Table 1: ATPase activity of labeled and unlabeled monocysteine SecA mutants			
SecA protein	Endogenous [Pi] (nmole min^{-1} µg $SecA^{-1}$)[1]	Membrane [Pi] (nmole min^{-1} µg $SecA^{-1}$)	Translocation [Pi] (nmole min^{-1} µg $SecA^{-1}$)
WT	0.23	0.37	1.06
59C	0.20	0.06	0.45
59C-IAE	0.63	0.22	0.34
59C-IAN	0.37	0.06	0.60
59C-AF568	0.12	0.08	0.20
59C-AF647	0.24	0.07	0.14
340C	0.15	0.09	0.36
340C-IAE	0.22	0.08	0.27
340C-IAN	0.41	0.23	0.45
340C-AF488	0.65	0.16	0.75
340C-AF568	0.35	0.09	0.56
402C	0.05	0.12	0.61
402C-IAE	0.15	0.15	0.46
402C-IAN	0.16	0.19	0.45
402C-AF488	0.15	0.09	0.38
402C-AF568	0.16	0.01	0.36
427C	0.01	0.06	0.33
427C-IAE	0.23	0.08	0.35
427C-IAN	0.20	0.15	0.15
427C-AF488	0.98	-0.08	0.09
427C-AF568	0.71	0.04	-0.01
427C-AF647	0.43	0.10	-0.02
458C	0.19	0.09	0.64
458C-AF488	0.28	0.00	0.46
458C-AF568	0.31	0.01	0.10
470C	0.07	0.44	1.30
470C-IAE	1.08	0.08	0.40
470C-IAN	1.26	-0.12	0.14
470C-AF488	0.41	0.72	0.31
470C-AF568	0.73	0.76	1.47
470C-AF647	0.40	0.02	0.38
506C	0.16	0.22	0.77
506C-AF488	1.51	0.11	0.76
506C-AF568	0.88	0.55	0.59
506C-AF647	1.32	0.14	0.74
696C	0.13	0.15	0.30
696C-IAE	0.15	0.10	0.50
696C-IAN	0.11	0.15	0.40
696C-AF488	0.23	0.12	0.52
696C-AF568	0.18	0.06	0.36
696C-AF647	0.34	0.09	0.49
734C	0.20	0.29	1.06
734C-IAE	1.07	0.11	0.69
734C-IAN	0.53	0.35	0.52
734C-AF488	0.34	0.04	0.06
734C-AF568	0.14	0.01	0.06
734C-AF647	0.31	0.08	0.20

[1]Errors are not reported as they are 10% or less of the measured value.

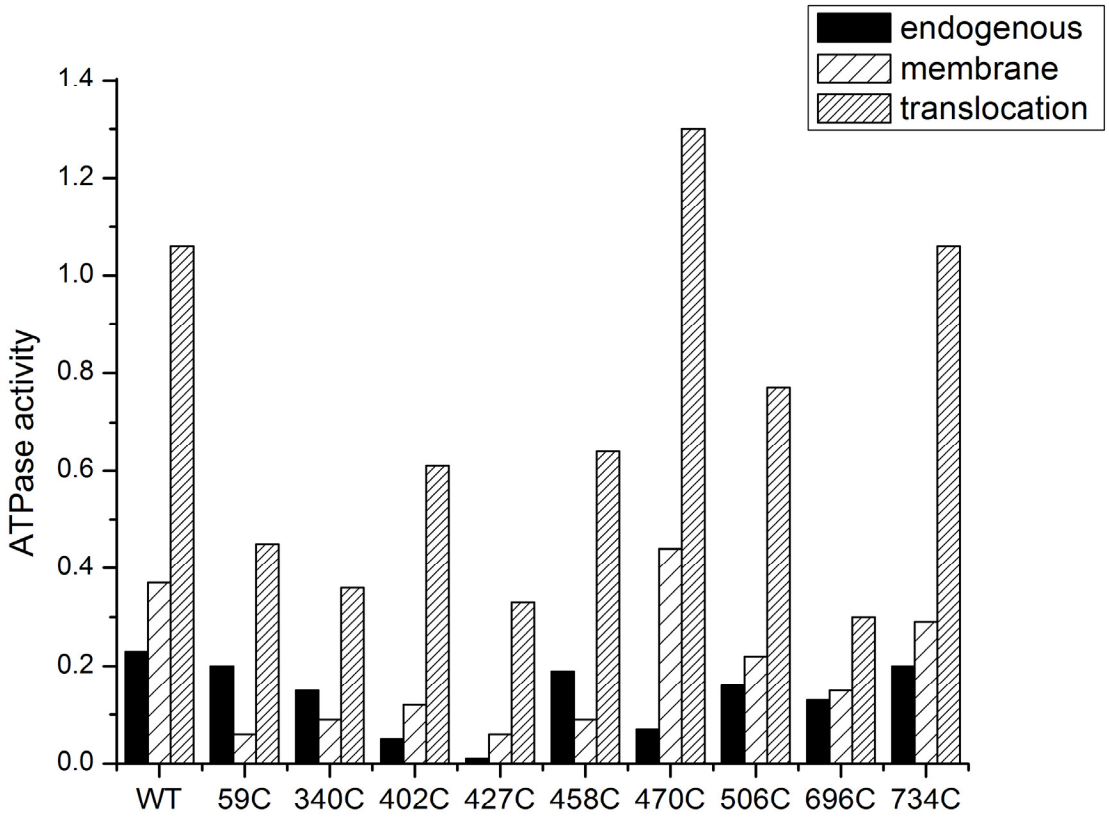

Figure 1: ATPase activity of monocysteine SecA mutants. ATPase activity was calculated using the following formulas: endogenous ATPase activity is the ATPase activity in the presence of SecA after subtracting ATPase activity in the absence of SecA; membrane ATPase activity is the ATPase activity in the presence of SecA and IMV after subtracting the endogenous ATPase activity; translocation ATPase activity is the ATPase activity in the presence of SecA, IMV, and preprotein after subtracting membrane ATPase activity.

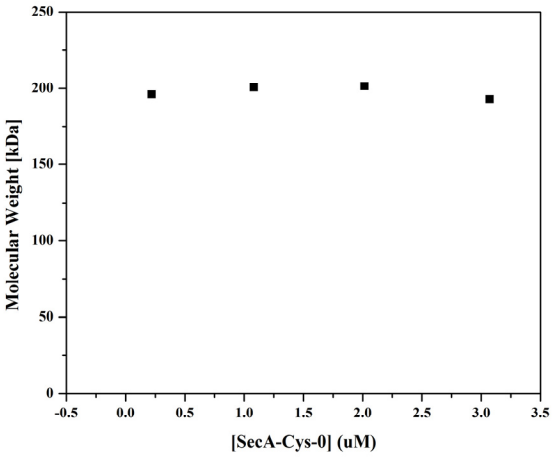

Figure 2. Assessment of SecA oligomeric state. Measurement of the weight average molecular mass (Molecular Weight) of SecA-0C at the indicated protein concentration in TKE buffer was performed by SEC and static light scattering as described in “Experimental Procedures”.

Table 2: Donor Quantum Yields (Φ_D)[1] and Associated R_0 values[2]		
FRET Pair	Φ_D	R_0 (Å)
59IAE-59IAN	0.05	31
59AF568-59AF657	0.76	84
402IAE-402IAN	0.10	34
402AF488-402AF568	0.16	48
59IAE-402IAN	0.05	31
340IAE-340IAN	0.01	22
340AF488-340AF568	0.51	56
427IAE-427IAN	0.08	33
427AF488-427AF568	0.21	51
427AF568-427AF647	0.09	57
458AF488-458AF568	0.63	59
470IAE-470IAN	0.08	33
470AF488-470AF568	0.58	59
470AF568-470AF647	0.58	79
506AF488-506AF568	0.67	61
506AF568-506AF647	0.52	78
696IAE-696IAN	0.15	37
696AF488-696AF548	0.69	62
696AF568-696AF647	0.52	78
734AF568-743AF647	0.68	81

[1] The quantum yield of IAE-labeled SecA was measured relative to quinine sulfate (Φ = 0.56), the quantum yield of AF488-labeled SecA was measured relative to fluorescein (Φ = 0.925) (Magde *et al.* 2002), and the quantum yield of AF568-labeled SecA was measured relative to cresyl violet (Φ = 0.54) (Magde *et al.* 1979). [2] R_0 values were calculated as previously described (Auclair *et al.* 2010)

Table 3: Evaluation of κ^2 Distributions and Effect on Distances using Steady State Anisotropy Values					
FRET pair	r_d [1]	r_a [1]	κ^2_{min}	κ^2_{max}	ΔR_{DA} [2]
59IAE-59IAN	0.09	0.15	0.31	1.95	-12.1…+19.6 %
59AF568-AF647	0.19	0.20	0.20	2.57	-18.1…+25.2%
402IAE-402IAN	0.15	0.15	0.26	2.23	-14.6…+22.3%
402AF488-402AF568	0.16	0.18	0.23	2.38	-16.1…+23.6%
59IAE-402IAN	0.09	0.15	0.31	1.95	-12.1…+19.6%
340IAE-340IAN	0.15	0.20	0.23	2.38	-16.1…+23.6%
340AF488-340AF568	0.15	0.20	0.22	2.42	-16.5…+23.9%
427AF488-427AF568	0.18	0.16	0.23	2.42	-16.5…+24.0%
427AF568-427AF647	0.16	0.19	0.22	2.46	-16.9…+24.3%
458AF488-458AF568	0.18	0.16	0.26	2.19	-14.2…+22.0%
470AF488-470AF568	0.18	0.17	0.25	2.26	-14.9…+22.6%
470AF568-470AF647	0.17	0.22	0.20	2.54	-17.8…+25.0%
506AF488-506AF568	0.13	0.18	0.25	2.25	-14.9…+22.5%
506AF568-506AF647	0.18	0.22	0.19	2.61	-18.5…+25.5%
696AF488-696AF568	0.15	0.18	0.24	2.34	-15.7…+23.3%
696AF568-696AF647	0.18	0.19	0.21	2.51	-17.4…+24.7%
734AF568-734AF647	0.18	0.19	0.21	2.51	-17.4…+24.7%
[1]Anisotropy values are reported for donor only or acceptor only solutions. [2]Percent error of distances calculated with κ^2 range relative to those calculated with κ^2=2/3.					

The κ^2_{min} and κ^2_{max} values were calculated using the following equations (Ivanov *et al.* 2009):

$$\kappa^2_{min} = \frac{2}{3}\left(1 - \frac{\left(\sqrt{\frac{5}{2}r_d} + \sqrt{\frac{5}{2}r_a}\right)}{2}\right)$$

$$\kappa^2_{max} = \frac{2}{3}\left(1 + \sqrt{\frac{5}{2}r_d} + \sqrt{\frac{5}{2}r_a} + 3\sqrt{\frac{5}{2}r_d}\sqrt{\frac{5}{2}r_a}\right)$$

where r_d and r_a are the steady state anisotropy values determined for donor only and acceptor only species.

The κ^2_{min} and κ^2_{max} values were used to estimate the error in the distance resulting from assuming an orientation factor of 2/3 using the following equation:

$$\Delta = \sqrt[6]{\frac{\kappa^2}{2/3}}$$

where $\Delta = \frac{R}{R_{app}}$ and R_{app} is the apparent distance calculated for the case when κ^2=2/3. These orientation factor errors were considered with measurement errors when reporting the uncertainties in FRET distance measurements in Table 1.

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Ivanov, V., Li, M. and Mizuuchi, K. (2009). "Impact of Emission Anisotropy on Fluorescence Spectroscopy and Fret Distance Measurements." *Biophys J* **97**(3): 922-929.

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