Voltage-dependent conformational changes of K$_v$AP S4 segment in bacterial membrane environment

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Abbreviations: K$_v$AP, voltage-dependent K$^+$ channel from Aeropyrum pernix; IPTG, isopropyl β-D-1-thiogalactopyranoside; MD, molecular dynamics; MTSET, [2-(trimethylammonium) ethyl] methane thiosulfonate bromide; [14C]-NEM, N-[ethyl-14C] maleimide; PMS, phenazine methosulfate; TPP$^+$, tetra-[3H]-phenylphosphonium bromide

The nature and magnitude of voltage sensor conformational changes during ion channel activation are controversial. We have analyzed the topology of the K$_v$AP voltage sensor domain in the absence and presence of a hyperpolarized voltage using native, right-side out membrane vesicles from E. coli. This approach does not disrupt the normal membrane environment of the channel protein and does not involve detergent solubilization. We found that voltage-dependent conformational changes are focused in the N-terminal half of the K$_v$AP S4 segment, in excellent agreement with results obtained with Shaker. Homologous residues in the K$_v$AP and Shaker S4 segments are transferred from the extracellular to the intracellular compartment upon hyperpolarization. Taken together with X-ray structures indicating that the K$_v$AP S4 segment is outwardly displaced at 0 mV compared to S4 in a mammalian Shaker channel, our results are consistent with the idea that S4 moves further during voltage-dependent activation in K$_v$AP than in Shaker.

Introduction

The S4 segment is the main functional component of the voltage sensor in K$^+$ channels. Positively-charged S4 residues drive voltage-dependent conformational changes that control the probability of channel opening. In Shaker K$^+$ channels, the equivalent of 12–13 elementary charges moves across the transmembrane electric field during activation of one channel. Most of this charge is carried by the first four arginine residues (R1–R4) in S4 segments from four channel subunits. R1–R4 move partly or entirely across the transmembrane field during activation.

The available evidence suggests that charge-moving S4 residues in Shaker traverse a focused electric field. The transmembrane field likely falls across a relatively narrow barrier that separates water-accessible crevices or vestibules that intrude into the voltage sensor domain from the external and internal surfaces of the membrane. Support for this idea comes from chemical modification of transmembrane residues by hydrophilic reagents, use of molecular calipers to estimate the distance S4 residues travel, measurement of the electric field profile near S4 using an electrochromic probe, and the high resolution X-ray structures of two eukaryotic K$^+$ channels, K$_v$1.2 and a K$_v$1.2/ K$_v$2.1 paddle chimera. Molecular dynamics (MD) simulations based on the K$_v$1.2 structure indicates that water fills internal and external gating crevices near S4, except for the narrow barrier region.

A focused electric field would be expected to reduce the distance that R1–R4 must travel during activation. However, the magnitude of voltage sensor conformational changes during activation is controversial. Data obtained using optical techniques and histidine scanning mutagenesis indicate that the Shaker S4 undergoes a vertical translation of 10 Å or less during activation. This distance is significantly less than the width of the bilayer, consistent with a focused electric field. In contrast, in the prokaryotic K$_v$AP K$^+$ channel, S4 has been estimated to move further, 15 to 20 Å, during activation. However, MD simulations using the X-ray structure of the K$_v$AP voltage sensor domain predict that it contains water-filled internal and external gating crevices, separated by a relatively narrow barrier. Indeed, MD results obtained using the K$_v$AP and K$_v$1.2 structures are strikingly consistent. Electron paramagnetic resonance spectroscopy has also provided evidence that water penetrates into deep crevices in the K$_v$AP voltage sensor domain. Therefore, the discrepancy between the amplitude of S4 movement in Shaker and K$_v$AP remains puzzling.

We have now investigated the topology of the K$_v$AP voltage sensor domain in the absence and presence of a hyperpolarized voltage in the intact bacterial membrane using a right side out vesicle preparation. In this approach, the protein is not solubilized in detergent and its environment is minimally disturbed. The vesicles contain the components of the electron transport...
with the idea that the S4 segment moves further in KvAP than in Shaker during voltage-dependent activation.

**Results**

**Calibration of the topology assay.** Single cysteine mutations were made in a cysteine-less KvAP background, C247S.37 Constructs were expressed in *E. coli*, which were grown to mid-log phase before induction of KvAP expression. *E. coli* were lysed to produce a uniform population of right-side out membrane vesicles using standard techniques.36,27,38

Right-side out vesicles were used directly to analyze KvAP topology according to the strategy illustrated in Figure 1A.29,31,39,40 Vesicles were first incubated in the presence or absence of MTSET, an impermeant, sulfhydryl-specific reagent. Extracellular cysteines, which face the outside of right side out vesicles, will react with MTSET, whereas intracellular cysteines, sequestered inside the vesicles, will not. Subsequently, the vesicles were treated with the idea that the S4 segment moves further in KvAP than in Shaker during voltage-dependent activation.
with a radioactively tagged, membrane-permeant, sulfhydryl-specific reagent, \([^{14}C]-N\)-ethylmaleimide ([\(^{14}C\)]-NEM). Without MTSET pretreatment, [\(^{14}C\)]-NEM will react with both intracellular and extracellular cysteines. With MTSET pretreatment, in contrast, extracellular cysteines are blocked and [\(^{14}C\)]-NEM will react with intracellular cysteine residues only. Therefore, the ratio of labeling by [\(^{14}C\)]-NEM with and without MTSET pretreatment will be high for intracellular cysteines but low for extracellular cysteines (Fig. 1A). Because cysteine residues react with maleimides and MTS reagents via a hydrophilic thiolate anion intermediate, this assay is useful for identifying residues located near the intracellular or extracellular surface of the protein or in aqueous crevices that intrude into the plane of the membrane.\(^{31,42}\) Labeling results were determined by purifying \(K_{\text{AP}}\) and subjecting it to electrophoresis, phosphorimaging and immunoblotting.

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**Figure 2.** Alignment of \(K_{\text{AP}}\), Shaker, and chimera sequences from S1 through S6 and generic nomenclature for voltage sensor residues. (A) Optimal alignment of the \(K_{\text{AP}}\), Shaker, and the chimera sequences is shown. This alignment maximizes sequence similarity over the length of the S4 segment, as is evident in the highly-conserved K5-R6-K7 pattern at the C terminal end, and corresponds unambiguously to an alignment of the \(K_{1.2}\) and chimera X-ray structures.\(^{1,13}\) K1.2, like Shaker, has an arginine at the R1 position in S4, whereas the chimera has a glutamine. Numbers above and below the sequences refer to \(K_{\text{AP}}\) and the \(K_{1.2}/K_{2.1}\) chimera, respectively.\(^{1,35}\) The K2.1 portion of the chimera sequence is shown in uppercase. Segments S1-S6 are identified by dashed red lines. The charged residues R1-K7 in S4 are indicated in blue. Green circles show the \(K_{\text{AP}}\) residues tested in this study. (B) The cartoon defines a generic nomenclature for equivalent residues in \(K_{\text{AP}}\), Shaker, and the \(K_{1.2}/K_{2.1}\) chimera for which a high resolution structure is available.\(^{11}\) A key correlating the generic nomenclature to the actual residues in these channels is provided in Table S1. The generic nomenclature is defined by the amino acids found in Shaker, with three exceptions: (1) S2-BS and S3b-BS correspond to residues that form a divalent cation binding site in the eag voltage sensor;\(^{2,14}\) (2) S3b-E\(^\circ\) corresponds to a residue that is negatively charged in \(K_{\text{AP}}\) but is neutral in Shaker and the \(K_{1.2}/K_{2.1}\) chimera; (3) S4-K5\(^\circ\), a lysine in Shaker and the chimera, is a neutral residue in \(K_{\text{AP}}\). The residues shown conform to the following color code: acidic residues, red; basic residues, blue; neutral residues, green.
K⁺ channel pores are well-conserved structures about which there is little controversy. Therefore, we chose two pore domain residues, one extracellular (I170C) and one intracellular (S246C), to calibrate the topology assay (Fig. 1B). We found that [¹⁴C]-NEM labeling of the extracellular residue I170C was dramatically reduced by pretreatment with MTSET. In contrast, significant [¹⁴C]-NEM labeling of the intracellular residue S246C occurred with and without MTSET pretreatment (Fig. 1C). These results establish guidelines for labeling ratios expected for extracellular and intracellular residues, respectively (Fig. 1D).

**Topology of the voltage sensor domain in the absence of a membrane potential.** Single cysteine mutations were made throughout the KvAP voltage sensor domain to assess its topology (Fig. 2A). The first set of experiments was conducted in the absence of a membrane potential (0 mV). Under these conditions, the conformation of the KvAP voltage sensor is expected to be similar to the high resolution X-ray structure of the isolated voltage sensor domain. Representative phosphorimages for selected positions are shown in **Figure 3A.** Residue F116 immediately precedes the first arginine in S4, R117 (S4-R1), whereas S135 immediately precedes the last lysine in S4, K136 (S4-K7) (Fig. 2B and Table S1). [¹⁴C]-NEM labeling of F116C was dramatically reduced by pretreatment with MTSET, indicating that this position is exposed to the extracellular environment. In contrast, substantial [¹⁴C]-NEM labeling of S135C remained after pretreatment with MTSET indicating that S135C fails to react with external MTSET. It is important to note that the cysteine at position 135 reacts with sulphydryl reagents, because S135C is readily labeled by [¹⁴C]-NEM. We conclude that S135C is not exposed to the external solution. This residue may be located on the intracellular side of the membrane or in an internal gating crevice. Residues with this pattern of labeling will be called intracellular. This terminology provides convenient shorthand but should not be interpreted as a rigorous description of the residue’s location.

Conversely, residues where [¹⁴C]-NEM labeling is substantially blocked by MTSET pretreatment will be referred to as extracellular. However, such residues could also be located in a water-filled external gating crevice rather than at the protein surface. The F116C and S135C results confirm that, as expected, S4 spans the membrane when KvAP is embedded in a native environment despite the apparently cytoplasmic location of S4 in the X-ray structure of full length KvAP.33 We investigated the topology of V66C in S2 (S2-BS) and G101C in S3b (S3b-BS) because the analogous positions in the ether-à-go-go (eag) channel are occupied by aspartate residues that form a divalent cation binding site (Fig. 2B and Table S1).47,48 We have previously shown that divalent cations can access this binding site whether the voltage sensor is in the resting or activated conformation.48 Ion binding to the eag site regulates voltage sensor conformational changes.49-52 As shown in **Figure 3A,** pretreatment with MTSET dramatically reduced [¹⁴C]-NEM labeling of V66C and G101C. These residues are likely to be located in an external gating crevice, as are their counterparts in eag.47,48 In eag, the binding site residues must be located within a few angstroms of each other to simultaneously coordinate a divalent cation.47 In contrast, the X-ray structure of the KvAP voltage sensor domain indicates that if
aspartate residues were positioned at 66 and 101, they would be too far apart to form an ion binding site.33

We analyzed the topological disposition of 25 positions in the K,AP voltage sensor domain (Figs. 2A and 3B). With a single exception, the tested residues fell into one of two groups. Ten residues were well labeled by [14C]-NEM with or without MTSET pretreatment, indicating that they are not exposed on the external side of the membrane (Fig. 3B). These residues were designated intracellular, with the cavets mentioned above. Interestingly, labeling ratios for these voltage sensor domain positions were higher than for the intracellular pore domain residue, S246C. In contrast, [14C]-NEM labeling of fourteen residues was dramatically reduced by MTSET pretreatment, indicating that they are exposed to the external aqueous solution (Fig. 3B). These residues were designated extracellular, although some of them are likely to reside in an external gating crevice. Although their labeling ratios were low, the values were typically higher than that obtained for the extracellular pore residue, I170C. Overall, higher labeling ratios may reflect greater conformational flexibility in the voltage sensor domain than in the pore, in accord with results of molecular dynamics simulations.12,13,22-24,53

One voltage sensor position, L125C in S4, did not fall clearly into either the intracellular or extracellular group (Fig. 3B). [14C]-NEM labeling was more sensitive to MTSET pretreatment than any of the intracellular residues, including S246C in the pore domain, but the residual labeling after MTSET pretreatment was greater than any of the extracellular residues. This may reflect a location that is poorly accessed by MTSET, such as the bottom of an external gating crevice, or transitions between intracellularly- and extracellularly-exposed locations at 0 mV. Individual voltage sensors may vary in a closed conformation at a low but finite frequency at 0 mV even though the overall probability of being open is high.54 If so, the data suggest that the L125 location is particularly sensitive to such excursions since it is the only residue that gave this pattern of reactivity. The data do not discriminate between these possibilities.

Effects of a hyperpolarized membrane potential on topology of voltage sensor domain. To assess the effect of a hyperpolarized voltage on K,AP topology, we used the electron transport chain to generate an inside-negative membrane potential in right-side out vesicles.29,30 Vesicles were incubated with the artificial electron donor system of ascorbate plus phenazine methosulfate (PMS).28 The membrane potential was measured using the lipophilic cation, [1H]-tetracycliphosphonium ion ([1H]TPP), which partitions into the vesicles as a function of voltage.50,53 The distribution of [1H]TPP was determined by flow dialysis (Fig. 4A).30 The membrane potential developed in less than 10 min and did not vary significantly over the course of the topology assay (Fig. 4A). The average voltage obtained under our experimental conditions was \(-75 \pm 0.8 \text{ mV} \) (mean \( \pm \text{SEM}, n = 24 \)). For comparison, the midpoint voltage \((V_{1/2})\) for K,AP activation has been reported to be \(-51 \text{ mV}\); ionic currents can be detected at \(--60 \text{ mV}\).37

Topology results obtained in the presence of a hyperpolarized membrane potential are shown in Figure 4B. To identify more readily residues that undergo voltage-dependent changes in topology, results obtained at 0 mV are shown on the same graph. In general, the [14C]-NEM labeling ratio decreased among residues in the intracellular group in the presence of a hyperpolarized membrane potential. This is likely due, at least in part, to the effect of the inside-negative electrostatic potential on the formation of the thiolate anion intermediate at intracellular positions. Because this trend was widespread among intracellular residues, it is likely to be non-specific.

In contrast, [14C]-NEM labeling ratios significantly increased at two positions in the N-terminal half of S4, L122C and L125C, upon exposure to a negative membrane potential (Fig. 4B, arrowheads). Both residues experienced a voltage-dependent change in environment. Upon hyperpolarization, L122C moved from an extracellular to an intracellular location. L125C moved from its intermediate location, unique among the tested positions, to an intracellular environment. These changes in topology were localized to the N-terminal half of S4.

**Discussion**

Voltage-dependent conformational changes in K,AP localized to S4. Significant inward movement of L122C and L125C occurred in response to hyperpolarization, with L122C moving from extracellular to intracellular and L125C moving from an intermediate position to intracellular (Figs. 4B and 5). These results are in excellent agreement with previous analysis of the Shaker S4 segment using MTS reagents.7 Voltage-dependent transfer of residues between external and internal aqueous compartments is localized to L366 and V367 in the Shaker S4.7,32 Significantly, V367 in Shaker corresponds to L122 in K,AP (Fig. 2A). L125 is located between R3 and R4 in the K,AP S4 segment (Fig. 2B and Table S1). Like L125, residues between R3 and R4 in Shaker move from an intermediate location in the activated state to the intracellular compartment upon hyperpolarization.7,52 We conclude that these homologous residues in K,AP and Shaker undergo similar changes in environment during activation, with voltage-dependent conformational changes localized to the N-terminal half of S4.7,32

Our results are compatible with previous analysis of K,AP topology using protein that had been solubilized with detergent, purified, biotinylated and reconstituted into liposomes prior to analysis in planar lipid bilayers using an avidin trap approach.20,21

Our data support the existence of water-filled gating crevices in the K,AP voltage sensor domain (Fig. 5). Cysteines in transmembrane segments, including V66C in S2 and A100C, G101C and A104C in S3, reacted with external MTSET. Residues in S4 exhibited a graded sensitivity to MTSET pretreatment. At 0 mV, F116C, R120C and L122C in the N-terminal half of S4 were exposed to the external solution. L125C near the middle of S4 had a unique pattern of reactivity that did not fall unambiguously into the extracellular or intracellular groups. S135C near the C-terminal end of S4 showed an intracellular labeling pattern. The presence of aqueous gating crevices in K,AP is consistent with the results of electron paramagnetic resonance experiments and with MD simulations.22,25

Voltage sensor domains in K,AP and Shaker stabilized by distinct charge pair networks. Several recent models postulate...
that the Shaker S4 undergoes translation, changes in tilt angle, and substantial rotation during voltage-dependent activation.25-19,56-58 These models can accommodate much of the available evidence concerning KvAP activation, including ours, but are unable to account for an apparent discrepancy in the distance S4 moves during activation. The KvAP S4 segment has been reported to move 15 to 20 Å during activation whereas distances of 10 Å or less have been estimated for Shaker.34-19,20,21

The available evidence supports the idea that charge-moving residues in S4 cross a focused electric field at a relatively narrow barrier that separates external and internal gating crevices.4-6 In response to changes in voltage, the homologous residues L122 in KvAP and V367 in Shaker are transferred between extracellular and intracellular compartments.7,32 This implies that L122 and V367 cross the barrier that divides the internal and external gating crevices during voltage-dependent gating.

In Shaker, several hydrophobic residues in S1 and S2, corresponding to S1-I, S2-SB and S2-F, have been implicated in forming the tight seal between the gating crevices (Fig. 2B).17,59 At hyperpolarized potentials, a salt bridge between S2-E1 and S4-R1 is required to maintain the impermeability of the barrier.17 At 0 mV, a salt bridge formed by S2-E1 and S4-R4 is located between the external and internal gating crevices according to MD simulations using the K_v1.2 structure.12,13 In contrast, MD simulations using the KvAP voltage sensor structure indicate that S4-R6, not S4-R4, participates in the salt bridge with S2-E1 that separates the aqueous compartments at 0 mV.22-24

Substantial evidence supports the idea that S2-E1 acts as an electrostatic partner of S4 arginine residues during activation. In Shaker, functional and biochemical data indicate that S2-E1 interacts with S4-R3 in an intermediate closed state and with S4-R4 in the activated state (Fig. 2B).34-56 This conclusion is supported by the X-ray structure of the K_v1.2/K_v2.1 paddle chimera, in which the side chains of S2-E1 and R4 are oriented to maximize their electrostatic interaction, whereas R3 is oriented to maximize its interaction with S1-E (Fig. 6A; Tables S1 and S2).33

In the KvAP structure, S2-E1 interacts with S4-R6.33 This charge pair has been seen in multiple structures and is stable in MD simulations, suggesting that it is not an artifact of crystallization (Figs. 2B and 6A; Tables S1 and S3).12,22,33,53 Consistent with this conclusion, other tertiary contacts of the KvAP S4 segment are distinct from those found in Shaker (Fig. 6A).33

The charge pairs between S2-E1 and S4 arginines contribute to networks of electrostatic interactions that stabilize the KvAP and Shaker voltage sensor domains (Fig. 6A).11,33-36 Because S2-E1 interacts with distinct S4 arginine residues in KvAP and the K_v1.2/K_v2.1 chimera, we compared in detail the charged interactions in their voltage sensor domains.11,33-36 Although the electrostatic networks in KvAP and the chimera are similar in
with evidence that S4 moves further in response to changes in voltage in KVAP than in Shaker.14-21

Upon hyperpolarization, inward movement of S4 is thought to exert force on the S4-S5 linker, which in turn pushes the C-terminal ends of S6 together, closing the pore.10 Compared to Shaker, topological and structural data suggest that the KVAP S4 must travel further to exert equivalent force on the S4-S5 linker.

Materials and Methods

KVAP mutagenesis and expression. A pQE-60 subclone of KVAP containing a hexahistidine tag was kindly provided by Dr. Rod MacKinnon (Rockefeller University).37 Endogenous cysteine, C247, was mutated to serine prior to generating single cysteine mutations using QuikChange® (Stratagene). Constructs were expressed in E. coli XL10-Gold® (Stratagene).
Figure 6. Structural differences in the KvAP and Kv1.2/Kv2.1 chimera voltage sensor domains. (A) Circled residues are involved in short range electrostatic interactions in the voltage sensor domains of the chimera (left) and KvAP (right) (accession number 2R9R) (Tables S2 and S3). Labeling of residues follows the generic nomenclature of Figure 2B and Table S1. Side chain atoms of charged residues as well as S2-BS and S3b-BS are shown. Color code: S2-R and S4 positively charged residues, gray; negatively charged residues in S1, S2 and S3, yellow; S2-BS and S3b-BS, green. S2-BS and S3b-BS are connected by dashed lines. In the chimera, R1 is a glutamine, which has been mutated to arginine in silico. (B) A different view of the voltage sensor domains of the chimera (left) and KvAP (right) is shown to illustrate the outer and inner charge networks relative to the barrier that separates the external and internal gating crevices. The images are vertically aligned at the position of S2-E1. The positions of V367 in Shaker and L122 in KvAP are indicated (blue side chains marked by *). Side chain atoms of charged and hydrophobic barrier residues are shown. Color code: S4 positively charged residues, gray; negatively charged residues in S1, S2 and S3, yellow; hydrophobic barrier residues, green. In the chimera, R1 is a glutamine and ‘L122/V367’ is a methionine (Fig. 2A). These positions have been mutated in silico to correspond to the Shaker sequence.
KvAP expression was induced for 2 h with 1 mM IPTG in the presence of 10 mM BaCl2.33,37

Preparation of right-side out vesicles. Right-side out membrane vesicles were prepared from 1 L cultures. E. coli were pelleted, incubated for 45 min at room temperature with 10 mM EDTA and 50 μg/ml lysozyme, and lysed by osmotic shock.26,37 These standard methods produce a uniform population of vesicles, approximately 95% of which have a right-side-out orientation.27,38 Vesicles were resuspended in 10 mM MgSO4, 100 mM potassium phosphate, pH 7.2, at a protein concentration of 10 mg/ml, frozen in liquid nitrogen, and stored at -80°C until use.

Topology assay. Right-side out vesicles (50 μl) were incubated for 5 min at room temperature with or without 200 μM [2-(trimethylammonium) ethyl]methane thiosulfonate bromide (MTSET) (Toronto Research Chemicals).31,39,40 N-[ethyl-14C]maleimide ([14C]-NEM, 40 μCi/ml) (Perkin Elmer) was then added to a final concentration of 0.4 mM and the incubation was continued for 8 min. The reaction was quenched with 25 mM dithiothreitol. Topology assays were conducted in parallel on identical samples in the presence and absence of a hyperpolarized membrane potential. Membrane potentials were measured in parallel experiments.

Generation and measurement of hyperpolarized membrane potential in right side out vesicles. Right-side out vesicles form from the resealed plasma membranes of lysed cells and therefore lack a membrane potential (0 mV).27,31,38 To generate an inside-negative membrane potential, vesicles were incubated under oxygen with an artificial electron donor system consisting of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS) for 10 min before and throughout the topology assay.24,30

Membrane potentials were measured using tetra-[3H]-phenylphosphonium bromide ([3H]-TPP+) (Amersham Biosciences).30,59 Voltage-dependent partitioning of [3H]-TPP+ into the vesicles was determined by flow dialysis in an apparatus consisting of two stirred chambers separated by dialysis tubing.60 Vesicles were added to the upper chamber and aerated by constant oxygen flow (~200 ml/min). After 2 min, [3H]-TPP+ was added to the upper chamber. Samples were collected at regular intervals from the lower chamber and [3H]-TPP+ was quantified using a liquid scintillation counter. A stable baseline was established for 15 min and then 20 mM ascorbate and 0.2 mM PMS were added to the upper chamber. Sampling from the lower chamber was continued during and after the development of the membrane potential. Generation of an inwardly directed, hyperpolarized membrane potential was indicated by a decrease in the amount of [3H]-TPP+ in samples taken from the lower chamber due to partitioning of [3H]-TPP+ into the vesicles in the upper chamber. Membrane potentials were calculated from the distribution of [3H]-TPP+ assuming an intravesicular volume of 2.2 μl/mg protein.61

Membrane potentials developed rapidly and were stable throughout the topology assay (see Fig. 3A). The value of the membrane potential obtained in the presence of MTSET and NEM, averaged over the time course of the topology assay, was -75 mV ± 0.8 mV (mean ± SEM, n = 24). The value of the membrane potential obtained in the absence of MTSET and NEM did not differ significantly, averaging -76 mV ± 0.9 mV (mean ± SEM, n = 24).

Cysteine reactivity was reduced by ~30% in the presence of PMS and ascorbate, but this effect was canceled out by expressing the data as the ratio of [14C]-NEM labeling with and without MTSET pretreatment.

Purification, immunoblotting and phosphorimaging of KvAP protein. KvAP was solubilized in 1% decyl-β-D-maltopyranoside (Anatrace) in the presence of protease inhibitors (0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 1 μg/ml aprotinin and 1 mM APMSF). KvAP was purified on Ni2+-Sepharose resin (Amersham Biosciences) and eluted with 200 mM imidazole, 50 mM Tris-Cl, pH 8.0.33,37 Duplicate samples of purified protein were subjected to SDS-polyacrylamide gel electrophoresis. Gels were divided into identical halves. One half was used to determine [14C]-NEM incorporation with a Storm Phosphorimager (Amersham Biosciences). The other half of the gel was immunoblotted to provide a loading control for the total amount of KvAP protein in the sample. Immunoblots were probed with an antibody directed against the hexahistidine tag (Bethyl Laboratories) and developed using enhanced chemiluminescence (Amersham Biosciences).

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/KoagCHAN3-5-Sup. pdf

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