Surface Structure and Its Dynamic Rearrangements of the KcsA Potassium Channel upon Gating and Tetrabutylammonium Blocking*

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KcsA is the first potassium channel for which the molecular structure was revealed. However, the high resolution structural information is limited to the transmembrane domain, and the dynamic picture of the full KcsA channel remains unsolved. We have developed a new approach to investigate the surface structure of proteins, and we applied this method to investigate the full length of the KcsA channel. Single-cysteine substitution was introduced into 25 sites, and specific reaction of these mutated channels to a bare surface of a flat gold plate was evaluated by surface plasmon resonance measurements. The surface plasmon resonance signals revealed the highest exposure for the mutant of the C-terminal end. When the gate of the KcsA channel is kept closed at pH 7.5, the extent of exposure showed periodic patterns for the consecutive sites located in the cytoplasmic (CP) and N-terminal domain. This suggests that these stretches take the α-helical structure. When the channel was actively gated at pH 4.0, many sites in the CP domain became exposed. Compared with the rigid structure in pH 7.5, these results indicate that the CP domain became loosely packed upon active gating. The C-terminal end of the M2 helix is a moving part of the gate, and it is exposed to the outer surface slightly at pH 4.0. By adding a channel blocker, tetrabutylammonium, the gate is further exposed. This suggests that in the active gating tetrabutylammonium keeps the gate open rather than being trapped in the central cavity.

Conformational changes of a protein rearrange the surface structure. Some residues become exposed, whereas others are enclosed in the interior. Thus the detection of changes in the exposure of residues will give information on the underlying conformational changes. Various methods, such as the substituted cysteine accessibility method (1–4), and various types of probe molecules have been used to reveal the geometry of the surface for various proteins. When a probe binds to the reactive site on the protein, the reactivity depends strongly on the size of the probe. Small probes detect whether an amino acid residue is either exposed or buried. Intermediate probes explore cavities in proteins. Large probes outline the outer surfaces of proteins. What would happen if the size of the probe becomes infinite? A "probe" with an infinite size, a bare flat gold piece was used, and reactions of cysteine-mutated proteins to the gold plate were detected by surface plasmon resonance (SPR) signals. This method should provide information about the rough surface structure of proteins.

In ion channels, domains extending out from the transmembrane domain (TM domain), either to the extracellular or intracellular space, pick up chemical signals and transfer information to the TM domain (5). In addition to this role as sensor, the cytoplasmic domains (CP domains) are known to regulate the assembly of the oligomeric structure (5). Several CP domains have been extracted, and their structures have been analyzed at the atomic resolution (6–9). For the KcsA channel (10–14), however, the high resolution structure of the CP domain remains unsolved, and only predicted structures (15, 16) are available. In this study we applied the SPR method to investigate the global structure of the KcsA channel into which cysteine mutations were introduced. First, the method was evaluated by comparing the data with the published structure (12, 15, 16). The results gave general agreements between structural information obtained by SPR measurements and those of previous reports, which allowed quantitative evaluation of SPR signals. We then focused on the surface structure of the CP domain. The KcsA channel opens at cytoplasmic acidic pH (13), and so the surface structures were examined at two pH values. The results indicate the structure of the full KcsA channel and its dynamic rearrangements upon gating. These changes were distributed throughout the CP domain.

For the KcsA channel, a crystal structure with a channel blocker, TBA, was resolved at pH 5–7 (17, 18), in which TBA is trapped in the closed structure. To see the effect of TBA under

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2 The abbreviations used are: SPR, surface plasmon resonance; TM domain, transmembrane domain; CP domain, cytoplasmic domain; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; 11-HUT, 11-hydroxy-1-undecanethiol; TBA, tetrabutylammonium; RU, resonance unit.
the actively gated channel, SPR experiments were performed at pH 4.0. No significant changes in the CP domain were detected. Surprisingly, we found that the C-terminal end of the M2 helix became much more exposed than that without TBA. We will discuss the dynamic picture of the KcsA channel revealed by the SPR method.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis, Expression, and Purification of KcsA—*Cysteine mutants of KcsA were prepared for the following 25 sites: Met-1, Ala-10, Arg-11, Leu-12, Val-13, Ala-23, Val-37, Ile-38, Arg-52, Gly-53, Gly-56, Leu-86, Thr-107, Gly-116, Gln-119, Arg-122, Gly-123, Lys-131, Ala-132, Ala-133, Glu-134, Leu-155, Asp-156, and Asp-157. In addition, the 161st site was added just behind the original C-terminal residue Arg-160. QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used for the site-directed mutagenesis of the KcsA protein. The sequence was confirmed by dideoxy DNA sequencing. Genes of wild-type and mutant channels were inserted into the pQE-82L vector (Qiagen, Valencia, CA), which was used to transform *Escherichia coli* M15 cells. Protein expression was induced by addition of 0.5 mM isopropyl β-d-thiogalactopyranoside. *E. coli* cells expressing KcsA channels were broken up by sonication, and membrane fractions were solubilized in buffer (20 mM potassium phosphate, 200 mM KCl, 20 mM 2-mercaptoethanol, 50 mM imidazole) containing 1% n-dodecyl β-d-maltoside (Dojindo, Kumamoto, Japan). Histidine-tagged channels were purified with a Co2+-based metal-chelate chromatography resin. Purified channels were eluted by 100–400 mM imidazole at a protein concentration of 0.5–3 mg/ml.

*SDS-PAGE Analysis—*SDS-PAGE was performed on a 15% gel. Sample buffer was modified slightly from the normal composition by reducing SDS to 0.5%. Approximately 1 μg of protein was loaded on each lane without boiling. Protein bands were stained by Coomassie Brilliant Blue R-250.

*Single Channel Current Recording—*Single channel currents were measured by the planar lipid bilayer technique. KcsA and its mutant channels were reconstituted into liposomes as follows. Purified channels were diluted with a reconstitution buffer (200 mM KCl, 10 mM HEPES, 10 mM 2-mercaptoethanol, 0.06% n-dodecyl β-d-maltoside, pH 7.5) to a final protein concentration of 20 μg/ml. A 3:1 mixture (1 mg/ml final phospholipid concentration) of 1-palmitoyl-2-oleoylphosphatidylcholine and 1-palmitoyl-2-oleoylphosphatic acid (Avanti Polar Lipids, Alabaster, AL) was added to the channels in the reconstitution buffer. Proteoliposomes were prepared by passing the protein/phospholipid mixtures through a BioSpin®30 column (Bio-Rad) equilibrated with the reconstitution buffer after incubation for 30 min at room temperature. The reference electrode was placed in the cis chamber, to which the reconstituted channels were added. A Teflon sheet with a thickness of 0.5 mm divided the two chambers. A hole (100–150 μm diameter) was made on the partition by the melt-and-shave method. The cis and trans chambers were asymmetrically buffered by 10 mM HEPES, pH 7.5, and 10 mM succinic acid, pH 4.0, respectively, in the presence of 200 mM KCl. Current data were collected and stored in a PC by using pCLAMP software (Axon Instruments, Sunnyvale, CA) through Axopatch 200B amplifier and Digidata 1322A digitizer (Axon Instruments). The low pass filter of the amplifier was set to 1 kHz, and data were sampled at 5 kHz. For the TBA block measurement, a small amount of concentrated TBA (Nacalai Tesque, Kyoto, Japan) solution was added to the cis or trans chamber to the desired final concentration.

*SPR Signal Measurement—*In this study, direct binding of cysteine-mutated channels to the bare gold surface was evaluated by Biacore®X instrument (Biacore AB, Uppsala, Sweden). Sensor chips bearing a gold surface were assembled from SIA kit Au (Biacore AB). The surfaces of the sensor chips were washed chemically by Piranha solution (3:1 mixture of sulfuric acid and 30% hydrogen peroxide) immediately before the measurements. Running buffers contained 200 mM KCl and 20 mM CHAPS buffered by 10 mM HEPES, pH 7.5, and 10 mM succinic acid for pH 4.0. The buffers were filtered and degassed before use. Purified channels were diluted to 20 μg/ml.
final protein concentration with the buffers of the desired pH values immediately before measurement. 80 μl of the diluted channels were loaded into the flow cell and the flow rate was 20 μl/min. After sample loading, the flow cell was washed three times with 80 μl of 0.5% SDS to remove nonspecifically adsorbed channels. Independent experiments for each sample were repeated 3–5 times, and the data were presented as means ± S.E. S.E. values for the difference plots were calculated as root mean square of two S.E. values. Data for the difference plots were analyzed by the unpaired Student’s t test, and a p value of <0.05 was considered statistically significant.

RESULTS

Stability of Tetrameric Structure of Site-directed Cysteine Mutants of KcsA Channel—The wild-type KcsA channel bares no cysteine residues. Cysteine was introduced into 25 sites throughout the full length of the KcsA channel (Fig. 1). The mutant channels have four cysteine residues in symmetrical positions in the tetrameric channel. To test whether the mutated channels maintain the tetrameric structure, SDS-PAGE analysis was carried out (Fig. 2). The predicted molecular masses of KcsA were ~19 kDa for the monomer and 77 kDa for the homotetramer. All the mutants showed the bands at 77 kDa. The results indicate that almost all of the mutants retained a tertiary structure even in the presence of 0.5% SDS. Partial dissociation of tetramer was found for V37C and D157C.

Electrophysiological Properties of Site-directed Cysteine Mutants of KcsA—Mutants were reconstituted into planar lipid bilayers, and single channel currents were recorded in symmetrical 200 mM KCl solutions. The bath solutions were set asymmetric in terms of pH (cis side, pH 7.5; trans side, pH 4.0) to record currents from functionally oriented channels with the CP domain facing the trans side. This was enabled by the sensitivity of the KcsA channel gating...
to the cytoplasmic pH. All mutated channels exhibited the characteristic fast gating at +200 mV and flickered gating at −200 mV (Fig. 3A). The open probability was very low at +200 mV, and it was reduced further at more negative membrane potentials. The single channel current-voltage relationships for all mutants showed slightly outward rectification (Fig. 3B). The conductance of the mutated channels at +200 mV ranged from 77 to 122 pS (the conductance of the wild-type channel was 108 pS). These results show that the gating and ion permeation properties were not affected significantly by the cysteine mutations.

Detecting Surface-exposing Sites by Using SPR Signals —The direct adsorption of the cysteine-mutated channels to the bare gold surface was evaluated by the SPR signal. The flat gold plate without any surface modification imposes the hard surface with simple geometry for the gold-thiol reaction. Thus, reaction proceeds only when residues are exposed to the outer surface.

Representative charts of SPR signal are shown in Fig. 4A. Adsorption was started immediately after the sample loading of cysteine-mutated channels. Considerable amounts of adsorption appeared even for the wild-type channel, barring no cysteine residue. The fraction of adsorption was not changed even when the concentration of the protein was reduced (not shown). However, the bound fraction of the wild-type was eliminated by washing with 0.5% SDS solution (Fig. 4A, gray trace). This washable fraction was assigned as nonspecific. A nonspecific binding was also observed for cysteine-baring mutants (Fig. 4A, red and blue traces). The fraction remaining after washing indicates the specific binding. As the loading time was increased, the specific binding fraction increased and reached saturation, indicating that the loading time of 240 s was enough for the gold-thiol reaction (Fig. 4B).

During the SDS washing, the amount of adsorption did not increase, indicating that the gold-thiol reaction had been completed before the washing. It should be noted that almost all of the mutated channels maintained their tetrameric structure even in the presence of 0.5% SDS as evidenced by the SDS-PAGE analysis (Fig. 1). Therefore, the KcsA channel retains its tertiary structure throughout the measurements, including the washing period.

Surface Accessibility of KcsA Channel at pH 7.5 —The KcsA channel is activated by acidic cytoplasmic pH (13), whereas at neutral pH the open state probability is practically zero (not shown). The extent of surface exposure of 25 positions in the KcsA channel was evaluated (Fig. 5A).
Mutants of M1C for the N terminus, G53C and G56C for the extracellular loop region, and L155C and 161C for the C terminus adsorbed well to the gold surface, indicating the presence of structural projections at these positions. The highest exposure of the C terminus indicates that it is located at the end of the molecule, which is the first observation by this method. In contrast, cysteine residues of V37C and I38C at the outer (M1) helix and at T107C and G116C at the inner (M2) helix were not reactive to the gold surface. The crystal structure of the TM domain (12) reveals that these residues on the M2 helix are buried or face the pore, whereas those on the M1 helix are exposed to the surface. The latter should be covered by detergent molecules and so have no access to the gold surface. The general agreement between the results for the TM domain with the information from the crystal structure supports the rationale for our method.

By focusing on the local structure, it was found that neighboring sites displayed significant differences in reactivity. For example, L155C in the CP domain exhibited high reactivity relative to those of neighboring D156C and D157C (Fig. 5A). A similar pattern, or periodicity, was also observed for consecutive sites in the N terminus (A10C, R11C, L12C, and V13C). These patterns of the reactivity along a short stretch of the sequence suggest the presence of an α-helix structure, as has been shown by EPR measurements (15).

Surface Accessibility of KcsA Channel at pH 4.0—Similar experiments to those performed at pH 7.5 were also carried out at pH 4.0 (Fig. 5B), where the channel is activated and single channel currents were recorded (Fig. 3). There were dramatic changes in the reactivity for the CP domain. The reactivity showed an overall increase throughout the CP domain. In fact the residues at Gln-119, Arg-122, Gly-123, Lys-131, Ala-132, Ala-133, Glu-134, Asp-156, and Asp-157 became exposed at pH 4.0.

Effect of pH on the Reactivity of Gold-Thiol Reaction—To evaluate the pH dependence of the gold-thiol reaction per se, quantitatively, the specific binding of 11-hydroxy-1-undecanethiol (Fig. 6A, 11-HUT) to the gold surface at pH values of 7.5 and 4.0 was compared. The specific binding of 11-HUT at pH 4.0 was slightly larger, by a factor of 1.16, than that at pH 7.5 (Fig. 6B). The results indicate that the reactivity of the gold-thiol reaction itself was little affected by the pH.

Comparison of the Surface Exposure at pH 7.5 and 4.0—To extract the geometrical changes or conformational changes around the mutated site under active gating, differences in reactivity at different pH values for each mutated site were evaluated quantitatively (Fig. 7A). The data measured at pH 7.5 (the closed state) were subtracted from those at pH 4.0 (the activated state). Reactivity differences (summarized by the color code on each site of the residues Fig. 7B) for mutants introduced into the extracellular loop and TM domain were negligible except for G116C. Gly-116, located at the C-terminal end of M2 helix, showed slight but significant increases in the reactivity. In the N-terminal domain, the reactivity difference showed changes in the pattern as follows: Ala-10 was closed and Ala-23 was exposed. There were large increases in reactivity differences in the CP domain. It should be noted that L155C became much less reactive. Thus, the local structure around the C terminus is maintained. The dramatic changes in reactivity in the CP domain indicate global structural changes and increased flexibility under loosely packed structure.
blocked at low concentration (10 μM) when the blocker was added to the cytoplasmic side (Fig. 8A, upper traces). No changes in the single channel conductance suggest TBA blocks the KcsA channel with the slow blocking mechanism (5). Micromolar concentrations of TBA showed no effect when the blocker was added to the extracellular side, and at 1 mM the channel was blocked (Fig. 8A, lower traces).

To explore the structural changes of KcsA channel under active gating in the presence of 10 mM TBA, the SPR method was applied. In this concentration TBA binds to both intracellular and extracellular sites. Fig. 8B indicates that the pattern of the reactivity was similar to that without TBA. However, the difference plot (Fig. 8C) elucidated the significant changes at and around the Gly-116 site. The Gly-116 site is the C-terminal end of the M2 helix and is located at a linker region between TM and CP domains. The Gly-116 site was not exposed at pH 7.5 but increased its exposure significantly at 4.0 (Fig. 7A), even though the absolute reactivity was still low compared with the sites in the following CP domain. In the presence of TBA the Gly-116 site showed increased exposure to the extent similar to the sites in the CP domain. In contrast to the Gly-116 site, the Gln-119 site decreased the reactivity, suggesting the rearrangement of the C-terminal part of the M2 helix.

DISCUSSION

In this study, the exposure of sites of the KcsA channel was examined with a simple experimental approach. The direct reaction of cysteine-introduced KcsA channel proteins to a bare gold surface was evaluated using SPR signals. The gold plate provides a unique reaction field with a hard, flat surface. Recently SPR measurements have been widely used for detecting various specific reactions, for which the surface of the gold is modified. However, the bare surface without any modifications is, by itself, a specific site for the gold-thiol
reaction. This approach has never been applied for investigating surface structure.

The development of the method required that some technical challenges had to be circumvented. Nonspecific binding was observed, which might have originated from the hydrophobicity of the gold surface and/or the KcsA channel. In our preliminary study, however, phoborhodopsin (sensory rhodopsin II) (19) did not adsorb to the gold surface, indicating nonspecific adsorption is not a general property of membrane proteins. To address the problem of nonspecific binding, we distinguished the specifically bound fraction from the nonspecific fraction by additional washing steps using SDS (Fig. 4A). It is likely that specific bonds between the thiol groups of the cysteine residue and the gold on the sensor chip surface would not be dissociated by washing with detergent solution. Also detergent washing did not increase the specific binding, indicating that gold-thiol reaction of intact channels had been completed in the absence of SDS before washing (Fig. 4A).

25 different single-cysteine mutants of KcsA potassium channels were investigated. All of the mutants were examined by single channel current recordings in planar lipid bilayers. The permeation properties and, especially, gating properties of the mutants were similar to those of the wild-type channels (Fig. 3). Conformational changes experienced upon gating are unlikely to be modified significantly by the mutations. Therefore, differences in the reactivities of the mutant channels should represent topo-specific reactions of the mutated sites. The sites with higher reactivity project structurally, whereas those with lower reactivity are buried. Our results from the SPR measurements at pH 7.5 provide clear topographic maps of the reactivity (Fig. 5A). The high reactivity of the N and C termini and the extracellular loop region and the low reactivity of the Thr-107 site were consistent with the results from x-ray crystallography (12) for the TM domain and those from EPR studies (15) for the N terminus and CP domains. In the results of the high resolution crystal structure (12), the side chain of Val-37 and Ile-38 points to the outer surface of the protein, whereas their accessibility was low in our measurement. This discrepancy can be explained by the detergent molecules covering the TM domain. Previous EPR study (15) predicted that stretches with amino acid positions 5–21 in the N-terminal region and 129–149 and 154–160 in the CP domain take an α-helical structure. Recent NMR study (16), however, suggested that stretch of 142–155 was the only α-helix in the CP domain. We support the EPR structure (15) from the exposure patterns recognized within the amino acid positions 10–13, 131–134, and 155–157 in this study.

Changes in the surface exposure of KcsA channel were detected under active gating at pH 4.0. The drastic changes, mostly an increased reactivity, were distributed in the CP domain (Fig. 7). These differences in reactivity between two conformations can be evaluated quantitatively because the gold-thiol reaction was little affected by pH changes (Fig. 6). We could detect small but significant increases in the reactivity for Gly-116, which is located at the C-terminal end of the M2 helix. This is important because M2 is the inner helix and may hardly be exposed in the closed conformation. However, recent structural information on open gated channels revealed that the M2 helix is bent and the C-terminal half of the helix may be flipped out (20, 21). Therefore, our results support such conformational changes in the M2 helix.

For the CP domain the large enhancement in reactivity leads to the loss of the pattern in consecutive sites. It is important to note that the highly exposed Leu-155 site at pH 7.5 became much less exposed at pH 4.0, indicating local structure of the CP domain is retained. These results suggest that the CP domain undergoes conformational changes and takes flexible and loosely packed conformations at pH 4.0. Exposing different faces at different conformations may result in the apparent loss of the local pattern of reactivity.

Neutron and x-ray small-angle solution scattering study (22) detected apparent dimerization of tetrameric KcsA channels at pH 5.0. They suggested that conformational rearrangement at the CP domain would cause the dimerization at the C-terminal end of the molecule. If it were the case, mutants substituted around the C terminus never bind to the gold surface. Our experimental results demonstrated that the C-terminal mutants showed the highest reactivity (Fig. 5B). In our experimental procedure, the pH was changed to 4.0 immediately before SPR measurements and structural changes in intact tetrameric KcsA channels could be studied.

The structure of the N terminus has not been solved in the crystal structure of the KcsA channel. EPR measurements (15) predicted the α-helical structure, and here we call it as the M0 helix. In this study SPR signals revealed periodicity in the reactivity and supported the α-helical structure. For the orientation of the M0 helix, the crystal structure of other types of potassium channels (23, 24) demonstrated that the helices are aligned tangentially around the pore domain. This is in contrast to the predicted structure from the EPR measurements (15), positioning the M0 helices radially. In this study, some positions in the M0 helix changed their reactivity upon gating, and the pattern of the reactivity changed significantly, suggesting the rotation around the axis of the M0 helix upon gating (Fig. 7). This further supports the tangential orientation of the M0 helix, facing one side of the helix to the transmembrane helices.

The role of the CP domain as a pH sensor has been examined. The pH dependence was retained by the CP domain-deleted channels (15, 25). Therefore, the main pH sensor seems to exist in the TM domain. However, successive deletions of the CP domain from the C terminus shifted the pK values of pH sensitivity significantly (15), suggesting that the CP domain contributed to the pH-sensitive gating. Our results of the flexible and loosely packed CP domain at pH 4.0, compared with the rigid structure in pH 7.5, may allow conformational changes of the TM domain upon gating. In our preliminary studies, the open probability of the KcsA channel at pH 4.0 was decreased significantly by deleting the CP domain. Thus, the CP domain may stabilize the open conformation.

Physiological relevance of the pH-dependent gating of KcsA channel remains unanswered because the intracellular pH should be controlled tightly at around neutral pH (26). It is suggested that the flexible structure at low pH may mimic those
occuring upon signal transduction, in which unknown physiological signaling molecules may induce such conformational change upon binding.

Upon signal transduction, the CP domain and the TM domain interplay. Also the gating movement of the TM domain may be transferred to the CP domain. What will happen if the gate is stopped by channel blockers? Recent crystal structure of KcsA channel with TBA (17, 18) indicated that TBA is trapped in the central cavity at pH 5–7. We applied our method to explore the effect of TBA upon active gating at pH 4.0. There were no global changes in structure. Surprisingly, however, local changes around the Gly-116 site were elucidated. This site was exposed slightly by lowering pH, and in the presence of TBA it is exposed further (Fig. 9). Gly-116 located at the C-terminal end of M2 helix can be exposed when the C-terminal half of the helix flips out. Why did the blocker induce more exposure of the gate? It is less likely that TBA forced the gate open. The mechanism of this phenomenon may be related to familiar channel behavior that burst length (the sum of open and blocked probability) is prolonged as the blocker concentration is increased (27). If the open conformation and the blocked-open conformation are indistinguishable at the Gly-116 site, the lumped “open” conformation stays for longer time in the presence of the blocker. The open probability of KcsA channel is very low at pH 4.0, and the transient exposure of this site was not long enough for reacting with the gold surface. Longer residence in the open state should facilitate the gold-thiol reaction.

Our method gave unprecedented information on the structure of the KcsA channel. This method with its simple and wide applicability would further provide structural information of proteins.

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