Specific Modulation of Kex2/Furin Family Proteases by Potassium*

Nathan C. Rockwell‡ and Robert S. Fuller¶

From the Department of Biological Chemistry, University of Michigan Medical Center, Rm. 5413 Med. Sci. I, 1301 E. Catherine, Ann Arbor, MI 48109.

‡ and Department of Biochemistry, B400 Beckman Center, Stanford University, Stanford, CA 94305. Present address: c/o Thorner lab, 401 Barker Hall, MCB Department, University of California, Berkeley, CA 94720.

¶ To whom correspondence should be addressed: Department of Biological Chemistry, University of Michigan Medical Center, Rm. 5413 Med. Sci. I, 1301 E. Catherine, Ann Arbor, MI 48109. Tel: (734) 936-9764; Fax: (734) 763-7799; E-mail: bfuller@umich.edu

running title: Potassium Modulates Prohormone Convertases
Summary: Kex2 protease is the prototype for a family of proteases responsible for endoproteolytic cleavage at multi-basic motifs in the eukaryotic secretory pathway. Here we demonstrate that potassium ion can act as a modulator of Kex2 activity with an apparent affinity of approximately 20 mM. Other monovalent cations (Li+, Na+, etc.) display similar effects, but affinities are all over 20-fold lower. Potassium ion binding stimulates turnover at physiologically relevant Lys-Arg cleavage sites but reduces turnover with at least one incorrect sequence. Furthermore, the mammalian Kex2 homolog furin displays similar effects. In contrast, the neuroendocrine homolog PC2 is inhibited by potassium ion with all substrates examined. The pre-steady-state behavior of Kex2 is also altered upon binding of potassium ion, with opposite effects on acylation and deacylation rates. These biochemical data indicate that potassium ion concentration may function as a regulator of processing protease specificity and activity in the eukaryotic secretory pathway, with such enzymes potentially encountering compartments high in potassium ion due to the action of antiporters such as yeast NHX1 (VPS44) or the mammalian NHE7.
In eukaryotes, many proteins and polypeptides in the secretory pathway are first synthesized as precursors which are then activated by specific endoproteolytic cleavage in the late secretory pathway. The majority of such processing reactions are catalyzed by members of the Kex2/furin family of processing proteases, which belong to the subtilisin superfamily of serine proteases (1-5). Metazoan members of the Kex2/furin family are found in both the constitutive secretory pathway (e.g., furin and PC7: 6, 7) and the regulated secretory pathway (e.g., PC1/3 and PC2: 8, 9).

Like all members of the subtilisin superfamily examined to date, processing enzymes of the Kex2/furin family require calcium ions for maximal activity (10, 11). Calcium is required for stable folding of mature subtilisin, and it is possible to generate thermostable forms of subtilisin by engineering additional calcium binding sites (12-14). Additionally, enzymes of the Kex2/furin family are sensitive to inhibition by heavy metals including Zn$^{2+}$ (15-17). However, allosteric interactions between these proteases and cations have not been reported, and potential interactions between monovalent cations and the enzymes of the Kex2/furin family have not been studied to date.

The sole member of this family in *S. cerevisiae* is Kex2 protease (kexin, EC 3.4.21.61), which has become the prototype for understanding the basis of substrate specificity in this family of enzymes (18-24). Kex2 cycles between late Golgi compartments and the pre-vacuolar compartment (yeast late endosome, 25) and exhibits stringent specificity for Arg at P$_1$ with less selective, but energetically important, accessory contacts at P$_2$ and P$_4$ (20, 21). The pre-steady-state behavior of Kex2 and related enzymes has recently been the focus of additional work (4, 23, 24, 26, 27). The processing proteases of the constitutive secretory pathway (Kex2 and furin) are thought to exhibit rate-limiting deacylation in cleavage of correct sequences (P$_1$ Arg), while incorrect sequences (P$_1$ Lys) result in a substantial defect in acylation such that it becomes rate-limiting (4, 24, 27). These enzymes are thus thought to accumulate the acylenzyme intermediate at the steady state *in vivo*. This behavior is in contrast to the related enzyme PC2, which is
localized to the regulated secretory pathway of neuroendocrine cells and which exhibits rate-limiting acylation (8, 27). The rate-limiting deacylation seen with Kex2 and furin is also in contrast to the more distantly related proteases of the subtilisin family, which display rate-limiting acylation and less stringent substrate specificity (28, 29).

The advantage conferred in vivo by the rate-limiting deacylation seen with Kex2 is not yet apparent. Such behavior reduces the concentration of free enzyme, thereby lowering the rate of cleavage of incorrect substrates, but it must also reduce the cleavage of correct substrates as well (23). It is possible that this behavior maintains the rate of incorrect cleavage below some toxic threshold, or it may serve as a means of maintaining a reserve pool of protease as a sequestered intermediate, as it is known that Kex2 is in excess in vivo (23). However, such a reserve population would only be advantageous if there were some means of mobilizing it, for example by a pH change in the processing compartment or by the binding of an allosteric effector. It is known that slightly acidic pH does not affect the pre-steady-state behavior of Kex2 (24), whereas pH>8 results in rapid inactivation of the enzyme (19). For there to be an effective reserve pool of Kex2 in vivo, it therefore seems plausible to propose that there should be some type of allosteric effector which is capable of modulating the pre-steady-state behavior of the enzyme.

Through an adventitious observation, we discovered that Kex2 exhibits substantial activation by monovalent cations. We demonstrate that potassium ion can stimulate $k_{\text{cat}}$ for Kex2 cleavage of multiple substrates with an apparent affinity of approximately 20 mM and maximal activity at 200-250 mM. Moreover, potassium ion is found to stimulate the homologous enzyme furin but inhibit the neuroendocrine homolog PC2. Cleavage of different substrates displays different responses for furin and Kex2, and potassium ion binding alters the pre-steady-state behavior of Kex2 by accelerating deacylation but slowing acylation. These results raise the possibility that processing proteases of the constitutive secretory pathway can be regulated by lumenal potassium ion concentrations in vivo.
MATERIALS AND METHODS

Substrates, Enzymes, and Reagents. Ac-Pro-Met-Tyr-Lys-Arg↓MCA was the generous gift of Jeremy Thorner. Boc-Arg-Val-Arg-Arg↓MCA and pyr-Arg-Thr-Lys-Arg↓MCA were purchased from commercial sources. Other AMC substrates have been described elsewhere (20, 21, 23, 26). Preparation of Z-Nle-Tyr-Lys-Lys↓MCE has been described elsewhere (27). Kex2 was prepared as described previously (20), and several preparations of enzyme were tested for potassium ion response without significant differences in steady-state reponse. Furin was prepared as described previously (11, 26), and was the gift of Luke Carter (Glaxo Wellcome). PC2 was prepared and pre-activated by incubation at low pH as described (30), and was the generous gift of Iris Lindberg (University of Louisiana Medical Center). Active-site titration of furin was carried out by initial burst titration using a Kintek RQF-3 rapid-quenched flow mixer and the substrate Ac-Arg-Tyr-Arg-Phe-Lys-Arg↓MCA (26), and enzyme concentration was found to be 1.9 µM. Active-site titration of PC2 was carried out by initial burst titration in the rapid-quenched-flow apparatus using Z-Nle-Tyr-Lys-Lys↓MCE as described (27), and enzyme concentration was found to be 2.7 µM. Reagents were from Sigma, Aldrich, or Fisher. All regression analyses were performed with Kaleidagograph (Synergy Software).

Steady-state kinetics. Assays were carried out at substrate concentrations well in excess of the known $K_M$ values for each substrate, so that the effect of monovalent ions on $k_{cat}^{obs}$ could be assessed independent of potential changes in substrate binding caused by the changing nature of the bulk solvent. We examined cleavage in the presence of potassium ion at multiple substrate concentrations in excess of $K_M$ and confirmed that the observed $V_{max}$ was independent of substrate concentration, indicating that the reaction was saturated.
For Kex2, assays were carried out in 0.2 M Bistris, pH 7.26, 1 mM CaCl$_2$, 0.1% Triton X-100 at 37°C as described previously (20, 21) except that different concentrations of monovalent cations were added (0-1.6 M). Since all monovalent cations tested displayed at least some effect, differential effects of different ions at equal concentrations were compared to assess the possible effects of changing total ionic strength. All assays were carried out for ≤10 minutes. Control reactions indicated that Kex2 was not as stable in the presence of potassium ion, possibly as a result of increased autoproteolysis (data not shown), but reactions were linear for >10 minutes, indicating that endpoint assays of ≤10 minutes would be unaffected by loss of activity or by substrate depletion.

Furin and PC2 assays were carried out as for Kex2 using previously described conditions (26, 30). Again, total ionic strength was not kept constant. Reaction times were ≤10 minutes, and control reactions indicated that both enzymes were stable in the presence of potassium on this timescale, as was the case with Kex2.

**Pre-steady-state kinetics.** For initial burst measurements with amide substrates, Kex2 protease was reacted with substrate at 21°C in a Kintek RQF-3 rapid-quenched-flow mixer as described previously (21), with 0.2 M KCl added to both enzyme and substrate syringes as appropriate. For single-turnover reactions, 1.5-7.5 µM Kex2 protease was reacted with 0.5-5.0 µM $d_3$-Ac-Nle-Tyr-Lys-Arg↓MCA in the rapid-quenched-flow mixer; again, potassium ion was added to both enzyme and substrate where it was present. Formation of free AMC followed a single exponential (Equation 1), where $S_0$ is the initial substrate concentration, $t$ is time, and $k_{app}$ is the observed first-order rate constant.

$$\text{Product} = S_0 (1 - e^{-k_{app}t})$$

(Equation 1)
Burst titration of Kex2 with the ester substrate Z-Nle-Tyr-Lys-Lys↓MCE was carried out exactly as described (27) except that enzyme was prepared in 200 mM sodium acetate, pH 6, 1 mM CaCl$_2$, 0.1% Triton X-100, 0.5 M KCl. Substrate was prepared in aqueous 0.1% trifluoroacetic acid (v/v) at a concentration of 560 µM. Final potassium ion concentration was 0.25 M. Re-equilibration of potassium ion is predicted to be rapid on the millisecond timescale accessible by rapid-quench.

**Analysis of ion response.** For Kex2 and furin cleavage of good substrates, examining $k_{cat}^{obs}$ as a function of monovalent cation concentration showed stimulation at low concentrations and inhibition at higher concentrations. These data could not be fitted by a two-state binding model, nor were they well described by changes in dielectric screening, which is not a bimodal function. This suggested direct interaction with at least a three-state model, with stimulation caused by binding a high-affinity site and inhibition caused by binding at least one low-affinity site (Scheme 1, with $M^+$ as any monovalent cation).

$$E \overset{k_1}{\longrightarrow} E^{M^+} \overset{k_2}{\longrightarrow} E^{2M^+}$$  \hspace{1cm} \text{(Scheme 1)}

Here, $k_1 = ([E][M^+])/[E^{M^+}]$ and $k_2 = ([E^{M^+}][M^+])/[E^{2M^+}]$, and the total enzyme concentration $E_0$ must obey $[E_0] = [E] + [E^{M^+}] + [E^{2M^+}]$ due to conservation of enzyme. Under saturating substrate, $k_{cat}^{obs}$ should simply reflect the fraction of total enzyme in each of the three states, obeying equation 2:

$$k_{cat}^{obs} = k_{cat} ([E]/[E_0]) + k_{cat}^{M^+} ([E^{M^+}]/[E_0]) + k_{cat}^{2M^+} ([E^{2M^+}]/[E_0])$$  \hspace{1cm} \text{(Equation 2)}$$
To simplify this further, one can substitute the definitions of the binding constants $K_j$ and $K_2$ and factor out the common $E_0$ term to derive equation 2A:

$$
k_{cat}^{obs} = \frac{1}{[E_0]} \left( k_{cat}[E] + \frac{k_{cat}^{M^+}}{K_1} [E][M^+] + \frac{k_{cat}^{2M^+}}{K_2} [E^{M^+}][M^+] \right)
$$

(Equation 2A)

The process of substitution and factoring can be repeated to give the contributions from all three states in terms of the unbound enzyme population $[E]$ (equation 2B):

$$
k_{cat}^{obs} = \frac{[E]}{[E_0]} \left( k_{cat} + \frac{k_{cat}^{M^+}}{K_1} [M^+] + \frac{k_{cat}^{2M^+}}{K_1 K_2} [M^+]^2 \right)
$$

(Equation 2B)

Similarly, the definitions of $K_j$ and $K_2$ may be used with conservation of enzyme to define the ratio $[E]/[E_0]$ in terms of monovalent cation concentration and the affinity constants $K_j$ and $K_2$ (equation 2C):

$$
\frac{[E]}{[E_0]} = \frac{1}{1 + \frac{[M^+]}{K_1} + \frac{[M^+]^2}{K_1 K_2}}
$$

(Equation 2C)

Equation 2B may be combined with equation 2C to give equation 2D, which describes the behavior of $k_{cat}^{obs}$ in terms of the binding constants $K_j$ and $K_2$, the rate constants for each state, and the concentration of monovalent cation:
\[ k_{\text{cat}}^{\text{obs}} = \frac{k_{\text{cat}} + \frac{k_{\text{cat}}^{M^+}}{K_1} [M^+] + \frac{k_{\text{cat}}^{2M^+}}{K_1 K_2} [M^+]^2}{1 + \frac{[M^+]}{K_1} + \frac{[M^+]^2}{K_1 K_2}} \]  
(Equation 2D)

Equation 2D can be simplified by multiplying numerator and denominator by the quantity \( K_1 K_2 \) to yield equation 3:

\[ k_{\text{cat}}^{\text{obs}} = \frac{k_{\text{cat}} K_1 K_2 + k_{\text{cat}}^{M^+} K_2 [M^+] + k_{\text{cat}}^{2M^+} [M^+]^2}{K_1 K_2 + K_2 [M^+] + [M^+]^2} \]  
(Equation 3)

In the case of our data sets, we find no evidence that the postulated multiply-bound species \( E^{2M^+} \) retained activity, so we were able to simplify equation 3 to yield equation 4, which was used for nonlinear regression:

\[ k_{\text{cat}}^{\text{obs}} = \frac{k_{\text{cat}} K_1 K_2 + k_{\text{cat}}^{M^+} K_2 [M^+] + k_{\text{cat}}^{2M^+} [M^+]^2}{K_1 K_2 + K_2 [M^+] + [M^+]^2} \]  
(Equation 4)

For certain cases, data at high ion concentrations were not well fit by this model. In these cases, inhibition at high concentrations was better described by a model in which two ions were responsible for the inhibitory limb of the observed dependence. A similar treatment could be used to derive equation 5, which was used for regression analysis:

\[ k_{\text{cat}}^{\text{obs}} = \frac{k_{\text{cat}} K_1 K_2 K_3 + k_{\text{cat}}^{M^+} K_2 K_3 [M^+] + k_{\text{cat}}^{2M^+} K_3 [M^+]^2}{K_1 K_2 K_3 + K_2 K_3 [M^+] + K_3 [M^+]^2} \]  
(Equation 5)
This model was used for analyzing the response of Kex2 cleavage to added Li\(^+\), NH\(_4\)^+, and NaBr. In the case of NaBr response, the predicted values of affinity (\(K_r\)) and maximal response (\(k_{\text{cat}}^{M^+}\)) obtained with NaCl were used with this model to deconvolute the effects of the bromide anion.

For cleavage of poor substrates by Kex2 and furin, as well as for PC2 cleavage of all substrates tested, titration of potassium ion resulted in inhibition with no apparent stimulation. This behavior could be described by a simple 2-state model and was fit by equation 6:

\[
\frac{k_{\text{cat}}^\text{obs}}{k_{\text{cat}}} = \frac{k_{\text{cat}}K_1 + k_{\text{cat}}^{M^+}[M^+]}{K_1 + [M^+]} \quad \text{(Equation 6)}
\]

For all ions except Me\(_4\)N\(^+\), these equations gave fits with \(r^2>0.94\) and affinity constants for the high-affinity site with errors of \(\pm 50\%\) or better (for Kex2 binding of potassium ion error was \(\pm 20\%\)). Errors for binding of additional ions or for predicted maximal stimulation were much larger because these values were poorly determined in our data sets. Errors for Me\(_4\)N\(^+\) were larger and regression analysis for this ion was much poorer (\(r^2=0.86\)) due to the very small changes observed with this ion, resulting in poor signal-to-noise for nonlinear regression.

In all cases, fits of relative values of \(k_{\text{cat}}^\text{obs}\) were obtained by setting the value of \(k_{\text{cat}}\) (without any monovalent cation) to 1. This analysis assumes ordered cation binding, with the low-affinity (inhibitory) site becoming occupied only after the high-affinity (stimulatory) site has been filled. It is not possible to exclude random-binding models on the basis of current data; indeed, the observed inhibition at high ion concentrations may well be an indirect effect and not due to binding of further monovalent cation. In any case, the behavior in question is only seen at monovalent cation concentrations well in excess of 0.5 M, so it is unlikely to have physiological relevance.
RESULTS

Monovalent cations stimulate cleavage by Kex2 protease. We examined the effect of different monovalent cations on Kex2 activity in the presence of saturating substrate (Fig. 1), allowing us to measure specific effects on $k_{cat}$ (which reflects catalysis and not product release: 23, 24) rather than substrate binding. All group one cations tested (lithium, sodium, potassium, rubidium, and cesium) stimulated cleavage. All of these ions displayed stimulation at lower concentrations and varying degrees of inhibition at higher concentrations (Fig. 1A, B), though inhibition at high concentrations of NaCl was very weak. We also examined a series of ammonium ions (Fig. 1C). Ammonium and tetramethylammonium gave similar effects, though tetramethylammonium response was very small. However, tetraethylammonium gave a more complicated response suggesting cooperative effects on enzyme stability (data not shown). Comparison of NaCl and NaBr (Fig. 1D) indicated that the observed stimulation was due to the cation; furthermore, because almost no inhibition was seen with NaCl over the concentration range examined (0-1.54 M), the additional inhibition seen at high concentrations with other salts (KCl, LiCl, etc.) must also be due to added cation.

There were marked differences in response to various cations. For instance, sodium produced <2-fold stimulation with a maximal effect seen above 1 M, while lithium activated the enzyme ~2-fold with a maximal stimulation at 0.5 M LiCl and pronounced inhibition by 1.54 M (Figure 1A). The larger ions rubidium and cesium both displayed stimulation at concentrations below 1 M and weak inhibition at higher concentrations, as did ammonium and tetramethylammonium (Figure 1B, 1C). Potassium ion exhibited the most profound effects, with greater than 2.5-fold stimulation at 0.2 M KCl and weak inhibition at concentrations above 0.5 M. With potassium ion, half-maximal stimulation was observed at approximately 20 mM KCl (Figure 1).
These data suggested the presence of at least two binding sites for monovalent cations on Kex2 protease: a high-affinity site whose occupancy stimulates activity, and a low-affinity site responsible for inhibition at high ionic concentrations. This would lead to a three-state model for ion response, with 0, 1, or 2 bound ions. Fitting such a model to the data gave good results for cations other than lithium and ammonium (Equation 4, Table 1). For these ions and for NaBr, data were fit to a model which assumed inhibition was caused by two ions, necessitating a four-state model (Equation 5, Table 1). A more complicated fit for NaBr seemed appropriate, since bromide clearly had an additional inhibitory effect apart from the effects of the sodium cation (Fig. 1D).

This analysis permitted us to estimate the affinity of the stimulatory site for various ions (Table 1). The results show that potassium ion binds >20-fold more tightly than any other ion tested, with a predicted binding constant of 22 mM. Larger or smaller cations bound with much lower affinity. Additionally, counter-titration of sodium and potassium ions at a constant concentration of added cation gave non-linear results, suggesting competition for a common site (data not shown). This effect is selective for monovalent cations; barium, which has a very similar ionic radius to potassium but is divalent, gave essentially no effect between 0 and 0.2 M (data not shown).

All monovalent ions tested stimulated the enzyme to at least some extent. Therefore, we did not attempt to repeat these measurements at constant ionic strength, because there was no known ion that could be used to hold ionic strength constant over the desired range without affecting the enzyme. However, the weak effect seen with tetramethylammonium provides a conservative estimate of the maximum effect of total ionic strength over this concentration range and cannot explain the much more dramatic effects seen with other ions. Furthermore, the apparent competition between sodium and potassium ion suggests that at least the stimulation seen at low concentrations arises from a specific interaction between Kex2 and a monovalent cation, presumably due to a monovalent cation binding site on the enzyme. We chose to focus on potassium effects for
further study, because the apparent affinity of potassium ion for this postulated site was >20-fold higher than any other ion and could be physiologically relevant.

**Kex2 exhibits different response to potassium ion with different substrates.** The processing proteases of the Kex2/furin family are noted for their exceptionally high substrate specificity (4, 5, 20, 21, 26, 31). We were therefore interested in whether potassium ion would exert similar effects on Kex2 cleavage of multiple substrates, acting as a simple activator, or whether instead different effects would be seen with different substrates.

To examine this question, we compared Kex2 cleavage of the substrates Ac-Nle-Tyr-Lys-Arg↓MCA and Ac-Nle-Tyr-Lys-Lys↓MCA under saturating conditions. The substitution of Lys for Arg at P$_1$ has been shown to result in a 10-fold drop in $k_{cat}$ as well as a change in rate-limiting step from deacylation to acylation (21, 24), but results in a ≤4-fold drop in affinity (24). Interestingly, addition of potassium ion did not stimulate Kex2 cleavage of this Lys-Lys substrate. Instead, only an inhibitory effect was observed, with $k_{cat}^{obs}$ lowered by approximately 5-fold over the K$^+$ concentration range examined (Fig. 2). The net effect of these changes is to increase discrimination at the level of $k_{cat}$ in favor of the physiologically correct Lys-Arg cleavage site from 10-fold to approximately 80-fold in the presence of >0.1 M potassium ion. Furthermore, the affinity of the enzyme for potassium ion in the presence of saturating Ac-Nle-Tyr-Lys-Lys↓MCA is lower than that seen with saturating Ac-Nle-Tyr-Lys-Arg↓MCA (22 mM for P$_1$ Arg, 74 mM for P$_1$ Lys), supporting the idea that there is an interplay between enzyme-substrate and enzyme-ion interactions.

**The Kex2 homologs furin and PC2 also exhibit potassium ion effects.** To explore the possibility that potassium ion could function as a modulator of processing proteases in
general, we also examined potassium response in two homologous enzymes from metazoans, furin and PC2. Furin cleavage of a good substrate, Ac-Arg-Tyr-Arg-Phe-Lys-Arg↓MCA (26), displayed a similar pattern of activation and then inhibition with increasing potassium ion concentration (Fig. 3A). However, cleavage of a poorer substrate, boc-Arg-Val-Arg-Arg↓MCA (11), was inhibited, comparable to the Kex2 inhibition seen with the poor substrate Ac-Nle-Tyr-Lys-Lys↓MCA (data not shown). The apparent affinity of furin for potassium ion was somewhat lower than that observed with Kex2, with affinities ranging from 90-200 mM.

The potassium ion response seen with PC2 was quite different (Fig. 3B). We examined PC2 cleavage of three substrates: pyr-Arg-Thr-Lys-Arg↓MCA, d₃-Ac-Nle-Tyr-Lys-Arg↓MCA, and Ac-Arg-Tyr-Arg-Phe-Lys-Arg↓MCA. Cleavage of all three substrates was inhibited by potassium ion (Fig. 3B), although the enzyme retained greater residual activity against the hexapeptide substrate than against either tetrapeptide. The apparent affinity of PC2 (~200 mM) for potassium ion was significantly lower than that seen with Kex2 and was similar to that seen for furin.

**Potassium ion changes the pre-steady-state behavior of Kex2.** For cleavage of physiologically correct sites (P₁ Arg) by Kex2 protease, the acylation step is rapid, but the deacylation step is rate-limiting and is insensitive to the enzyme-substrate interactions that accelerate acylation (23, 24). We were interested in whether potassium ion could influence the pre-steady-state behavior of the enzyme. Interestingly, addition of 0.2 M KCl resulted in a loss of burst kinetics in Kex2 cleavage of multiple Lys-Arg substrates (data not shown), indicating that deacylation was no longer rate-limiting. This behavior was somewhat variable among different preparations of enzyme, but observed burst size was always substoichiometric. However, the initial burst in Kex2 cleavage of the ester
substrate Z-Nle-Tyr-Lys-Lys↓MCE was unaffected by potassium ion (data not shown). These results indicate that acylation is rate-limiting for Kex2 protease in the presence of potassium ion, but the overall stimulation of steady-state turnover by potassium ion (increase in $k_{cat}^{obs}$: Fig. 1) indicates that the deacylation rate must be increased by potassium ion binding so that it becomes faster than acylation. Thus, the observed stimulation of $k_{cat}^{obs}$ in Kex2 cleavage of a correct substrate at low concentrations of potassium ion probably reflects a change from a potassium-free enzyme with rapid acylation and slow deacylation to a potassium-bound enzyme with slower acylation but faster deacylation.

To test the hypothesis that potassium-bound and potassium-unbound species were behaving differently under pre-steady-state conditions, we also examined Kex2 cleavage of $d_3$-Ac-Nle-Tyr-Lys-Arg↓MCA, a fluorogenic tetrapeptide substrate based on the physiological yeast cleavage site in pro-α-factor (23, 24, 32), under single-turnover conditions. Under these conditions, with enzyme in molar excess relative to substrate (7.5 μM Kex2 versus 0.5-5.0 μM substrate), the conversion of substrate to product follows a single exponential with an apparent rate constant $k_{app}$ (Fig. 4). The observed rate constant should be independent of substrate concentration, as is the case without potassium ion (Table 2). However, a decrease in $k_{app}$ was observed with increasing substrate concentration in the presence of 0.2 M KCl (Fig. 4B; Table 2). Indeed, as substrate concentration was increased, the observed rate constant $k_{app}$ dropped from the value of $k_{app}$ observed in the absence of potassium ion to the value of the steady-state $k_{cat}^{obs}$ seen in the presence of potassium ion with saturating substrate under the same conditions (29 s$^{-1}$ at 21°C as determined in the burst experiment: Fig. 4B & Table 2). This indicates that there are two populations of enzyme in the presence of potassium ion, with increasing substrate concentration favoring the potassium-bound species, which has a slower acylation rate.
We have used single-turnover conditions with enzyme only slightly in excess relative to substrate, due to problems with autoproteolysis (data not shown). Examination of a range of enzyme concentrations between 1.4 and 7 µM at constant substrate concentration (0.5 µM) showed a linear relationship between enzyme concentration and observed rate, confirming that the observed first-order rate constant ($k_{\text{app}}$) describes a reaction which still possesses bimolecular character (Fig. 4C). Thus, both substrate and potassium binding equilibria are involved in determining the observed single-turnover rate (Fig. 5). Moreover, the initial burst result indicates that potassium-bound and potassium-unbound species have very different acylation rates: the acylation rate without potassium ion is $\geq550$ s$^{-1}$ at 21°C (24), and the rate with potassium ion is 29 s$^{-1}$ under otherwise identical conditions (as discussed above), so that the observed rate is a composite of the acylation rates for these two populations. Thus, increasing substrate concentration will lead to increased amounts of the enzyme-substrate complex. However, if this complex has a higher affinity for potassium ion than does the free enzyme, increasing amounts of substrate will also act to drive potassium binding to the enzyme-substrate complex and thereby increase the relative contribution of the potassium-bound species to the observed rate. As the potassium-bound species has a lower acylation rate than the unbound species, the increased contribution of this species will lower the observed rate constant. Attempts to simulate these experiments with the program KINSIM (33) have qualitatively reproduced the trends in Figure 4 and Table 2 (i.e., substrate-insensitive rate in the absence of potassium ion and substrate-induced decrease in rate in the presence of potassium ion), although our current knowledge of microscopic rate constants is insufficient to allow quantitative simulation of these processes. However, it is very difficult to envision that an indirect effect of ionic strength on Kex2 activity could result in such a situation. We therefore conclude that Kex2 binds potassium ion and that this binding modulates the pre-steady-state behavior of this enzyme, affecting both substrate binding and catalysis.
DISCUSSION

We have shown that the kinetic behavior of the Kex2/furin family of proteases can be modulated by potassium ion. Two proteases which function within the constitutive secretory pathway or its yeast equivalent, furin and Kex2, were stimulated at low potassium ion concentrations in cleavage of good substrates but inhibited in cleavage of poor substrates. However, a homologous enzyme which normally functions in the regulated secretory pathway, PC2, was inhibited by potassium ion with all substrates examined. The pre-steady-state behavior of Kex2 was altered by maximally stimulatory concentrations of potassium ion, suggesting that potassium could act as the postulated effector which could mobilize a reserve pool of Kex2 in vivo (23).

At higher concentrations of potassium ion, we observed inhibition of Kex2 and furin cleavage of good substrates as well as poor ones. We have gathered little information about this latter behavior. Even though the observed response is well fit by 3-state or 4-state models, we certainly cannot rule out the idea that these effects do not involve additional binding sites, but rather are indirect and stem from changes in the behavior of bulk solvent at high salt concentrations. However, the high concentrations of monovalent cations required for this effect to be detectable (>1 M potassium ion: Figure 1) make it unlikely that this inhibitory limb is physiologically relevant.

Although the lack of a crystal structure for Kex2, furin, or another processing protease precludes identifying potassium binding sites, several lines of evidence suggest that the stimulatory effects we observe at lower concentrations stem from a direct interaction between an ion and a specific binding site on the enzyme. First, the apparent affinity of Kex2 for potassium ion is quite high for binding a monovalent cation. This effect is also quite selective for ionic radius, as no other monovalent ion has a comparable affinity (Table 1), and for ionic charge, as the similarly sized but divalent barium has no effect (although its orbital structure is of course rather different). Moreover, counter-
titration of sodium and potassium ion in Kex2 cleavage of a good substrate at constant ionic strength resulted in a non-linear response, suggesting that these ions are competing for a common binding site rather than merely changing the character of the bulk solvent. Additionally, the apparent affinity of Kex2 for potassium ion in the presence of saturating substrate varies with the substrate (Figure 2), indicating cooperativity between substrate binding and potassium binding. Finally, a substrate titration under single-turnover conditions in the presence of potassium ion showed a decrease in the observed rate of Kex2 catalysis with increasing substrate (Figure 4, Table 2), again consistent with the idea that there is cooperativity between substrate binding and potassium binding.

Potassium ion response varied with substrate for both furin and Kex2. In both cases, cleavage of relatively poor substrates was inhibited by potassium ion, while cleavage of good substrates was stimulated. These enzymes exhibit burst kinetics with good substrates in the absence of potassium ion (4). For Kex2, potassium ion binding causes a loss of burst kinetics with amide substrates but not esters, indicating that acylation has become rate limiting for all amide substrates upon potassium binding. Thus, the observed value of $k_{cat}$ with maximally stimulating potassium ion present is approximately equal to the acylation rate constant under these conditions. However, this value is much lower than that in the absence of potassium ion (24). Nevertheless, the steady-state stimulation of Kex2 cleavage of good substrates by potassium ion indicates that potassium binding must stimulate the formerly rate-determining deacylation step. Therefore, potassium binding accelerates deacylation and slows acylation, effectively converting Kex2 into a more conventional subtilisin.

Unlike Kex2 and furin, PC2 was inhibited by potassium ion (Figure 3). This enzyme differs from Kex2 and furin in a number of other respects, including localization, less stringent specificity at P₁ and P₂, and pre-steady-state behavior (8, 27, 34, 35). The difference in pre-steady-state behavior is thought to arise due to a substitution in PC2 which replaces a conserved Asn required for transition state stabilization in subtilisin BPN'.
with an Asp (36). Introducing the corresponding substitution in Kex2 results in a large
defect in catalysis (37), and this mutant form of Kex2 exhibits a turnover number similar to
the known acylation rate for PC2 cleavage of amide substrates (27). Therefore, it seems
likely that PC2 is effectively crippled at the acylation step and is a much less proficient
enzyme. It is interesting that the apparent affinity of PC2 for potassium ion is lower than
that seen with Kex2 but is comparable to that seen with furin (~200 mM for PC2 and 90-200 mM for furin). It is thus possible that inhibition of furin cleavage of poor substrates
by potassium ion follows a very similar molecular mechanism to the inhibition of PC2
cleavage by potassium ion, with the crippled PC2 active site being unable to adopt the
stimulated conformation seen in Kex2 and furin cleavage of good substrates.

The in vivo implications of this in vitro modulation of Kex2 and furin by
potassium ion are currently unclear. Certainly, the <3-fold activation seen with these
enzymes is unlikely to have profound implications for processing, and currently available
data do not suggest profound changes in specificity associated with potassium ion binding.
However, the change in pre-steady-state behavior observed with Kex2 may itself have
implications for cleavage of authentic substrates in vivo. The rate-limiting deacylation seen
in Kex2 cleavage of correct substrates in the absence of potassium ion serves to reduce the
free enzyme concentration (23), and potassium ion binding would thus be equivalent to
increasing the effective concentration of protease in any compartment with a high
concentration of potassium ion. This would permit a higher overall turnover of substrates
in such a compartment, both because of the steady-state stimulation and because of the
increased concentration of free enzyme which is competent to bind and cleave substrate,
which could be substantial (>90% of the enzyme could be sequestered as acylenzyme in
vivo on the basis of known data about acylation and deacylation rates: 24). Thus, either
pumping potassium ions into processing compartments or sorting enzymes such as Kex2
or furin into high-potassium compartments could allow a rapid increase in processing
capacity. Further characterization of this point would best be achieved by mutagenesis of
Kex2 or furin to alter the pre-steady-state behavior and ablate the monovalent cation binding site, as such mutants could then be expressed as the sole source of enzyme in appropriate cells. However, such experiments must await a crystal structure for one of the members of this family.

Data on in vivo concentrations of monovalent cations in compartments of the late constitutive secretory pathway is somewhat sparse. Early characterization of secretory granule constituents from rat insulinoma cells (38) indicated that potassium ion was present at a concentration of approximately 16 mM in isolated granules, a lower level than was present in the insulinoma tissue as a whole. This level is well below the affinity of PC2 for potassium, as would be expected for PC2 function in such compartments. However, a similar characterization of compartments of the constitutive secretory pathway has not been reported. It is thus hard to know whether the observed affinities would allow saturation of the potassium ion binding sites in Kex2 and furin in vivo. In the case of Kex2, it is possible to estimate the volume of processing compartments at 0.1-1 fL (23). This allows one to calculate that approximately $10^8$ potassium ions ($<0.2$ fmol) would suffice to generate a lumenal concentration of 100 mM, even for the higher size estimate. It is therefore not unreasonable to speculate that such a concentration could be achieved in vivo.

Recent evidence also raises the possibility that Kex2 and furin may both cycle between compartments with widely varying potassium ion concentrations. The ubiquitously expressed putative Na$^+$/H$^+$ antiporter NHE7 has recently been localized to the trans Golgi network (TGN) in mammalian cells (39). Significantly, this protein is able to catalyze the uptake of both Na$^+$ and Rb$^+$ in a permeabilized cell system (39). Moreover, potassium ion was an effective competitor for rubidium in this assay (39). As potassium is the main monovalent cation in the cytosol and the TGN is acidified by the action of the vacuolar ATPase (40, 41), the physiologically relevant reaction for NHE7 is likely to be the import of potassium ion into the lumen of the mammalian TGN in exchange for proton transport to the cytosol (39). Thus, furin is likely to encounter high potassium ion concentrations.
concentrations in the TGN but low potassium ion concentrations in the endosome, which lacks \textit{NHE7}, and at the cell surface (2, 39).

Less is known about possible potassium ion gradients in the yeast secretory pathway. The functional yeast homolog of \textit{NHE7} is probably \textit{NHX1} (also named \textit{VPS44}: 42, 43), which is localized to the yeast endosome and is required for proper protein sorting in the late secretory pathway (43). No data on the ion selectivity of this protein are currently available. However, should \textit{NHX1/VPS44} behave similarly to its mammalian ortholog, it is possible that Kex2 would also cycle between a low-potassium compartment (in this case, the yeast TGN) and a high-potassium compartment, the yeast endosome. In that case, the putative reserve pool of Kex2 would be presented to its allosteric effector, potassium ion, in the course of trafficking in the secretory pathway.
REFERENCES

1. Steiner, D. F. (2001) in *The Enzymes*, Vol. XXII, 3rd Ed., pp. 163-198, Dalbey, R. E., & Sigman, D. S., eds., Academic Press, San Diego, CA

2. Molloy, S. S., and Thomas, G. (2001) in *The Enzymes*, Vol. XXII, 3rd Ed., pp. 199-235, Dalbey, R. E., and Sigman, D. S., eds., Academic Press, San Diego, CA

3. Seidah, N. G. (2001) in *The Enzymes*, Vol. XXII, 3rd Ed., pp. 237-258, Dalbey, R. E., and Sigman, D. S., eds., Academic Press, San Diego, CA

4. Rockwell, N. C., and Fuller, R. S. (2001) in *The Enzymes*, Vol. XXII, 3rd Ed., pp. 259-289, Dalbey, R. E., and Sigman, D. S., eds., Academic Press, San Diego, CA

5. Cameron, A., Apletalina, E. V., and Lindberg, I. (2001) in *The Enzymes*, Vol. XXII, 3rd Ed., pp. 291-332, Dalbey, R. E., and Sigman, D. S., eds., Academic Press, San Diego, CA

6. Takahashi, S., Nakagawa, T., Banno, T., Watanabe, T., Murakami, K., and Nakayama, K. (1995) *J. Biol. Chem.* 270, 28397-28401

7. Wan, L., Molloy, S. S., Thomas, L., Liu, G., Xiang, Y., Rybak, S. L., and Thomas, G. (1998) *Cell* 94, 205-216

8. Muller, L., and Lindberg, I. (1999) in *Progress in Nucleic Acids Research*, Moldave, K., ed., Academic Press, San Diego, CA

9. Uehara, M., Yaoi, Y., Suzuki, M., Takata, K., and Tanaka, S. (2001) *Cell Tissue Res.* 304, 43-49

10. Fuller, R. S., Brake, A., and Thorner, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 1434-1438

11. Bravo, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) *J. Biol. Chem.* 269, 25830-25837

12. Gallagher, T., Bryan, P., and Gilliland, G. L. (1993) *Proteins* 16, 205-213
13. Bryan, P., Alexander, P., Strausberg, S., Schwarz, F., Lan, W., Gilliland, G., and Gallagher, D. T. (1992) *Biochemistry* **31**, 4937-4945

14. Braxton, S., and Wells, J. A. (1992) *Biochemistry* **31**, 7796-7801

15. Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984) *Cell* **37**, 1075-1089

16. Fuller, R. S., Brake, A. J., and Thorner, J. (1986) in *Microbiology--1986*, (Lieve, L., ed), pp. 273-278, American Society for Microbiology, Washington, D.C.

17. Hatsuzawa, K., Nagahama, M., Takahashi, S., Takada, K., Murakami, K., Nakayama, K. (1992) *J. Biol. Chem.* **267**, 16094-16099

18. Brenner, C., and Fuller, R. S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 922-926

19. Brenner, C., Bevan, A., and Fuller, R. S. (1994) *Methods Enzymol.* **244**, 152-167

20. Rockwell, N. C., Wang, G., Krafft, G., and Fuller, R. S. (1997) *Biochemistry* **36**, 1912-1917

21. Rockwell, N. C., and Fuller, R. S. (1998) *Biochemistry* **37**, 3386-3391

22. Bevan, A., Brenner, C., and Fuller, R. S. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 922-926

23. Rockwell, N. C., and Fuller, R. S. (2001) *Biochemistry* **40**, 3657-3665

24. Rockwell, N. C., and Fuller, R. S. (2001) *J. Biol. Chem.* **276**, 38394-38399

25. Brickner, J. H., and Fuller, R. S. (1997) *J. Cell Biol.* **139**, 23-36

26. Krysan, D. J., Rockwell, N. C., and Fuller, R. S. (1999) *J. Biol. Chem.* **274**, 23229-23234

27. Rockwell, N. C., Krysan, D. J., and Fuller, R. S. (2000) *Anal. Biochem.* **280**, 201-208

28. Philipp, M., and Bender, M. L. (1983) *Mol. Cell. Biochem.* **51**, 5-32

29. Grøn, H., Meldal, M., and Breddam, K. (1992) *Biochemistry* **31**, 6011-6018
30. Lamango, N. S., Zhu, X., and Lindberg, I. (1996) Arch. Biochem. Biophys. 330, 238-250
31. Jean, F., Boudreault, A., Basak, A., Seidah, N. G., and Lazure, C. (1995) J. Biol. Chem. 270, 19225-19231
32. Fuller, R. S., Sterne, R. E., and Thorner, J. (1988) Annu. Rev. Physiol. 50, 345-362
33. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1983) Anal. Biochem. 130, 134-145
34. Johanning, K., Juliano, M. A., Juliano, L., Lazure, C., Lamango, N. S., Steiner, D. F., and Lindberg, I. (1998) J. Biol. Chem. 273, 22672-22680
35. Day, R., Lazure, C., Basak, A., Boudreault, A., Limperis, P., Dong, W., and Lindberg, I. (1998) J. Biol. Chem. 273, 829-836
36. Wells, J. A., Cunningham, B. C., Graycar, T. P., and Estell, D. A. (1986) Philos. Trans. R. Soc. London 317, 415-423
37. Brenner, C., Bevan, A., and Fuller, R. S. (1993) Curr. Biol. 3, 498-506
38. Hutton, J. C., Penn, E. J., and Peshavaria, M. (1983) Biochem. J. 210, 297-305
39. Numata, M., and Orlowski, J. (2001) J. Biol. Chem. 276, 17387-17394
40. Mellman, I., Fuchs, R., and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663-700
41. Forgac, M. (1999) J. Biol. Chem. 274, 12951-12954
42. Nass, R., and Rao, R. (1998) J. Biol. Chem. 273, 21054-21060
43. Bowers, K., Levi, B. P., Patel, F. I., and Stevens, T. H. (2000) Mol. Biol. Cell 11, 4277-4294
44. Zimmerman, M., Ashe, B., Yurewicz, E. C., and Patel, G. (1977) Anal. Biochem. 78, 47-51
45. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157-162
FOOTNOTES:

*This work was supported in part by NIH Training Grant 2T32GM07599 (N. R.) and NIH GM 39697 (R. S. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Abbreviations: Ac, acetyl; AMC, 7-amino-4-methylcoumarin; boc, tert-butoxycarbonyl; $d_3$-Ac, trideuteroacetyl; $k_{\text{app}}$, the apparent first-order rate constant in single-turnover experiments; $k_{\text{cat}}^{\text{obs}}$, the observed value of $k_{\text{cat}}$ under given conditions; MCA, C-terminal methylcoumarinamide (44); MCE, C-terminal methylcoumarinester (27); Nle, norleucine; pyr, pyroglutamate; TGN, trans Golgi network; Z, benzyloxycarbonyl. Throughout we use the nomenclature of Schechter and Berger (45) in designating the cleavage site as $--P_3-P_2-P_1\downarrow P_1'-P_2'--$.

Acknowledgement-The authors wish to thank Alison Bevan, Michael S. Kay, Jack Thomas, & Geoff Stamper for assistance with different Kex2 preparations, Luke Carter for the gift of furin, Iris Lindberg for the generous gift of PC2, and Dan Herschlag and the members of the Fuller lab for helpful discussions.
Table 1: Interaction of monovalent cations with Kex2 Protease

| Ion     | Affinity (M) | Maximal Stimulation (fold) | Ionic Radius (Å) |
|---------|--------------|----------------------------|------------------|
| Li⁺     | 2.6          | 13                         | 0.68             |
| Na⁺     | 0.58         | 2.3                        | 0.97             |
| K⁺      | 0.022        | 2.8                        | 1.33             |
| NH₄⁺    | 0.60         | 4.2                        | 1.43             |
| Rb⁺     | 0.45         | 4.5                        | 1.47             |
| Cs⁺     | 0.64         | 3.3                        | 1.67             |

*Parameters from fits in Fig. 1A-C. Affinity is the affinity for the high-affinity, stimulatory binding event (Kᵢ in equations 4 and 5), and maximal stimulation is the predicted maximum in the absence of inhibition at high concentration, defined as (kₐₜ M⁺)/(kₐₜ) for each ion (using equations 4 or 5). The regression analysis is described in the Methods. *b* Response evaluated using equation 5 to account for the additional inhibition observed at higher concentrations (Figure 1). *c* Response evaluated using equation 4.
Table 2: Potassium effects on single-turnover behavior of Kex2

| [S], µM | $k_{app}$, s$^{-1}$ (0.2 M KCl) | $k_{app}$, s$^{-1}$ (no KCl) |
|---------|---------------------------------|-----------------------------|
| 0.5     | 79±14                           | 87±4                        |
| 2.0     | 44±3                            | 86±5                        |
| 2.3     | n/d$^b$                         | 85±6                        |
| 5.0     | 29±4                            | n/d$^b$                     |

$^a$Single-turnover reactions were carried out at 7.5 µM Kex2 protease as described in Materials and Methods. Under these conditions, $k_{cat}$ was 16±2 s$^{-1}$ without potassium and $k_{cat}^{obs}$ was 29±1 s$^{-1}$ in the presence of 0.2 M KCl. $^b$n/d, not determined.
FIGURE LEGENDS

Fig. 1. Stimulation of Kex2 protease by monovalent cations. Relative values of $k_{cat}^{obs}$ were determined at 200 µM Ac-Pro-Met-Tyr-Lys-Arg↓MCA (A, B, D), 370 µM Ac-Pro-Nle-Tyr-Lys-Arg↓MCA (NH$_4^+$, C), or 20 µM Ac-Nle-Tyr-Lys-Arg↓MCA (Me$_4$N$^+$, C) at varying concentrations of different cations. A, Li$^+$ (open circles), Na$^+$ (filled triangles), and K$^+$ (filled circles). B, Rb$^+$ (filled squares), and Cs$^+$ (open squares). C, NH$_4^+$ (filled diamonds), and Me$_4$N$^+$ (open diamonds). The K$^+$ curve from A is shown as a dashed trace in B and C for comparison. Cations in A-C were all added as chloride salts. D, NaCl (dashed curve; same data as in A) and NaBr (open triangles) were examined to test the effects of different anions. Data were fit to a three-state model (Equation 4) or a four-state model (Equation 5) as described in Table 1 and the text. All substrate concentrations were at least 20-fold above the known $K_M$ values for these substrates, and $k_{cat}$ for Kex2 cleavage of all of these substrates was 45±5 s$^{-1}$.

Fig. 2. Potassium affects Kex2 specificity. $k_{cat}^{obs}$ was determined for $d_3$-Ac-Nle-Tyr-Lys-Arg↓MCA (200 µM, A) or Ac-Nle-Tyr-Lys-Lys↓MCA (400 µM, B) at varying concentrations of KCl as described in Materials and Methods. C, relative values of $k_{cat}^{obs}$ were plotted for the data sets from A (filled circles) and B (open circles). Data for Ac-Nle-Tyr-Lys-Lys↓MCA were fitted to equation 6, with an apparent affinity of 74 mM and a predicted reduction in activity of approximately 10-fold upon potassium binding. Data for Ac-Nle-Tyr-Lys-Arg↓MCA were fitted to equation 4, with similar results to those seen with Ac-Pro-Met-Tyr-Lys-Arg↓MCA (Table 1).
Fig. 3. Potassium effects on furin and PC2. A, the effect of potassium on furin activity was assessed with the substrates Ac-Arg-Tyr-Arg-Phe-Lys-Arg↓MCA (filled circles) and boc-Arg-Val-Arg-Arg↓MCA (filled squares). Substrate concentrations were well in excess of $K_M$, and data were fitted to equation 4 (Ac-Arg-Tyr-Arg-Phe-Lys-Arg↓MCA) and equation 6 (boc-Arg-Val-Arg-Arg↓MCA). $k_{cat}$ for furin cleavage of boc-Arg-Val-Arg-Arg↓MCA in the absence of potassium was 0.5 s$^{-1}$ under these conditions, within 2-fold of published data (11). B, the effect of potassium on PC2 activity was assessed with pyr-Arg-Thr-Lys-Arg↓MCA (open circles), $d_3$-Ac-Nle-Tyr-Lys-Arg↓MCA (open squares), or Ac-Arg-Tyr-Arg-Phe-Lys-Arg↓MCA (filled circles). All substrate concentrations were well in excess of $K_M$. Data were fitted to equation 6. $K_M$ for PC2 cleavage of pyr-Arg-Thr-Lys-Arg↓MCA was approximately 40 µM and $k_{cat}$ was 0.2 s$^{-1}$. Both values are within 3-fold of published values for PC2 cleavage of this substrate (34).

Fig. 4. Pre-steady-state behavior of Kex2 protease is affected by potassium. A, single-turnover cleavage of $d_3$-Ac-Nle-Tyr-Lys-Arg↓MCA in the presence (filled circles) or absence (open circles) of 0.2 M KCl. Substrate concentration was 2 µM and enzyme concentration was 7.5 µM. Data were fit to equation 1, and parameters are in Table 2. B, semilog decay plots of single-turnover Kex2 cleavage of $d_3$-Ac-Nle-Tyr-Lys-Arg↓MCA at increasing substrate concentrations in the presence (filled symbols) or absence (open symbols) of potassium. Enzyme concentration and other conditions were as in A, and substrate concentrations were 0.5 µM (triangles), 2.0 µM (circles), and 5.0 µM (squares). The data for 2.0 µM substrate are the same as shown in A. C, varying
concentrations of Kex2 were reacted with 0.5 µM substrate, and the apparent rate constants were plotted against enzyme concentration.

Fig. 5. Scheme for potassium and substrate binding equilibria in single-turnover reactions. Under single-turnover conditions, product is formed from substrate in a single exponential phase with observed rate constant $k_{app} = k_2 \times ([ES]/[E_0]) + k_2 K^+ \times ([ES^K_+]/[E_0])$. Thus, binding of both substrate and potassium play a role in determining $k_{app}$. 
Rockwell & Fuller Fig. 1
Figure 2 from Rockwell & Fuller:

A) Plot of $k_{cat,obs}$ vs. [KCl], M.

B) Plot of $k_{cat,obs}$ vs. [KCl], M.

C) Plot of Relative $k_{cat,obs}$ vs. [KCl], M.
Rockwell & Fuller Fig. 3
Rockwell & Fuller Fig. 4
Rockwell & Fuller, Fig. 5
