Identification and Characterization of the Slowly Exchanging pH-dependent Conformational Rearrangement in KcsA*

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Gating of ion channels is strictly regulated by physiological conditions as well as intra/extracellular ligands. To understand the underlying structures mediating ion channel gating, we investigated the pH-dependent gating of the K⁺ channel KcsA under near-physiological conditions, using solution-state NMR. In a series of 1H-15N-TROSY HSQC (transverse relaxation optimized spectroscopy-heteronuclear single quantum coherence) spectra measured at various pH values, significant chemical shift changes were detected between pH 3.9 and 5.2, reflecting a conformational rearrangement associated with the gating. The pH-dependent chemical shift changes were mainly observed for the resonances from the residues near the intracellular helix bundle, which has been considered to form the primary gate in the K⁺ channel, as well as the intracellular extension of the inner helix. The substitution of His-25 by Ala abolished this pH-dependent conformational rearrangement, indicating that the residue serves as a “pH-sensor” for the channel. Although the electrophysiological open probability of KcsA is less than 10%, the conformations of the intracellular helix bundle between the acidic and neutral conditions seem to be remarkably different. This supports the recently proposed “dual gating” properties of the K⁺ channel, in which the activation-coupled inactivation at the selectivity filter determines the channel open probability of the channel. Indeed, a pH-dependent chemical shift change was also observed for the signal from the Trp-67 indole, which is involved in a hydrogen bond network related to the activation-coupled inactivation. The slow kinetic parameter obtained for the intracellular bundle seems to fit better into the time scale for burst duration than very fast fluctuations within a burst period, indicating the existence of another gating element with faster kinetic properties.

Ion channels play crucial roles in regulating membrane potential, signal transduction, and various physiological events. Therefore, the opening and closing of an ion channel (gating) is strictly controlled by physiological conditions as well as intra/extracellular ligands. The recent determinations of the crystal structures of K⁺ channels, including KcsA (a pH-dependent K⁺ channel) (1, 2), MthK (a Ca²⁺-gated channel) (3), KirBac (an inward rectifier K⁺ channel) (4), and KvAP (5, 6) and Kv1.2 (7, 8) (voltage-gated K⁺ channels), as well as extensive investigations using other structural biological methods (9, 10), have enhanced our understanding of the gating mechanisms of the ion channels. Although the locations and structures of the regulatory regions differ significantly among the K⁺ channels, depending on their activation mechanisms, all K⁺ channels harbor a central pore formed by a symmetrical homotetramer and share an overall topology with two transmembrane helices in each subunit. The pore region contains a potassium-selectivity filter on its extracellular side, a region critical for the selective ion permeation, and the extracellular regions, including the selectivity filter, are quite similar among the K⁺ channels. The second transmembrane helices (or inner helices) of KcsA are straight and form a helix bundle near the membrane/aqueous interface (1, 2). The ion permeation pathway is thus closed at the helix bundle, and this is considered to represent the non-conductive conformation. This is quite reasonable, because the crystal structure was determined at a neutral pH, at which KcsA is expected to be closed (11–13). In contrast, the inner helices of MthK, crystallized in the Ca²⁺-bound state, are bent near the middle of the inner helices (3, 14). As a result, the inner helices splay out toward the intracellular side, thus opening the ion permeation pathway. The other potassium channel structures could be classified as being in either a KcsA-type nonconductive conformation (KirbAC) or an MthK-type conductive conformation (KvAP and Kv1.2) (5, 8, 15). This observation and other experimental evidence support the idea that the intracellular helix bundle serves as the primary gate of the potassium channel (14, 16). However, there are several pieces of evidence suggesting that the gating of K⁺ channels is not solely determined by the conformational rearrangements within the intracellular helix bundle. In fact, mutations near the selectivity filter are known to affect the gating kinetics of K⁺ channels (17–20). Recent detailed analyses of the gating of KcsA also claimed that the selectivity filter controls the electrophysiological open probability of the channel via an activation-coupled inactivation (21, 22). Under such circumstances, the role of each gating element must be identified for an accurate understanding of potassium channel gating.

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Gating of a \( K^+ \) Channel, KcsA

The large scale conformational rearrangement associated with the gating implies intrinsic flexibility within the corresponding regions of the \( K^+ \) channels, and thus emphasizes the importance of studying \( K^+ \) channel structures in motion. Although some electron polarization resonance spectroscopy has been successfully applied to mutated and spin-labeled \( K^+ \) channels (21, 23), NMR spectroscopy would be invaluable to study the structures and dynamics of the unmodified channel at atomic resolution. Here we directly examined the conformational rearrangement associated with the gating by analyzing a \( pH \)-dependent \( K^+ \) channel, KcsA, which is closed at a neutral \( pH \) and open at an acidic \( pH \) (11–13), using solution NMR techniques. We found that KcsA adapts distinctly different in conformations between the closed (\( pH \) 5.2) and open (\( pH \) 3.9) conditions. The conformational rearrangement was mainly observed on the intracellular side, especially for the intracellular gate region, which is consistent with the gate movement suggested by the x-ray crystallographic studies. An Ala substitution of His-25 abolished the conformational rearrangement, showing that the residue serves as a “\( pH \) sensor” for KcsA. The higher rate of the conformational rearrangement than the electrophysiological open probability (\( P_o \)) supports the idea that the activation-coupled inactivation of the selectivity filter, rather than the opening of the intracellular helix bundle, acts as a determining factor for the electrophysiological open probability of the channel. On the basis of these results from NMR experiments and several mutation studies, we will discuss the structural elements that control the gating of \( K^+ \) channels.

EXPERIMENTAL PROCEDURES

Construction of the KcsA \( K^+ \) Channel Expression Vector—The C-terminal deletion construct, \( \Delta 125-160 \) KcsA \( K^+ \) channel, was generated from the wild type KcsA construct reported previously (24). The wild type KcsA vector contains the gene encoding the KcsA \( K^+ \) channel with optimal codon usage for *Escherichia coli*. An additional sequence, including the decahistidine tag and the PreScission\textsuperscript{TM} protease recognition site, was added immediately before the initial methionine residue. The resultant gene was transferred into the pET24d vector, using the Ncol and SalI restriction sites. To generate the \( \Delta 125-160 \) KcsA \( K^+ \) channel, a stop codon (TGA) was introduced by PCR mutagenesis, using *Pfu* DNA polymerase. In all of the constructs, the KcsA \( K^+ \) channel coding regions were completely sequenced, using the T7 promoter and T7 terminator primers, for verification.

Expression and Purification of the KcsA \( K^+ \) Channel—The KcsA \( K^+ \) channel was expressed and purified according to the previous report, with minor modifications (24). *E. coli* strain BL21 (DE3) was transformed with the expression construct. The KcsA protein uniformly labeled with \( ^1H \) and \( ^15N \) was obtained by growing the *E. coli* in M9 minimal medium, in 99.8% \( D_2O \) with 50 \( \mu \)g/ml kanamycin, prepared with 500 mg liter\(^{-1} \) \( ^{15}NH_4Cl \) (99.9% enriched), 2 g liter\(^{-1} \) \( ^2H_6 \) glucose (97% enriched), and 1 g liter\(^{-1} \) \( ^1H_6 \) glucose (97% enriched), and 2 g liter\(^{-1} \) \( ^2H \) (>97% enriched), and \( ^{15}N \) (>98% enriched) Celtone\textsuperscript{TM} base powder. To obtain the partially deuterated \( ^{15}N \)-labeled KcsA protein, M9 minimal medium in \( H_2O \), with 50 \( \mu \)g/ml kanamycin, was prepared with 500 mg liter\(^{-1} \) \( ^{15}NH_4Cl \) (99.9% enriched), 1.5 g liter\(^{-1} \) \( ^2H_6 \) glucose (97% enriched), and 2 g liter\(^{-1} \) \( ^2H \) (>97% enriched), and \( ^{15}N \) (>98% enriched) Celtone\textsuperscript{TM} base powder was used. To obtain Lys and His selectively labeled KcsA, M9 minimal medium in \( H_2O \), supplemented with 19 amino acids other than the targeted amino acid, was prepared with 1 g liter\(^{-1} \) \( NH_4Cl \) (99.9% enriched), 3 g liter\(^{-1} \) glucose, and 50 \( \mu \)g/ml kanamycin, and then 100 mg liter\(^{-1} \) of \( ^{15}N \)-labeled Lys or His was added 15 min prior to the induction of protein expression. The cells were incubated at 37 \( ^\circ \)C, and after reaching an \( A_{590} \) = 0.6, the expression was induced by the addition of 1 mm isopropyl 1-thio-\( \beta \)-\( D \)-galactopyranoside. The cells were harvested after 4–6 h of induction and were resuspended in 60 ml of buffer A (50 mm Tris-HCl (pH 8.0), 150 mm NaCl, and 150 mm KCl) with 0.1 mg/ml PefaBloc at 4 \( ^\circ \)C. The suspended cells were disrupted by sonication. The resultant solution was solubilized with 20 mm of \( n \)-dodecyl \( \beta \)-\( D \)-maltoside (DDM)\textsuperscript{3} for 1 h at room temperature, and the insoluble fraction was removed by centrifugation for 20 min at 15,000 \( \times \) g. The supernatant was applied to a 5-ml column of nickel-nitrilotriacetic acid-agarose. After washing the resin with 40 ml of buffer A, containing 30–100 mm imidazole and 5 mm DDM, the KcsA \( K^+ \) channel protein was eluted with 40 ml of buffer A, containing 400 mm imidazole and 1 mm DDM. The elution fraction was concentrated to 20 ml by a 30,000 molecular weight cut-off membrane ultrafiltration. PreScission protease (0.5 units/20 \( \mu \)g of KcsA) and 1 mm dithiothreitol were added to the concentrated elution fraction, and the solution was dialyzed against 1 liter of buffer B (20 mm NaPi (pH 8.0), 150 mm KCl, and 30 mm imidazole), containing 1 mm dithiothreitol and 0.25 mm DDM, for 15 h. The digested solution was dialyzed twice against 1 liter of buffer B, containing 150 mm NaCl and 0.25 mm DDM, and was continuously applied to a 1.5-ml bed volume of glutathione-Sepharose and a 3-ml bed volume of nickel-nitrilotriacetic acid-agarose, to remove the PreScission protease and the undigested KcsA, respectively. The final yield of the \( \Delta 125-160 \) KcsA \( K^+ \) channel was 2 mg/liter culture.

NMR Spectroscopy—For NMR experiments, the purified KcsA was concentrated to 250 \( \mu \)l by a 30,000 molecular weight cut-off membrane ultrafiltration. The concentration of DDM was controlled by the addition of DDM-containing or DDM-deficient buffer. Experiments were performed on a Bruker Avance 500, 600, or 800 spectrometer equipped with a cryogenic probe. For the spectra, a 200–800 \( \mu \)m concentration of \( ^2H,^{15}N \)-labeled or amino acid selectively labeled KcsA \( K^+ \) channel (as the monomer), solubilized with DDM, was prepared in 10 mm potassium phosphate buffer, containing 150 mm KCl and 90% \( H_2O \), 10% \( D_2O \). DDM-solubilized KcsA is known to form a stable tetramer, even at an elevated temperature (25). Therefore, we performed all measurements at 323 K. The chemical shifts of the resonances at 323 K were almost identical to those observed at room temperature, whereas the line widths of each resonance were significantly improved (data not shown). The concentration of DDM in the sample was estimated from the standard sample with a known amount of the

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\( ^3 \) The abbreviations used are: DDM, \( n \)-dodecyl \( \beta \)-\( D \)-maltoside; TROSY, transverse relaxation optimized spectroscopy; HSQC, heteronuclear single quantum coherence.
detergent. Assuming all DDM molecules in the sample form a complex with KcsA, the total molecular mass of the KcsA-DDM complex under the experimental conditions was estimated to be 106–132 kDa, from the DDM concentration in the sample. The molecular weight of the complex is also consistent with gradient diffusion measurements (26). To observe the amide signals resonating from the structured region of such a large protein complex, maximization of the TROSY effect by the almost complete deuteration of the nonexchangeable protons would be essential (27). Unfortunately, the expression level of H9004125–160 KcsA in D2O-based medium was quite low (less than 100 μg liter⁻¹). Therefore, we produced the protein in H2O-based medium supplemented with a deuterated amino acid mixture, as reported previously (28, 29).

RESULTS AND DISCUSSION

pH Titration Behavior of KcsA, Monitored by TROSY HSQC—The NMR spectra presented in this paper were acquired using the C-terminal deletion construct of KcsA (H9004125–160), which was solubilized in DDM. Fig. 1 shows a comparison of the 1H,15N-TROSY HSQC spectra of H9004125–160 and the full-length KcsA at pH 6 (closed condition). The distributions of the dispersed signals are almost identical for both of the constructs, indicating that the truncation of the C-terminal region does not perturb the overall structure of the protein, and thus we chose Δ125–160 KcsA for further analyses. The observed HSQC spectra showed overall resonance dispersions similar to what is obtained for the SDS-solubilized KcsA (10), indicating the similarity in the secondary structure elements. However, the exact chemical shifts of the resonances are not identical, including those from the intracellular gate region, which we will focus on later. Because the SDS-solubilized KcsA could not tolerate lower pH conditions, the use of a strong ionic detergent for the solubilization might induce some structural instability or pH-dependent morphology change in the protein, which would not be the case with the mild nonionic detergent DDM. Although the solubilization in detergent can cause functional deficiency in a membrane protein, this does not seem to be the case for the DDM-solubilized KcsA. In fact, the interaction of KcsA with an inhibitor, agitoxin2, was not different between the DDM-solubilized and membrane-reconstituted conditions (30), and the pH-dependent opening of the channel was intact in the DDM-solubilized condition (16).

The pH titration behaviors of Δ125–160 KcsA, monitored by TROSY HSQC spectra, are shown in Fig. 2. The line widths of the signals from H9004125–160 were reasonably narrow, and out of 123 expected resonances, 110 resonances were observed in the TROSY HSQC spectra. At pH 3.9, the dispersion of the resonances was quite different from that at pH 5.2. The chemical shift changes were predominantly observed in this pH range, because the spectra recorded at pH 3.6 and pH 6.0 were almost identical to the spectra obtained at pH 3.9 and pH 5.2, respectively (data not shown). The apparent pKa value of the spectral transition was estimated to be pH 4.5. Because the pH

![Figure 1](https://example.com/fig1.png)

**FIGURE 1.** 1H,15N-TROSY HSQC spectra of the H9004 partially protonated/2H/15N-labeled Δ125–160 KcsA (A) and the full-length KcsA (B). Both spectra were recorded at 323 K, in a buffer containing 10 mM KPi (pH 6.1), 150 mM KCl, 0.05% NaN₃, and 10% D₂O. All spectra were recorded at 800 MHz. Both the Δ125–160 and full-length KcsA proteins were dissolved in DDM micelles. The KcsA protein concentrations were 300 μM as the monomer. The spectra were recorded for 64 transients per t₁ increment, and the inter-scan delay was set to 1.1 s.

![Figure 2](https://example.com/fig2.png)

**FIGURE 2.** 1H,15N-TROSY HSQC spectra of H9004 partially protonated/2H/15N-labeled Δ125–160 KcsA at various pH values. Counter plots in black, green, and red correspond to the spectra recorded at pH 5.2, pH 4.4, and pH 3.9, respectively. Typical slow and intermediate exchanging resonances are circled by a broken blue line and indicated by a blue arrow, respectively. Each set of these resonances was enlarged. The experimental conditions, except for the pH value, were the same as in Fig. 1.
range of the chemical shift transitions overlapped with that of the open/closed transition, the observed spectral change most likely reflects a conformational rearrangement that is accompanied by the gating of KcsA. About 30 resonances showed apparent shifts in a slow to intermediate exchanging fashion, as typically observed for the cross-peaks indicated in Fig. 2 by the blue dotted circles (slow exchange) and the arrows (intermediate exchange). On the other hand, the total number of resonances was almost identical under both pH conditions. In the spectrum recorded at pH 4.4, two sets of signals were observed, corresponding to both sets of slowly exchanging signals recorded at pH 3.9 and pH 5.2. These facts indicate that only a limited region in the protein undergoes a pH-dependent conformational rearrangement, and that the conformational rearrangement of this region is relatively slow (in the range of second-millisecond motion). Assuming two-site exchange between these signals, then an exchange rate, slower than 150 s$^{-1}$, can be estimated from the line shapes of these resonances (Fig. 3).

**pH Titration Behavior of Trp Indole Signals and Amino Acid Selectively Labeled KcsA—**To identify the region undergoing the pH-dependent conformational rearrangement, we extensively analyzed the pH titration behavior of the Trp indole signals, as well as the main chain amide signals from Lys and His residues in an amino acid selectively labeled KcsA. KcsA has only a small number of these residues (1 for Lys residues and 4 for His residues), and the main chain amide moieties of these residues are known to be less scrambled with the other amino acids; thus, these residues are suitable for obtaining information about the conformational rearrangement of KcsA without ambiguity. As shown in Fig. 3A, these residues are distributed in both the proximal (His-25, Trp-26, and Trp-113) and distal (His-1, His-20, His-67, Trp-68, Trp-87, and His-124) regions relative to the intracellular gate of KcsA. The distributions also make these residues good probes to identify the conformational rearrangement associated with the gating of KcsA. Fig. 4 (B–D) shows close up views of the pH titration behaviors of the Trp indole resonances and the main chain amide signals of Lys and His residues in amino acid selectively labeled Asp-125 to Asp-160 KcsA. Δ125–160 KcsA possesses only one Lys residue, Lys-14, and thus the resonance observed in the Lys-selective spectrum was unambiguously assigned. The assignments of the other resonances were established by comparisons between the wild type and mutant spectra (H1A, H20A, H25A, W26F, W67F, W87Y, W113F, and H124A) under each pH condition. Overall, the spectra of the wild type and mutant KcsA were similar, indicating that these substitutions did not alter the global conformation of the proteins (data not shown). At neutral pH, Trp-26 and Trp-67 showed significantly higher and lower field proton chemical shifts, respectively. The high field shift of Trp-26 is possibly a consequence of the tilted stacking of the residue toward Trp-113 (Fig. 4A), whereas the lower field shift of the Trp-67 resonance might reflect a hydrogen bond network involving the indole proton, a water molecule, and the carboxyl moieties of Glu-71 and Asp-80 (2).

With decreasing pH, the signals resonating from the residues near the intracellular helix bundle (His-25, Trp-26, and Trp-113) drastically shifted their positions (change in normalized chemical shift >0.2 ppm) in a slowly exchanging fashion (see chemical shift differences in Table 1). Smaller chemical shift changes were observed for His-20 and His-124, which are located at the N terminus of the outer helix (7 Å apart from the most significantly shifted His-25 to the cytoplasmic side) and the C terminus of the inner helix (17 Å apart from His-25 to the cytoplasmic side), respectively. A slowly exchanging pH-dependent chemical shift was also observed for the C-2 position of His-124 (data not shown). No chemical shift perturbation was observed for the N-terminal distal region (His-1 and Lys-14) and the extracellular side (Trp-68 and Trp-87) of KcsA over the pH range recorded in this experiment. These observations show that the pH-dependent structural rearrangement occurs over a rather wide range on the intracellular side, especially around the region close to the helix bundle. This agrees well with the proposed gating model, based upon the x-ray structures of KcsA and MthK (14). In this model, the bending of the inner helices, at a Gly residue in the middle of the inner helix, induces the simultaneous disassembly of the inner helix bundle through a rigid body movement (14), and thus anticipates the conformational rearrangements in the N-terminal half of the outer-helix, the C-terminal half of the inner-helix, and the intracellular helix bundle. The direction of the Trp-26 chemical shift change is also explained well by the model, i.e. the stacking between Trp-26 and Trp-113, which could
induce a high field shift, would be lost when the intracellular helix bundle opens. The NMR data are also consistent with an extensive experiment analyzing the effect of site-specific mutations on the voltage-dependent opening of the Shaker K⁺ channel, which indicated that the gating-sensitive amino acids were clustered near the inner helix bundle and were in both the outer and inner helices (Lys-390 (Ala-23 in KcsA), Ala-391 (Leu-24), Met-393 (Trp-26), Glu-395 (Ala-28), Val-476 (Ala-109), Ile-477 (Leu-110), and Asn-482 (Val-115)) (31).

It is also worth noting that Trp-67 also shows a slowly exchanging chemical shift. A recent paper revealed that the Ala mutation of Glu-71 abolished the activation-coupled inactivation of KcsA (21). Because Glu-71 forms a hydrogen bond network with the Trp-67 indole behind the selectivity filter, a possible explanation for the observed chemical shift change would be a structural transition of the selectivity filter to the inactivated conformation. The electrophysiological characterization revealed that the activation-coupled inactivation emerges slowly after the activation of the channel (time cause between 0.5 and 1 s) and became dominant in the steady-state condition (21). Although further characterization would be needed, the slowly exchanging pH-dependent chemical shift change observed for the Trp-67 resonance agrees well with the structural characteristics, which can be predicted from the electrophysiological experiments. In the x-ray structure of the E71A mutant of KcsA, Trp-67 adopts two conformations, a wild type-unflipped conformation and a flipped conformation (21), among which the latter could not have a hydrogen bond network lining the selectivity filter. Judging from the low field chemical shift of the Trp-67 indole resonance, it seems that the hydrogen bond network is still present in the acidic condition. This might indicate the importance of the hydrogen bond network to maintain the inactivated state, and the transition to the inactivated conformation might not be accompanied by a large structural change, such as the flipping of the Trp-67 side chain.

Identification of a "pH Sensor" in KcsA—KcsA opens at an acidic pH and is closed at a neutral pH, suggesting that one or some acidic or His residues would serve as a pH sensor of the channel. The truncation of the cytoplasmic region at Glu-120 had only a modest effect (shifted the pKₐ between the open and closed conditions by 0.5) indicating that the pH sensor of KcsA would be in the core domain of the channel (32). There are two acidic (Glu-118 and Glu-120) and three His residues (His-20, His-25, and His-124) on the intracellular side of the KcsA molecule. We substituted each of the residues with an Ala residue and analyzed the effect of the substitutions on the pH dependence of the Trp indole signals, as shown in Fig. 5. Although the substitutions of the acidic residues, His-20, and His-124 did not affect the pH dependence of the Trp indole signals, the substitution of His-25 abolished the pH dependence. These results indicate that His-25 is the pH sensor, which controls the pH-dependent gating of KcsA.

His-25 is located at the interface of the inner and outer helices, near the intracellular helix bundle (2). This buried location might also explain the unusually low pKₐ of His-25. A comparison with experiments performed with other K⁺ channels suggests the indispensable role of this region in the K⁺ channel gating. In the Shaker K⁺ channel, several outer helix residues that are involved in the packing between the inner helix (Lys-390 (Ala-23 in KcsA), Ala-391 (Leu-24), Met-393 (Trp-26), Glu-395 (Ala-28) and Leu-396 (Ala-29)) were found to be gating-sensitive (31). In addition, when the residue corresponding to His-25 in KcsA was substituted with Ala in the Shaker voltage-gated potassium channel, no functional expression was observed for this mutant (31). In the type 2 G-protein gated, inwardly rectifying K⁺ channel, the substitution of the residue corresponding to His-25 in KcsA (Asn-94) increased the open probability of the channel (31). An NMR study using the SDS-solubilized KcsA also detected subtle chemical shift changes between pH 6 and 8 for the outer helix residues, including His-25, and the inner helix residues 113–117 near the helix bundle, which may represent possible cross-talk between the inner and outer helices and account for the drastic structural change observed in this study (10).

Although the locations and structures of the regulatory domains of K⁺ channels are diverse, the structures of the pore domains, which contain the gates of these channels, are conserved among all types of potassium channels. Therefore, it is reasonable to propose that the information received by the various regulatory regions, located in different ports of the K⁺ channels, would be integrated at a specific site of a channel before it is passed to the gate. The conserved localization of the gating-sensitive residues, at the interface of the inner and outer helices near the intracellular helix bundle, suggested that this site might participate in such a role. The location of the site in the structure of the K⁺ channel is quite reasonable, if one considers the proximity of this region to both the N- and C-terminal regulatory regions of the K⁺ channels.

Gating of KcsA—The steady-state electrophysiological gating of KcsA is characterized by long closures interrupted by the occasional short bursting periods, where the channel undergoes the fast equilibrium between the open and closed states. Because of the long closures, the electrophysiological Pₒ of KcsA remains low, even under the open condition at an acidic pH. On the other hand, as shown here by the structural study using the wild type KcsA, the region around the intracellular helix bundle adopts different conformations at an acidic pH, as compared with a neutral pH. This has also been detected by electron polarization resonance studies, using a chemically modified mutant KcsA channel (9, 21, 23). These observations confirm that the KcsA channel could not assume a conductive conformation by merely opening the inner helix bundle.

The recent finding of the activation-coupled inactivation of KcsA is a key toward solving this inconsistency between the structural and electrophysiological data (21, 22). These studies revealed that KcsA gives larger macroscopic currents just after the pH is dropped from 7.5 to 4, and the current slowly relaxes to 5–10% of the initial current after 2–3 s, which corresponds to the steady-state electrophysiological activity of the channel. The inactivation was abolished in the E71A mutant channel, which shows almost 100% open probability even at the steady state. From this observation, the wild type KcsA is...
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A

B

C

D

$\text{^1H chemical shift (ppm)}$

$\text{^1N chemical shift (ppm)}$
considered to predominantly reside in the inactivated conformation at an acidic pH, and occasional recoveries from the inactive conformation generate burst openings of the channel. In the model, the selectivity filter forms an inactivation gate to control the open probability, whereas the intracellular gate is considered to be a determinant for other properties, such as burst duration or rapid open/closed exchange in the burst periods. As calculated from the known mean electrophysiological open (1.1 ms) and closed times (29 ms) during the burst periods, the exchange rate for the rapid fluctuation between the open and closed conformations would be 950 s$^{-1}$ (33); however, the exchange rate estimated for the conformational rearrangement of the intracellular gate was much slower than that (150 s$^{-1}$; see Fig. 3). The $^{15}$N transverse relaxation rates of the Trp-26 and Trp-113 indole resonances were not different under the counts/min $g$ spin echo sequence (34), with the delay between 180° pulses ($\tau_{mw}$) ranging from 0.5 to 3 ms in both acidic (pH 3.9) and neutral (pH 5.8) conditions, indicating that there is no chemical exchange corresponding to a 950 s$^{-1}$ exchange rate in the internal gate. The kinetic parameters rather support the idea that the intracellular helix bundle is a determinant for slower motion, for example, the burst duration. This idea is consistent with the finding that a mutation in the intracellular crossing region prolonged the “burst” period in Kir6.2 (35).

This also leads to the thought that there would be another gate element that participates in an intra-burst short open/closed equilibrium. The KcsA filter is quite flexible and can change its conformation, depending on the potassium concentration (2, 36) or Fab binding (2, 21). Although the structural changes identified thus far are considered to represent the open (or inactive) conformation, it might also be possible that the selective filter adopts the closed conformation in the burst durations.

**TABLE 1**

| Residue | $\Delta\delta N$ | $\Delta\delta H$ | Normalized chemical shift change | Exchange mode |
|---------|------------------|------------------|---------------------------------|---------------|
| Trp-25  | +0.62 ppm        | +1.97 ppm        | 0.73 ppm                        | Slow          |
| Trp-67  | −0.08 ppm        | +0.68 ppm        | 0.16 ppm                        | Slow          |
| Trp-68  | +0.02 ppm        | 0.00 ppm         | 0.02 ppm                        | Slow          |
| Trp-87  | +0.02 ppm        | 0.00 ppm         | 0.02 ppm                        | Slow          |
| Trp-113 | −0.24 ppm        | +0.01 ppm        | 0.24 ppm                        | Slow          |
| His-1   | +0.01 ppm        | 0.00 ppm         | 0.01 ppm                        | Slow          |
| His-20  | −0.07 ppm        | −0.87 ppm        | 0.19 ppm                        | Intermediate  |
| His-25  | +0.38 ppm        | +0.45 ppm        | 1.34 ppm                        | Slow          |
| His-124 | +0.03 ppm        | +0.12 ppm        | 0.04 ppm                        | Intermediate  |
| Lys-14  | 0.00 ppm         | 0.00 ppm         | 0.00 ppm                        | Slow          |

*Chemical shift difference between acidic and neutral conditions. The chemical shift at neutral pH was subtracted from that at acidic pH.

*Normalized chemical shift values calculated by $c(\Delta\delta H)^2 + (\Delta\delta N)^2/25)^{1/2}$.

**Conclusion**—In summary, using solution NMR spectroscopy, we directly observed the conformational rearrangement associated with the gating of the potassium channel KcsA. The conformational rearrangement was primarily observed for the residues near the intracellular helix bundle and the C-terminal regions of the inner transmembrane helices. Our results agree well with the intracellular gate movement, proposed on the basis of the x-ray structures of potassium channels. From the effects of the substitutions on the pH-dependent conformational rearrangement, we identified His-25 as the pH-sensor that controls the pH-dependent gating of KcsA. His-25 is located on the interface of the inner/outer helices, near the inner helix bundle and the cluster of “gate-sensitive” residues in the region, suggesting the conserved role of this region in the gating of K$^+$ channels. The intracellular helix bundle adopts a different conformation, whereas the steady-state electrophysiological open probability of the channel is known to be less than 10%. This discordance adds another piece of evidence that supports the idea that the inner helix bundle is not the only gate element of the channel. The slow exchange kinetics of the region might exclude the possibility that the intracellular gate would be a fast gate that undergoes intra-burst electrophysi-

**FIGURE 4.** Distributions of Trp, Lys, and His residues in KcsA (A) and close up views of the Trp indole (B), Lys (C), and His (D) main chain amide resonances in the $^{15}$N HSQC spectra. A, distributions of Trp, Lys, and His residues are mapped on the crystal structure of KcsA (2). $\Delta 125$–160 KcsA contains four Trp residues per subunit. Two of them are located on the intracellular side near the helix bundle (Trp-26 and Trp-113), and the others (Trp-67, Trp-68, and Trp-87) are on the extracellular side of the protein. Trp-67 forms the pore-inner helix interface, and Trp-68 is located in the protein core between the selectivity filter and the pore helix, and Trp-87 is at the N terminus of the inner helix. $\Delta 125$–160 KcsA possesses only one Lys residue, Lys-14, in the N-terminal intracellular region, and this residue was not observed in the crystal structure of KcsA. For the As residues, $\Delta 125$–160 KcsA contains four residues as follows: His-1 is in the N-terminal sequence left after the enzymatic digestion; His-20 is at the end of the N-terminal intracellular region; His-25 is in the outer/inner helix interface near the helix bundle; and His-124 is at the C terminus of the protein. His-1 and His-20 do not exist in the crystal structure of KcsA. Close up view of the Trp indole region of the $^{15}$N-TROSY HSQC spectra of Hu partially protonated $^{15}$N-labeled $\Delta 125$–160 KcsA (B), $^{1}$H$^{15}$N-HSQC spectra of Lys (C), and His (D) selectively $^{15}$N-labeled $\Delta 125$–160 KcsA at various pH values. The resonances from Trp-68 and Trp-87 overlap each other. Assignments were established by site-specific mutagenesis. B–D, the dashed lines connect corresponding signals. The experimental conditions were the same as in Fig. 1.
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ological fluctuations, suggesting the existence of another gating element. This obviously needs further characterization. For example, a kinetic study of the selective filter, by using NMR or electron polarization resonance, might be an important step toward fully understand the gating mechanism of the $K^+$ channel.

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