GLIC is a homopentameric proton-gated, prokaryotic homologue of the Cys-loop receptor family of neurotransmitter-gated ion channels. Recently, crystal structures of GLIC hypothesized to represent an open channel state were published. To explore the channel structure in functional GLIC channels, we tested the ability of p-chloromercuribenzenesulfonate (pCMBS) to react with 30 individual cysteine substitution mutants in and flanking the M2 channel-lining segment in the closed state (pH 7.5) and in a submaximally activated state (pH 5.0). Nine mutants did not tolerate cysteine substitution and were not functional. From positions 10' to 27', pCMBS significantly modified the currents at pH 7.5 and 5.0 in all mutants except H234C (11'), I235C (12'), V241C (18'), T243C (20'), L245C (22'), and Y250C (27'), which were not functional, except for 12'. Currents for P246C (23') and K247C (24') were only significantly altered at pH 5.0. Reaction rates were all >1000 M⁻¹s⁻¹. The reactive residues were more accessible in the activated than in the resting state. We infer that M2 is tightly associated with the adjacent transmembrane helices at the intracellular end but is more loosely packed from 10' to the extracellular end than the x-ray structures suggest. We infer that the charge-selectivity filter is in the cytoplasmic half of the channel. We also show that below pH 5.0 GLIC desensitizes on a minutes time scale and infer that the crystal structures may represent a desensitized state.

Fast chemical synaptic signaling in the nervous system is dependent on ligand-gated ion channels. The Cys-loop receptor family of neurotransmitter-gated channels encompasses anionic channels that mediate inhibitory signaling [e.g. γ-aminobutyric acid type A (GABA<sub>A</sub>) and glycine receptors] and cationic channels that mediate excitatory signaling [e.g. nicotinic acetylcholine (nACh) and serotonin type 3 (5-HT<sub>3</sub>) receptors] (1-5). These channels are pentameric assemblies in which each subunit has an extracellular domain, 4 transmembrane α-helices (M1 – M4), and a large cytoplasmic domain between the M3 and M4 helices. The channel pore is formed along the radial axis and is lined by the five M2 segments, one from each subunit (6-10). Ligand binding in the extracellular domain induces conformational changes in the transmembrane domain that opens the gate and allows ions to pass through the channel. The gate is located in the middle of the membrane (11-13). Using cryo-electron microscopy Unwin solved the Torpedo marmorata nAChR structure in the absence of agonist at 4 Å resolution (12). The structure was consistent with the subunit organization and topology proposed by earlier studies (1,2,14,5). To date, a high-resolution crystal structure of a full-length eukaryotic Cys-loop receptor is not available.

Recently, prokaryotic Cys-loop receptor homologues were identified based on predicted structural similarities in the extracellular ligand binding domain (15). The Gloeobacter violaceus homologue (GLIC) was functionally expressed in eukaryotic cells and shown to be a proton-gated cation channel (16). GLIC lacks some of the conserved features found in the eukaryotic Cys-loop receptors, including the large cytoplasmic M3M4 loop domain and the pair of Cys that form the eponymous extracellular disulfide-link in the C-ys-loop. GLIC was crystallized at pH 4.6 and 4 and the structures solved to 2.9 and 3.1 Å resolution, respectively (17,18). The GLIC structure shows substantial similarities to the nAChR structure indicating that despite the low degree of amino acid sequence identity GLIC is a Cys-loop receptor homologue. Thus, GLIC may serve as a model in which to study the mechanisms of channel function and the conformational changes that the channels undergo as they transition between the closed, open and desensitized states. However, there are some significant differences between the crystal structures of GLIC and the nAChR (Parikh et al., in preparation). The M2 segments are 1-2 helical turns
longer in the nAChR than in GLIC. This alters the position of aligned residues in the M2-M3 loop and thus interactions between the extracellular and transmembrane domains. The interface between the extracellular and transmembrane domains is critical for signal transduction from the binding site to the gate. Furthermore, while crystal structures provide a tremendous amount of information, it is unclear whether the GLIC structures correspond to an open or desensitized state of the channel. It was reported that GLIC does not desensitize (16,17). This supported the contention that the crystal structures prepared in low pH solutions represent an open state. However, a recent report suggests that GLIC does desensitize (19). Furthermore, to crystallize GLIC the protein was extracted from its native lipid membrane environment and crystallized from detergent solution at a pH that should activate the channel. The effects of detergent solubilization on the structure and desensitization rates are unknown for the GLIC channels. Detergent solubilization can have a significant impact on membrane protein structure (20). Thus, functional studies of GLIC channels in a lipid membrane environment can provide important information about the structure of the functional channel.

In this study, we used the substituted-cysteine accessibility method (SCAM) (21) to evaluate the chemical reactivity of Cys substituted, one at a time, for residues in the GLIC M2 segment and M2-M3 loop from E221 (-2') to Y250 (27'). We screened each for residues in the GLIC M2 segment and M2-M3 accessibility method (SCAM) (21) to evaluate the chemical reactivity of Cys substituted, one at a time, for residues in the GLIC M2 segment and M2-M3 loop from E221 (-2') to Y250 (27'). We screened each mutant for reactivity with the sulfhydryl-specific mercurial reagent p-chloromercuribenzenesulfonate (pCMBS). We found that pCMBS reacted with almost every functional Cys mutant on the extracellular half of M2, from 10' to 27'. This suggested that the extracellular half of the M2 helix is loosely packed with the adjacent transmembrane helices. This, combined with the desensitization of GLIC that we observed with prolonged exposure to pH < 5.0, suggests that the crystal structures may represent a desensitized or non-physiological state of the channel.

Experimental Procedures

Cysteine Mutagenesis and mRNA Production- The GLIC coding sequence was cloned by PCR from a Gloeobacter violaceus (ATCC 29082) culture and inserted into the pXOON dual-function vector for protein expression in Xenopus laevis oocytes or mammalian cells (22). The unique endogenous Cys at position 26 was mutated to leucine (C26L) by QuikChange (Agilent, Santa Clara, CA) site-directed mutagenesis to produce a cysteine-free GLIC.

Electrophysiology- Oocytes were prepared as described previously and injected with 10 ng mRNA (24). One to 3 days after mRNA injection, currents were recorded using two-electrode voltage clamp at a holding potential of -40 or -60 mV via a TEV-200 amplifier (Dagan Corp., Minneapolis, MN) and digitized with a Digidata 1440A A-D converter and pClamp 10.2 software (Molecular Devices, Sunnyvale, CA). Agarose-tipped glass microelectrodes were filled with 3 M KCl (0.3-0.8 MΩ tip resistance). The ground electrode connected to the bath via a 3 M KCl-agar bridge. During recording, oocytes were continuously perfused at 5 ml/min with GLV buffer (100 mM NaCl, 20 mM NaOH, 2.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM HEPES, 5 mM Citric Acid, pH adjusted with HCl) at room temperature. All reagents were dissolved in GLV buffer.

Complete pH response curves could not be obtained because the oocytes became unstable at pH < 4. Oocytes tolerated repeated exposures to pH 5.0 or, to a lesser extent, pH 4.5. Proton-induced currents were measured for each mutant at pH 5.0 and 4.5 to determine if the mutant was functional and to verify that pH 5.0 induced a submaximal current response. For mutants that had current amplitudes similar to those elicited from water-injected oocytes, we tested for the presence of spontaneously-formed disulfide bonds between engineered Cys, by applying 10 mM DTT + 2 mM tris(2-carboxyethyl)phosphine (TCEP) for 2 min at pH 7.5 followed by 2 min at pH 5.0.
pCMBS Accessibility- We evaluated the accessibility of each Cys-substitution mutant in the resting (pH 7.5) and submaximally activated states (pH 5.0 for all Cys mutants except 12' where we used pH 6.0 because of the increase pH sensitivity of this mutant). Given that the Cys thiol has a pKa of 8.14 in free solution (25), at pH 5.0 Cys thiols are unlikely to be significantly ionized. Therefore, we did not use methanethiosulphonate reagents because they react at a negligible rate with unionized thiols (26,27). Due to the low pH activation of GLIC channels, we used pCMBS as our sulfhydryl reactive reagent because the organomercurial reagents react with both ionized thiocyanates (-S-) and with unionized thiols (-SH), albeit at different rates. pCMBS is an anion in the pH range 5.0 to 7.5. It is 313 times more soluble in water than in n-octanol (28). pCMBS is essentially impermeable through the lipid bilayer (29). Thus, its concentration in lipophilic regions of the protein should be negligible during application. Therefore, pCMBS should not react with buried Cys or should do so at a very slow rate. It should only modify sulfhydryl groups when they are in an aqueous environment. A potential problem in using pCMBS is that it is an anion and thus may have limited access to residues in the region of the charge selectivity filter in this cation-selective channel.

pCMBS reaction with Cys mutants was assayed functionally. We measured the peak current amplitude evoked by low pH test pulses applied before and after pCMBS application. Low pH test pulses were applied for 2 min, with 2 min pH 7.5 washes between pulses to allow recovery from desensitization. Occasionally we saw run up of the current amplitude induced by sequential low pH test pulses. When the current amplitudes of two consecutive low pH test pulses were within 10% the response was considered stable, and the effect of pCMBS was tested. Following a 2 min prewash in buffer at the same pH as the pCMBS solution (pH 7.5, 6.0, or 5.0), 200 µM pCMBS was applied for 2 min. When pCMBS was applied at low pH, it was washed out for 20 s with buffer at the same pH before returning to the pH 7.5 buffer. Additional 2 min low pH test pulses were recorded at 2 min intervals until the response was stable, as was done before pCMBS treatment. The effect of modification was calculated as the ratio of the average peak current of the two pulses following pCMBS application to the average of the two pulses immediately before application.

pCMBS Reaction Rates- We measured the reaction rate of pCMBS with the reactive Cys mutants. Reaction rates at pH 7.5 were determined by alternating applications of pCMBS at pH 7.5 with 2 min low pH test pulses. Between the pCMBS and low pH test pulses the oocytes were washed for 2 min with pH 7.5 buffer. The pCMBS exposure from each application could be controlled by varying the pCMBS concentration (0.2–200 µM) or the time of application (10 – 120 s). As expected, similar rates were obtained whether duration or concentration of pCMBS was varied. Five or more sequential pCMBS applications and test pulses were recorded. The test currents following pCMBS application were normalized to the initial pH 5.0 current and plotted against cumulative pCMBS exposure (in units of µM*s).

Because GLIC is a homopentamer, there are five potential reactive sites in each mutant channel. The observed functional effect may require modification of only one Cys or may require reaction at several Cys before a functional effect occurs. The reaction rate data was fit to a single exponential function, representing a single bimolecular reaction, or to a kinetic model representing two consecutive reactions:

\[
 f = a e^{-k_{x}t} + b \frac{e^{-k_{x}t} - e^{-k_{y}t}}{k_{2} - k_{1}} + c \left(1 + \frac{-k_{2} e^{-k_{x}t} + k_{1} e^{-k_{y}t}}{k_{2} - k_{1}}\right)
\]

(Equation 1)

Because pH 5.0 produced a stable current that did not appear to desensitize, reactions at pH 5.0 were observed in real-time. pH 5.0 buffer alone was applied for 2 min, until a steady-state current was reached, then pCMBS was added to the buffer at a concentration that would allow the reaction to proceed to completion during a 5 - 10 min period. Changes in the recorded current during pCMBS application at pH 5.0 were attributed to sulphhydryl modification, and the recorded curve was fit to single or sequential reaction kinetics.

Optical Absorption and Stopped Flow Kinetic Measurements- To identify spectral differences between pCMBS and the mercury-mercaptide product, UV-Vis absorption spectra of pCMBS were measured before and after reaction with L-cysteine on an Agilent 8453 UV-Visible spectrophotometer (Agilent Technologies, Santa Clara, CA) over the pH range 3.0 to 12.0. The GLV buffer used for electrophysiological recordings had a strong optical absorption at wavelengths shorter than ~240 nm. Therefore, reagents were prepared in phosphate-
acetate buffered saline (150 mM NaCl, 5 mM NaCH2COO-3H2O, 2.5 mM Na2HPO4, 2.5 mM NaH2PO4-H2O, pH adjusted with HCl or NaOH) to minimize the interference of buffer.

We determined that the optical absorbance at 236 nm changed during reaction of pCMBS with Cys in phosphate-acetate buffered saline over the pH range from 3.0 to 12.0 (data not shown). Reaction between pCMBS and cysteine was followed at 236 nm on a π*180 stopped flow spectrometer equipped with a Hg-Xe lamp and a photomultiplier tube detector (Applied Photophysics, Surrey, UK). Reactions were carried out with a 10x excess of cysteine to give pseudo first-order kinetics. The recorded curves were fit to a single exponential function, and second-order rate constants were calculated. The reaction rate was measured over the pH range from 3.0 to 6.5.

Data Analysis and Statistics- All experiments were performed in at least three oocytes from at least two separate frogs. Data are presented as mean ± sem. Statistical significance was determined by one-way ANOVA with Tukey's post hoc test using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA). Curve fitting to determine pCMBS reaction rates was performed with SigmaPlot 11 software (Systat Software Inc., San Jose, CA) or pClamp 10.2 software (Molecular Devices). Curve fitting for stopped-flow kinetics experiments was performed with Prism version 5.02 (GraphPad Software).

RESULTS

GLIC expression and electrophysiology. Increasing the buffer proton concentration by decreasing the buffer pH to values under 6.5 elicited currents in Xenopus oocytes expressing wild type GLIC. For buffer pH ≥ 5.0 the pH-activated current response was similar to previously published results (16,18). However, in contrast to previously published statements that GLIC does not desensitize, the wild type GLIC currents desensitized with prolonged (minutes) exposure to pH < 5.0 (Fig. 1A). The rate of desensitization was faster at pH 4.0 than at 4.5 (Fig. 1A). The desensitization induced at pH 4.0 at a holding potential of -50 mV was fitted with an exponential function with a mean time constant of 27.9 ± 2.9 s (n=5). A recent report also noted that when expressed in HEK293 cells, macroscopic GLIC currents showed desensitization (19).

We tested the effect of a 2-min application of 200 µM pCMBS on wild type GLIC. pCMBS application significantly inhibited the subsequent pH 5.0 test currents (Fig. 1B). We inferred that pCMBS reacted with the sole endogenous cysteine in wild-type GLIC, Cys26. We mutated Cys26 to leucine (C26L) to produce a Cys-free construct that was insensitive to pCMBS (Fig. 1D). Cys26 lies in extracellular domain in the middle of the β1 strand. It faces into the β sandwich. Cys26 is in close proximity to the β8-β9 loop and residues in the β2 and β9 strands (Fig. 2A and B). There are several possible mechanisms by which pCMBS modification of Cys26 might inhibit GLIC function. The β1-β2 loop plays an important role in signal transduction between the extracellular and transmembrane domains (30). Modification of Cys26 might disrupt this transduction pathway or it might disrupt the β sandwich. At present we cannot distinguish amongst these possibilities.

We could not measure a complete pH response curve to determine the proton EC50 (the pH that gives rise to 50% of the maximal current) because the oocytes did not tolerate prolonged (minutes) exposure to pH < 4. The Cys-free GLIC mutant had a pH-response that was shifted to lower pH compared to wild type. Like the wild-type, Cys-free GLIC did not appear to desensitize during a 2 min pH 5.0 pulse but did desensitize with exposure to pH < 5.0 (Fig. 1C). All of the subsequent Cys substitutions were made in the Cys-free background construct.

Effects of cysteine mutagenesis. For each of the 30 individual Cys mutants, we measured the currents induced by low pH test pulses to determine the effect of the mutation on proton-induced channel activation (Fig. 3). The currents induced by pH 5.0 application were not significantly different from those recorded from water-injected oocytes for 9 of the 30 cysteine mutants: -1' (A222C), 6' (S229C), 11' (H234C), 13' (A236C), 15' (N238C), 18' (V241C), 20' (T243C), 22' (L245C), and 27' (Y250C) (Fig. 2C, 3). Because each mutant channel possesses five disulfide bonds, we sought to determine whether the lack of function was due to spontaneous disulfide bond formation between engineered Cys. We applied a solution containing the reducing agents DTT (10 mM) and TCEP (2 mM) at both pH 7.5 and 5.0 but this did not rescue channel function in any of the nonconducting mutants (data not shown). Either disulfide bonds were not responsible for the lack of function of these mutants or steric factors limited reducing agent access to the disulfide bonds so they could not be reduced or they reformed very rapidly after reduction.

The 8' (L231C), 9' (I232C), and 10' (A233C) mutants exhibited significant changes in the pH-
dependent gating process. The 8' (L231C) mutant was constitutively conductive at pH 7.5. Most oocytes injected with L231C mRNA were dead within two days. These oocytes displayed irregular patterns of pigmentation on their surface (31) or released their cytoplasmic contents into the storage media. Electrophysiological recordings from 8' expressing oocytes that appeared healthy on visual inspection >36 hours after mRNA injection showed holding currents in excess of 6 µA when clamped at -60 mV. We recorded from oocytes injected with the 8' L231C mutant 12 – 24 hours after injection, when we expected lower expression levels. In these oocytes holding currents ranged from 1 – 5 µA. The holding current was not significantly reduced by application of pH 9.0 buffer or 100 mM QX-222 at pH 7.5. Surprisingly, the holding currents were significantly reduced by application of pH 5.0 or pH 4.5 buffer (data not shown).

Following pH 5.0 exposure, the currents in oocytes expressing the 9' (I232C) mutant closed very slowly, and did not return to the initial baseline current even after several minutes of pH 7.5 wash. The pH-response of 12' (I235C) was shifted to higher pH such that the pH 5.0 current amplitudes were of similar magnitude to those at pH 4.5. For this mutant, pH 6.0 was used to elicit a submaximally open state in subsequent experiments.

Effects of pCMBS modification. We evaluated the ability of pCMBS to react with each Cys mutant in the closed (pH 7.5) and submaximally opened (pH 5.0 or 6.0) states (Fig. 4). Covalent modification by pCMBS could have multiple effects on an individual mutant; the subsequent current amplitudes could be altered and/or the channel’s gating kinetics or pH dependence could change. We infer that pCMBS reacted with Cys mutants whose subsequent currents or gating kinetics were significantly altered following pCMBS application.

For the mutants ranging from 13' to 27', a 2 min application of 200 µM pCMBS at pH 7.5 significantly altered the subsequent pH 5.0 test pulse current amplitudes for 13' (A236C), 14' (F237C), 15' (N238C), 16' (I239C), 17' (L240C), 19' (E242C), 21' (N244C), 23' (P246C), 24' (K247C), and 26' (P249C) (Fig. 4A). For the 13' (A236C) and 15' (N238C) mutants, prior to pCMBS application the low pH test pulses did not elicit currents that were significantly different than water-injected oocytes. However, pCMBS modification significantly increased the subsequent currents elicited by low pH test pulses for both mutants (Fig. 2C, purple residues). Thus, we infer that the Cys substitutions at 13' (A236C) and 15' (N238C) inhibited channel function, but pCMBS modification of the engineered Cys restored channel function. This implies that for these two mutants the lack of function prior to pCMBS modification was not due to disulfide bond formation between the engineered Cys. For the four other non-functional mutants between 13’ and 27’, 18’ (V241C), 20’ (T243C), 22’ (L245C), and 27’ (Y250C) there were no changes following pCMBS application at either pH 7.5 or 5.0.

All of the mutants that reacted with pCMBS at pH 7.5 also reacted with 200 µM pCMBS applied at pH 5.0 (Fig. 4B). In addition, although pCMBS application at pH 7.5 did not have a significant effect on the 23' (P246C) and 24' (K247C) mutants, application of 200 µM pCMBS at pH 5.0 significantly altered the subsequent pH 5.0 test currents (Fig. 4B). Covalent modification of a Cys may potentiate or inhibit subsequent currents. At the 11 mutants between 13’ and 27’ where pCMBS modification significantly altered the currents, the currents were potentiated at 13’ (A236C), 14’ (F237C), 15’ (N238C), 17’ (L240C), and 25’ (T248C) and were inhibited at 16’ (I239C), 19’ (E242C), 21’ (N244C), 23’ (P246C), 24’ (K247C), and 26’ (P249C) (Fig. 2D-G and 4). It is difficult to infer the structural or mechanistic basis for the functional effects of modification and we have not pursued such studies (Fig. 2D). Note that the pCMBS-reactive residues on the backside of M2 are not accessible from the lipid bilayer in the crystal structure (compare Fig. 2F and G, M1, M3 and M4 removed in panel G to visualize the location of the M2 residues buried in the subunit interior).

From -2' to 12', the only pCMBS-reactive residue was A233C (10') where pCMBS application caused a change in gating kinetics (Fig. 5). Application of pCMBS at pH 7.5 slowed down subsequent channel deactivation after the pH was changed from 5.0 to 7.5 and increased the amplitude of the holding current at pH 7.5. Proton-induced currents were increased by 23 ± 7% following pCMBS application (n=4), but this change was not statistically significant (Fig. 5A). In contrast, following application of pCMBS at pH 5.0 the current remained stable with little reduction in the holding current after the bath pH was returned to pH 7.5 over a 10 minute period (Fig. 5B). The Cys mutants at -1’ (A222C), 6’ (S229C), 11’ (H234C) were non-functional (Fig. 2C and 3) and application of either the reducing agents, DTT/TCEP, or pCMBS had no effects on these mutants (Fig. 4 and data not shown).

Thus, of the 30 Cys mutants tested in and flanking the M2 segment, 7 Cys mutants resulted in non-functional channels that were not altered by
either reduction or pCMBS modification. pCMBS modified 12 of the 30 Cys mutants. All of the pCMBS reactive residues were more extracellular than 10'. For all of the residues more cytoplasmic than 10', pCMBS application had no functional effect.

Second-order rate constants for cysteine modification. Measuring the pCMBS reaction rate with the engineered Cys mutants provides information about the relative reactivity of different residues and of the reactivity of the same residue in different functional states, i.e., pH 7.5 (resting) vs 5.0 (sub-maximally activated). For GLIC channels, one complication in measuring the reaction rate is that each channel contains five engineered Cys residues. We do not know if the functional effects occur with modification of the first Cys or require modification of two or more of the subunits. For several positions the initiation of the functional response appeared to be delayed. This suggested that one or more functionally “silent” reactions occurred before the onset of the functional effect of pCMBS modification. It is important to note that we could only detect a “silent” modification when the second-order rate constants for each modification, silent or functional, were similar. If the silent modification occurred much faster than the second “functional” reaction, we would not have observed a delay in the functional response. If the functional modification occurred much faster than the silent reaction, we would have observed a functional change limited by the rate of the silent reaction. Thus, we fit the current-response data as a function of the cumulative concentration*time of pCMBS application, with both a single reaction model and a sequential reaction model.

We measured the second-order reaction rate constants for pCMBS with the reactive Cys mutants at pH 7.5 and 5.0 (Fig. 6 and 7). At pH 7.5, the reactions with 16', 19', 21' and 26' were consistent with the functional effects arising from modification of a single subunit. In contrast, the reactions with the 13', 14', 15', 17' and 25' mutants indicated that modification at more than one subunit was required to produce the effect seen with the 2 min application of 200 µM pCMBS. We could not fit the reaction with 25' to either kinetic model. At pH 7.5, reaction rates with all positions except 10' were >1000 M⁻¹s⁻¹ (Fig. 7). For 10', we could not quantify the changes in closing kinetics following modification to determine the reaction rate. A second 2 min application of 200 µM pCMBS further slowed channel closing. We infer that the 10' reaction did not go to completion with a 24000 µM·s pCMBS exposure and that the second-order rate constant at 10' at pH 7.5 was <100 M⁻¹s⁻¹.

At pH 5.0, the reaction rate data at 14', 19', 23' and 24' were best fit by a single exponential function consistent with the functional effects arising from modification of a single subunit (Fig. 6 and 7). For the 10', 13', 15', 16', 17' and 26' mutants the reaction rate data was best fit with a sequential reaction model. The reaction at 21' was best fit by a double exponential function. At 25' the reaction at 5.0 was not well fit by either single or sequential reaction kinetic models. Reaction rates were all >1000 M⁻¹s⁻¹. It should be noted that pH 5.0 induces submaximal activation of GLIC channels. Thus, the reaction rates at pH 5.0 represent a combination of reaction rates with channels in the open, closed and, presumably, desensitized states. The proportion of channels in each state is unknown. The rates with the fully open state may be significantly different.

pH dependence of second-order rate constants for pCMBS modification of cysteine. Comparison of pCMBS reaction rates with a given Cys mutant at different pH's is complicated by the fact that 1) the ratio of thiols (-SH) to thiolates (-S⁻) varies with the solution pH, and 2) pCMBS reacts at different rates with thiols and thiolates. To normalize for these effects, we measured the reactivity of pCMBS with Cys in free solution as a function of pH. We measured the reaction of pCMBS with Cys using a spectrophotometer equipped with a stopped-flow, rapid-mixing apparatus. We fit the recorded absorption traces (data not shown) to single exponential functions to calculate the second-order rate constants for the reaction between pCMBS and free cysteine in solutions buffered at different pH values (Fig. 8). At pH > 6.5 the reaction proceeded too rapidly to be measured accurately using the available apparatus.

To account for the difference in the pCMBS reaction rate with thiols and thiolates we used the following reaction scheme,

\[
X + R-SH \xrightarrow{k_1} X-S-R
\]

(Scheme 1)

where X is pCMBS, k₁ and k₂ are the second order rate constants, and Kₐ is the acid dissociation constant of the sulfhydryl group. In this reaction scheme, the observed second-order rate constant, k_{obs}, is given by:
Using $pK_a = 8.14$ for Cys (25) and fitting the data to Equation 2, we determined that for the reaction between Cys and pCMBS, $k_1 = 3.5 \times 10^5$ M$^{-1}$s$^{-1}$ and $k_2 = 1.2 \times 10^9$ M$^{-1}$s$^{-1}$. This gives values for $k_{obs}$ of $1.2 \times 10^6$ M$^{-1}$s$^{-1}$ at pH 5.0 and $2.2 \times 10^8$ M$^{-1}$s$^{-1}$ at pH 7.5. Thus, the intrinsic reactivity of pCMBS with Cys was 183 times greater at pH 7.5 than at pH 5.0. This is consistent with the differences in second-order rate constants measured for other mercurial reagents with thiol and thiolate forms of other sulfhydryl compounds (32).

To account for this 183-fold increase in the intrinsic reactivity of pCMBS at pH 7.5 compared to pH 5.0, we normalized the measured reaction rates for pCMBS with the engineered Cys residues by their intrinsic reaction rates (i.e., for the pH 5.0 rate constants, apparent reactivity = $k_{measured}/k_{obs, pH 5.0}$). With this correction, it is apparent that at every reactive Cys residue, pCMBS reacted faster at pH 5.0 than at pH 7.5 (Fig. 9).

DISCUSSION

While the crystal structures of GLIC provide valuable new information, little is known about how the crystal structures compare with the structure of a functional channel in a lipid bilayer membrane. Multiple experimental approaches are necessary to gain a clearer view of structure/function relationship in functional GLIC channels. Previous reports stated that wild type GLIC did not desensitize (16,18). However at pH < 5.0, we show that the GLIC channels desensitized on a timescale of minutes and the rate of desensitization increased with decreasing pH (Fig. 1A). A recent report also noted that GLIC expressed in HEK293 cells desensitizes at pH < 5.0 albeit with a rate significantly faster than what we observed (19). This may relate to differences in the lipid composition of oocyte and HEK293 cell membranes. The crystals for both of the published GLIC structures were grown at pH < 5.0 (17,18). Under these conditions, our data indicate that the GLIC channels would be in a desensitized state on the time-scale needed for crystallization. While we cannot rule out the possibility that the rate of desensitization is different in detergent solubilized GLIC, we conclude that the GLIC crystal structures may represent a desensitized rather than an open state of the channel.

Consistent with the conclusion that the GLIC structures represent a non-conducting state of the channel, the structures in the absence and presence of propofol are identical (33). Propofol inhibits GLIC channels. Thus, it is likely to bind to a non-conducting state with greater affinity than to an open state. The fact that its binding produced no change in the GLIC structure suggests that the structure may be a non-conducting state. Furthermore, the location of the propofol binding site in GLIC in the interior of the subunit is inconsistent with photoaffinity labeling and mutagenesis studies in GABA$_A$ receptors that show the propofol binding site to be in the intersubunit interface between M3 and M1 (34-36). Propofol is rather promiscuous in its protein binding and has also been crystallized bound to other proteins including albumin and apoferitin (37,38). Thus, the propofol-bound GLIC crystal structure may not provide insight into propofol binding to pharmacologically relevant targets, such as the GABA$_A$ receptor. In addition, it was recently suggested that the ELIC structure, another prokaryotic Cys-loop homologue, does not represent a resting closed state but rather a non-conducting, non-functional state (19). Thus, studies trying to elucidate the conformational changes occurring during Cys-loop receptor gating by comparing the ELIC and GLIC structures as representative of the closed and open states may not be fruitful (17,18).

Although Cys accessibility studies of ion channels lack the high resolution of x-ray crystallography, they have the advantage that they probe the protein structure in functional channels. We determined the ability of pCMBS to react with Cys substituted for residues in and flanking the GLIC M2 segment to assess solvent accessibility of the amino acid side chains. Seven of the Cys substitutions (-1', 6', 11', 18', 20', 22', and 27') resulted in non-functional channels (Fig. 2C, 3). Neither reduction with DTT/TCEP nor application of pCMBS restored the function of these channels (Fig. 2C, 3). Neither reduction with DTT/TCEP nor application of pCMBS restored the function of these channels (Fig. 2C, 3). Neither reduction with DTT/TCEP nor application of pCMBS restored the function of these channels (Fig. 2C, 3). Neither reduction with DTT/TCEP nor application of pCMBS restored the function of these channels (Fig. 2C, 3). Neither reduction with DTT/TCEP nor application of pCMBS restored the function of these channels (Fig. 2C, 3).
every functional Cys mutant from 10’ to 26’ except 12’ (Fig. 2, 4). For all positions except 10’ the second order reaction rate of pCMBS was greater than 1000 M⁻¹s⁻¹ (Fig. 7).

There were several unexpected aspects of our results. M2 ends at ~22’ in the GLIC crystal structure. So we expected an α-helical pattern in either the chemical accessibility data or in the reaction rates from 10’ to 22’ (Fig. 9). In fact, given the tightness of protein packing of this region in the crystal structures we expected that pCMBS would only have access to channel-lining residues (Fig. 2F, G). Previous SCAM studies of the M2 segment of eukaryotic Cys-loop receptors have shown an α-helical pattern of sulfhydryl accessibility or reaction rates in the extracellular half of M2 (21,9,39,40,11,41,42).

Surprisingly, in GLIC M2 we observed comparable reaction rates for pCMBS (Fig. 7, 9) with Cys substituted for channel-lining and non channel-lining residues in both the resting and sub-maximally activated state (Fig. 2E-G). The failure to observe a difference in the reaction rates is unlikely to be due to a modified pKa value of buried Cys residues. The pKa of residues within a protein may vary significantly from the pKa of the corresponding amino acid in bulk solution (10). In the AChR M2 segment, Cyimes et al., (2005) showed that the pKa’s of lysines substituted for channel-lining residues were close to the pKa of lysine in free solution. In contrast, the pKa’s of lysines substituted for buried residues were altered significantly to favor the uncharged state. This suggests that the Cys pKa for buried residues might be significantly greater than the pKa of 8.14 in bulk solution (25). This would have reduced the measured reaction rate for buried residues by increasing the percent of the time spent in the less reactive thiol form. Thus, we conclude that in functional GLIC channels embedded in a lipid bilayer membrane the top half of the M2 segment is less tightly packed than suggested by the crystal structure. This region of GLIC M2 may more loosely packed than in the eukaryotic Cys-loop receptors that have been similarly studied in the past.

Based on their reactivity, we infer that ostensibly buried Cys more extracellular than 12’ are at least transiently exposed to the water-accessible protein surface. Perhaps thermal protein motion results in the formation of aqueous crevices into the interior of the transmembrane domain. These crevices must be large enough to accommodate pCMBS, which would fit into a right cylinder 6 Å in diameter and 10 Å in length. We previously suggested that such crevices form in the GABAᵦ receptor during channel gating (43,41). We conclude that when GLIC is embedded in a lipid membrane, the extracellular half of the GLIC M2 segment is significantly more mobile than suggested by the crystal structures. A corollary to this is that for M2 to be that mobile, the protein packing in the transmembrane regions must be significantly looser than that seen in the crystal structures.

Consistent with the idea of loose packing, for several of the GLIC Cys mutants the reaction rate data is most consistent with a model where pCMBS modification of more than one subunit was necessary to cause the observed functional effects (Fig. 8). The fact that pCMBS could modify one or more M2 Cys with no functional effect suggests that the extracellular half of the M2 segment of GLIC has sufficient flexibility to accommodate the pCMBS-modified engineered Cys.

In contrast to what we observed with the upper part of M2, the cytoplasmic half of M2 was tightly packed against the adjacent membrane-spanning segments. pCMBS application did not have a significant effect on any of the Cys mutant from -2’ to 9’ (Fig. 4). For the channel-lining residues in this region, it is possible that pCMBS is too large to enter this region of the channel. Alternatively, this region may form the charge selectivity filter that might exclude the negatively charged pCMBS from this region. For the buried residues, their lack of reactivity suggests that the aqueous crevices do not extend this deep into the membrane. This is consistent with previous SCAM studies of this region in eukaryotic channels (41).

Differences in the pCMBS reaction rates with Cys in the resting and activated states of the channel provides evidence for structural changes associated with channel gating. A complication for this comparison in GLIC is that the channel is activated by low pH: The intrinsic reactivity of pCMBS with thiols depends on the ionization state of the thiol. We measured that pCMBS reacts 183 times faster with the ionized thiolate than with the unionized thiol. The relative accessibility of each mutant at pH 7.5 and pH 5.0 was inferred by normalizing the rate constants measured for the mutant to the rate constant for reaction with free Cys in solution at the same pH (Fig. 9). This normalization accounts for the intrinsic difference in reaction rate at pH 7.5 and pH 5.0. With this correction, we found that pCMBS reacted faster with all of the engineered Cys in the sub-maximally activated state than in the resting state (Fig. 9). The increase in reaction rates with activation suggests a dramatic increase in mutant accessibility when the
channels are submaximally activated. It should be noted that at pH 5.0 the channels are submaximally activated; the channels will be in a variety of states including closed, open and perhaps desensitized. Thus, the reaction rates with Cys in the open channel state may actually be faster than the rates we have measured at pH 5.0. We infer that the transmembrane helices are more loosely packed, more mobile, or more flexible during channel gating than at rest. This is in contrast to the tightly packed structures and gating model proposed based on the ELIC and GLIC crystal structures (17,18).

In the GLIC crystal structures M2 is tightly packed against the other transmembrane segments (17,18). It is important to note that the GLIC crystal structures were produced from detergent-solubilized protein. The effect of detergent solubilization on membrane protein structure is unknown but can be significant (20). In the _Torpedo_ nAChR cryo-electron microscopy structure, which was derived from protein in its native lipid membrane, the transmembrane segments are more loosely packed (12,17,18). A recently published NMR structure of the transmembrane domains of the nAChR suggests that the transmembrane segments are tightly packed (44). However, the NMR structure was produced in detergent solution from a truncated construct that was missing the N-terminal extracellular domain and the large cytoplasmic loop between M3 and M4. The effects of these modifications on protein structure are unknown. Furthermore, the construct was monomeric in detergent solution raising questions about its relevance to the native protein structure. Indications of loose packing consistent with the cryo-electron microscopy structure of the _Torpedo_ nAChR has previously been observed in SCAM studies of the nAChR and the GABA<sub>A</sub>R (40-42). Our current data implies that when embedded in a lipid membrane the GLIC channel has a structure that is also more consistent with the loose packing observed in the _Torpedo_ nAChR structure.

GLIC is a cation-selective channel (16). Our data suggest that the primary determinants of charge selectivity in the GLIC channel are at the cytoplasmic end of the channel. The high rates of reaction that we observed with residues in the extracellular half of M2 and in the M2M3 loop suggest that pCMBS, which is an anion in the pH ranges utilized in these experiments, had free access to these regions in the GLIC channel. Thus, the extracellular vestibule does not contribute significantly to the charge-selectivity filter in GLIC. We did not detect pCMBS modification at any residue below 8' at pH 7.5 or at pH 5.0. Access to residues below this region may be blocked by the charge-selectivity filter or by steric factors. Our results are consistent with chemical accessibility studies in the nAChR and the GABA<sub>A</sub>R which inferred that charge selectivity occurred near the cytoplasmic end of the channel (21,9) and with the location of an binding site for extracellularly applied Zn<sup>2+</sup> at the M2 17' βHis in the anion-selective GABA<sub>A</sub> channel (45). The location of the charge selectivity filter near the cytoplasmic end of the channel is also consistent with experiments that showed that swapping three residues in and flanking the cytoplasmic end of M2 flips the charge selectivity of eukaryotic Cys-loop receptor channels (46-50). It is notable that the GLIC channel lacks the 0′ Arg or Lys found in virtually all eukaryotic family members. Clearly cation selectivity can be obtained without this residue which has been inferred to be deprotonated in nAChR channels (10). In contrast to the charge selectivity filter, the determinants of single channel conductance are more widely distributed along the length of the pathway that ions traverse through these proteins. Residues influencing conductance have been identified in the extracellular vestibule, the extracellular end of M2, the cytoplasmic end of M2 and in the cytoplasmic MA helices (6,51,46,52-55). These regions may serve minor roles in determining charge selectivity, but our data suggests that residues near the cytoplasmic end of the channel are the primary determinants of charge selectivity in GLIC.

In conclusion, the GLIC channel desensitizes at pH < 5.0. Cysteine accessibility studies indicate that in contrast to the GLIC x-ray crystal structures, the extracellular half of M2 and the M2-M3 loop are highly accessible to water soluble, charged sulfhydryl reactive reagents. This implies that the extracellular half of the transmembrane domain is more loosely packed and highly mobile than suggested by the GLIC crystal structures. Access of negatively charged pCMBS to the channel-lining residues in the cytoplasmic half of M2 is limited either by charge or size selectivity filters or both. The cytoplasmic half of M2 is tightly packed limiting access of pCMBS to residues on the back side of M2. These results suggest that the crystal structures are more likely to represent a desensitized state of the channel than the open state.
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FOOTNOTES

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#The abbreviations used are: AChR, acetylcholine receptor; DTT, dithiothreitol; GABA<sub>r</sub>, γ-aminobutyric acid Type A; GLIC, *Gloeobacter violaceus* ligand-gated ion channel; pCMBS, p-chloromercuribenzenesulfonate; SCAM, substituted cysteine accessibility method; TCEP, tris(2-carboxyethyl)phosphine; 5-HT<sub>3</sub>, serotonin (5-HT) Type 3;

FIGURE LEGENDS

Fig. 1. pH response and pCMBS effects on wild-type GLIC and C26L GLIC. A, application of low pH pulses to oocytes expressing wild-type GLIC as indicated by the black bars above the traces. Channels desensitize on the minutes time-scale with exposure to pH < 5.0. The perfusion buffer pH is 7.5 unless otherwise indicated. B,
Fig. 2. Illustrates the location of Cys mutants in the GLIC channel structure using 3EAM PDB coordinates. 

A. Location of endogenous Cys26 in the GLIC extracellular domain. View from the GLIC channel, four subunits have been removed. Color scheme: The subunit is light purple, Cys26 red in space-filling format, β1 strand is cyan, β8-β9 loop and β9 strand are yellow, M2 segment blue. B. Ninety degree rotation of the view in panel A. Color scheme same as in panel A. C. View of the membrane-spanning, channel-lining region showing the positions of the non-functional Cys mutants. Residues for which the Cys mutants were non-functional are shown on the middle subunit in space filling format. Cyan, residues for which no function was elicited. Purple, 13’ and 15’ residues that were functional following pCMBS modification. To visualize the channel-lining residues the front two subunits have been removed. The remaining three subunits are color coded to distinguish them in this and in all subsequent panels in this figure. The middle subunit is colored light purple, with the M2 and M2-M3 loop regions that were mutated to Cys in this study in blue. The two flanking subunits are colored light green, except for the region studied that is dark green. Note that the Cys mutants were present in all subunits but is only shown in one to simplify the figure. D. Close up view of the extracellular end of M2 to illustrate the position of the pCMBS reactive residues in space filling format. Orange, pCMBS modification potentiated subsequent currents. Yellow, pCMBS modification inhibited subsequent currents. Red, 10’ residue where pCMBS modified gating kinetics. Cyan, non-functional mutants. Blue, Cys mutants not affected by pCMBS. E. View of the M2 segments looking into the channel. All residues are shown in space filling format. The extracellular domain of the subunit on the right is shown in ribbon format to clarify the extent of that domain in the other subunits. Note that based on the crystal structure many of the pCMBS-reactive residues are buried in the protein interior. F. View of the lipid bilayer facing side of the same three subunits seen in panel E. This view results from a 180º rotation around the vertical axis of panel E. Color scheme as in panel D. Note that only one of the pCMBS reactive residues, P249 (26’), has significant accessibility from the external surface of the protein. G. Same view as in panel F, except that the M1, M3 and M4 segments have been removed from the light purple subunit to reveal the “buried”, pCMBS-reactive residues on the non-channel facing side of the M2 segment.

Fig. 3. Average proton-induced currents at pH 5.0 for the constructs used. Black bars indicate currents that were significantly different from water injected oocytes by one-way ANOVA with Tukey's post hoc test. White bars indicate currents that were not significantly different from water injected oocytes. L231C (8’) was constitutively conductive at pH 7.5. Note that the x-axis has a log scale.

Fig. 4. Irreversible effects of a 2 min application of 200 µM pCMBS on proton-induced currents. A, application of pCMBS at pH=7.5. B, application of pCMBS at pH=5.0. Black bars indicate mutants in which the average ratio of the proton-induced currents following pCMBS exposure to those before exposure was significantly different than the average ratio in C26L GLIC by one-way ANOVA with Tukey's post test. Application of pCMBS to oocytes expressing A233C (10’) did not significantly alter the magnitude of the proton-induced current, but the response was altered as shown in Fig. 5. The effect of pCMBS application at pH 7.5 or 5.0 was not determined in oocytes expressing L231C (8’) or I232C (9’), and the effect of pCMBS application at pH 5.0 was not determined in oocytes expressing wild type GLIC. Note that the x-axis has a log scale.

Fig. 5. Application of 200 µM pCMBS to oocytes expressing A233C (10’) for 2 min at pH 7.5 (A) and pH 5.0 (B). The oocytes were continuously perfused with pH 7.5 buffer except as indicated by the black bars above the current traces. The zero current level is indicated by the dashed line. The holding potentials were -60 mV.

Fig. 6. Measurement of pCMBS reaction rates. A, Example of a position where the modification by pCMBS inhibits the pH-induced current. Currents from an oocyte expressing K247C elicited by alternating 2 min applications of pH 5.0 test pulses and 0.2 µM pCMBS in pH 7.5 buffer as indicated by the black bars over the current traces. B, The pH 5.0 induced currents from panel A were normalized to the initial current and plotted as
a function of cumulative concentration*duration of pCMBS exposure. The solid line is the single
exponential fit of the data. The calculated second-order reaction rate constant is 103,000 ± 3200 M⁻¹s⁻¹. C. Example of a
position where the modification by pCMBS potentiates the pH-induced current. Currents from an illustrative
oocyte expressing the F237C mutant elicited by alternating 2 min applications of pH 5.0 test pulses and 2 µM or
20 µM pCMBS in pH 7.5 buffer as indicated by the black bars over the current traces. D, The pH 5.0 currents
from panel C were normalized by the initial current, plotted as a function of the cumulative concentration-
duration of pCMBS exposure and fit to the shown equation describing the kinetics of two consecutive pseudo-
first order reactions. The calculated reaction rates were 4,600 ± 220 M⁻¹s⁻¹. E, Example of a measurement of the
rate of reaction of pCMBS in the activated state induced by applying pCMBS at pH 5.0. The currents shown are
from an oocyte expressing the F237C mutant. The reaction rate was obtained by fitting the current change
following pCMBS application with a single exponential decay function. Application of 0.1 µM pCMBS at pH
5.0 is indicated by the gray bar above the current traces. The portion of the current trace during pCMBS
exposure was fit with a single exponential function (indicated by the circles) to calculate the reaction rate
constant of 89000 ± 29 M⁻¹s⁻¹.

Fig. 7. Second order rate constants for pCMBS modification of reactive residues. We could not fit the data for
modification at 25' (T248C) to either kinetic model. Note that the x-axis has a log scale.

Fig. 8. The pH dependence of the second-order rate constant for reaction between pCMBS and free cysteine. The
experimental data points (dots) are the measured second-order rate constants. Each data point is the average of at
least 8 reactions. Error bars are smaller than the data points. The reaction proceeded too rapidly to measure at pH
> 6.5. The curve is a 1/Y²-weighted nonlinear regression of the data with Equation 2 using GraphPad Prism
version 5.02 for Windows. Since we expect the error in our data to be relative to the magnitude, this weighting is
applicable. Note that the y-axis has a log scale.

Fig. 9. Apparent accessibility of cysteine mutants to pCMBS. The second-order rate constants reported in Figure
7 have been normalized to the second-order rate constant for the reaction between pCMBS and free cysteine at
pH 7.5 or pH 5.0. Note that the x-axis has a log scale.
Fig. 3

mutant

I (nA)
Fig. 7
Fig. 8

$K_{app} \text{ (M}^{-1}\text{S}^{-1})$

$\text{pH}$
Fig. 9

normalized reactivity

\( \frac{k_{\text{meas}}}{k_{\text{cys}}} \)

○ pH 5.0
● pH 7.5
Structure of M2 transmembrane segment of GLIC, a prokaryotic Cys-loop receptor homologue from *Gloeobacter violaceus*, probed by substituted cysteine accessibility

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