Characterization of the C-terminal Domain of a Potassium Channel from *Streptomyces lividans* (KcsA)*

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Victor P. T. Pau‡, Yongfang Zhu†, Zhiguang Yuchi‡, Quyen Q. Hoang§, and Daniel S. C. Yang††

From the ‡Department of Biochemistry and Biomedical Sciences, Faculty of Health Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada and the §Rosenstiel Basic Medical Research Center, Brandeis University, Waltham, Massachusetts 02454

KcsA, a potassium channel from *Streptomyces lividans*, is a good model for probing the general working mechanism of potassium channels. To date, the physiological activator of KcsA is still unknown, but *in vitro* studies showed that it could be opened by lowering the pH of the cytoplasmic compartment to 4. The C-terminal domain (CTD, residues 112–160) was proposed to be the modulator for this pH-responsive event. Here, we support this proposal by examining the pH profiles of: (a) thermal stability of KcsA with and without its CTD and (b) aggregation properties of a recombinant fragment of CTD. We found that the presence of the CTD weakened and enhanced the stability of KcsA at acidic and basic pH values, respectively. In addition, the CTD fragment oligomerized at basic pH values with a transition profile close to that of channel opening. Our results are consistent with the CTD being a pH modulator. We propose herein a mechanism on how this domain may contribute to the pH-dependent opening of KcsA.

Permeation of ions across cellular membranes is essential to life, but it is energetically unfavorable due to the dielectric barrier formed by the lipidic components of the membrane. Ion channels provide means to overcome this barrier. These channels allow high flux of ions across the membrane while maintaining high selectivity in a well regulated manner (1, 2). The mechanisms of efficiency, selectivity, and open-closed switching of channels have been the focus of many scientists in the field (3–8). Toward these goals, studies on the potassium channel KcsA from *Streptomyces lividans* have provided a wealth of information.

KcsA exists as a homotetramer with each subunit consisting of 160 residues (9, 10). Crystal structure of a truncated KcsA (consisting of residues 22–124 without the N- and C-terminal domains) (PDB id 1K4C) showed two transmembrane α-helices separated by a P loop in each subunit (11, 12). The tetrameric structure resembles an inverted teepee with the base facing the extracellular side. The wall of the teepee is made up of eight helices (two from each monomer), and the body of the teepee is filled with four P loops (one from each monomer) forming a narrow passage that is selective for K⁺ ions. A mechanism for efficiency and selectivity was proposed based on this structure (11, 13). The mechanism for open-closed switching of KcsA, however, was not immediately evident from the structure of the truncated molecule in the crystal.

KcsA has been demonstrated to be a pH-dependent channel *in vitro*, and it has been demonstrated that its opening probability increases as the intracellular pH decreases (14,15) (Fig. 1). The gating site is located at the narrow opening of the teepee that faces the intracellular space (5). Although the N-terminal and C-terminal domains (CTD) are absent in the crystal structure (Protein Data Bank id 1K4C), they are predicted to face toward the cytosol (14, 16). The N-terminal domain was proposed to be a membrane anchor (14, 16). When it was truncated, the activity of the channel was not altered (22). The CTD was proposed to be a modulator for open-closed switching (14, 17). Its partial removal (last 35 residues) destabilized the tetramer and increased ion flux through the channel at neutral pH, suggesting that it stabilized the closed state (14). In addition, the deletion mutant was still able to open in a pH-dependent manner but with an altered transition profile, indicating that the primary pH sensor is not located in the last 35 residues (14). A recent study suggested that the pH sensor is located at the N terminus end of the first transmembrane helix (H25 (18)). To date, the detailed mechanism of gate opening remains elusive and requires further investigation. We investigated the role of CTD in gate opening by examining (a) its contribution to the thermal stability of KcsA tetramer and (b) its oligomerization, at various pH values. We also proposed a mechanism to relate our findings to the open-closed switching of KcsA.

**EXPERIMENTAL PROCEDURES**

**Material**—Electrophoresis setups were purchased from Bio-Rad Laboratories Ltd. (Ontario, Canada). All restriction enzymes, Taq polymerase, *Pfu* polymerase, and T4 ligase were purchased from Fermentas Canada Inc. (Ontario, Canada). HiTrap chelating HP and HiTrap heparin columns and pET28a

‡ The abbreviations used are: CTD, C-terminal Domain of KcsA; rCTD, recombinant fragment of the C-terminal domain of KcsA; rHCTD, His*-tagged recombinant fragment of the C-terminal domain of KcsA; rKcsA, recombinant KcsA; KcsA3ACTD, residues 1–125 of rKcsA generated by chymotrypsin digestion; chKcsA, recombinant KcsA with C-terminal His tag; rcKcsA, residues 1–125 of chKcsA generated by chymotrypsin digestion with purification; LDAO, Lauryldimethylamine N-oxide; MWCO, molecular weight cut-off; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-glycerol); CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine.

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† To whom correspondence should be addressed: Dept. of Biochemistry and Biomedical Sciences, Faculty of Health Sciences, McMaster University, 1200 Main St. West, Hamilton, Ontario L8N 3Z5, Canada. Tel.: 905-525-9140, Ext. 22455; Fax: 905-522-9033; E-mail: yang@mcmaster.ca.
were purchased from Amersham Biosciences and Novagen, respectively. Sep-Pak Vac 12CC C_{18} Cartridge and high pressure liquid chromatography Delta-Pak C_{18} columns were purchased from Waters Corp. (Milford, MA). Thrombin was either purchased from Sigma-Aldrich Canada Ltd. (Ontario, Canada) or purified from Thrombostat (Warner-Lambert Canada Inc., Ontario, Canada) with HiTrap heparin column (19). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-rac-(1-glycerol)) (sodium salt) (POPG) and Specta/Por dialysis membrane with MWCO: 3,500 and Vivaspin concentrator with MWCO: 50,000 were bought from Avanti Polar Lipid, Inc. (Alabaster, AL) and VWR (Ontario, Canada), respectively. Other chemicals were purchased from Sigma-Aldrich Canada Ltd.

Cloning of KcsA (rKcsA), C-terminal His-tagged KcsA (chKcsA), and N-terminal His-tagged rCTD of KcsA—The DNA coding for KcsA was amplified by PCR with TaqDNA polymerase (Fermentas) from pQE60/kcsa (a gift from Dr. MacKinnon) with an alanine inserted at the second position to form rKcsA and chKcsA. The amplified products were cloned into a pET28a expression vector (Novagen) at its respective restriction enzyme recognition sites.

Cloning of recombinant CTD fragment (rCTD) of KcsA (residues 116–160) was also amplified by PCR from pQE60/kcsa using BamHI and EcoRI restriction enzyme recognition sites. The amplified product was cloned into a modified pET28a expression vector using BamHI and EcoRI sites. The modified pET28a expression vector has a BamHI restriction enzyme recognition site incorporated into the codons of the thrombin recognition site at the glycine and serine positions. Positive clones were selected by kanamycin. The plasmids for rKcsA, chKcsA, and rCTD were sequenced (MOBIX, McMaster University) and named pET28a/rkcsa, pET28a/chkcsa, and pET28a/hctd, respectively.

Overexpression of rKcsA and chKcsA—Overnight culture of BL21(DE3) transformed with either pET28a/rkcsa or pET28a/chkcsa was diluted 10 times with LB containing 100 μg/ml kanamycin and incubated at 37 °C. After 1.5 h, isopropyl-1-thio-β-D-galactopyranoside was added to the culture to a final

![FIGURE 1. Measurement of current conducted by rKcsA and rctKcsA. A, a typical record of current conducted by rKcsA at pH 3.8 for a period of 3 s with an applied voltage of 100 mV. The open and closed states are indicated by symbols C and O on the right side of the figure. B, histograms of current conducted by rKcsA and rctKcsA at various pH values and recorded for periods of 20 s. The open and closed states are indicated by the symbols C and O on the bottom of each histogram. No open state was observed at pH values (6.2 and 7.0) for rKcsA and at pH 7.0 for rctKcsA. C, pH profile of current conducted by rKcsA and rctKcsA in its open states determined by fitting Gaussian curves to histograms in panel B. The current measured at different pH values for each construct was normalized to the current measured at pH 3.8. Currents of the closed state were used for pH values (6.2 and 7.2) of rKcsA and pH 7.0 of rctKcsA because no open state was observed. Standard derivations of the Gaussian peaks were calculated from the current values at the half-height positions and shown as error bars.](image-url)
concentration of 1 mM for induction. The culture was further incubated at 37 °C for 3 h. Cells were harvested by centrifuging the culture at 6,000 × g for 15 min.

Purification of rKcsA—The cell pellet was resuspended in the lysis buffer (0.8% w/v NaCl, 0.04% w/v KCl, 0.12% w/v Na2HPO4, 0.04% w/v KH2PO4, 7.5 mM phenylmethylsulfonyl fluoride, pH 7.4) and lysed by French press three times at 10,000 p.s.i. The cell lysate was spun at 10,000 × g for 60 min. rKcsA were purified from the pellet and the supernatant. rKcsA in the pellet was solubilized as follows. (a) The pellet was resuspended in the binding buffer (see below) with 0.8% w/v LDAO; (b) the resuspension was incubated at room temperature with gentle mixing for 1 h; (c) the mixture was centrifuged at 100,000 × g for 60 min at 22 °C; and (d) the supernatant containing rKcsA was collected for further purification. rKcsA in the supernatant of the low speed centrifugation (10,000 × g) was further centrifuged at 100,000 × g for 60 min at 22 °C. rKcsA in this pellet was solubilized as stated previously. Solubilized rKcsA was purified using a HiTrap S column. The column was first equilibrated with binding buffer (25 mM Bicine, K+, pH 8.0, 50 mM KCl, 0.1% w/v LDAO), and the detergent-solubilized rKcsA was subsequently loaded onto the column. rKcsA was eluted with a linear gradient mixture of the binding buffer and elution buffer (25 mM Bicine, K+, pH 8.0, 1.0 M KCl, 0.1% w/v LDAO). The protein came out at 0.2 M KCl.

Purification of chKcsA—The cell pellet was resuspended in the lysis buffer (20 mM Bicine, K+, pH 8.0, 150 mM KCl, 7.5 mM phenylmethylsulfonyl fluoride) and lysed by French press three times at 10,000 p.s.i. The cell lysate was spun at 100,000 × g for 60 min at 22 °C. rKcsA in this pellet was solubilized as stated previously. Solubilized rKcsA was purified using a HiTrap S column. The column was first equilibrated with binding buffer (25 mM Bicine, K+, pH 8.0, 50 mM KCl, 0.1% w/v LDAO), and the detergent-solubilized rKcsA was subsequently loaded onto the column. rKcsA was eluted with a linear gradient mixture of the binding buffer and elution buffer (25 mM Bicine, K+, pH 8.0, 1.0 M KCl, 0.1% w/v LDAO). The protein came out at 0.2 M KCl.

Chymotrypsin Digestion of rKcsA and chKcsA—80 µl of chymotrypsin (0.1 mg/ml) was added to 4 ml of purified rKcsA or chKcsA (0.25 mg/ml) and incubated at 37 °C for 2 h. Subse-
subsequently, the reaction was quenched by the addition of 40 μl of 0.3 M phenylmethylsulfonyl fluoride and further incubated for 30 min. Then, the sample was concentrated with a Vivaspin concentrator (MWCO 50,000 Da) to a final volume of 1 ml. The rKcsA sample was used without further purification as CTD-truncated KcsA (KcsA<sub>CTD</sub>). The chKcsA sample was further purified by using a Ni<sup>2+</sup>-charged HiTrap chelating HP column to remove the cleaved C-terminal domain as well as any uncleaved chKcsA to generate the C-terminal truncated KcsA (rctKcsA).

Reconstitution of rKcsA and rct-KcsA in Liposome and Formation of Giant Liposome—Micellar solution of lipid detergent (7.5 mg/ml POPE, 2.5 mg/ml POPG, 21 mg/ml CHAPS) in reconstitution buffer (450 mM KCl, 10 mM HEPES, pH 7.0) was prepared according to Heginbotham et al. (15, 24). Purified rKcsA or rctKcsA was added to the micellar solution to a final concentration of 30–60 μg/ml. A mixture of lipid detergent and rKcsA or chKcsA (1 ml) was dialyzed against reconstitution buffer (1 liter) in a dialysis bag (MWCO of 3,500 Da) at room temperature for 12 h with the buffer replaced once at the 6th h. Liposomes containing rKcsA or rctKcsA were fused to form giant liposomes for electrophysiological studies according to the freeze-thaw method of Kasahara and Hinkle (25). In brief, a glass culture tube with 0.5 ml of rKcsA- or rctKcsA-containing liposomes was put into liquid nitrogen for 2 min and thawed back to room temperature. This freeze-thaw cycle was repeated 5–6 times to produce giant liposomes.

Channel Current Measurement by Cell-attached Patch Clamp Technique—All buffer solutions except the one at pH 7 contain 1 mM citric acid, 0.01 mM CaCl<sub>2</sub>. The pH values (3.8, 4.7, 5.2, and 6.2) of each solution were adjusted by the addition of KOH and KCl to yield a final solution with exactly 100 mM [K<sup>+</sup>]. The buffer solution at pH 7 contains
95 mM KCl, 5 mM KOH, 10 mM Hepes, and 0.01 mM CaCl₂. The pH of this buffer was adjusted to 7 by the addition of HCl. 5 μl of giant liposomes was deposited onto a Petri dish (3.5 cm diameter) containing 200–300 μl of anchoring buffer (10 mM HEPES, 100 mM KCl, 0.1 mM CaCl₂, pH 7.4). The Petri dish was incubated at room temperature for 20 min to allow liposomes to anchor to the bottom of the dish. The Petri dish was rinsed extensively using the pH 3.8 buffer to remove unanchored liposomes. Then, the pH 3.8 buffer was used as the bath solution.

The tip of a patch pipette containing a microelectrode and filled with the pH 3.8 buffer (10–20 megohms of resistance for the open electrode) was apposed to an anchored liposome with the assistance of a micromanipulator to form a gigaseal (1–10 gigaohms) between the pipette and the liposome. 100 mV was applied across the gigaseal using a Model 2400 patch clamp amplifier (A-M Systems Inc., Carlsborg, WA) with the reference electrode sitting in the Petri dish. Cell-attached current signal (filtered at 2 KHz) was sampled at a rate of 4,096 Hz by a personal computer equipped with a USB-linked LabJack U12 DAQ device (LabJack Corp., Lakewood, CO). Different liposomes would be patched if no channel activity was detected within 30 min. Cell-attached channel currents at different pH values were measured on the same liposome with the bath solution changed to corresponding buffers. The data set was analyzed using the Molecular Devices pClamp 9.0 software (Molecular Devices Corp., Sunnyvale, CA).

**Thermal Stability Determination**—200-μl samples of rKcsA and KcsAΔCTD were dialyzed in dialysis bags (MWCO 3,500 Da) at room temperature overnight against 200-ml solutions of 150 mM KCl, 0.1% v/v LDAO, and 15 mM buffers (pH 3–6, K⁺-citrate, pH 7, K⁺-phosphate, pH 8, K⁺-Hepes). 2 μl of thermal stability test solutions (10% w/v SDS, 9.3% w/v dithiothreitol, and 38% w/v glycerol) was added to 8 μl of dialyzed samples and heated at 30 °C for 30 min at constant temperatures ranging from 30 to 100 °C at 10 °C-intervals. The heated samples were allowed to cool to room temperature and analyzed on 15% SDS-PAGE. Scanned images of the gels were analyzed using imageJ in integrated intensity mode to determine the amounts of tetramer and monomer in the samples. Fractional tetramer content was calculated by dividing the integrated density of tetramer by the combined integrated densities of tetramer and monomer. The thermal denaturation profiles (plots of fractional tetramer content versus temperature) for all pH values were analyzed to determine the temperatures at which half of the tetramers were dissociated (T_m) directly from the plot without curve fitting. Three independent experiments were performed for each pH and construct.

**Overexpression and Purification of His-tagged rCTD, rHCTD**—Procedures for overexpression of rHCTD and cell lysis were the same as those for rKcsA except that the Escherichia coli BL21(DE3) was transformed with pET28a/hctd instead. Cell lysate was spun at 10,000 × g, and the supernatant was loaded onto a Ni²⁺-charged HiTrap chelating HP column. The column was washed with the lysis buffer with the addition of 0.1 M imidazole. The bound protein was eluted with 0.5 M imidazole in the lysis buffer.

**Thrombin Digestion of rHCTD and Purification of rCTD**—Thrombin (144 units) was added to a 12-ml solution of purified rHCTD (1 mg/ml), and the reaction mixture was incubated at 37 °C for 15 h to remove the His tag from the rHCTD. Then, the digested mixture was loaded onto a Sep-Pak Vac 12CC C₁₈ cartridge pre-equilibrated with 0.1% v/v trifluoroacetic acid. After sample loading, the cartridge was washed with 12 ml of 0.1% v/v trifluoroacetic acid and followed by 12 ml of 0.1% v/v trifluoroacetic acid in 20% v/v acetonitrile. rCTD was eluted from the cartridge by 12 ml of 0.1% v/v trifluoroacetic acid in 50% v/v acetonitrile. The eluted protein fraction was diluted three times with 0.1% v/v trifluoroacetic acid, and rCTD was further purified using a Delta-Pak C₁₈ column. The purified rCTD was lyophilized and analyzed on SDS-PAGE. Its identity was confirmed by mass spectrometry (McMaster Regional Center for Mass Spectrometry, McMaster University).

**Oligomerization State Determination of the rCTD**—Sedimentation equilibrium experiments were performed on a Beckman Optima XL-I analytical ultracentrifuge (Beckman-Coulter) at 22 °C. A cell with six channels of 12-mm optical path was used. The lyophilized rCTD was dissolved in water to a concentration of 10 mg/ml. The protein solutions of different concentrations (1, 2, and 3 mg/ml) at different pH values were prepared by adding KCl and corresponding buffers (pH 4–6, K⁺-citrate, pH 7, K⁺-phosphate/Tris-HCl, pH 8–9, Tris-HCl; the citrate and phosphate buffers were prepared by titrating citric acid and KH₂PO₄ against KOH to the desired pH values) to a final concentration of 227 mM KCl and 54 mM buffers. Sample channels were filled with 110 μl of protein solutions, and the reference channels were loaded with 120 μl of corresponding solutions without protein. All runs were performed at two speeds: 23,000 and 30,000 rpm. Absorption profiles at 280 nm of each centrifuged samples were collected after 17 and 19 h of centrifugation. The absorption profiles at the two time points were compared to ensure that equilibrium had been achieved. Absorption profiles were analyzed using the XL-A/XL-I data analysis software (Beckman Coulter). The partial specific volume of rCTD and the density of buffers were estimated with the program SEDNTERP (20).

**RESULTS**

**Overexpression, Purification, and Characterization of rKcsA and chKcsA**—rKcsA was expressed in E. coli and purified to homogeneity as analyzed on SDS-PAGE (Fig. 2, A and C). KcsAΔCTD and rctKcsA were generated by limited chymotrypsin digestion as it was previously reported that KcsA can be cleaved specifically after residue 125 by chymotrypsin (21). The
activities of rKcsA and rctKcsA were confirmed by cell-attached patch clamp technique (Fig. 1, A–C). The pH profiles of rKcsA and rctKcsA were similar to that reported by Cortes et al. (14) by Rb⁺ flux assay.

Thermal Stability of Tetrameric rKcsA and KcsAΔCTD at Various pH Values—Tetrameric KcsA is stable even in the presence of harsh detergent such as SDS, and heating is required to dissociate it into monomer. Its thermal stability in SDS was used by Cortes et al. (14, 22) to determine the effect of various mutations (including CTD truncation) on the overall stability of the channel. It was reported that truncation of the CTD destabilized the channel. In addition, Rb⁺ flux assay was also used to characterize the CTD deletion mutants, and increased ion leakage at basic pH was observed. Cortes et al. (14) concluded that the CTD stabilized the closed form of the channel. To have a better understanding of the molecular basis of this observed phenomenon and to settle a recent claim by Molina et al. (21) that truncation of the CTD did not destabilize KcsA tetramer, we extended the previous thermal stability studies carried out at a single pH to include a wide range of pH values.

The tetramer–monomer dissociation temperature \( T_m \) for rKcsA was pH-dependent. At pH 5.0 or higher, it was about 80.0 °C and shifted dramatically to about 37.4 °C when pH was lowered to 4.0 (Fig. 2E). At pH 3.0, rKcsA existed mainly as monomer at all temperatures tested (data not shown). The pH-dependent \( T_m \) profile of KcsAΔCTD was quite different from its full-length counterpart. \( T_m \) of KcsAΔCTD was about 20.0 °C higher and 25.0 °C lower than rKcsA at pH 4.0 and 8.0, respectively. When pH was lowered to 3.0, KcsAΔCTD also existed as monomer.

Oligomerization of rCTD at Various pH Values—The observed contradicting contribution of the CTD to the stability of tetrameric KcsA at pH 4.0 and 8.0 can be related to its modulating role in the open-closed switching of the channel. rKcsA is destabilized in the pH range where the gate is opened and stabilized by its CTD in the pH range where the gate is closed. This hypothetical role of the CTD requires it to oligomerize in a pH-dependent manner. We therefore constructed a His-tagged recombinant fragment of the CTD (residues 116–160, rHCTD) and expressed it in E. coli. The expressed protein was purified to homogeneity as shown on 15% SDS-PAGE (Fig. 3). After removal of the His tag by thrombin digestion, the mass of the rCTD was determined by mass spectrometry to be 5,729.0 Da, which is close to the predicted value of 5,727.2 Da.

We used sedimentation equilibrium to monitor the apparent molecular weight of the rCTD at various pH values, and the results are shown in Fig. 4. When we fit the data to a single species model, the apparent molecular masses of the rCTD at acidic pH values of 4.5, and 56 were 5,049 ± 312, 5,551 ± 373, and 5,409 ± 298 Da (mean ± 95% confidence level generated by weighted function), respectively. These values were close to the expected mass of the rCTD in its monomeric form (5,727.2 Da). The apparent molecular mass of the sample increased to about 17,000 Da at neutral and basic pH values (Fig. 4C). It was more than three times the molecular mass of a monomer at acidic pH values (e.g. 3 x 5,409 Da). When the concentration of KCl was increased from 227 mM to 1.5 M, the apparent molecular mass increased and plateaued at 20,000 Da, which is close to that of a tetramer (Fig. 5). Since rKcsA exists as homotetramer in nature, its CTD is likely to form tetramer. When we fit the data to monomer-tetramer, monomer-dimer-tetramer, and monomer-trimer-tetramer models, all three models yielded similar \( K_d \) values for tetramer (3.0 × 10⁻¹² M⁻³). Since much higher \( K_d \) values for other oligomers (trimer, 1.2 x 10⁴ M⁻², and dimer, 1.4 x 10¹² M⁻²; data not shown) were obtained, the relative amount of these oligomers may be negligibly small.

DISCUSSION

Cortes et al. (14) proposed that the CTD of KcsA may be involved in modulating the open-closed switching of the channel. Here, we examined this hypothesis by characterizing the biochemical and biophysical properties of the CTD. The primary structure of the CTD predicted that it is highly charged with a theoretical pI value around 10. About 44% of the CTD (22 out of 48 residues) are charged at neutral pH, with 12 positive and 10 negative residues, resulting in a theoretical net charge of about +2. Previous EPR studies showed close contacts among the CTDs at pH above neutral (14) despite its theoretical net charge of +2, indicating the existence of substantial attraction among the CTDs. One can imagine when the environment was acidified such that the net charge in this region is further increased, a stronger repulsion force will override the attraction force and prevent the CTDs from staying together. This pH/charge-based repulsion among the CTDs may contribute to channel opening, whereas the attraction among the CTDs may contribute to the enhanced stability of the closed form as observed by Cortes et al. (14).

To test the hypothesis mentioned above, we measured the pH-dependent thermal stability of tetrameric KcsA and found that its denaturation profile was pH-dependent with close resemblance to the pH profile of its opening. The channel is more stable at neutral and basic pH values, where the gate of KcsA is mostly closed, than at acidic pH values, where the gate opens (15, 23). This close correlation between the denaturation and opening pH profiles suggests that factors that affect the stability of KcsA tetramer may also have an influence on channel opening. We therefore investigated the role of the CTD in
gating by comparing the thermal stability of rKcsA and that of KcsAΔCTD at various pH values. We found that the full-length channel is more stable than its CTD truncated counterpart at basic and neutral pH values, presumably due to the extra contacts among the CTDs. Interestingly, the full-length channel is less stable than the CTD truncated form at acidic pH when the channel is supposed to be in its open state. This is in agreement with the hypothesis that the principal molecular interactions responsible for stabilizing the KcsA tetramer are contained within the transmembrane domain and that the CTD is involved in modulating channel opening.

If gate opening and closing are modulated by the dissociation and association of the CTD as our data suggest, then the interactions between the CTDs have to be strong enough to stabilize its own oligomerization independent of other parts of the channel. We examined the oligomeric state of rCTD at various pH values by sedimentation equilibrium to determine whether rCTD alone was sufficient to form oligomer in a pH-dependent manner. As expected, we found that rCTD formed oligomer (probably tetramer) at basic pH values and that no oligomer was detected at acidic pH values.

Taken together, our data support the concept that interactions between the CTDs of rKcsA modulate gate opening and that the ionization state of residues within the CTD confers the pH dependence of this process. The transition pH for the oligomerization of rCTD is about 2 pH units higher than the reported value for channel opening (15, 23). This discrepancy may be due to the extra force required to pull the transmembrane domain apart, and additional ionizable residues located at the CTD likely play a role in providing the required supplemental force.

We set out to determine the residues involved in the observed pH-dependent thermal stability of rKcsA. The transition pH for gating (pH 5.2–6.5 (14, 15, 23)) and oligomerization (pH 6.5) are close to the theoretical pKas of glutamic acid (4.25), aspartic acid (3.65), and histidine (6.00), which are abundant in the C-terminal domain (7 Glu, 3 Asp, 3 His). Among these three types of amino acids, the number of the histidines located in this region is the smallest, and its theoretical pK is close to the pH transition point for channel opening. Thus, the histidine residues (His-124, His-128, and His-145) were chosen as targets for our mutation studies. Since removal of the last 20 residues from KcsA did not alter its pH profile of opening, whereas removal of the last 35 residues did (14), we only mutated His-124 and His-128. No observable changes to the thermal stability of all mutants (H124A, H128A, and H124A-H128A; data not shown) were detected. The pH profiles of H124A in terms of gating as a full-length channel and oligomerization as an rCTD were also examined. Both profiles were the same as that of wild type (data not shown). These observations suggest that His-124 and His-128 are not the key residues in the pH sensor and that other ionizable residues in the CTD may be responsible for the observed pH-dependent thermal stability. Nevertheless, our data provide compelling evidence to support that the pH-dependent oligomerization of the C-terminal domain plays a significant role in modulating the gating of KcsA in a pH-dependent manner.

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