Thermal Stability of the K^+ Channel Tetramer: Cation Interactions and the Conserved Threonine Residue at the Innermost Site (S_4) of the KcsA Selectivity Filter†

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ABSTRACT: The selectivity filter of most K^+ channels contains a highly conserved Thr residue that uniquely forms the S_4 binding site for K^+ by dual coordination with the backbone carbonyl oxygen and side chain hydroxyl of the same residue. This study examines the effect of mutations of Thr75 in the S_4 site of the KcsA K^+ channel on the cation dependence of the thermal stability of the tetramer, a phenomenon that reflects the structural role of cations in the filter. Conservative mutations of Thr75 destabilize the tetramer and alter its temperature dependence. Replacement of Thr with Ala or Cys lowers the apparent affinity of K^+, Rb^+, and Cs^+ for tetramer stabilization by factors ranging from 4- to 14-fold. These same mutations lower the apparent affinity of Ba^{2+} by $\sim 10^3$- or $\sim 10^4$-fold for Ala and Cys substitution, respectively, consistent with the known preference of the S_4 site for Ba^{2+}. In contrast, substitution of Ala or Cys at T75 anomalously enhances the ability of Na^+ to stabilize the tetramer, suggesting that the native Thr residue at S_4 is important for ultrahigh K^+/Na^+ selectivity of K^+ channel pores. Elevated temperature or Cu^{2+} cation catalyzes formation of covalent dimers of the T75C mutant of KcsA via formation of disulfide bonds between Cys residues of adjacent subunits. Thiophilic cations such as Hg^{2+} and Ag^+ specifically protect the T75C tetramer against heat-induced dimer formation, demonstrating the contribution of cation interactions to tetramer stability in a channel with a non-native S_4 site engineered to bind foreign cations.

K^+ channels belong to the voltage-gated-like channel superfamily (VGL)[1] of cation-selective ion channel proteins that include voltage-gated Na^+ and Ca^{2+} channels, cyclic nucleotide-gated channels (CNG), hyperpolarization and cyclic nucleotide-gated channels (HCN), and transient receptor potential channels (TRP) (*1*). Recognized by a homologous pore domain coupled to diverse gating and regulatory domains, the VGL is an example of modular protein evolution. By controlling cation currents across the cell membrane, VGL channels are key effectors of numerous physiological processes such as the electrical behavior of excitable cells, neurotransmitter release, activation of the immune response, and sensory perception. A hallmark feature of VGL channel architecture is the tetrameric (homotetrameric, heterotetrameric, or pseudotetrameric) quaternary structure of the pore-forming subunit. Given that integrity of the tetramer is essential to the channel function of these proteins, the molecular basis of intersubunit recognition, oligomerization, and stability of the quaternary complex of VGL channels is a crucial aspect of their membrane biochemistry. For some K^+ channels, the specificity of monomer association, tetramer oligomerization, and tetramer stability is known to be influenced by intersubunit interactions of conserved cytoplasmic domains such as the N-terminal T1 domain of certain Kv channels (*2*−*4*) and the C-terminal RCK domain of the MthK channel (*5, 6*). However, structure−function analyses of K^+ channels have also revealed a prominent role for the membrane-spanning regions and selectivity filter in the tetramer stability of the pore domain (*7*−*9*).

As first described in the X-ray crystal structure of KcsA, a prototypical K^+ channel protein from *Streptomyces lividans* (*10, 11*), the selectivity filter is a pipelike molecular device approximately 12 Å in length that is situated toward the outer leaflet of the lipid bilayer. In KcsA and related K^+ channels of known structure, the filter contains multiple binding sites for K^+ ions arranged single file along the pore axis formed at the center of a radially symmetric tetramer. The most intimate ion−protein interactions of the filter involve KcsA residues T75, V76, G77, and Y78 (Figure 1). As illustrated by the structure of the seven-residue segment from two diagonal subunits of the tetramer shown in Figure 1A (left), four K^+ binding sites named S_1−S_4 are formed by the apposition of 20 oxygen atoms that face the central cavity of the filter. At each of these sites, K^+ is effectively coordinated by four oxygen atoms lying in a plane above the cation and four oxygen atoms in a plane below

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[1] Abbreviations: $C_{12}M$, *n*-dodecyl β-maltoside; CNG, cyclic nucleotide-gated channels; DTT, dithiothreitol; HCN, hyperpolarization and cyclic nucleotide-gated channels; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MD, molecular dynamics; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SK, small conductance Ca^{2+}-activated K^+ channel; TCEP, tris(2-carboxyethyl)phosphine; Tris, tris(hydroxymethyl)aminomethane; TRP, transient receptor potential channels; VGL, voltage-gated-like channel superfamily.

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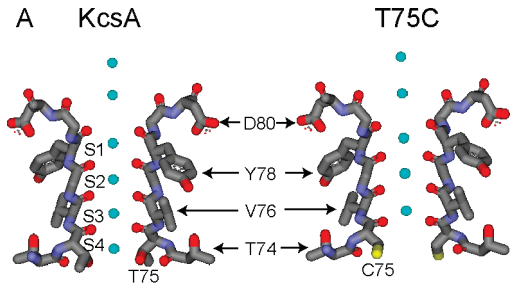

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KcsA	T	T	V	G	Y	G	D
Kir2.4	T	S	I	G	Y	G	V
SK(K_{Ca}2.1)	L	S	I	G	Y	G	D
HERG(Kv11.1)	T	S	V	G	F	G	N
HCN4	L	C	I	G	Y	G	R

FIGURE 1: Selectivity filter of KcsA and natural sequence variations found in K^+ channels. (A) Comparison of the filter structure of KcsA [left, Protein Data Bank (PDB) entry 1K4C] and the T75C mutant of KcsA (right, PDB entry 1S5H) showing corresponding segments of two diagonal subunits of the tetramer (residues T74−D80) with labeled side chains and K^+ ions (cyan spheres). (B) Natural sequence variations at residue T75. The top row (KcsA, NCBI accession number P0A334) is the partial sequence (T74−D80) forming the native KcsA selectivity filter shown in panel A. The bottom four rows of the alignment are sequences of human K^+ channel genes (NCBI accession numbers in parentheses): Kir2.4 (NM_ 013348), SK or KCa2.1 (NM_002248), HERG or Kv 11.1 (NM_000238), and HCN4 (NM_005477). Boxed residues include T75−Y78 of KcsA which contribute five layers of oxygen atoms that form the four numbered K^+ sites, S_1−S_4, in the filter. Residues colored red are variations from the KcsA sequence.

the cation, where "above" refers to the external side of the membrane. Three sites, S_1−S_3, are formed by carbonyl oxygen atoms from the peptide backbone of respective pairs of residues Y78 and G77, G77 and V76, and V76 and T75, whereas the innermost S_4 site is uniquely formed by the backbone carbonyl oxygen of Thr75 above and the oxygen atom of the side chain hydroxyl group of Thr75 below the K^+ ion (*11*).

Numerous electrophysiological studies lead to the conclusion that binding of K^+ to the filter is coupled to conformational changes involved in gating or collapse of the external pore of K^+ channels: (1) C-type and P-type inactivation gating behavior modulated by changes in K^+ concentration (*12*−*15*), (2) loss of strict K^+ selectivity of ion conductance upon removal of external K^+ (*16, 17*), and (3) long-lived nonconducting states (*18*) and irreversible conversion to a "defunct" state triggered by removal of K^+ from the medium (*19*−*21*). Structural correlation to these electrophysiological observations can be drawn from the observation that the KcsA selectivity filter converts from an open, conducting conformation in the presence of a high K^+ concentration to a disordered and presumably inactivated (nonconducting) conformation under conditions with a low K^+ concentration where ionic strength is compensated by a normally impermeant cation such as Na^+ (*11*). Biochemical analyses have correspondingly demonstrated that native KcsA is a highly stable tetramer resistant to dissociation in the presence of SDS (*8, 22*); however, tetramer stability is vulnerable to mutation of various residues of the pore domain and selectivity filter (*8, 9*).

The notion that ion-dependent conformational changes of the selectivity filter may affect tetramer stability and oligomerization of K^+ channels previously led us to investigate the cation dependence of quaternary structure (*23*). These studies showed that KcsA exhibits ion-dependent stability as demonstrated by cation protection against thermal dissociation of the tetramer monitored by SDS−PAGE. Cations known to permeate or strongly block K^+ channels such as K^+, Rb^+, Cs^+, Tl^+, NH_4^+, and Ba^{2+} are much more effective in conferring tetramer stability to KcsA than Na^+, Li^+, choline$^+$, and Tris$^+$, cations that do not readily permeate or weakly block the channel (*23*). A similar phenomenon was recently reported for Kcv, a 94-residue K^+ channel protein from the chorella virus, PBCV-1 (*24*). A recombinant form of the Kcv protein that displays characteristic ion conduction properties of K^+ channels also migrates as a stable tetramer assayed by SDS−PAGE. Highly permeant cations such as K^+ and Rb^+ or the blocker cation, Cs^+, protect against tetramer dissociation at 95 °C, while the smaller impermeant alkali cations, Na^+ and Li^+, fail to stabilize the tetramer (*24*). The similar cation dependence of tetramer stability can be attributed to the conserved selectivity filter sequence of Kcv (TVGFGD) compared to KcsA (TVGYGD). Since Kcv completely lacks a cytoplasmic C-terminal domain following the second transmembrane helix that forms the inner pore, oligomeric stability can be solely attributed to intersubunit interactions within the membrane. Thus, cation-dependent tetramer stability may be a characteristic structural feature of diverse K^+ channel proteins.

In the work reported here, we sought to extend these observations by investigating the specific contribution of the side chain hydroxyl group of the Thr residue at the S_4 site of the KcsA selectivity filter to tetramer stability. The unique features of Thr75 described above in forming the S_4 site for K^+ and other inorganic cations, as well as forming a preferred binding site for the blocker, Ba^{2+} (*25*), led us to hypothesize that mutation of this residue may specifically perturb the cation dependence of tetramer stability. Indeed, we find that conservative mutations of Thr75 weaken tetramer stability, greatly reduce the apparent affinity of Ba^{2+} in protecting against tetramer destabilization, and anomalously enhance the ability of Na^+ and Li^+ to support tetramer stability. Furthermore, substitution of Thr75 with a Cys residue that occurs naturally in the HCN subfamily of VGL channels confers upon KcsA the ability to form stable dimers by disulfide formation in the presence of Cu^{2+} or elevated temperatures. The T75C mutant of KcsA also exhibits unique protection of tetramer stability by thiophilic cations such as Hg^{2+} and Ag^+. These observations add new insight into the special structural role of Thr75 in the K^+ channel selectivity filter and the strong evolutionary conservation of this residue.

MATERIALS AND METHODS

Expression and Mutagenesis. KcsA was expressed in *Escherichia coli* using an expression vector (pASK90) containing a synthetic gene modified by insertion of a coding sequence for hexahistidine following the initial Met residue of KcsA as described previously (*23*). Mutation of KcsA residue T75 to C, A, V, S, or N was performed using the QuickChange site-directed mutagenesis kit of Stratagene according to the manufacturer's instructions. Mutations were confirmed by DNA sequencing.

Protein Production and Purification of KscA and Mutants. A culture (7 L) of *E. coli* strain BL21 transformed with the

pASK90-KcsA native or mutant expression plasmid was grown in LB medium to an absorbance of 0.5 at 550 nm. KcsA expression was induced by addition of 200 μg/L anhydrotetracycline for 1 h, and cells were harvested by centrifugation (5500*g*, 10 min). The pellet was resuspended in phosphate buffer [100 mM NaP_i and 5 mM KCl (pH 7.0)] at a level of 5 mL of buffer/L of original culture and sonicated (Branson Sonifier 450, three pulses of 40 s spaced by 1 min intervals on ice) or homogenized with an EmulsiFlex C-5 high-pressure cell disruptor apparatus (Avestin, Ottawa, ON) at 15000−20000 psi. After centrifugation of the homogenate (110000*g*, 1 h), the membrane pellet was resuspended in phosphate buffer containing 20 mM $C_{12}M$ (*n*-dodecyl β-maltoside from Anatrace) and 2.5% Triton X-100 (v/v). Following gentle shaking for 2 h at 4 °C, the suspension was centrifuged at 85000*g* for 45 min and the supernatant was incubated with 50 μL of Ni-NTA agarose (Qiagen) per millilitre of extract. After the mixture was gently shaken for 4 h at 4 °C, Ni-NTA agarose was collected into a small column and washed extensively (50 times the bed volume) with phosphate buffer with 1 mM $C_{12}M$ and 40 mM imidazole (pH 7.0). KcsA protein was eluted in 20 times the bed volume of phosphate buffer containing 1 mM $C_{12}M$ and 400 mM imidazole (pH 7.0).

Dependence of the KcsA Tetramer on Temperature or Cation Concentration. The tetramer content of unheated or heated KcsA was analyzed by SDS−PAGE using precast 12% acrylamide Tris-HCl Ready Gels run on a MiniProtean apparatus (Bio-Rad) as previously described (*23*). Exchange of purified native or mutant KcsA protein into solutions with a defined cation composition was performed by five to seven rounds of concentration and dilution with an Amicon Ultra-4 centrifugal ultrafiltration device (*23*). Assay and quantitation of the dependence of KcsA tetramer on temperature or cation concentration were performed as previously described (*23*) or with modifications of temperature and other conditions noted in the text. In particular, the standard chloride-containing buffer for the assay of tetramer stability [10 mM Hepes-Tris (pH 7.4) and 100 mM choline Cl or other Cl^- salt of the test cation] was modified for use with thiophilic cations (Ag^+, Hg^{2+}, Pb^{2+}, and Zn^{2+}) that are known to form a variety of soluble and insoluble aqueous complexes with Cl^-. In such experiments, a nominally chloride-free buffer [10 mM Hepes-Tris and 100 mM Tris-NO_3 (pH 7.4)] was prepared with HNO_3 as the acid titrant for use in these experiments (e.g., Figures 5 and 6). Normalized titration data for various cations were fit to the following empirical Hill equation:

$$f_T = [X]^N/(K_{0.5}{}^N + [X]^N) \quad (1)$$

where f_T is the fraction of the tetramer band relative to an appropriate control sample, [X] is the cation concentration, N is a numerical coefficient, and $K_{0.5}$ is the cation concentration at half-maximal effect.

RESULTS

Tetramer Stability of KcsA Is Sensitive to Residue Substitution at T75. As described in the introductory section, the subtle divergence in molecular construction of the S_4 site versus sites S_1−S_3 in the KcsA filter raises the question of whether the Thr residue at position 75 has special structural or functional significance for the operation of KcsA or other K^+ channels. A survey of the protein database shows that T75 is a highly conserved residue within K^+ channel sequences. From a sample of 155 diverse K^+ channels in a comprehensive sequence alignment and 83 K^+ channels identified in the human genome (*1, 26*), only two substitutions, Ser and Cys, are found to naturally replace Thr at the position equivalent to T75 of KcsA. In this combined nonredundant sample of 214 K^+ channel sequences, Ser occurs at position T75 in 23 (10.7%) sequences and Cys occurs in only nine (4.2%) of the sequences. As illustrated in the alignment of Figure 1B, Ser at position T75 is found in only one of the 15 members of the human inward rectifier class of K^+ channels ($K_{ir}2.4$, human gene KCNJ14), in all three SK or low-conductance Ca^{2+}-activated K^+ channels ($K_{Ca}2.1$−2.3, human genes KCNN1−3, respectively), and in seven of the eight members of the combined *eag* (Kv10.1), *erg* (Kv11.1−11.3), and *elk* (Kv12.1−12.3) family of K^+ channels such as HERG ($K_V11.1$, human gene KCNH2). In humans, the natural occurrence of Cys at position T75 is limited to the family of four HCN K^+ channels (human genes HCN1−4).

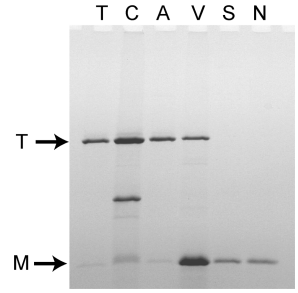

FIGURE 2: Dependence of tetramer stability of KcsA on residue substitutions at T75. KcsA (lane T) and five mutants with substitution of T75 with Cys (lane C), Ala (lane A), Val (lane V), Ser (lane S), or Asn (lane N) were expressed in *E. coli* and purified as described in Materials and Methods. Purified protein was eluted from the Ni-NTA column in 5 mM KCl, 100 mM NaP_i, 400 mM imidazole, and 1 mM $C_{12}M$ (pH 7.0), and samples of each (0.5−1 μg) were analyzed for tetramer composition by SDS−PAGE. T, tetramer band; M, monomer band.

We investigated the consequences of mutation of T75 on quaternary structure by examining the tetramer stability of KscA mutants with conservative substitutions of Thr at this position. Typical behavior of His_6-tagged KcsA on SDS−PAGE directly after affinity purification in standard buffer [100 mM NaP_i (pH 7.0), 5 mM KCl, 1 mM $C_{12}M$, and 400 mM imidazole] is shown in Figure 2 (lane T). As previously documented (*8, 22*), such preparations of KcsA consist of a prominent tetramer band (74 kDa) and variable but small amounts of a low-molecular mass band corresponding to monomer (18.5 kDa). Our attempts to similarly prepare detergent-solubilized, tetrameric form of various T75 substitution mutants of KcsA met with limited success. As shown in Figure 2, significant tetramer content was obtained for only T75C, T75A, and T75V, whereas mutants T75S and T75N consisted of only monomer band.

Mutations of the T75 residue chosen for study included the two naturally occurring replacement residues, Ser and Cys, and three other neutral amino acids (Ala, Val, and Asn) with small side chains comparable to Thr in molecular volume and steric configuration. Of the five tested mutants, robust tetramer stability is characteristic of only T75C and

T75A, as judged by the small amount of monomer band after purification (Figure 2). As described later in more detail, preparations of purified T75C often contain a prominent third band of intermediate size between monomer and tetramer (Figure 2, lane C) which corresponds to a covalent dimer of two monomers tethered by formation of an intersubunit disulfide bond. We attempted to enhance the tetramer content of T75V, T75S, and T75N preparations by adding 100 mM KCl or 10 mM $BaCl_2$ to the purification buffer, ionic conditions known to strongly stabilize native KcsA tetramer (*23*). However, these manipulations failed to increase the proportion of tetramer obtained for these mutants as monitored by SDS−PAGE. Since the tetramer content of T75V, T75S, and T75N was too low or short-lived to permit routine study, we focused on detailed analysis of the temperature and cation dependence of the T75C and T75A mutants. The results in Figure 2 nevertheless demonstrate that tetramer stability of KscA as solubilized and purified in $C_{12}M$ micelles is quite sensitive to the chemical nature of the amino acid side chain at T75.

Temperature Dependence of Tetramer Stability. Results of experiments investigating the temperature dependence of tetramer stability of the T75C and T75A mutants in the presence of various cations are summarized in Figure 3. To perform this analysis, purified KcsA and mutant proteins were first thoroughly exchanged into solutions containing 10 mM Hepes-Tris (pH 7.4) and one of the following salts: 100 mM choline Cl, 100 mM KCl, 100 mM RbCl, 100 mM CsCl, 100 mM NaCl, 100 mM LiCl, 10 mM Tl acetate, 10 mM $BaCl_2$, or 25 mM $SrCl_2$. The temperature dependence of KcsA equilibrated with the different cations was assessed by heating the samples at various temperatures for 10 min and assaying for tetramer content by SDS−PAGE.

Thermal stability of native and mutant tetramer in the presence of 100 mM choline$^+$ versus 100 mM K^+ is compared in panels A and B of Figure 3, respectively. This comparison shows that K^+ strongly enhances the stability of KcsA and both mutants by increasing the midpoint temperature for 50% tetramer dissociation by at least 50 °C. Choline$^+$ is an organic cation that is expected to be excluded from the native selectivity filter on the basis of molecular size. Assuming that choline$^+$ does not have specific effects on protein structure, 100 mM choline Cl may be considered as a control condition for assessing the extra degree of stabilization that can be attributed to specific interaction of inorganic cations with the selectivity filter. Comparison of the temperature dependence of native KcsA and the two mutants shows that both mutants have markedly lower thermal stability than the wild-type tetramer with the Cys substitution at T75 being more stable than the Ala substitution in the presence of both choline$^+$ and K^+.

T75C and T75A mutations also have strong effects on tetramer stability mediated by the divalent cations, Ba^{2+} and Sr^{2+}. These two divalent inorganic cations, particularly Ba^{2+}, are known to block a wide variety of K^+ channels (examples cited in refs *23* and *25*). Ba^{2+} was also previously found to exhibit the highest apparent affinity among a variety of inorganic cations for protection against thermal destabilization of native KcsA tetramer (*23*). The strong interaction of Ba^{2+} with the K^+ channel selectivity filter reflects both the similarity of its Pauling ionic radius (1.35 Å) to that of K^+ (1.33 Å) and its divalent charge. As shown in Figure 3D,

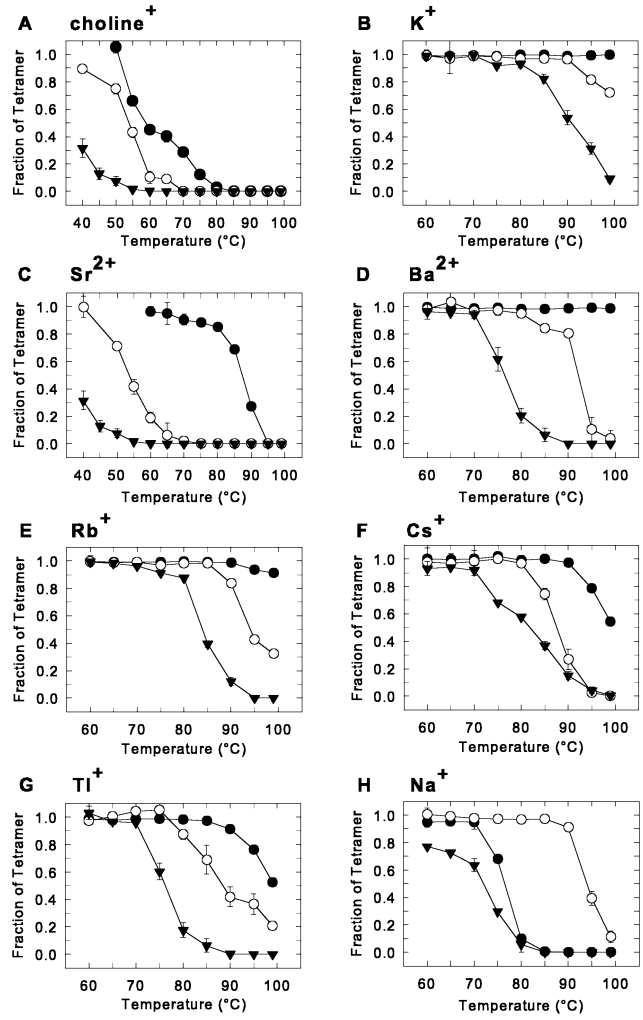

FIGURE 3: Temperature dependence of tetramer stability of KcsA and T75 mutants in the presence of various cations. KcsA (●) and mutant proteins, T75C (○) and T75A (▼), were purified and exchanged into a buffer containing 10 mM Hepes-Tris (pH 7.4) ~4 mM $C_{12}M$, and the following salts: (A) 100 mM choline Cl, (B) 100 mM KCl, (C) 25 mM $SrCl_2$, (D) 10 mM $BaCl_2$, (E) 100 mM RbCl, (F) 100 mM CsCl, (G) 10 mM Tl acetate, or (H) 100 mM NaCl. For each temperature curve, identical samples of protein (~0.5 μg) were incubated for 10 min at the indicated temperatures, cooled to 0 °C, and analyzed for tetramer content by SDS−PAGE. Data points correspond to the fraction of tetramer [mean ± standard deviation (SD); $N = 3$] as determined by densitometric scanning and normalization of the measured tetramer band area to that of an unheated sample. Comparison data for native KcsA are from ref *23*. Note that data in panels A and C are plotted over a wider temperature range (40−99 °C) than data in the other panels (60−99 °C).

wild-type KcsA tetramer pre-equilibrated with 10 mM Ba^{2+} is highly resistant to dissociation at temperatures tested up to 99 °C. In contrast, tetramer band of T75C and T75A pre-equilibrated with Ba^{2+} is lost at lower temperatures, with midpoint transitions near ~93 and 77 °C, respectively. The stronger destabilizing effect of T75C and T75A mutations on tetramer integrity mediated by Ba^{2+} (Figure 3D) compared to that of K^+ (Figure 3B) is consistent with crystallographic evidence that S_4 is a preferred binding site for Ba^{2+} in KcsA (*25*). Sr^{2+} stabilizes native KcsA tetramer (Figure 3C) at a midpoint temperature of ~87 °C, but it does not significantly enhance thermal stability of the T75C and T75A mutants over that exhibited in the presence of 100 mM choline$^+$ (compare panels A and C of Figure 3). These results imply

that the native S_4 site is a preferred binding site for Sr^{2+} as well as Ba^{2+}.

The monovalent inorganic cations, Rb^+, Cs^+, and Tl^+, are known to bind within the selectivity filter of K^+ channels as inferred from many electrophysiological studies of ion conduction and block and also by direct crystallographic observations on KcsA (*27*). The data in panels E−G of Figure 3 show that Rb^+, Cs^+, and Tl^+, respectively, are only slightly less effective than K^+ in enhancing the thermal stability of KcsA tetramer and the two T75 mutants by raising the midpoint temperature relative to that of choline$^+$ control (Figure 3A) by ∼40 °C or more. The results also indicate that the T75C and T75A mutations inhibit the ability of each of these cations to stabilize the tetramer as indicated by a shift toward a weaker temperature dependence relative to wild-type KcsA. For the inorganic cations discussed thus far, the temperature dependence of tetramer stability consistently follows this sequence: wild-type T75 > T75C > T75A. The destabilizing effect of the two S_4 site mutations on protection against thermal dissociation of the tetramer by K^+, Rb^+, Cs^+, Tl^+, Ba^{2+}, and Sr^{2+} is consistent with the previous interpretation that these effects are mediated by specific binding of these cations to the selectivity filter (*23*). These results are also compatible with crystallographic data on ion binding to the S_1−S_4 filter sites, including observations that the T75C mutation reduces K^+ occupancy at the S_4 and S_2 sites (*28*), and that Rb^+, Cs^+, and Tl^+ readily occupy the native S_4 site (*27, 29*). The weaker temperature dependence of tetramer stability for the two S_4 site mutants can be explained by weaker ion binding and lower ion occupancy of the filter, resulting in reduction of the intersubunit stabilization energy that can be attributed to the filter region.

In the case of Na^+ and Li^+, a surprisingly different behavior was observed. Na^+ actually increased the midpoint temperature of tetramer stability of the T75C mutant by ∼18 °C over that of wild-type KcsA (Figure 3H) in contrast to the destabilizing effect of this mutation for the other tested cations (Figure 3A−G). The midpoint temperature for tetramer equilibrated with 100 mM Na^+ (Figure 3H) is also ∼39 °C higher for T75C than the control condition of 100 mM choline$^+$ (Figure 3A) and similarly ∼36 °C higher for T75A in this same comparison. In contrast, there is a smaller shift of only ∼16 °C for Na^+-dependent stabilization of native KcsA tetramer versus choline$^+$ control. Li^+ is less effective than Na^+ in stabilizing native KcsA tetramer against temperature dissociation (*23*); however, the midpoint temperature of the T75C mutant in 100 mM Li^+ is ∼10 °C higher than that of native KcsA (data not shown). These results clearly indicate that tetramers formed by the T75C and T75A mutants are more stable to high temperature in the presence of Na^+ and Li^+ than the wild-type tetramer. Such a "gain-of-function" effect of the two T75 mutations in specifically enhancing the stability of the tetramer to Na^+ and Li^+ suggests that the T75 residue at the S_4 site may have a special role in maintaining the key physiological property of strong discrimination of the K^+ channel pore in favor of K^+ against Na^+.

Concentration Dependence of Monovalent and Divalent Cations. To investigate changes in the apparent affinity of cations produced by the T75C and T75A mutations, we performed titrations of several cations (K^+, Rb^+, Cs^+, Tl^+, Na^+, and Ba^{2+}) which protect against thermal dissociation of KcsA tetramer. These experiments were carried out by pre-equilibrating samples of KcsA protein in 100 mM choline Cl and variable concentrations of the test cation, heating the protein samples for 10 min at 70 °C, rapidly cooling the samples to stop tetramer dissociation, and analyzing the remaining tetramer content by SDS−PAGE. Figure 4 presents results of such cation titrations for the two mutant proteins in comparison to previously reported titration curves for wild-type KcsA (*23*).

Panels A−C of Figure 4 show that the T75C and T75A mutations have similar effects on the concentration dependence of K^+, Rb^+, and Cs^+, respectively, for tetramer protection. Both mutations shift the titration curves to a concentration range higher than that of wild-type KcsA, indicating the lower apparent affinity of the mutants for these cations. To facilitate comparison of the data, titration curves in Figure 4 were fit to an empirical Hill function (eq 1) with best-fit $K_{0.5}$ parameters summarized in the legend of Figure 4. $K_{0.5}$ values for tetramer stabilization by K^+, Rb^+, and Cs^+ are increased by factors ranging from 3.8-fold to 13.8-fold for the T75C and T75A mutants, respectively, relative to native KcsA. Hill parameters (N) for these cations are also typically lower for the mutants (range of 1.0−2.2) than wild-type KcsA (range of 2.9−3.3), reflecting a noticeably shallower cation concentration dependence for the mutants. As discussed previously (*23*), $K_{0.5}$ parameters measured in this experiment do not correspond to equilibrium constants for cation binding since thermal dissociation of the tetramer is an irreversible process under these conditions. The protective effect of K^+ on tetramer stability reflects K^+-dependent inhibition of the rate of tetramer dissociation (*23*). Together, the results of Figure 3 and 4 imply that the T75C and T75A mutations decrease the thermal stability of the tetramer in the presence of K^+, Rb^+, and Cs^+ by reducing ion binding affinity and filter occupancy, as reflected in the kinetics of irreversible tetramer dissociation.

Titration experiments also reveal selective changes in the apparent affinity for Tl^+, Na^+, and Ba^{2+}. Unlike results for K^+, Rb^+, and Cs^+, the titration curve of the T75C mutant for stabilization by Tl^+ is practically indistinguishable from that of the wild-type channel (Figure 4D). The different behavior of Tl^+ in this experiment may reflect a chemical preference of Tl^+ for interaction with thiol groups relative to alkali cations (*30*). Na^+ dependence of tetramer stability of the two S_4 site mutants exhibits $K_{0.5}$ values of 6.8 mM Na^+ for T75C and 4.3 mM Na^+ for T75A (Figure 4E). A comparable Na^+ titration cannot be readily performed for wild-type KcsA (*23*) due to poor tetramer stabilization by Na^+. However, previous work showed that inhibition of wild-type tetramer stability by Na^+ in the presence of 5 mM K^+ exhibits a $K_{0.5}$ value of 109 mM Na^+ (*23*). The latter value undoubtedly reflects binding competition of Na^+ with the higher-affinity K^+ ion. However, an equilibrium dissociation constant of 190 mM for the interaction of Na^+ with wild-type KcsA was recently measured by direct monitoring of intrinsic tryptophan fluorescence (*31*). Considering the low intrinsic affinity of KcsA for Na^+ implied by the latter measurements, the data in Figure 4E further support the idea that the Na^+ binding by T75C and T75A mutants is anomalously enhanced over that of native KcsA.

In the case of Ba^{2+}, $K_{0.5}$ values for thermal protection of the tetramer by this divalent cation are 2.7 mM for T75C

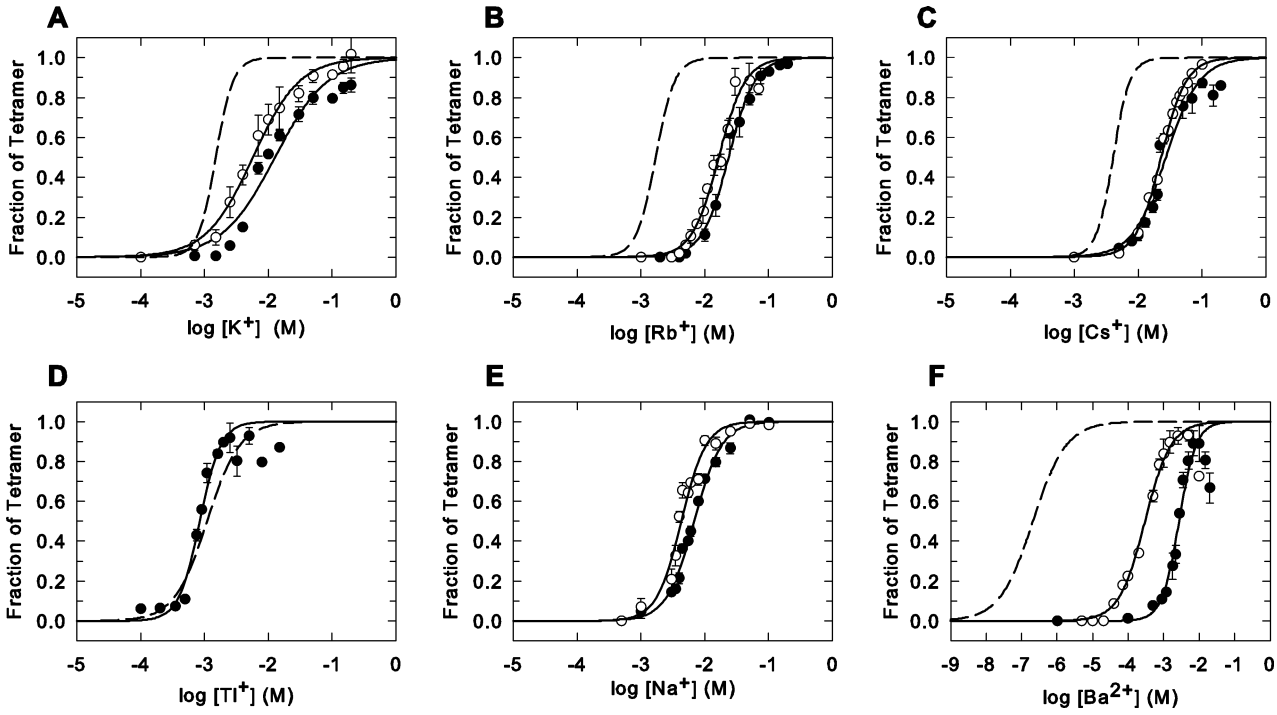

FIGURE 4: Concentration dependence of various cations for protection against thermal dissociation of KcsA tetramer. For each titration curve, identical 550 ng samples of KscA mutants T75C (●) and T75A (○) in choline buffer [10 mM Hepes-Tris (pH 7.4), 100 mM choline Cl, and ~4 mM $C_{12}M$] with the indicated concentrations of various inorganic cations were equilibrated for 1 h at room temperature. Samples were then heated at 70 °C (T75C or T75A) or 90 °C (KcsA) for 10 min, rapidly cooled to 0 °C, and analyzed at room temperature by SDS−PAGE. Panels correspond to titrations of following salts: (A) KCl, (B) RbCl, (C) CsCl, (D) Tl acetate, (E) NaCl, and (F) $BaCl_2$. Data points indicate fraction of tetramer (mean ± SD; $N = 3$) determined by densitometric scanning and normalization of the tetramer band area to that of an unheated sample. The dashed line comparison is a fit of data previously measured for KcsA (*23*). Solid lines correspond to fits of the mutant data to eq 1 with the following parameters (N, $K_{0.5}$) for KscA: (K^+) 3.2, 1.5 mM; (Rb^+) 2.9, 1.7 mM; (Cs^+) 3.3, 4.1 mM; (Tl^+) 1.7, 1.1 mM; (Ba^{2+}) 0.93, 2.1 × 10^{-4} mM. For mutant T75C: (K^+) 1.0, 12.6 mM; (Rb^+) 2.1, 23.5 mM; (Cs^+) 1.7, 27.4 mM; (Tl^+) 2.6, 0.85 mM; (Na^+) 2.0, 6.8 mM; (Ba^{2+}) 1.8, 2.7 mM. For mutant T75A: (K^+) 1.2, 5.7 mM; (Rb^+) 2.1, 16.4 mM; (Cs^+) 2.2, 23.0 mM; (Na^+) 2.2, 4.3 mM; (Ba^{2+}) 1.2, 0.3 mM.

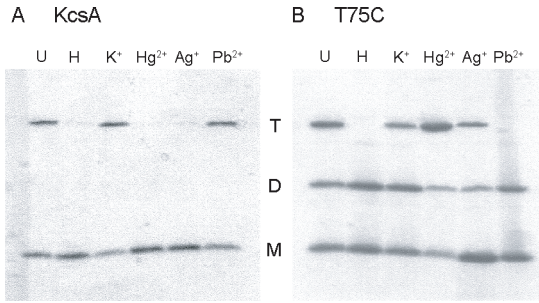

FIGURE 5: Effect of thiophilic cations on the thermal stability of KcsA and the T75C mutant. KcsA (A) and T75C (B) proteins were purified and exchanged into buffer containing 10 mM Hepes-Tris, 100 mM Tris-NO_3 (pH 7.4), and ~4 mM $C_{12}M$. Protein samples (~0.5 μg) were equilibrated at room temperature for 1 h in this same buffer with either no addition (lanes U and H) or buffer containing 1 mM KCl (lane K^+), 1 mM $Hg(NO_3)_2$ (lane Hg^{2+}), 1 mM $Ag(NO_3)$ (lane Ag^+), or 1 mM $Pb(NO_3)_2$ (lane Pb^{2+}). Samples were analyzed at room temperature by SDS−PAGE either without heating (lane U) or after heating at 65 °C for 10 min (all other lanes).

and 0.3 mM for T75A (Figure 4F). These values are 13000- and ~1400-fold higher, respectively, than the $K_{0.5}$ value of 210 nM previously measured for a similar Ba^{2+} titration of wild-type KcsA tetramer (*23*). The markedly stronger effect of T75C and T75A mutations on the apparent affinity for Ba^{2+} versus that of monovalent alkali cations (Figures 4A−C) is consistent with the preference of the S_4 filter site for this divalent cation as found in the Ba^{2+}−KcsA crystal structure (*25*).

Interaction of Thiophilic Cations at the S_4 Site and Dimer Formation in the T75C Mutant. X-ray crystallographic studies of the T75C mutant of KcsA previously showed that the tertiary structure of the mutant channel is virtually identical to that of native KcsA except for alteration of the S_4 site (*28*). In the filter region of the K^+ complex of T75C (Figure 1A, right), the sulfhydryl (SH) group of the Cys75 side chain points away from the pore and assumes a location analogous to that of the side chain methyl group of Thr75 in native KcsA, resulting in a defective S_4 site that lacks a lower set of oxygen ligands (*28*). Although the sulfhydryl (SH) group of Cys75 is apparently not oriented toward the pore in the K^+ complex of T75C, it is nevertheless possible that certain thiophilic metal ions may coordinate with one or more of these sulfhydryl groups by binding within the central cavity of the channel located just intracellular to the filter or by facilitating reorientation of the SH group(s) toward the filter. To investigate this possibility, we studied the effect of various transition and heavy metal cations on the quaternary structure and thermal stability of the T75C mutant by SDS−PAGE.

As previously described with respect to the experiment depicted in Figure 3, the tetramer band of the T75C mutant disappears from its normal location on the SDS−PAGE gel at elevated temperatures in the absence of protecting cations. Heating of this mutant protein results in enhanced formation of a prominent dimer band (Figure 5B), a phenomenon that is not observed for native KcsA (Figure 5A) or other T75

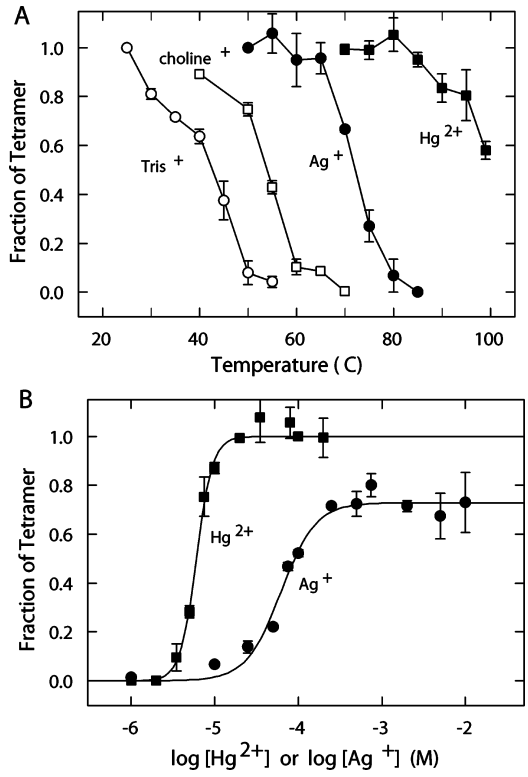

FIGURE 6: Characterization of the effect of Hg^{2+} and Ag^{+} on the tetramer stability of the T75C mutant. (A) Temperature dependence of T75C tetramer in buffer containing 10 mM Hepes-Tris (pH 7.4), ~4 mM $C_{12}M$, and either 100 mM choline Cl alone, 100 mM Tris-NO_3 alone, 100 mM Tris-NO_3 with 1 mM $AgNO_3$, or 1 mM $Hg(NO_3)_2$ as indicated. Protein samples (~0.5 μg) were equilibrated for 1 h in the latter solutions at 22 °C prior to being heated for 10 min at the indicated temperature. (B) Stabilization of T75C tetramer by increasing concentrations of Hg^{2+} and Ag^{+}. T75C protein was equilibrated for 1 h at 22 °C in 10 mM Hepes-Tris, 100 mM Tris-NO_3 (pH 7.4), ~4 mM $C_{12}M$, and indicated concentrations of $Hg(NO_3)_2$ or $AgNO_3$ prior to being heated at 70 °C for 10 min and analysis by SDS−PAGE. For both panels A and B, tetramer band measured by SDS−PAGE was normalized to an unheated control sample. Solid lines correspond to a fit of Hg^{2+} data to eq 1 with an N of 4.4 and a $K_{0.5}$ of 6.0 μM Hg^{2+} and a fit of Ag^{+} data to a modified form of eq 1 with a fixed tetramer plateau value of 0.73, an N of 2.1, and a $K_{0.5}$ of 62 μM Ag^{+}.

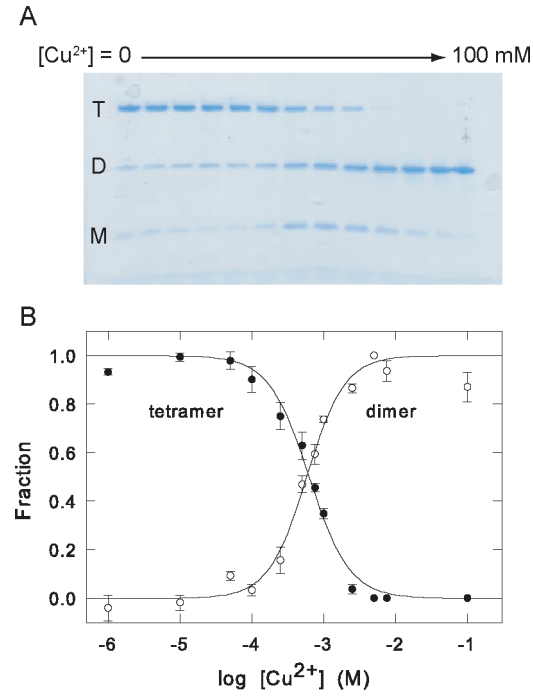

FIGURE 7: Formation of a dimer of the T75C mutant of KcsA catalyzed by Cu^{2+}. (A) SDS−PAGE analysis of dimer formation. Identical samples of T75C protein (~0.5 μg) were incubated in 10 mM Hepes-Tris (pH 7.4), 100 mM choline Cl, 4 mM $C_{12}M$, and the following concentrations of $CuCl_2$ corresponding to individual lanes of the gel proceeding from left to right: 0, 1, 10, 50, 100, 250, 500, and 750 μM and 1, 2.5, 5, 7.5, and 100 mM. Samples were subjected to SDS−PAGE after incubation for 1 h at 22 °C. (B) Fraction of tetramer (●) normalized to tetramer band measured in the absence of Cu^{2+} and fraction of dimer (○) normalized to dimer band measured in the presence of 5 mM Cu^{2+}. Solid lines correspond to fits of data to eq 1 with an N of 1.5 and a $K_{0.5}$ of 630 μM Cu^{2+} for tetramer and an N of 1.6 and a $K_{0.5}$ of 580 μM Cu^{2+} for dimer.

mutants (Figure 2). We found that certain thiophilic metal ions, such as Hg^{2+} and Ag^{+}, stabilize the T75C tetramer against dimer formation (Figure 5B). As shown in Figure 5B, 1 mM Hg^{2+} or Ag^{+} is slightly more effective that 1 mM K^{+} in protecting T75C against tetramer dissociation and dimer formation promoted by heating for 10 min at 65 °C. In contrast, neither 1 mM Hg^{2+} nor Ag^{+} protects native KcsA tetramer against thermal dissociation (Figure 5A), indicating that tetramer stabilization by these heavy metal cations is specifically conferred by the thiol group at Cys75. Curiously, another heavy metal cation, Pb^{2+}, was found to have a weak protective effect on the tetramer stability of native KcsA (Figure 5A), but not for the T75C mutant (Figure 5B). We tested various other transition metal cations in this same assay and found that 1 mM Ni^{2+} and Cd^{2+} weakly protected native KscA against tetramer dissociation and also weakly protected the T75C mutant against heat-induced dimer formation; however, 1 mM Mn^{2+}, Co^{2+}, and Zn^{2+} had no effect (data not shown). Taken together, these results indicate that tetramer stabilization by Ag^{+} and Hg^{2+} is a rather unique interaction of these particular thiophilic metal cations and the T75C mutant. Figure 6A further documents this behavior by showing that 1 mM Ag^{+} and Hg^{2+} substantially increase the midpoint temperature dependence of the T75C tetramer by approximately 30 and 58 °C, respectively, as compared to the control condition of 100 mM Tris-HCl. The apparent affinity ($K_{0.5}$) of these same heavy metal cations in promoting the thermal stability of the T75C tetramer is 6.0 μM Hg^{2+} and 62 μM Ag^{+} (Figure 6B), considerably stronger than the apparent affinity of native KcsA for tetramer stabilization by K^{+} ($K_{0.5}$ = 1.5 mM), Rb^{+} ($K_{0.5}$ = 1.7 mM), and Cs^{+} ($K_{0.5}$ = 4.1 mM).

In addition to enhanced dimer formation of the T75C mutant with the simple elevation of temperature (Figure 5B), we also observed that Cu^{2+}, a known catalyst of disulfide formation of neighboring Cys residues by air oxidation (*32, 33*), readily promotes formation of dimer by T75C at 22 °C (Figure 7). Figure 7 summarizes the results of a titration experiment in which increasing concentrations of $CuCl_2$ were incubated with identical samples of T75C protein for 1 h at 22 °C. Analysis of these samples by SDS−PAGE shows that the concentration at which half of the original tetramer band is lost is ~630 μM Cu^{2+} and the concentration at which the half-maximal level of dimer band occurs is ~580 μM Cu^{2+} (Figure 7B). The reciprocal nature of tetramer loss and dimer formation seen in this experiment is consistent with conversion of the tetramer to a covalent dimer stabilized by formation of a disulfide bond between adjacent Cys residues

of the monomer. This hypothesis was further tested by adding the known disulfide reducing agents dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) to samples of T75C dimer formed by initial treatment with 10 mM Cu^{2+}. Samples of confirmed T75C dimer were heated for 10 min at 60 °C with either 20 mM DTT or TCEP. Subsequent analysis showed that both of these treatments eliminated the T75C dimer band with a concomitant increase in the density of the monomer band on SDS−PAGE (data not shown). The fact that the T75C dimer band is sensitive to treatment by these two disulfide reducing agents verifies that the dimer band consists of two T75C monomers covalently linked by a disulfide bond.

DISCUSSION

Effect of Mutations at the S4 Filter Site on the Cation Dependence of Tetramer Stability. In this work, we examined the impact of rather conservative mutations of a single residue (T75) on the tetramer stability of KcsA, an assay of K^+ channel quaternary structure intimately related to cation interactions with the selectivity filter. Crystallographic studies of KcsA reveal that the four S_1-S_4 sites simultaneously bind at least two K^+, Rb^+, Cs^+, or Tl^+ cations (*10, 11, 27, 29*). Corresponding structural analysis of the T75C mutant of KcsA indicates that fractional occupancy by K^+ at the S_4 and S_2 sites is reduced by 56 and 23%, respectively, while K^+ occupancy at the S_1 and S_3 sites is not significantly diminished by this mutation (*28*). The definite but relatively modest effect of T75C and T75A mutations on K^+-dependent thermal stability (Figure 3B) and the K^+ concentration dependence of the tetramer (Figure 4A) imply that tetramer stability is dependent upon K^+ binding to all four S_1-S_4 sites of the filter. Assuming that the T75C and T75A mutations do not affect the structure of the filter except for the change in amino acid side chain at the S_4 site, the residual K^+ dependence of the tetramer stability of these mutants observed in our studies reflects K^+ binding to the unaltered S_1-S_3 filter sites.

The curiously coupled reduction in K^+ occupancy at the S_2 and S_4 sites resulting from the T75C mutation has been interpreted by an ion binding and conduction model involving alternating pairwise occupancy of filter sites by inorganic cations and intervening water molecules (*27*−*29*). In planar bilayers, the T75C mutant of KcsA exhibits a major reduction in single-channel conductance for K^+ but has the same conductance for Rb^+ as native KscA (*28*). The differential effect of this mutation on K^+ versus Rb^+ conductance appears to be related to the observation that the S_2 site does not readily bind Rb^+ or Cs^+ (*27*). Apparently, the four-site filter tolerates only certain linear configurations of water ion chains of the larger two alkali cations, and on the basis of the crystallographic data, the four sites seem to be energetically balanced only for K^+ and Tl^+ (*27*). Here we find that tetramer-stabilizing interactions of Rb^+ and Cs^+ are affected somewhat more strongly by mutations at T75 than the interaction of K^+ and Tl^+ as seen by the relative increase in magnitude of $K_{0.5}$ values and the reduction in the slope of cation titrations for the mutant channels as compared to wild-type channels (Figure 4). The stronger destabilizing effect of the T75 mutations on the tetramer formed in the presence of Rb^+ and Cs^+ versus K^+ may be related to the proportionally greater reduction in overall filter occupancy by Rb^+ and Cs^+ that would result from mutations at the S_4 site for cations that exhibit stable binding to only three (S_1, S_3, and S_4) of the four filter sites. Since Tl^+ readily binds to all four filter sites (*27*), the observation that the Tl^+ titration curve is barely affected by the T75C mutation compared to that of wild-type KcsA (Figure 4D) may be due to a gain of a compensating interaction of Tl^+ with one or more thiolate groups of Cys75. The possibility of such a stabilizing chemical interaction of Tl^+ with thiol groups of Cys residues has been previously proposed to explain results of single-channel studies and mutagenesis experiments at a different Cys residue in the Kir2.1 K^+ channel (*30*).

The more prominent effect of T75 mutations on stabilization of the tetramer by the divalent cations, Ba^{2+} and Sr^{2+} (Figure 3), correlates with crystallographic evidence for preferential binding of Ba^{2+} to the S_4 site (*25*). Figure 4 shows that the reduction in Ba^{2+} affinity (increase in $K_{0.5}$) for tetramer stabilization of the T75 mutants versus the wild type is at least 2 orders of magnitude greater than the corresponding effect on stabilization by K^+, Rb^+, Cs^+, and Tl^+. The fact that Ba^{2+} is nevertheless still able to stabilize the tetramer with a low apparent affinity in T75C and T75A mutants suggests that mutation of the S_4 site unmasks the ability of the mutant channel to bind Ba^{2+} with low affinity at a defective S_4 site lacking the Thr hydroxyl ligands or at one of the other filter sites, S_1-S_3. In fact, Ba^{2+} was recently found to bind to both the S_4 site and the S_2 site in a high-resolution crystal structure of the Ba^{2+} complex of KcsA (*34*). In the case of Sr^{2+}, it appears that both the T75C and T75A mutations practically abolish the ability of this divalent cation to stabilize the tetramer above the temperature range observed in choline$^+$ (Figure 3A,C), as if the native S_4 site is the only site that is able to effectively bind Sr^{2+}. Electrostatic calculations based on dipole moments of the four pore helices provide a plausible basis for understanding the preference of the central cavity site of KscA for monovalent ions over divalent metal cations (*35*); however, the physical basis of preferential binding of divalent Ba^{2+} to the S_4 and S_2 sites versus the other filter sites has not yet been elucidated. A possible interpretation is that specific electrostatic contributions and steric mobility of Thr hydroxyl groups at the S_4 site of native KcsA underlie the higher affinity for Ba^{2+} relative to the other filter sites which are formed solely by carbonyl oxygen atoms.

The enhanced ability of Na^+ (Figures 3H and 4E) to stabilize tetramers of the T75C and T75A mutants was not anticipated from previous work on KcsA. As one indication of the feeble binding interaction of Na^+ with K^+ channel proteins possessing the standard TVGY filter motif, Na^+ binds to native KscA bout 20-fold more weakly than K^+ on the basis of apparent macroscopic dissociation constants of 190 mM for Na^+ and 8 mM for K^+ measured by a spectroscopic method on purified KcsA (*31*). Reduction of the K^+ concentration below 2 mM in the presence of Na^+ also results in a collapsed conformation of the KcsA selectivity filter (*11*), implying that the conductive structure of the native filter is not stable in the presence of Na^+ alone. The effects observed here on tetramer stability suggest that mutation of the S_4 site disrupts the ability of the native filter

to discriminate against Na^+ and allows this smaller cation to interact more effectively with the remaining ion binding sites.

Overall, the relative changes in cation affinity for tetramer protection shown in Figure 4 support the conclusion that the chemical identity of the amino acid side chain at the T75 position is important for cation discrimination and, by extension, the physiological conduction and gating properties of the native K^+ channel selectivity filter. The two substitution mutations assessed in this study (T75C and T75A) have the following major effects. (1) They moderately weaken the intrinsic affinity of KcsA for monovalent alkali cations (K^+ and Rb^+) that readily permeate or block (Cs^+) the native channel. (2) They severely weaken the interaction of the channel with Ba^{2+}, a universal blocker of K^+ channels. (3) They anomalously enhance the apparent affinity of KcsA for tetramer stabilization by Na^+, a cation which is normally impermeant under physiological conditions.

Interaction of Cys75 with Thiophilic Metal Cations. The natural ability of the sulfur atom of the Cys sulfhydryl group to coordinate with a variety of transition metal and heavy metal cations has been widely exploited as a method of identifying amino acid residues whose side chains line the permeation pathway of ion channels. Binding of Cd^{2+}, Zn^{2+}, and Ag^+ to Cys sulfhydryl groups located in the ion conduction pathway domain of various native Cys-containing isoforms or Cys substitution mutants of Na^+ channels (*36, 37*) and K^+ channels (*38*−*40*) results in partial or complete blockage of current flow of permeant cations. Such blocking interactions of thiophilic cations with Cys pore residues have been successfully used to probe mechanisms of channel permeation, gating, and pharmacology. Here we used a similar approach to examine a question of intrinsic theoretical interest: do various thiophilic metal cations affect the stability of the quaternary structure of the T75C mutant of KcsA?

In pursuing this question, we found that the proximity of the four sulfhydryl groups at the S_4 site of T75C renders this tetramer naturally susceptible to covalent dimer formation either spontaneously in the course of bacterial expression and protein purification or as enhanced by elevated temperature and Cu^{2+}. This behavior is characteristic of Cys disulfide formation as further demonstrated by the sensitivity of the dimer band to treatment with the disulfide reducing agents, DTT and TCEP. The measured distance relationships of Cys75 residues in the published crystal structure (*28*) of the nondimerized T75C mutant of KcsA (PDB entry 1S5H) also support this conclusion. The Cα−Cα′ distance for Cys75 between adjacent subunits is 6.43 Å, and that for diagonal subunits is 9.10 Å. Since the Cα−Cα′ distance of 85−95% of disulfide bonds in a large collection of known protein structures falls in the range of 4.4−6.8 Å (*41*), it is most likely that the dimer band is a result of formation of a disulfide between Cys75 residues of adjacent KcsA monomers in the tetramer.

Our studies of the interaction of metal ions with T75C reveal that only certain thiophilic cations such as Ag^+ and Hg^{2+} are capable of effectively stabilizing this mutant tetramer against dissociation to monomers and dimer formation at high temperatures. Two other cations, Cd^{2+} and Ni^{2+}, stabilized the T75C tetramer less effectively, whereas Mn^{2+}, Co^{2+}, Zn^{2+}, and Pb^{2+} were ineffective. In the absence of crystal structures showing how these different metal ions actually bind to the S_4 site, we speculate that the relative effectiveness of thiophilic cations in this assay reflects their different avidity for single thiol groups, their propensity to bridge between two thiols, and their preferred coordination geometries with four thiol ligands as observed in various proteins that bind these metal ions (*42*). However, the finding that the T75C mutant exhibits a unique pattern of tetramer-stabilizing interactions with thiophilic cations is further evidence that inorganic cations are capable of making very specific structural contributions to K^+ channel stability. These results also suggest that interactions of thiophilic cations with various types of K^+ channels mutated to contain Cys residues at the residue homologous to T75 may be a useful experimental approach to investigating the role of the selectivity filter in C- and P-type inactivation gating (*12*−*15*). In fact, HCN channels, the only members of the K^+ channel family that contain a natural Cys substitution at the residue homologous to T75 of KcsA, exhibit unique sensitivity to block by Cd^{2+} and irreversible inhibition by treatment with the thiol oxidizing agent, Cu^{2+}-orthophenanthroline (*43, 44*). The latter Cu^{2+}-dependent phenomenon appears to reflect disulfide formation as directly observed here for mutant KcsA.

Functional Effect of T75 Substitutions on Various K^+ Channels. In a pioneering study of the *Drosophila* Shaker K_V channel "signature sequence" that forms the external pore, Heginbotham et al. (*45*) found that substitutions of the "T4" residue (Shaker residue T442 equivalent to T75 in KcsA) with Ala, Asn, Gly, or Ser yielded functional voltage-activated K^+ current with little effect on ionic selectivity as measured by permeability ratios. Notably, however, electrophysiological recordings of the Ala substitution mutant displayed prominent inward current carried by external Na^+ and Cs^+, a feature not exhibited by wild-type Shaker (*45*). The latter observation suggests that substitution of Shaker residue T442 subtly modifies the interactions of cations with the K^+ channel selectivity filter corresponding to crystallographic evidence that the T75C mutant of KcsA reduces K^+ occupancy at the S_4 and S_2 sites but not at the S_1 and S_3 sites (*28*). Other studies of the Shaker K_V channel indicate that very few mutations are tolerated well at Shaker residue T442, as judged by the failure of such mutants to express robust currents in *Xenopus* oocytes (*45, 46*). In Shaker B, the T442S mutation appears to strongly suppress fast inactivation mediated by the N-terminal inactivation peptide (*47*). This basis of this latter phenomenon has not been fully elucidated or explained, but it suggests that the inactivation peptide interacts directly or indirectly with the innermost S_4 site of the selectivity filter. In an N-terminally truncated Shaker variant deficient in fast inactivation, the T442S mutation stabilizes the open state of the channel by greatly slowing the time course of deactivation (*46*). At the single-channel level, this mutant exhibits enhanced occurrence of two prominent subconductance states with conductance selectivity for K^+, Rb^+, and NH_4^+ different from that of the main open state (*46*). Subconductance behavior of the T442S mutant associated with coupled changes in gating and ion selectivity may be explained by asymmetric conformational changes in pore structure associated with intermediate steps in the activation process (*46, 48*). The poor tolerance for expression of functional K^+ current and the subtle changes in ion selectivity associated with mutations of Shaker T442

are consistent with the observations of tetrameric stability of KcsA described in this paper. Poor expression may reflect unstable tetramer formation. The apparent contradiction in the fact that the T442S mutant of Shaker is functional in *Xenopus* oocytes (*45, 46*) and the T75S KcsA mutant is defective in tetramer stability (Figure 2) may be explained by compensatory sequence differences at filter positions neighboring T75 (see Figure 1B), other structural differences between Shaker and KcsA, or the harsher conditions of detergent solubilization employed in our biochemical assay of quaternary structure that may differentially affect the stability of different K^+ channels.

The natural ability of Na^+ to permeate more readily through HCN, SK, and HERG channels (*49*−*52*) relative to other families of K^+ channels may also be related to natural substitutions of Cys (HCN) and Ser (SK, HERG) at the residue equivalent to T75 of KcsA (Figure 1B). Curiously, among the eight evolutionarily distinct families of K^+-selective channel proteins, HCN channels have the weakest ionic preference for K^+, as indicated by a permeability ratio, P_{Na}/P_K, of 0.24−0.31 (*49, 50*) in contrast to the P_{Na}/P_K of <0.03 typically found for other K^+ channels (*53*). Weak Na^+/K^+ discrimination of HCN channels would appear to be important for spontaneous electrical rhythm of cardiac pacemaker cells and certain neurons; however, it is not clear whether the Cys residue at position T75 solely determines this behavior. On the basis of a hypothetical molecular model of the pore region of the HCN channel, Giogetti et al. (*44*) suggest that the loss of ion selectivity is related to the reduced rigidity of the selectivity filter in HCN channels compared to KcsA, due to a reduction in stabilizing hydrogen bonds in the filter region.

The native HERG channel (human $K_V11.1$) with a Ser residue (S624) at the corresponding T75 position of KcsA does not pass measurable Na^+ current in the presence of symmetrical Na^+ solutions; however, the S624A mutant of HERG does readily conduct Na^+ under these conditions (*52*). The lack of Na^+ current through the HERG channels in physiological salt solutions is due to a potent blocking effect of external Na^+ in the native channel that is eliminated by the S624A mutation (*52, 54*). The complex effects of Na^+ on the HERG channel appear to reflect binding interactions of Na^+ and K^+ in the selectivity filter as well as rapid conformational changes of the filter associated with P-type inactivation gating (*52*). Mullins et al. (*54*) have also suggested that the hydroxyl and carbonyl oxygen groups of the four T75 residues of KcsA participate in a octagonal ring of stabilizing hydrogen bonds that would be absent in K^+ channels that contain a Cys or Ala residue at this position. The lower thermal stability of the T75C and T75A tetramers observed in our study may partly reflect the absence of such an intersubunit hydrogen bond network.

Functional studies of small conductance SK channels activated by Ca^{2+}/calmodulin identify the native Ser residue (S359 in rSK2) at the S_4 filter site residue equivalent to T75 of KcsA as an important determinant of divalent cation block and monovalent ion selectivity. For example, the S359A mutation in the rat SK channel, rSK2, reduced the apparent affinity for internal block by both Ba^{2+} and Sr^{2+} by 50-fold (*55*). The wild-type rSK2 channel also exhibits an usually high permeability to Na^+ and Li^+ ($P_{Na}/P_K = 0.12$; $P_{Li}/P_K = 0.14$), compared to the P_{Na}/P_K of <0.03 typical of most other K^+ channels with a conserved Thr residue at the S_4 filter site location (*51*). Substitution of the native S359 residue with Ala increases relative Na^+ permeability to 0.40 (P_{Na}/P_K) and Li^+ permeability to 0.22 (P_{Li}/P_K), whereas the more conservative S359T mutation did not alter these permeability ratios from wild-type values (*51*). The S359 position in rSk2 also appears to have special structural significance in the SK subfamily of K^+ channels since substitution of this position with residues other than Ala or Thr did not yield measurable K^+ current for seven other mutants (*55*). The cited data on the functional role of the S359 residue in SK channels correlate well with tetramer analysis of KcsA in this paper showing the role of T75 in the tetramer stability, reduced affinity for tetramer stabilization by Ba^{2+}, and enhanced tetramer stabilization by Na^+ and Li^+ in the T75C and T75A mutants. Taken together, current evidence of the role of the S_4 site residue in various K^+ channels reinforces the concept that T75 (KscA) is not only an important element of tetramer stability but also a determinant of selectivity filter interactions of inorganic cations with respect to permeation and block.

Structural Constraints and Dynamics of the T75 Residue. The various effects on tetramer stability and K^+ channel function discussed above point to a special role of the T75 residue. The structural significance of this residue in KcsA can be attributed to its unique location just at the transitional turn of the peptide backbone from the so-called pore α-helix to the unusual extended strand of T75−Y78 that forms the selectivity filter. As described by Valivayeetil et al. (*56*), the severe structural constraints on this residue can be appreciated by the fact that the φ and ψ dihedral angles of T75 of KcsA fall into a sterically unfavorable left-handed helical region of the Ramachandran plot. Molecular dynamics simulations also indicate that reorientation of the V76 peptide carbonyl followed by conformational rearrangement of the T75 residue in one of the four KcsA subunits can act as a gate, effectively blocking the selectivity filter to ion conduction (*57*).

Other conformational changes of the selectivity filter are also linked to intrinsic slow inactivation gating behavior of KcsA. Cordero-Morales et al. (*58*) described a native carboxyl−carboxylate hydrogen bond between residues E71 and D80 that comprises a springlike molecular interaction favoring a gating transition of the open state to a nonconducting state of the filter. Weakening of this interaction in KcsA mutants such as E71A virtually eliminates such inactivation gating (*58*). Since most eukaryotic K^+ channels lack conserved residues equivalent to E71 and D80, there must be other molecular interactions of the selectivity filter that govern C-type inactivation gating in diverse K^+ channels. The unfavorable bond geometry and susceptibility to reorientation of the T75 residue at the S_4 site may provide a clue to the explanation of several phenomena: effects of T75 on tetramer stability of KcsA reported here, effects of T75 on permeant cation selectivity inferred from studies of other K^+ channels, and mechanisms that determine C-type inactivation. More detailed structural studies on engineered and native K^+ channels with substitutions at this position may provide further insight. As noted above, natural replacement of T75 with Ser or Cys is typically accompanied by other local sequence changes (Figure 1B), suggesting that compensatory

structural changes may be necessary to allow monomers with these substitutions to form stable or properly functioning tetramers.

Implications for the Structural Basis of Ion Selectivity in K^+ Channels. The preceding discussion correlates the effect of T75 mutations on tetramer stability with known structure−function relationships of the selectivity filter of KcsA and other K^+ channels. In this section, we attempt more broadly to reconcile our biochemical observations of cation interactions of KcsA with current views of the mechanism of ion selectivity.

It must first be acknowledged that this study of ion dependence of tetramer stability represents a rather limited view of the complex landscape of ion selectivity. As often noted, the definition of ion selectivity of a channel depends upon how it is measured (*34, 53, 59*). Electrophysiologists typically define Na^+/K^+ selectivity of cation channels by the permeability ratio of Na^+ to K^+, P_{Na}/P_K, measured from the reversal potential of ionic current with the test ions on the opposite side of the membrane. This assay essentially measures the relative ability the channel to pass voltage-driven current of one ion in the competing presence of the other ion. For many K^+ channels, the permeability ratio of Na^+ to K^+ (P_{Na}/P_K) is gauged at <0.03 (*53*), but it has been estimated to be <0.006 for KcsA (*60*) and <0.001 for the BK Ca^{2+}-activated K^+ channel (*61*). However, certain members of the K^+ channel protein family noted above such as SK channels and HCN channels exhibit less selective P_{Na}/P_K ratios of 0.12 and 0.24−0.31, respectively (*49*−*51*). Another functional assay of cation selectivity in K^+ channels is the relative maximal conductance of unitary current measured in pure solutions of different ions. K^+ channels typically exhibit the highest conductance for K^+, a substantially lower conductance for Rb^+ (and Cs^+ if it is measurable), and an immeasurably low conductance for Na^+ and Li^+ (*60, 62*). Functional definitions of ion selectivity based on permeability and conductance essentially pertain to the nonequilibrium flow of ions through the pore, which is a complex function of the concentration of the ions, the number of sites, and the detailed kinetics of ion movement in single-file, multi-ion channels such as K^+ channels (*53, 59*).

On the other hand, the simplest definition of ion selectivity from the standpoint of protein biochemistry is the ratio of equilibrium dissociation constants (K_D values) for binding of two different ions at a given site in a protein. As noted above, the tetramer stability assay used in this work reports on the rate of irreversible dissociation of the KcsA tetramer. This assay is clearly related to the equilibria of ion binding to the selectivity filter, but since we have not yet been able to measure initial rates of tetramer dissociation, these ion titrations cannot be analyzed to extract true equilibrium K_D values.

From the perspective of enzymology, kinetic theory states that the relative values of k_{cat}/K_M, the ratio of the maximal turnover number (k_{cat}) to the Michaelis constant (K_M), determine the relative rate of enzyme action for two different substrates (*63*). The latter ratio known as the substrate "specificity constant" can be directly related to the ion permeability parameter, P, of Nernst−Planck electrodiffusion theory. The enzyme specificity constant corresponds to the g_{max}/K_M parameter of Eyring rate theory of ion conduction (*59*), where g_{max}/K_M is the ratio of maximal conductance (g_{max}) of a one-site ion channel to the Michaelis constant for the ion (K_M). While this brief review of theory makes it clear that molecular studies of "biochemical" ion selectivity can contribute to an understanding of the underlying basis of "electrophysiological" selectivity, measurements reporting on protein structural phenomena related to ion binding reflect a very different weighting of states, transitions, and rate constants that contribute to the complex cycle of ion transport than functional assays of current. Thus, biochemical observations on tetramer stability cannot realistically be expected to account for K^+ selectivity of ion permeation and conduction just as the measurement of a substrate binding constants cannot account for the relative substrate specificity of catalysis and turnover of an enzyme. This important reminder of operational definitions of "selectivity" and caveats to interpretation can help to clarify apparent controversy concerning discussion of the classical size-based "geometrical" model (or "snug-fit" model) of ion selectivity of K^+ channels (*53, 64*) supported by static X-ray crystal structures (*10*) versus the "dynamic" model of selectivity supported by analysis of molecular dynamics simulations (*65*−*69*).

The perspective on selectivity ascribed to the geometrical model is supported by crystallographic data of MacKinnon and colleagues demonstrating that inorganic cations are preferentially bound by the KcsA selectivity filter in different structural configurations depending on the size of the cation (*11, 27, 29, 34*). K^+ (ionic radius, r, of 1.33 Å) and Tl^+ (r of 1.40 Å) bind uniformly to all four S_1−S_4 sites in the "conductive" conformation of the filter. The actual number of K^+ and Tl^+ ions that bind to these four sites at any given time is estimated to be near 2.0 on the basis of analysis of single-wavelength anomalous difference (SAD) refinement and the argument that cation occupancy at adjacent sites is highly unfavorable due to electrostatic repulsion (*11, 27, 29*). Thus, nearly equivalent density corresponding to four ions bound to the selectivity filter in the K^+−KcsA crystal structure is thought to reflect equal numbers of protein molecules in the crystal with dual S_1/S_3 and S_2/S_4 site occupancy. As mentioned above, the larger alkali cations, Rb^+ ($r = 1.48$ Å) and Cs^+ ($r = 1.69$ Å), are observed to physically occupy only three of the four filter sites, S_1, S_3, and S_4 (*27*). The divalent cation Ba^{2+} ($r = 1.35$ Å) binds to two locations in the filter, S_4 and a second location near the S_2 K^+-site, but slightly shifted toward S_1 (*25, 34*). The structural interaction of KcsA with smaller alkali cations such as Na^+ (and presumably Li^+) is quite different. In the presence of low K^+ and high Na^+ levels or in the presence of Na^+ alone, the KcsA filter adopts a "nonconductive" conformation of the filter with Na^+ bound in the S_1 and S_4 sites (*11, 34*). This nonconductive conformation has the backbone carbonyl oxygen atoms of V76 and G77 filter residues oriented away from the central pore, effectively destroying the native S_2 and S_3 coordination sites for K^+. Compelling evidence of a slow conformational transition from the nonconductive form of the KcsA filter in solution containing Na^+ as the major cation to the conductive form of the filter in a K^+ solution has recently been obtained by isothermal titration calorimetry (ITC) of KcsA titrated with various cations (*34*). Binding isotherms for titrations of exothermic binding of K^+, Rb^+, and Cs^+, and endothermic binding of Ba^{2+} to the Na^+ form of KcsA, are consistent with a major conformational change of the

filter from the nonconductive to conductive form observed by crystallography (*11, 34*).

On the basis of the structural manifestations of biochemical cation selectivity in KcsA apparent in the crystallographic and ITC data of MacKinnon and co-workers cited above, we propose that process of equilibrium binding of cations coupled to conformational changes of the filter underlies the changes in tetramer stability of KcsA demonstrated in this paper. Facile dissociation of the tetramer at lower temperatures in the presence of cations such as Na^+ and Li^+ versus greater stability at high temperatures conferred by cations such as K^+, Rb^+, Cs^+, Tl^+, and Ba^{2+} is nicely explained by the hypothesis that the conductive state of the tetramer is held together more strongly than the nonconductive state of the Na^+ complex of KcsA due to the 16 additional oxygen−cation interactions at the subunit interface contributed by properly positioned carbonyl oxygen groups of the S_2 and S_3 cation-binding sites. As suggested earlier, macroscopic changes in biochemical ion binding selectivity that arise from the effects of T75 KscA mutations on tetramer stability probably correspond to numerous microscopic changes in cation binding to the overall filter caused by replacement of the threonine hydroxyl group at S_4. This interpretation is further supported by the coupled reduction in K^+ occupancy at sites S_4 and S_2 seen in the crystal structure of the T75C mutant (*28*).

More detailed insight into the exact structural basis for the enhanced thermal stabilization by Na^+ of T75C and T75A tetramers as compared to native KcsA will require further investigations such as crystallographic and ITC studies of the interaction of Na^+ with these mutants. The exquisite dependence of binding selectivity for Na^+ versus K^+ on the conserved amino acid sequence of the filter can also be appreciated by the crystal structure of the NaK channel of *Bacillus cereus* (*70*). NaK is a K^+ channel protein homologous to KcsA that possesses a selectivity filter sequence of TVGDG instead of the canonical TVGYG signature sequence of most highly K^+ selective channels. The crystal structure of the NaK channel demonstrates cation binding to oxygen atoms of intact filter sites at S_4 and S_3 equivalent to those of KcsA but defective cation coordination at S_2 and S_1 (*70*). Furthermore, the NaK channel appears to bind both K^+ and Na^+ at the S_4 and S_3 sites equally well and does not exhibit the conformational change from conductive to nonconductive filter conformations in the presence of K^+ versus Na^+ characteristic of KcsA (*70*). The substitution of an Asp residue for the equivalent Tyr78 filter residue of KcsA appears to render the NaK channel biochemically nonselective for Na^+ and K^+ and also disrupts the Na^+ dependence of the filter conformation. This interesting example of altered ion binding to the NaK channel can be cited to speculate that substitution of the T75 residue of KcsA with Cys or Ala similarly alters a subtle structural interaction (perhaps the Na^+-dependent transition from conductive to nonconductive states) that affects binding of Na^+ to the KcsA filter.

Detailed assessment of the significance of Thr75 to permeation selectivity of ions will require electrophysiological current measurements of the T75C and T75A mutants in the presence of Na^+. Existing data on the T75C mutant of KcsA concerning the greatly reduced unitary conductance of the mutant for K^+ but little change in Rb^+ conductance compared to the wild-type value are consistent with the differential preference of the filter sites for these ions seen in the crystal structures (*27*). Cited evidence that SK, HERG, and HCN K^+ channels with natural variations of the Thr75 residue exhibit enhanced Na^+ permeability (*49*−*52*) supports the idea that altered ion selectivity may be associated with specific molecular interactions of the Thr75 hydroxyl group or other structural changes of the filter related to an altered S_4 site.

Current discussion of the mechanism of permeation selectivity in K^+ channels reflects controversy over whether interpretations based on ion binding to the conductive structure of the KcsA filter are consistent with a classic view of Na^+/K^+ selectivity based on precise geometrical positioning of oxygen atoms of the selectivity filter that provide a snug fit for the large K^+ ion ($r = 1.33$ Å) but not for the smaller Na^+ ion ($r = 0.95$ Å) (*34, 68, 69*). According to this interpretation, the energetic cost of deforming the filter structure to physically conform to the smaller Na^+ ion results in favorable compensation of the dehydration energy for K^+ but not for Na^+, giving rise to size dependence in the selectivity series for inorganic cations. At face value, this argument provides a nice rationale for the stable equilibrium binding of larger cations such as K^+, Rb^+, Cs^+, Tl^+, and Ba^{2+} to the conductive conformation of the filter and the observed conformational transition to a different nonconductive filter conformation required to accommodate stable Na^+ binding (*11, 34*). However, it does not fully explain the extraordinary permeation selectivity for K^+ of the conductive structure of the filter that is dominant under conditions of electrophysiological current assays. For example, size selectivity does not explain the abysmally low conductance of Ba^{2+}, which has the same size as K^+ and exhibits stable binding to only two of the four size-appropriate filter sites (S_4 and S_2) in the conductive filter (*25, 34*). Assuming that transient simultaneous binding of Ba^{2+} is possible at the S_2 and S_4 sites, one might expect that strong ion−ion repulsion would allow Ba^{2+} to move rapidly through the channel instead of merely acting as a blocker. Also, the snug-fit perspective of selectivity would seem to require differential deformability of the four filter sites to explain the nonuniform preference of the four filter sites for K^+, Rb^+, and Cs^+ seen in KcsA crystal structures (*27, 34*).

Inadequacy of the snug-fit paradigm in accounting for the nuances of electrophysiological measurements of permeation selectivity has been addressed by the powerful but computationally intensive approach of all-atom molecular dynamics (MD) simulation (for reviews, see refs *67*, *68*, and *71*). Whether one is interested in the equilibrium binding affinity of a particular site in the filter or relative rates of ion conduction through the whole filter, the ability of K^+ channels to discriminate K^+ from Na^+ ultimately arises as a consequence of two opposing free energy contributions: the higher dehydration energy of Na^+ versus K^+ and the more favorable ligand interaction energy of K^+ versus Na^+ with the 16 carbonyl oxygen atoms lining the selectivity filter (*65, 68, 69*). With the ability to effectively "turn off" each of the elementary forces that contribute to ligand interactions in proteins, MD simulation has the power to reveal the individual contribution of these forces to complex processes such as ion permeation (*65*).

The MD approach has already contributed many important insights to the understanding of ion selectivity in KcsA. One

of the most significant findings from MD studies is that protein structural dynamics plays a major role in ion discrimination of the KcsA filter sites. For example, the difference in the ionic radii of Na^+ versus K^+ is only 0.38 Å, whereas root-mean-square fluctuations of the selectivity filter atoms of KcsA lie in the range of 0.5−1.0 Å (*65, 68, 69*). This means that the filter sites can readily deform locally to accommodate either ion on the basis of size. In fact, carbonyl oxygens atoms of filter residue Val76 from opposing subunits in one crystal structure of KcsA (PDB entry 1K4C) define a pore radius of only 0.9 Å at this location in the filter, which is too narrow for passage of dehydrated K^+ ($r = 1.33$ Å) (*66*). Therefore, the permeation of alkali ions through the filter actually depends on thermal fluctuations that dynamically affect the "fit" of ions in the filter sites. MD simulations also show that Na^+/K^+ selectivity in the KcsA filter arises from the interplay of electrostatic attraction between the permeant cations and the coordinating oxygen atoms and the variation in electrostatic repulsion of dynamically fluctuating carbonyl oxygen atoms in the presence small versus large cations (*65, 68*). Other important results from MD studies include verification of a single-file conduction mechanism for K^+ and H_2O molecules involving alternating states of dual K^+ occupancy [(S_1, S_3) and (S_2, S_4)] (*71*−*73*), nonequivalence of the relative free energy of interaction of K^+ and Na^+ with the four filter sites (*69*), and conduction asymmetry corresponding to nonequivalence of the (S_1, S_3) and (S_2, S_4) states of K^+ occupancy for different K^+ channels (*74*). While it appears that MD simulations reported thus far have primarily focused on the role of the carbonyl oxygen groups lining the KcsA filter, this same computational approach may also help to interpret the major implication of our present study of tetramer stability that hydroxyl groups of Thr75 at the S4 site may also make a significant contribution to ionic selectivity of K^+ channels. We look forward to further results from both structure-based and MD investigations that seek to address the unique character of this site in the K^+ channel selectivity filter.

Summary and Conclusion. Considering that K^+ channels and related members of the VGL family of channels function as tetramers or pseudotetramers, the structural and dynamic basis of tetramer stability is fundamental to their mechanism. This study provides direct evidence that the side chain of a highly conserved Thr75 residue that forms the S_4 site of the KcsA selectivity filter is a significant determinant of cation selectivity as monitored by changes in the thermal stability of the tetramer. Replacement of Thr75 with Cys confers the ability to bind certain thiophilic metal ions to the filter and also allows formation of covalent dimers of KcsA monomers by Cu^{2+}-catalyzed disulfide formation. Engineering such features into K^+ channel pores features may provide useful approaches to investigation of certain mechanistic questions. Deeper insight into the role of the S_4 filter site in K^+ channels will require a combination of techniques, including electrophysiology, structural biochemistry, and computational molecular dynamics.

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