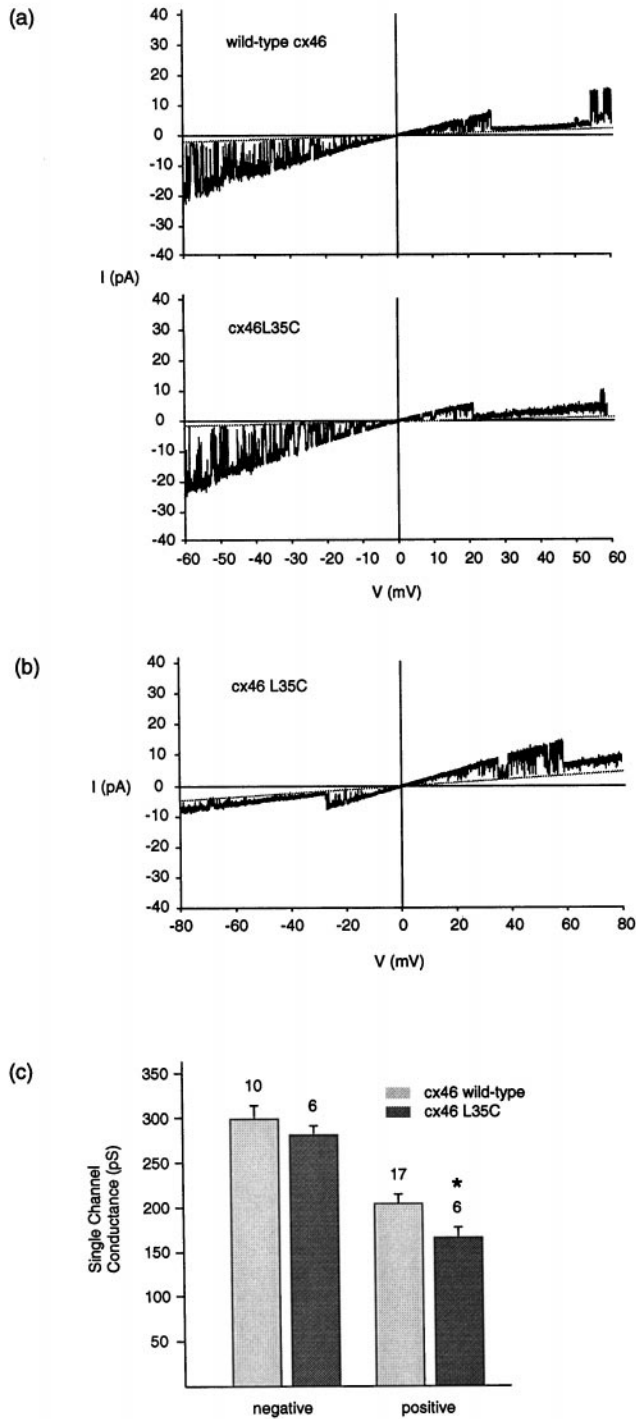

FIGURE 3 Characterization of cx46L35C gap junction hemichannels. (*a* and *b*) Recordings from inside-out excised membrane patches from oocytes expressing wild-type cx46 and cx46L35C. The dotted lines indicate leak conductance (with assumed linearity). The current records were obtained by applying 80-s voltage ramps from +60 mV to −60 mV. Because of the slow inactivation of the channel by negative voltages, activity can still be seen at potentials as low as −60 mV, where under steady-state conditions the channels would be closed. (*c*) Reversing the direction of the ramp (from −80 to +80 mV) shows that channels are closed up to approximately −30 mV. (The activity at potentials more negative than −30 mV is due to the presence of a second, small, nonconnexin channel in this particular patch.) Note that the channel resides mainly at a subconductance level at positive potentials. All patch recordings were obtained in symmetrical 140 mM potassium gluconate, 10 mM KCl, and 5 mM TES (pH 7.5) solutions. (*c*) Analysis of the single-channel conductances of wild-type and mutant channels showed a small but significantly ($p < 0.05$) reduced conductance of mutant channels at positive (+15 mV to +40 mV) potentials but not at negative (−40 mV to −15 mV) potentials. Only full conductance levels were considered for this comparison. The number above the bar indicates the number of patches analyzed. Recording conditions and filter settings are given in Materials and Methods.

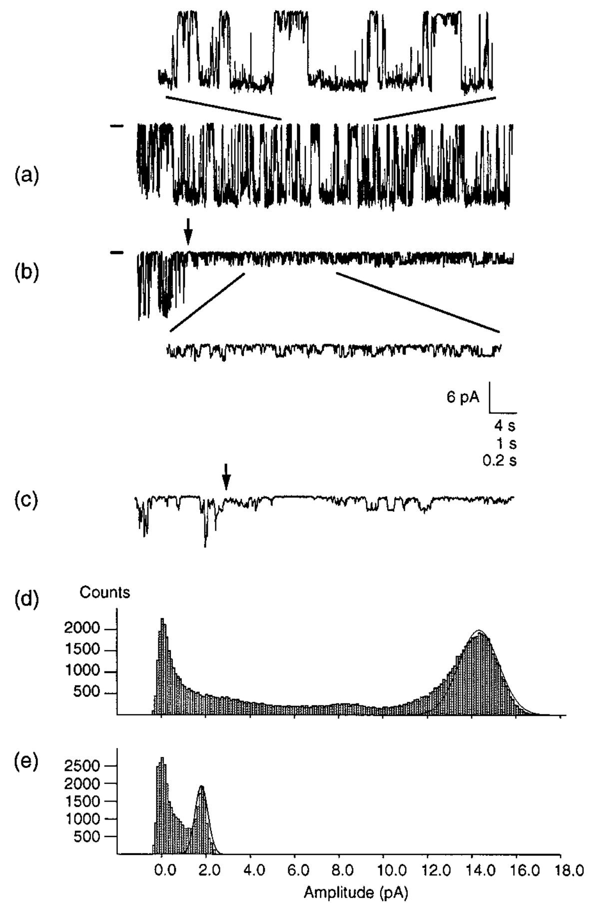

FIGURE 4 Effect of MBB on cx46L35C gap junction hemichannels in excised inside-out patches. MBB (0.1 mM) was applied from the intracellular side. (*a*) A representative 50-s segment showing activity at a −40 mV holding potential before MBB application. A segment with a time scale expanded four times is shown above. (*b*) A representative 50-s segment during MBB application to the same hemichannel held at −40 mV. The arrow indicates the time (74 s after the start of MBB application) at which the full conductance channel disappeared. Instead, a small conductance channel became apparent. A segment with a time scale expanded four times is shown below. The small conductance channel activity remained unchanged upon washout. Tic marks indicate closed level. (*c*) The transition from full channel activity to partial channel block at a time scale expanded 20 times. (*d* and *e*) Amplitude histograms representing 96 s of recorded activity of the same channel at −40 mV before application of MBB (*d*) and after block with MBB (*e*). The amplitude histograms show the absence of the full channel conductance after block (*e*). The partially blocked channel has a conductance ~1/5 that of the full channel.

ways occurred within 3 min. Application of MBB to wild-type channels, even for extended periods of time (5 min), did not affect these channels (Fig. 5).

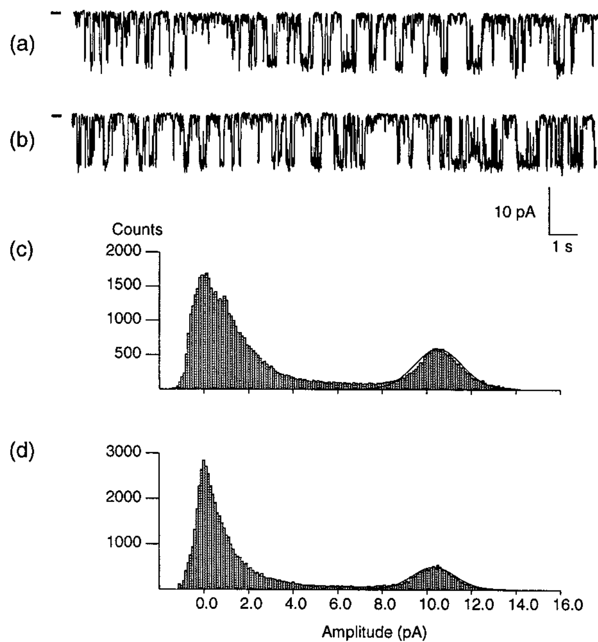

FIGURE 5 Wild-type cx46 gap junction hemichannels are unaffected by MBB. Recordings from an inside-out patch with bath applied MBB (0.1 mM) held at −35 mV. (*a*) An 18-s trace of activity before MBB application. (*b*) An 18-s trace recorded 310 s after the start of MBB application. (*c* and *d*) Amplitude histograms, each representing 96 s of recorded channel activity before and after MBB application. As observed in macroscopic recordings (Zhou et al., 1997), wild-type cx46 is unaffected by the thiol reagent.

The MBB-reacted cx46L35C channel still exhibited properties of the regular channel. The voltage dependence was preserved (Fig. 6): the conductance of the MBB reacted channel was higher at negative than at positive potentials, it gated to a subconductance level at positive potentials, and when held at negative potentials (below −30 mV) the channel closed. Furthermore, channel activity could be abolished by reducing the bath pH to <6.8 (Fig. 7). This effect was reversible, as with the unreacted channel. These data suggest that it is unlikely that MBB causes channels to operate at one of its subconductance levels. Instead MBB probably provides a steric hindrance within the pore.

Accessibility of MBB to closed channels

As shown in Fig. 1, an effect of the p_o gate on the accessibility of the 35 position for MBB was tested with extracellular and intracellular application of the thiol reagent. Extracellular application was performed on intact oocytes, and intracellular application was carried out on excised inside-out patches. As shown in Figs. 2 and 3 *b*, holding membrane potentials below −30 mV closes the cx46L35C channels. In intact oocytes shifting the membrane potential from −10 mV to −50 mV reduced the membrane conductance to levels seen in uninjected oocytes (Figs. 2 and 8), indicating that the majority of the cx46L35C channels were closed. While we held the potential at −50 mV, MBB was applied for 20 min and then washed out. The membrane potential was then switched back to −10 mV. The steady-state levels of membrane conductance at −10 mV before and after application of MBB were not significantly different. However, reapplication of MBB to the same oocytes at −10 mV caused a significant reduction in membrane conductance. These data (Fig. 8) suggest that with extracellular application of MBB the thiol reaction took place at depolarized potentials but not when the channels were in the closed state.

To test the accessibility of the 35 position for MBB from the cytoplasmic side when the channel is closed by the p_o gate, excised inside-out patches were used. Fig. 9 shows an experiment of this type. After recording channel activity at −30 mV (Fig. 9 *a*), the holding potential was shifted to −95 mV (Fig. 9 *b*), and then MBB was applied for 5 min (Fig. 9 *c*). After thorough washing, the holding potential was switched back to −30 mV. The only channel activity to be seen was that of the cx46L35C channel in the partially blocked state (Fig. 9 *d*). A quantitative analysis of the inhibition of channel conductance by MBB shows that irrespective of the holding potential (i.e., conductive state of the channel) at the time of MBB application to the cytoplasmic side of the channel, the thiol reaction results in an irreversible partial block of the channel (Fig. 9, *e* and *f*).

DISCUSSION

Amino acid position 35 of cx46 is pore lining

The pore lining of gap junction channels has long been a matter of speculation, and it had been held that the pore was formed by the third of four transmembrane segments of connexins (Bennett et al., 1991; Unwin, 1989). Only recently has it become feasible to address this question experimentally. Based on data obtained with cysteine scanning mutagenesis on gap junction hemichannels, Zhou et al. (1997) concluded that amino acids within the first transmembrane segment including position 35 (cx46) are part of the pore lining. The position equivalent to 35 in other connexins proved to be critical, inasmuch as mutation results in disease (Kelsell et al., 1997; Tan et al., 1996). Subsequently, Oh et al. (1997) found that mutations within the M1 segment resulted in changes in channel properties and concluded that the first transmembrane domain of cx32 contributes to the pore.

The data obtained in the present study lend further support to the notion that amino acid 35 of cx46 is part of the pore lining of gap junction channels. Reaction of the thiol reagent MBB with a cysteine substituting for a leucine in position 35 of cx46 results in a reduction in the single-channel conductance (γ) to ~20% of the full conductance level. Considering the size of an MBB molecule, which has an abaxial dimension of 11–12 Å, a considerable block of the pore would be expected if MBB were to be bound inside the pore. Consistent with the type of thiol reaction (maleimide), this process is irreversible. This finding corroborates

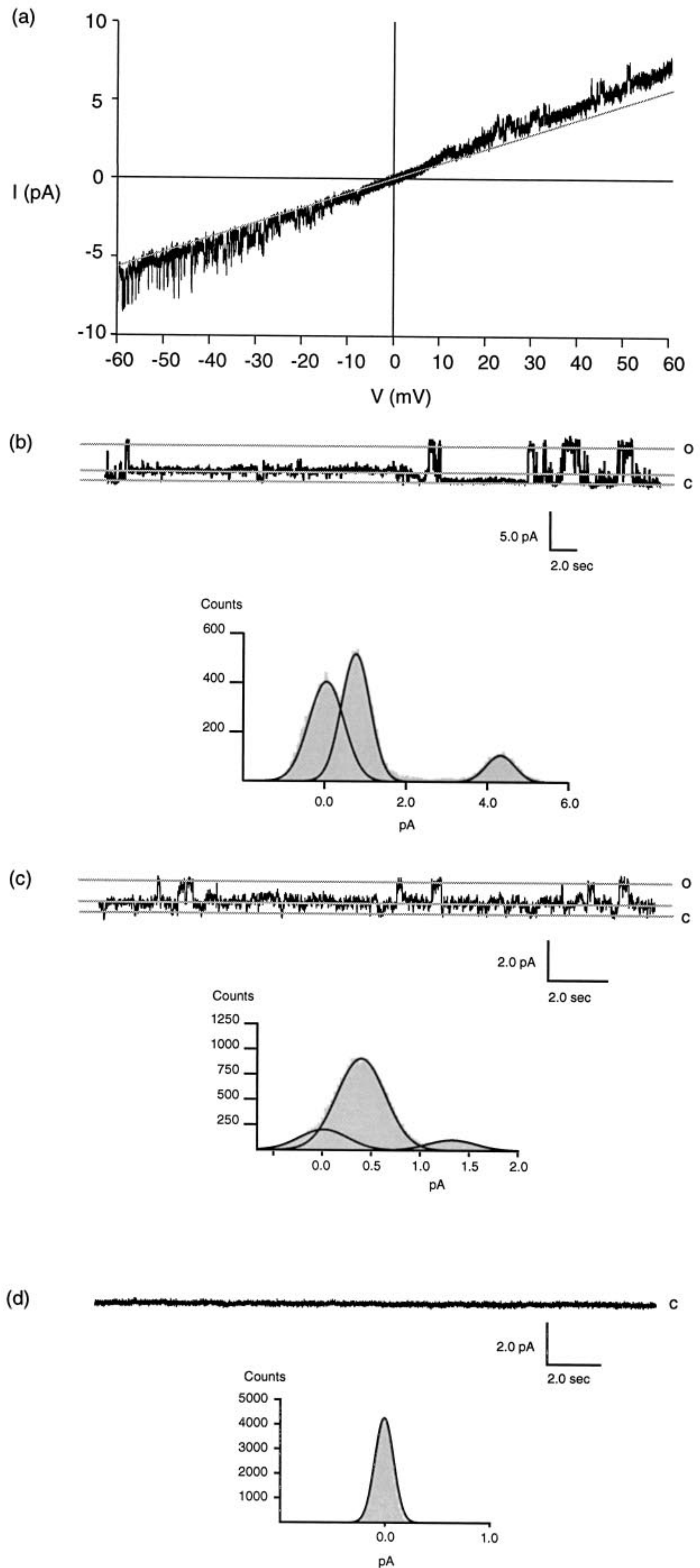

FIGURE 6 Characterization of MBB-reacted channels. (*a*) Voltage ramp (80 s, from +60 to −60 mV) applied to a channel after MBB reaction. The dotted line indicates leak conductance (with assumed linearity). Voltage effects are similar to those shown for the unreacted channel in Fig. 3 *a*, but occur at lower conductances. (*b*) Activity of unreacted cx46L35C channel held at positive potential (+25 mV). Open (o), closed (c), and subconductance levels are indicated by lines. The amplitude histogram is based on analysis of a 96-s segment of the same record. (*c*) Activity of MBB-reacted channel held at positive potential (+25 mV). The amplitude histogram is based on analysis of a 96-s segment of the same record. (*d*) Lack of activity of MBB-reacted channel held at −50 mV.

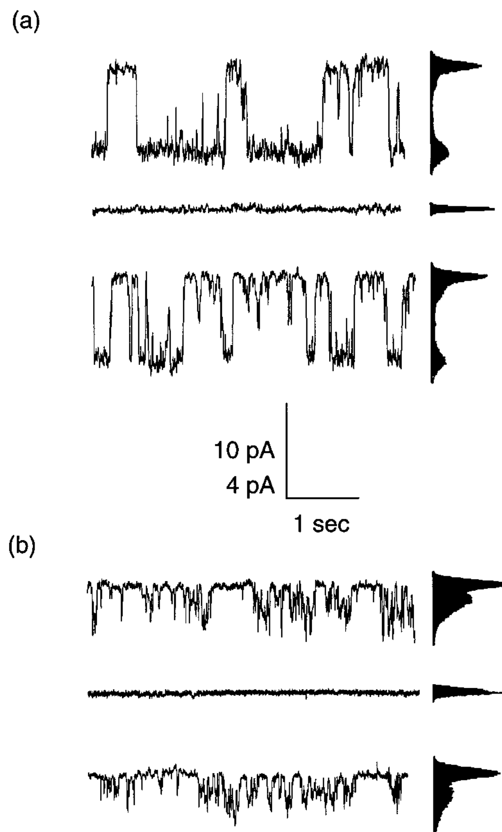

FIGURE 7 Effect of low pH on channel activity of unreacted (*a*) and MBB-reacted (*b*) cx46L35C channel held at −30 mV. *Upper traces*: activity at regular pH (7.5); *middle traces*: lack of activity at pH 6.0; *lower traces*: recovery of activity after return of pH to 7.5.

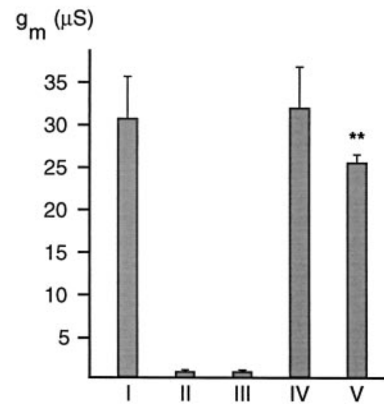

FIGURE 8 Whole-cell currents from single oocytes expressing cx46L35C gap junction hemichannels. (*I*) Oocytes held at −10 mV had a large membrane conductance. (*II*) The holding potential was changed to −60 mV, which closed the cx46L35C channels. (*III*) At −60 mV, the oocytes were superfused with reducing agent (TCEP) for 1 min followed by thiol reagent (MBB) (in OR2). After superfusion of the oocytes with MBB for 20 min the MBB was washed out, and then (*IV*) the holding potential was switched back to −10 mV. Next (*V*), continuing with the same oocytes at a holding potential of −10 mV, application of MBB resulted in an inhibition of membrane conductance. Membrane conductance was determined by applying 5-mV test pulses from the various holding potentials. $n = 5$, *I* and *IV* are not significantly different ($p > 0.05$); ** *V* is significantly different from *IV* ($p < 0.01$)

conclusions based on dye exclusion studies showing that the thiol reaction shifted the exclusion limit to lower levels (Zhou et al., 1997). The level of inhibition seen with single channels exceeds the levels found for inhibition of macroscopic conductance. The reasons for this apparent discrepancy are not clear. One contributing factor could be the peculiar nature of exogenous expression of proteins in oocytes, which entails the constant addition of new channels in the membrane, which in turn results in an underestimation of reacted cysteines. However, even with high expression, the rate of acquisition of new channels (<20% increase in conductance per hour) is too low to account for all of the observed disparity in inhibition levels. On the other hand, there may be a true difference in channel block based on the sidedness of MBB application.

Consistent with the slow decline in macroscopic membrane conductance caused by MBB, the reduction in conductance of single channels was slow to set in, requiring up to 3 min. However, the transition from the full to the reduced conductance state occurred within milliseconds. The present data indicate that the thiol reagent reacts with the cysteine from the inside of the channel pore rather than through the lipid phase, because closure of the channel by application of inside negative voltage prevents the inhibition of channel conductance by MBB applied extracellularly.

Whereas the effect of MBB on single-channel conductance is prominent, there might be an additional effect of MBB on the open probability. In most (but not all) records obtained in this study, the open probability of the MBB reacted channel was found to be lower than before the thiol reaction. This effect may be attributable to one or all of the following. The MBB reaction may indeed change the p_o gating in addition to the effect on γ. Alternatively, the bound MBB molecule within the pore could undergo conformational changes, resulting in a gating effect. Another possibility is that by chance, the channel was observed in a low-activity mode that also occasionally can be seen with unreacted cx46L35C or the wild-type cx46 gap junction hemichannels (unpublished).

Localization of a voltage gate

The data obtained in this study yielded information on the general structure of a voltage gate. Gating structures, particularly structures of voltage gating, have remained obscure, whereas the voltage sensor seems to be well characterized. After Hodgkin and Huxley (1952d), gates of ion channels have typically been presented in a simplified way in the form of a trap-door mechanism. This view dramatically changed after the proposal by Unwin (Unwin and Zampighi, 1980), who, based on diffraction data, depicted channel gating as a process in which subunits change their tilting angle in the membrane. Apparently the tilt angle is a variable (Unwin and Zampighi, 1980; Unwin and Ennis, 1984). This iris-like movement of subunits occludes all or

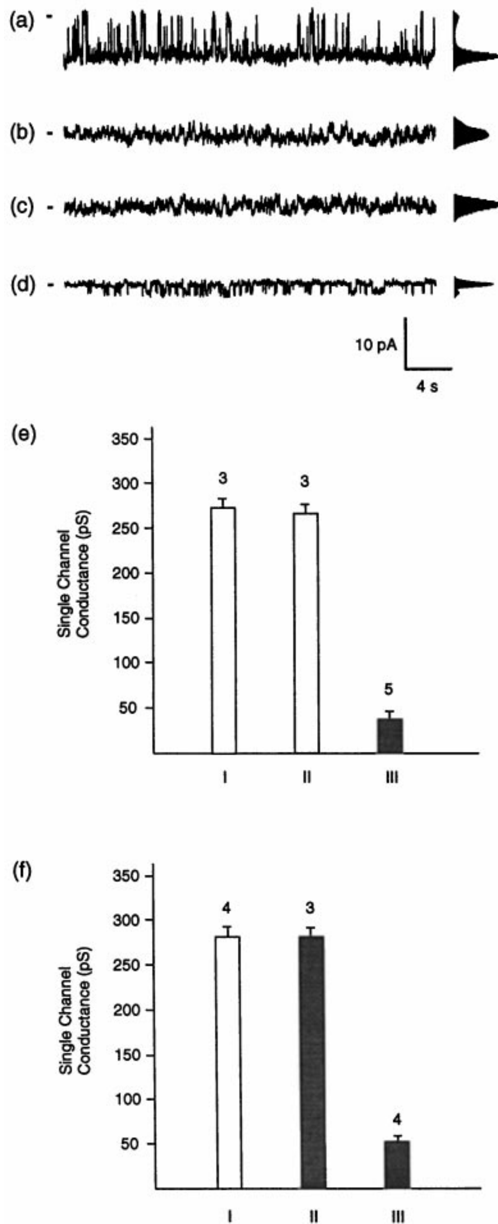

FIGURE 9 Effect of MBB on cx46L35C gap junction hemichannels closed by the p_o gate. (*a*) Activity of a gap junction hemichannel at −30 mV holding potential. (*b*) Holding potential switched to −95 mV, which closed the channel. (*c*) While holding at −95 mV, MBB was applied to the cytoplasmic side of the patch. Channel activity is not discernible in *b* and *c* within the noise amplified by the large driving force. After 5 min the MBB was washed out, and then (*d*) the holding potential was switched back to −30 mV. Although the channel was closed, the 35C position was reactive to MBB applied to the intracellular side of the channel. The amplitude histograms represent 96 s of channel activity. (*e* and *f*) Quantitative analysis of single-channel experiments with excised inside-out patches and the reagents applied to the cytoplasmic side. Plotted are the means (± SE) of single-channel conductances as determined by analysis of 96-s segments for each patch. The numbers of patches analyzed are given above the bars. *White bars*: Wild-type cx46; *hatched bars*: cx46L35C. (*e*) Conductance of wild-type cx46 channels held at −30 mV (*I*). Conductance of wild-type channels held at −30 mV after application of MBB while held at −90 mV (*II*). Conductance of MBB-reacted cx46L35C channels held at −30 mV. MBB was applied to the channels in the closed state (holding potential −95 mV to −50 mV), i.e., no excursion to the open state was seen during the exposure to the thiol reagent (*III*). (*f*) Conductance of wild-type cx46 channels held between −40 mV and −20 mV after exposure to MBB at the same potentials (*I*). Conductance of cx46L35C channels held between −40 and −20 mV after exposure to a reducing agent (TCEP) (*II*). Conductance of cx46L35C channels held between −40 and −20 mV after application of MBB at the same potentials (*III*). The effects of MBB were not significantly different from each other when MBB was applied to the closed channel (*e III*) or to the channel when $p_o > 0$ (*f III*) ($p > 0.05$).

large parts of the channel. Although localized gates like the "ball and chain" inactivation gate of voltage-sensitive ion channels and possibly gap junction channels have been documented (Hoshi et al., 1990; Ekvitorin et al., 1996), this has usually been seen as an exception. Another series of observations with open channel blockers (Holmgren et al., 1997; Liu et al., 1997; Armstrong, 1971) appears to favor a localized gate over a closing of the entire pore, although it cannot exclude a collapse of the pore around the test reagents.

The present study shows that an amino acid within the pore of cx46L35C hemichannels can be accessed from the cytoplasmic side by a large probe, despite the channel being in a closed state. This finding makes a closing of the entire pore unlikely. Instead the observation that the 35 position can be reacted with MBB from the cytoplasmic side but not from the extracellular side while the channel is not conducting (−90 mV holding potential) suggests that the p_o voltage gate of cx46 gap junction hemichannels is a localized gate. This gate apparently occludes only a fraction of the length of the pore, whereas the remainder continues to be accessible to molecules as large as MBB (MW 537).

The sidedness of exclusion and accessibility of MBB to the channel closed by the voltage gate indicates that the gate is localized extracellularly to the 35 position. The polarity of gating is the same as seen with the so-called loop gate (Trexler et al., 1996). However, at present it cannot be concluded what the precise location of the gate is; whether it indeed involves the extracellular loops or extracellular portions of transmembrane segments remains to be determined.

The approach taken here to localize the p_o voltage gate should be useful in mapping other gap junction gates. Both calcium and pH gates should be amenable to this approach. It has to be pointed out, though, that this approach can yield only a rough mapping. The gate can only be localized with respect to the reference point. The precise mapping will require other approaches, like site-directed mutagenesis in the targeted area.

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