

Stabilization of SecA ATPase by the primary cytoplasmic salt of *Escherichia coli*

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Abstract: Much is known about the structure, function, and stability of the SecA motor ATPase that powers the secretion of periplasmic proteins across the inner membrane of *Escherichia coli*. Most studies of SecA are carried out in buffered sodium or potassium chloride salt solutions. However, the principal intracellular salt of *E. coli* is potassium glutamate (KGlu), which is known to stabilize folded proteins and protein-nucleic acid complexes. Here we report that KGlu stabilizes SecA, including its dimeric state, and increases its ATPase activity, suggesting that SecA is likely fully folded, stable, and active *in vivo* at 37°C. Furthermore, KGlu also stabilizes a precursor form of the secreted maltose-binding protein.

Keywords: protein secretion; protein thermal stability; ATPase activity; SecYEG

SecA ATPase is an essential 102 kDa motor protein required for the secretion of periplasmic proteins across the plasma membrane of *Escherichia coli* via the SecYEG translocon complex. Proteins synthesized in the cytoplasm that are destined for export carry a cleavable N-terminal signal sequence that is recognized by SecA but subsequently cleaved by membrane-embedded signal peptidase.¹ Since its discovery^{2,3} in 1982, SecA has been intensively studied in many laboratories. Exhaustive biochemical and structural studies from numerous laboratories have revealed the essential features of SecA structure and function at the molecular level (see review by Crane and Randall⁴), although the structural details of its interactions with the SecYEG translocase are just now beginning to emerge.⁵ Since the first structure of

SecA from *Bacillus subtilis*,⁶ structures of SecA from several species have been determined (see reviews^{7,8}) and suggest, along with biochemical studies,^{9,10} that SecA is dimeric in the cytoplasm. However, little is known about SecA conformational states in the cytoplasm of *E. coli*, which are important for understanding *in vivo* conformational transitions that occur during secretion of preproteins across the inner membrane.¹¹

As far as we can establish, most physicochemical studies of SecA are carried out in buffered KCl or NaCl solutions. Analytical ultracentrifuge studies of SecA show that dimerization depends strongly on ionic strength.¹⁰ For example, in 100 mM KCl about 90% of SecA is dimeric while in 500 mM KCl, about 95% is monomeric.¹² Although there are wide variations in buffer compositions reported in the literature, SecA is typically studied as a dimer in buffered chloride solutions. Using 50 mM KCl and SecA extracted from *E. coli*, Song and Kim¹³ observed through tryptophan fluorescence measurements that SecA tertiary structure was 50% destabilized at 37°C while CD spectroscopy revealed a complete unfolding transition with a midpoint of about 45°C. Similar unfolding temperatures had been

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observed earlier by Ulbrandt et al.¹⁴ Den Blaauwen et al.¹⁵ found similar transition temperatures by calorimetry for *B. subtilis* SecA.

We found it interesting that SecA is apparently destabilized at the typical *E. coli* growth temperature of 37°C. This suggested either that destabilization might be important for function or that 50 mM KCl was a poor representative of the *E. coli* intracellular environment. Looking more closely at the latter possibility, we learned that when *E. coli* are grown in glucose media, their intracellular metabolome is dominated, on a molar basis, by amino acids (49%) and that glutamate is the most abundant with concentrations ranging from ≈ 100 mM under normal conditions¹⁶ and up to 300 mM in response to osmotic stress.^{17,18} Together, these data indicate that potassium glutamate (KGlu) is the primary cytoplasmic salt in *E. coli*. Furthermore, we learned that KGlu can stabilize soluble proteins, because Glu⁻ interacts unfavorably with apolar side-chains and amide groups.^{19–21} Given these facts, we examined the stability of SecA in KGlu solutions. We report here that KGlu significantly stabilizes SecA, including its dimeric state, and increases its ATPase activity.

We first confirmed the findings of Song and Kim,¹³ as shown in Figure 1 and Table S1. Panels a and c show thermal denaturation curves determined using Trp fluorescence and CD spectroscopy, respectively. In buffered 50 mM KCl or KF (black curves), the denaturation

midpoints are entirely consistent with Song and Kim's observations ($37.7 \pm 0.4^\circ\text{C}$ by Trp fluorescence and $43.6 \pm 0.5^\circ\text{C}$ by CD spectroscopy). In both cases, unfolding was irreversible if the denaturation midpoints were exceeded. We interpret these results to mean that as temperature is raised, tertiary structure is perturbed causing increased aqueous exposure of Trp residues followed by loss of secondary structure. We found negligible changes in the thermal transition midpoints for KCl concentrations as high as 750 mM, indicating that ionic strength had little effect on SecA stability (Fig. S1). In contrast, replacement of Cl⁻ by Glu⁻ caused dramatic increases in stability as judged by both Trp fluorescence and CD spectroscopy [Fig. 1(a, c), Table S1]. Stability progressively increased with increasing Glu⁻ concentration up to about 400 mM and then began to level off at about 42°C by Trp fluorescence and 48°C by CD spectroscopy [Fig. 1(b, d)]. These results indicate that SecA is likely to be stable at *E. coli* growth temperatures well beyond 37°C.

As noted earlier, high KCl concentrations are known to destabilize SecA dimers. We verified this well-established finding using size-exclusion chromatography for 5 μM SecA at 22 and 37°C (Fig. 2, black curves). In 500 mM KCl at 22°C, SecA exists mostly (90%) as monomers, as expected from the analytical ultracentrifuge measurements of Wowor et al.¹² But at 37°, the equilibrium shifts toward dimers, suggesting that the

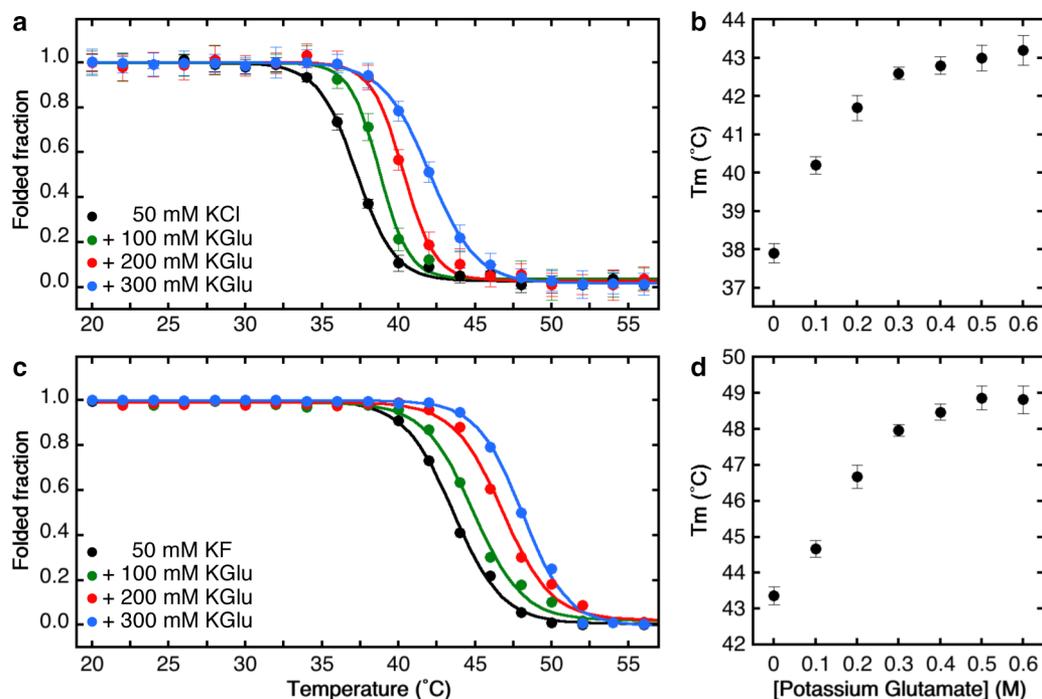


Figure 1. Cytoplasmic osmolytes stabilize SecA. (a) Changes in tertiary structure monitored by tryptophan fluorescence as a function of the temperature. The folded fraction was determined from the Trp fluorescence at 340 nm, normalized by setting the intensities of the native (20°C) and fully unfolded SecA (60°C) to be 1 and 0, respectively, after proper correction for the temperature dependence of the fluorescence intensity. (b) Effect of cytoplasmic osmolytes on the melting temperature of SecA determined by Trp fluorescence. (c) Changes in secondary structure monitored by circular dichroism as a function of the temperature and normalized using the molar ellipticity of native (20°C) and unfolded (60°C) SecA to be 1 and 0, respectively. (d) Effect of potassium glutamate on the stability of the secondary structure.

dimerization interface involves hydrophobic as well as electrostatic interactions. Results from measurements in KGlu (Fig. 2, red curves) were quite similar to the KCl results up to 300 mM. In 500 mM KGlu, however, dimers started to appear at 22°, becoming highly favored at 37°C. We conclude that the salt dependence of SecA dimerization is generally similar for both KCl and KGlu up to 300 mM, although close examination of the curves reveals a slight preference for dimer in KGlu compared to KCl. At 500 mM salt concentrations, the dimeric form of SecA is strongly favored in KGlu. Broadly speaking, high KGlu concentrations stabilize SecA dimers.

Given that Glu⁻ stabilizes SecA, what are the consequences for its ATPase activity? To answer this question, we measured the dependence of endogenous ATP hydrolysis on temperature using Glu⁻ concentrations ranging from 100 to 300 mM (Fig. 3). At each concentration, we measured ATPase activities (nmol P_i/μg SecA/min) at 17 temperatures between 22°C and 60°C (i.e., 2.5° steps). As expected, rates increased with temperature until the beginning of the thermal transition and then declined as SecA unfolded. The rate observed for 50 mM KCl at 22°C (0.038 ± 0.007 nmol P_i/μg SecA/min) is similar to values obtained earlier by other

investigators,²²⁻²⁴ as is the increase in rate with temperature. For 50 mM KCl, the rate was maximum at 37°C, declining to background values at about 50°C (panel a). Panels b, c, and d of Figure 3 reveal modest activity increases at 22°C as Glu⁻ was increased from 100 to 300 mM. For all concentrations, activities increased with temperature reaching a maximum of about 0.11 nmol P_i/μg SecA/min at 42°C in 300 mM KGlu⁻. These data are consistent with the thermal denaturation data (Fig. 1) and show that stabilization of SecA by Glu⁻ improves ATPase activity. KGlu therefore both stabilizes SecA tertiary structure and enhances its activity in solution.

Other investigators have shown that the conformation of SecA changes when it binds to the membrane²⁵ or the translocon SecYEG,^{26,27} resulting in a more open conformation. Solution studies involving low concentration of denaturant appear to mimic this “translocation-active SecA” and have shown that the destabilization of the tertiary structure by urea stimulates the ATPase activity of SecA.^{11,13} How the stabilization of SecA by KGlu in the cytoplasm will affect its binding and conformation when bound to the membrane or translocon remains to be determined.

Escherichia coli contains a complex mixture of osmolytes in its cytoplasm. Although glutamate is the

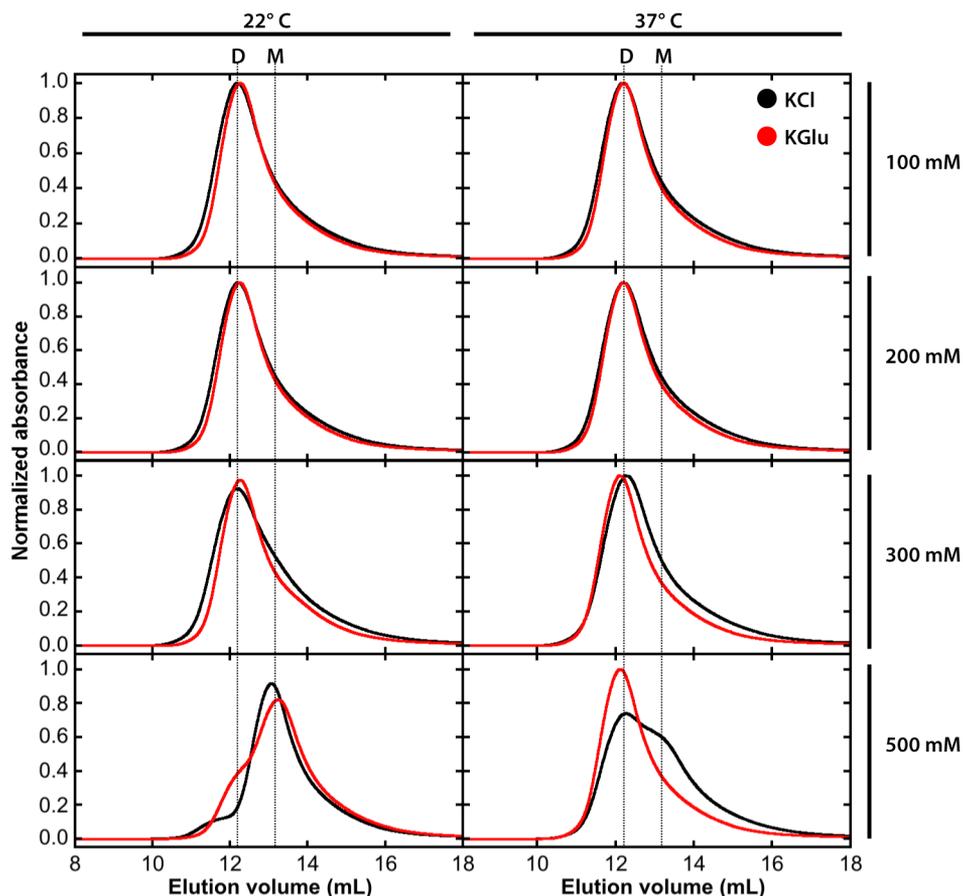


Figure 2. SecA is stabilized as a dimer at 37°C in the presence of potassium glutamate. The oligomeric state of 5 μM SecA protein was determined by size exclusion chromatography at 22°C (left-hand panels) or 37°C (right-hand panels) in KCl and KGlu concentrations ranging from 100 mM to 500 mM. KCl results are indicated by black curves and KGlu results by red curves.

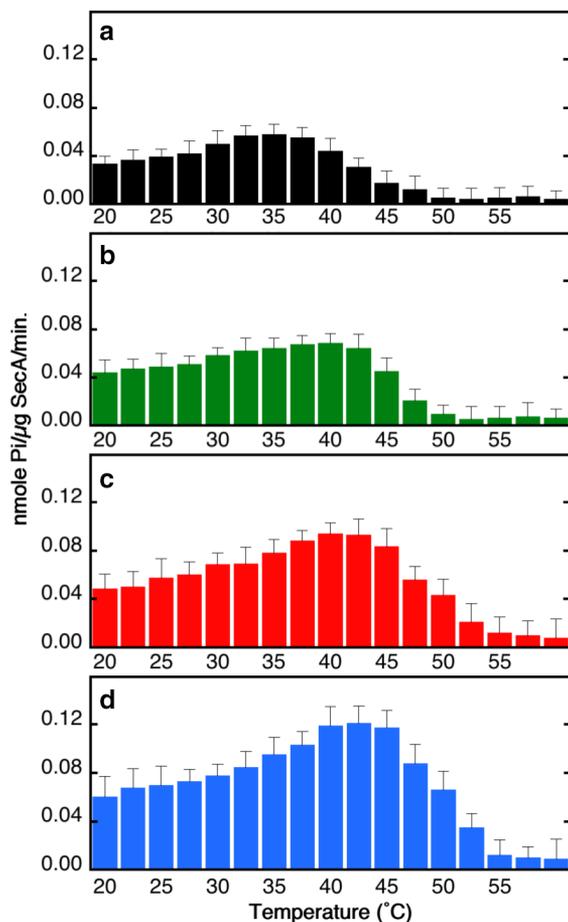


Figure 3. Potassium glutamate enhances the endogenous ATPase activity of SecA. The ATPase activities of SecA were determined in (a) 20 mM phosphate buffer pH 7.4, 50 mM KCl, 1 mM DTT, 5 mM MgCl₂, 2 mM ATP, and (b) 100, (c) 200, or (d) 300 mM potassium glutamate pH 7.4.

major component, several other osmolytes can also be present that also affect SecA stability and activity. For example, under anaerobic glucose-limiting conditions, as found in the digestive track when bacteria are passing through the colon, acetate can replace glutamate and accumulate in the cytoplasm at concentration up to 500 mM.^{28,29} Woodbury et al.³⁰ have shown that potassium acetate can shift the monomer–dimer equilibria of SecA toward the dimer. We found that potassium acetate also stabilizes SecA (Fig. S2, red line), suggesting that the protein is likely folded at physiologically relevant temperatures even when acetate replaces glutamate.

The above results suggest that other proteins within the cytoplasm might also be stabilized by primary cytoplasmic salts, as suggested by studies of the NTL9 ribosomal protein domain.¹⁹ A question of particular interest, of course, is how Glu⁻ might affect the cytoplasmic stability of proteins destined for secretion. The stability and folding of the *E. coli* periplasmic maltose-binding protein (MalE) and its cytoplasmic precursor (preMalE) have been studied extensively in the laboratories of Linda Randall^{31–33} and Raghavan Varadarajan.^{34,35} PreMalE is

a 396-residue protein carrying a 26-residue presequence (MKIKTGARILALSA₁₄LTTMMFSASALA⁻). Park et al.³¹ showed via guanidine hydrochloride denaturation and renaturation that the A14E signal sequence had no effect on the unfolding rate of the protein but dramatically slowed the refolding rate. This suggested that a key role for the signal sequence is to slow down intracellular folding as means of keeping preMalE competent for export and for the binding of the SecB chaperone that generally works in concert with SecA to facilitate export of preMalE.³⁶

Liu et al.³³ discovered a non-exportable mutant version (preMalE-A14E) with an Ala-to-Glu mutation at position 14. Because preMalE-A14E is not exported, it is readily expressed and purified for study as a stand-in for cytoplasmic preMalE. Beena et al.³⁴ characterized extensively the stability and folding kinetics of MalE and preMalE-A14E. Equilibrium thermal denaturation studies of MalE and preMalE-A14E using CD spectroscopy revealed that the mature form of MalE is more stable (denaturation temperature midpoint $\approx 63^\circ\text{C}$) than the immature form (midpoint $\approx 55^\circ\text{C}$). Beena et al. performed their experiments in buffered 150 mM sodium chloride. We carried out similar measurements using 50 mM KF and 200 mM KGlu (Fig. 4). We found the precursor to be less stable than the mature form in both KF (57.3 vs. 62.3 $^\circ\text{C}$) and KGlu (61.3 vs. 63.0 $^\circ\text{C}$). However, while KGlu had only a minor effect on the stability of the mature form, it stabilized the precursor by about 4 $^\circ\text{C}$. Thus, KGlu stabilizes at least one preprotein, whether it stabilizes others as well remains to be determined. An important question

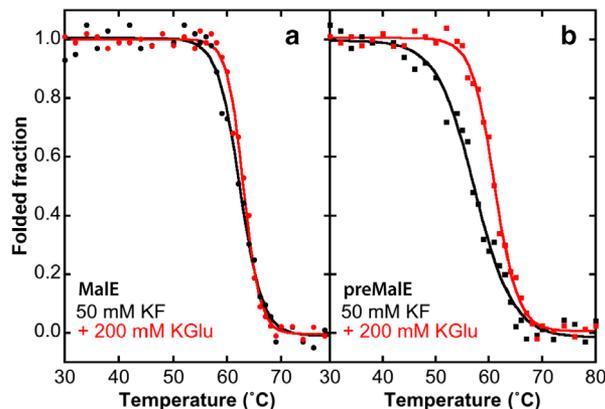


Figure 4. KGlu stabilizes a precursor form of MalE maltose-binding protein. Stability was measured in KF by CD spectroscopy at a wavelength of 222 nm. KF was used to reduce spectral absorption in the near UV.³⁷ (a) Stability of MalE. In potassium fluoride, the transition midpoint temperature is 62.3 \pm 0.2 $^\circ\text{C}$ while in KGlu it is 63.0 \pm 0.1 $^\circ\text{C}$. (b) Stability of preMalE-A14E. In potassium fluoride, the transition midpoint temperature is 57.3 \pm 0.1 $^\circ\text{C}$ while in KGlu it is 61.3 \pm 0.1 $^\circ\text{C}$. The precursor is thus less stable than the mature form in both KF and KGlu. However, while KGlu has only a minor effect on the stability of the mature form, it stabilizes the precursor by 4 $^\circ\text{C}$.

to be answered is how glutamate will affect the kinetics of folding of MalE and its interaction with SecB, which are believed to be of critical importance for successful secretion of preproteins.^{31–36}

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Conflict of Interest

The authors declare that they have no conflict of interest.

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