# Precursor Processing by Kex2/Furin Proteases

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## I. Introduction

Cell−cell interaction is a feature of even the simplest biological systems, as shown by the exchange of genetic material between bacteria and by mating in unicellular eukaryotes such as the budding yeast *Saccharomyces cerevisiae*. In multicellular organisms, the process of cell−cell signaling becomes even more important; even the formation of a fruiting body by the social bacterium *Myxococcus xanthus* requires multiple signals and the differentiation of multiple cell types,[1] while higher eukaryotes rely on extremely complicated networks of signals for development and differentiation, recognizing and responding to changes in the environment and interacting with each other.

Much cell−cell signaling relies on the production and secretion of soluble hormones which can then travel to receptors on a target cell. For a complicated organism such as *Homo sapiens*, the mature neuroendocrine system regulates a number of processes with many secreted effectors. The synthesis of these molecules is tightly regulated; for example, the insulin precursor proinsulin is synthesized and processed into mature insulin only in a particular cell type in a particular tissue in a particular organ (the $\beta$ islet cells of the pancreas). In a more complicated case, multiple secreted molecules can be synthesized from a single precursor such as proopiomelanocortin (POMC). Proinsulin, POMC, and many other molecules are synthesized as larger precursors which are proteolytically cleaved in late compartments of the eukaryotic secretory pathway such as the trans Golgi network (TGN) or the secretory granules of neuroendocrine cells to yield mature effector molecules (for recent reviews on this topic, see refs 2−4).

This excision of mature hormones and neuropeptides from larger precursors is a special case of a more general event in eukaryotic organisms, whereby specific endoproteolysis is used to generate a mature protein from a precursor (or proprotein). Additional examples include the maturation of hormone receptors,[5] the activation of digestive enzymes such as trypsin and chymotrypsin,[6] and the maturation of viral membrane glycoproteins,[7] to name a few. This specific endoproteolysis is distinct from the action of digestive proteases such as trypsin, chymotrypsin, proteinase K, and subtilisin because it occurs at very specific sites, generating a different protein rather than degrading the substrate into short peptides and amino acids. In eukaryotes ranging from fungi (e.g., *S. cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Aspergillus nidulans*) through *Hydra* and higher animals (e.g., *Caenorhabditis elegans*, *Aplysia*, *Drosophila melanogaster*, and mammals), such processing frequently occurs at motifs containing multiple basic residues. The existence of such a pathway has also been recently demonstrated in plants,[8,9] but

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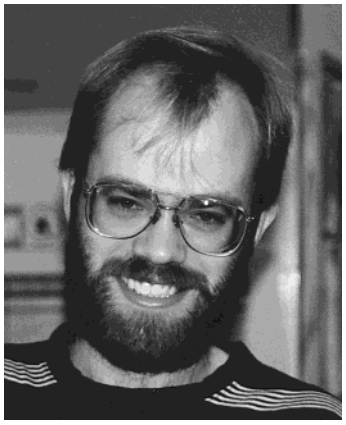
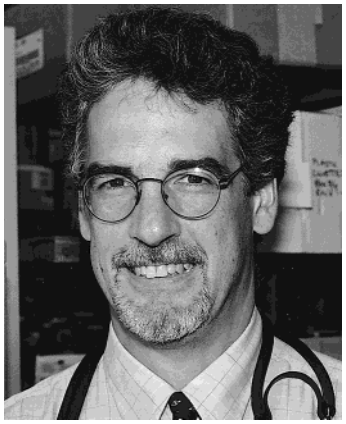


N. C. Rockwell was born in Newport Beach, California. He received his B.S. in Biology at the California Institute of Technology in 1992 and Ph.D. in Biochemistry at Stanford University in 1998, where he worked with R. S. Fuller on the interrelationship of specificity and mechanism in Kex2 protease. Since 1998, he has pursued postdoctoral work on ABC transporters with Jeremy Thorner in the Department of Cell and Molecular Biology at the University of California Cancer Research Laboratory.

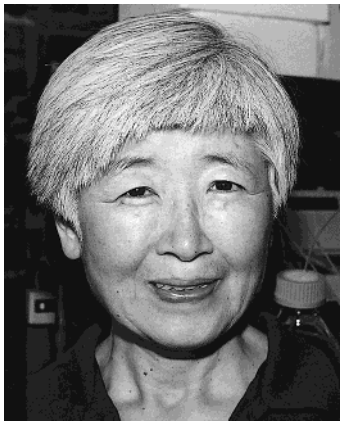


D. J. Krysan was born in Urbana, Illinois. He received his B.S. in Chemistry at Grinnell College in 1984 and his Ph.D. in Organic Chemistry from Northwestern University in 1989, where he researched the application of organometallic chemistry to asymmetric organic synthesis with Peter B. Mackenzie. After postdoctoral work in organic chemistry with Lanny S. Liebeskind at Emory University from 1989 to 1991, he served as Senior Research Chemist at Abbott Laboratories, Abbott Park, Illinois, from 1991 to 1994, where he applied asymmetric synthesis of amino-diol based peptidomimetics to drug synthesis. From 1994 to 1996, he was Senior Associate Chemist at Hoechst Marion Roussel working in asymmetric synthesis and heterocycle synthesis. He attended University of Michigan Medical School from 1996 to 2000, receiving his M.D. in 2000, and afterward pursued both internship and residency in Pediatrics at the C. S. Mott Children's Hospital at the University of Michigan. He is currently a Fellow in Pediatric Infectious Disease at the University of Michigan Medical School with research interests in fungal pathogenesis.

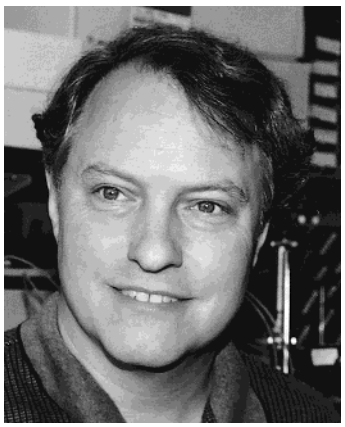
the enzymes responsible for such processing in plant cells have not yet been isolated, in contrast to other eukaryotes.

## *II. The Kex2/Furin Proteases or Proprotein Convertases*

The first proprotein processing protease to be discovered was Kex2 protease (kexin, E.C. 3.4.21.61) from *S. cerevisiae*. The *KEX2* gene was initially identified as a genetic locus required for *k*iller toxin *ex*pression.[10] It was subsequently shown to be required for production of α-factor, the mating pheromone secreted by *MAT*α haploid cells,[11] and subsequent characterization of the structures of these two effector molecules established that both were excised from larger precursors by specific endoproteolysis carboxyl to pairs of basic residues,[12] in a manner strikingly similar to the maturation of proinsulin by excision of the C-peptide at similar motifs.[2] Subsequent work demonstrated that *KEX2* encoded a $Ca^{2+}$-dependent serine protease distantly related to the digestive proteases of the subtilisin family (reviewed in ref 13). This protease (variously designated Kex2p,



T. Komiyama was born in Tokyo, Japan. She received her B.Sc. in Chemistry from Ochanomizu University in Tokyo in 1964, her Ms.D. in Chemistry from Tohoku University, Sendai, Japan, in 1966 and her Ph.D. in Chemistry from Tokyo Institute of Technology in 1979, where she studied fluorescence including use of intrinsic tryptophan fluorescence in studies of the Streptomyces subtilisin inhibitor. From 1974 to 1988, she was Research Associate and Lecturer in Industrial Chemistry at Seikei University in Tokyo and from 1988 to 1991 studied turkey ovomucoid third domain inhibitors with M. Laskowski, Jr., as Visiting Assistant Professor, at Purdue University. From 1991 to 1996, she was a Research Associate in the Departments of Pathology and Biochemistry where she studied the Cowpox Virus serpin CrmA with Guy Salvesen. From 1996 to present, she has been in the Department of Biological Chemistry at the University of Michigan where she is currently a Senior Research Associate. Her research interests are in inhibition of the Kex2/furin family enzymes.



R. S. Fuller was born in Ft. Belvoir, Virginia. He received his B.S. in Molecular Biophysics and Biochemistry from Yale University in 1978 and completed his Ph.D. in Biochemistry with Arthur Kornberg in 1984 studying initiation of DNA replication in *E. coli*. He was a postdoctoral fellow with Jeremy Thorner, support from the Helen Hay Whitney and Lucille P. Markey Foundations in the Departments of Microbiology and Immunology and Biochemistry at the University of California, Berkeley, from 1984 to 1987 and Assistant Professor of Biochemistry at Stanford University from 1987 to 1994. In 1994, he moved to the Department of Biological Chemistry at the University of Michigan, Ann Arbor, as Associate Professor and became Professor in 1999. His research interests are in the enzymology and cell biology of protein processing and localization within the eukaryotic secretory pathway.

YscF, or kexin: refs 13−15) is most frequently referred to as Kex2 or Kex2 protease, in keeping with the original yeast nomenclature. The enzyme has a single transmembrane domain followed by a cytosolic tail which is responsible for Kex2 localization to late compartments of the yeast secretory pathway.

It has subsequently become clear that the majority of proteolytic processing at basic motifs is carried out by close homologues of Kex2. In mammalian cells, three Kex2 homologues were rapidly identified. Two of these, PC1/3 and PC2, were the *p*rohormone *c*onvertases (or proprotein convertases) responsible for the processing of proinsulin and other prohormones and neuropeptide precursors.[2,16−19] These enzymes lack transmembrane domains, are expressed in neuroendocrine cells, and are localized to the regulated secretory pathway,[2,3] The third homologue, furin, is expressed ubiquitously and has a transmembrane domain. Furin functions in a manner more analogous to Kex2, cycling among late compartments of the constitutive secretory pathway.[4]

More recent work has identified several additional homologues in mammals, including PACE4, PC4, PC5/6, and PC7. Like Kex2, these enzymes are all synthesized as zymogens which undergo autoactivation.[3,4] Each contains a catalytic domain homologous to Kex2, furin, and the subtilisins, as well as an additional region of homology not present in subtilisins termed the P-domain or Homo B domain,[13,20] which is required for activity but whose function is not yet known. The mammalian family members seem to belong to two subfamilies distinguishable by the presence or absence of a transmembrane domain and by sorting to the regulated or constitutive secretory pathway. PC4 is expressed in testis and localized to the regulated secretory pathway,[21] while PACE4 and PC7 are expressed ubiquitously and localized to the constitutive secretory pathway.[2−4] PC5/6 is widely expressed and exists in two isoforms which differ in their sorting: one isoform, PC6A, lacks a transmembrane domain and is sorted to the regulated branch of the secretory pathway, while PC6B has a transmembrane domain and is sorted to the constitutive branch.[2−4] The presence of these two classes of prohormone convertase or Kex2/furin protease can be seen in lower animals as well. For example, *Drosophila* is known to have two genes for different furin isoforms[22,23] as well as a gene for PC2 and its activator protein, 7B2,[24,25] and a protein that closely resembles PC1/3 has been described in *Hydra*.[26]

In fungi, the absence of a regulated branch of the secretory pathway correlates with the absence of enzymes such as PC1/3 or PC2. Fungal members of this family include Kex2 itself as well as *krp1* from *Sch. pombe*,[27] KEX1 from *Kluyveromyces lactis*,[28] XPR6 from *Yarrowia lipolytica*[29]), and *KEX2* genes from *C. albicans*, *C. glabrata*, and *A. niger*,[30−32] all of which are close relatives of Kex2. Like Kex2, each of these enzymes is synthesized as a zymogen and contains a P-domain. Additionally, other fungi such as *Pichia pastoris* are able to correctly process precursors containing such motifs,[33] but Kex2/furin proteases have not yet been cloned from these organisms.

The two branches of this family must operate in substantially different environments. Enzymes such as furin or Kex2 cycle rapidly between several compartments in the secretory pathway, where they are transiently exposed to correct substrates in the presence of an excess of incorrect substrates on a time scale as brief as a minute. However, enzymes such as PC1/3 and PC2 are sorted into compartments such as the dense-core secretory granule, where they can be exposed to a protein environment very highly enriched for precursors on a much longer time scale of several hours.

All the members of this family are also members of the subtilase superfamily,[34] which also includes a wide variety of digestive enzymes such as the subtilisin family, proteinase K and its relatives, and more distantly related enzymes such as those involved in lantibiotic biosynthesis.[34] These enzymes all catalyze acyl transfer reactions by means of the classical serine protease mechanism,[35,36] utilizing a catalytic triad of serine, histidine, and aspartate also found in the enzymes of the trypsin superfamily.[36] The proteases of the subtilase superfamily also have a fourth conserved catalytic residue, the so-called oxyanion hole Asn, which acts as a hydrogen bond donor to stabilize the buildup of negative charge on the scissile carbonyl at the transition state.[34,36] Interestingly, the neuroendocrine processing protease PC2 has a conserved substitution of Asp for Asn at this position.[2] This substitution has been proposed to assist PC2 function in the acidic environment of the secretory granule,[2] but recent research raises the possibility that this substitution may simply reduce the specific activity of PC2 substantially (see the section on the mechanistic basis for specificity below). It is possible that such a crippled enzyme may actually be favorable in the environment of the secretory granule, where enzyme and substrate are colocalized for a period of hours. This debate highlights the complexity of precursor processing; for processing to occur, the substrate must be expressed in the right cell, sorted to the right compartment, and then presented to the right enzyme. For accurate processing, the enzyme must be able to cleave the correct site efficiently enough to ensure complete processing on the relevant time scale without significant cleavage at incorrect sites, which could result in degradation of the effector. Therefore, the ability of a given processing protease to cleave a precursor efficiently and correctly is the final determinant of the specificity of processing, in addition to the aforementioned cellular factors.

Thus, the enzymes of the Kex2/furin family face a challenge common to other proteases: they must specifically cleave correct substrates in the presence of a considerable excess of incorrect sites, some of which are in the same molecule. This challenge is more strenuous for these enzymes, however, because such incorrect cleavage could well prove toxic to the cell, either due to misprocessed proteins which could aggregate or through the cleavage and inactivation of essential proteins. Such processing could also prove toxic to the organism, through ectopic release of effectors which should not normally be generated in

a given tissue type, through the release of misprocessed forms which could then exert inappropriate biological effects or even aggregate in the bloodstream, or through the inability to generate a correct effector because it has itself been cleaved improperly. Thus, unlike degradative proteases, processing proteases must be exceptionally accurate. However, in many cases they must also be very efficient, because they are often only exposed to their substrates briefly, especially for those enzymes functioning in the constitutive secretory pathway or its fungal equivalents. Enzymes such as Kex2 and furin therefore must combine efficient catalysis (high $k_{cat}/K_M$ for correct sites) with extremely stringent specificity (high *ratio* of correct $k_{cat}/K_M$ to incorrect $k_{cat}/K_M$: ref 37). This is in contrast to the related subtilisins, which are digestive enzymes acting either in proteolytic compartments or in the extracellular medium. Such enzymes have instead been optimized for a broad specificity, with lower maximal values of $k_{cat}/K_M$ and a much broader range of substrate sequences giving values of $k_{cat}/K_M$ close to the maximal value.[38,39] The means by which proteases of the Kex2/furin family harness the well-known serine protease mechanism to achieve the required efficiency and specificity are not yet fully clear, in large part due to the continued lack of a crystal structure for one of these enzymes. However, biochemical methods have proven fruitful in elucidating substrate specificity for these enzymes, and more recent work has begun to extend the resulting picture to the pre-steady-state level. The remainder of this review will focus on current understanding of specificity and its mechanistic basis in these proteases. For further information about the cellular factors involved in processing, the sorting signals and mechanisms involved in the proper localization of these enzymes, and the important role other enzymes such as carboxypeptidases or peptide-amidating enzyme play in carrying out subsequent reactions to finish the biosynthesis of mature effectors, the reader is referred to recent reviews of these topics.[2−4,40−43]

A working model for understanding the basis for specificity in these proteases has been developed thanks to pre-steady-state characterization of Kex2 protease, which has been the subject of more detailed enzymological characterization than other members of this family. Preliminary results for other proteases, especially furin and PC2, have allowed the refinement of this model, and on the basis of this combined body of work it is possible to estimate that a more complete picture of the mechanistic basis for processing protease specificity could be available in five to 10 years. Obviously, the availability of a crystal structure will be a great aid in this regard.

## III. Experimental Approaches for Studying Processing Protease Specificity

A number of experimental approaches can be envisioned for tackling the problem of protease specificity, with a combination of in vitro and in vivo experiments providing a desirable combination of kinetic rigor and physiological relevance. For example, simply expressing a fusion protein with a presumptive Kex2 cleavage site in *S. cerevisiae* for the purpose of bulk purification is an experiment in measuring Kex2 specificity, albeit a trivial one. Preparing a library of different substitutions within the fusion protein in an attempt to optimize cleavage would provide a more sophisticated picture of protease specificity in vivo, and synthesizing substrates for in vitro characterization of such a library would provide more detail still but would entail the development of an in vitro system. Thus, a wide variety of approaches and assays can be employed. Conceptually, these can be divided into in vivo and in vitro experiments.

Examining the specificity of processing proteases in vivo has proven surprisingly fruitful. Essentially, three main approaches have emerged. In the first, a broad body of experiments from a number of labs is surveyed to obtain a picture of which cleavage sites are favored by the enzyme under study. This approach has provided empirical evidence for preferences in furin, Kex2, PC1, and PC2.[43−45] An alternative approach is to take a single substrate and mutagenize it extensively, an approach successfully employed to characterize $P_2$ specificity in the Kex2 substrate pro-α-factor.[46] This provides more detailed information within a single, otherwise consistent sequence context, which is desirable because of the known interactions among subsites in these enzymes (refs 47 and 48; Rozan, L., Krysan, D. J., Rockwell, N. C., and Fuller, R. S., unpublished data). Finally, one can instead mutagenize the enzyme and see how this affects processing of a panel of substrates. Such work has been successfully carried out with furin[49] and with Kex2 (Bevan, A., Rozan, L., and Fuller, R. S., unpublished data; Bevan, A., Thomas, J. N., and Fuller, R. S., unpublished data; Rozan, L., Krysan, D. J., Rockwell, N. C., and Fuller, R. S., unpublished data), but the current lack of crystal structures limits the application of this approach at the present time.

Characterization of specificity in vitro is obviously a different proposition altogether. Although it is possible to assay prohormone processing directly in vitro in some cases, there are a number of drawbacks to such an approach, including the limited amount of substrate that may be available, the low sensitivity and low throughput often encountered in assaying such substrates, and the complications imposed by having an additional protein in the experimental system (such as aggregation). A variety of synthetic substrates developed to allow spectrophotometric or spectrofluorometric assays in general protease research have been used successfully in characterizing members of the Kex2/furin family (Figure 1), including *p*-nitroanilides,[50] peptidyl methylcoumarinamides (peptidyl-MCA substrates: refs 47, 48, and 51−53), and internally quenched fluorogenic peptide substrates (IQ substrates: refs 52 and 54−56). Additionally, peptidyl methylcoumarinesters (peptidyl-MCE substrates: ref 57) have been synthesized and employed in characterizing two members of this family, Kex2 and PC2.

While the use of such substrates must obviously be approached with caution due to concerns about

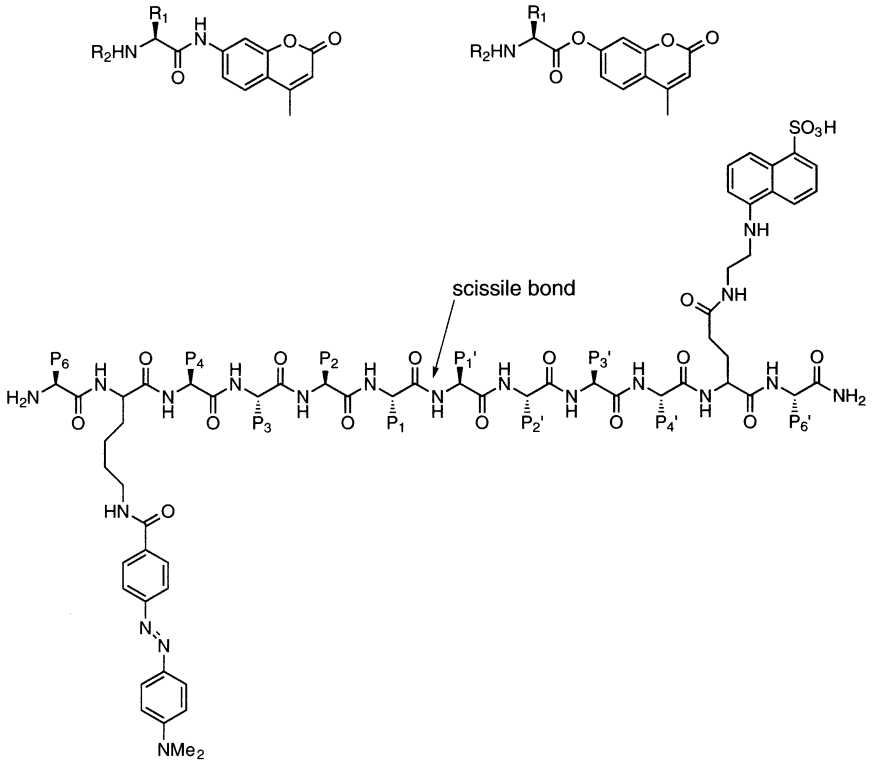



**Figure 1.** Structures of substrates for processing proteases. Peptidyl-methylcoumarinamides (peptidyl-MCA substrates, top left) allow fluorimetric detection of free 7-amino-4-methyl coumarin (AMC), which is released at the acylation step (Scheme 1). With peptidyl-methylcoumarinesters (peptidyl-MCE substrates, top right), hydrolysis occurs at a more labile ester linkage. In both cases, $R_1$ is the side chain of the $P_1$ residue and $R_2$ is the remainder of the peptide substrate. Internally quenched substrates (IQ substrates, bottom) allow fluorimetric detection of cleavage at an actual peptide bond; here, fluorescence of the EDANS chromophore (*e*thylene*dia*mine-*n*aphthalene *s*ulfonic acid) is quenched by the DABCYL chromophore (*d*imethylaminophenyl*azo*b*enzoyl*) until cleavage occurs. For the IQ substrate, the side chains are labeled according to the nomenclature of Schechter and Berger.[61]

physiological relevance, they offer a number of advantages that offset these potential disadvantages. First, such synthetic substrates offer high sensitivity and throughput. This allows rapid characterization of many sequences, which is vitally important to developing a complete understanding of steady-state specificity. Moreover, such substrates are not limited to the 20 side chains encompassed within the standard genetic code, permitting characterization with greater chemical detail. Additionally, such substrates permit the pre-steady-state dissection of the reaction mechanism, providing a detailed view of how specificity is generated, something not possible with physiological substrates. Furthermore, these molecules can also be examined using mass spectrometry, allowing the experimenter to bridge the gap between the behavior of the synthetic peptide and the authentic proprotein substrate. Finally, several approaches are available to allow active-site titration of these enzymes utilizing these small, sensitive substrates, allowing the measurement of true rate constants.[53,57−60]

The availability of both in vivo and in vitro techniques has benefited the field greatly. The combination of these various approaches and methodologies has permitted a detailed understanding of specificity and the kinetic basis for it in Kex2 protease and a steadily improving picture of the specificity of related enzymes.

## *IV. The Specificity of Kex2 Protease*

Kex2 specificity has been extensively studied both in vivo and in vitro, and its steady-state specificity has also been reviewed recently.[13] This enzyme has a number of known substrates, including the mating pheromone precursor pro-α-factor and pro-killer toxin. Pro-α-factor contains 2−4 copies of the α-factor pheromone (separated by Kex2 cleavage sites) in tandem behind a prodomain ending in another cleavage site, reminiscent of neuropeptide precursors such as POMC, while pro-killer-toxin is a folded precursor with multiple chains linked by disulfide bonds, reminiscent of proinsulin.[12] A number of authentic substrates for Kex2 are now known, and these suggest a preference for dibasic sites with Arg at $P_1$ (Table 1). The most important study of specificity in vivo utilized a library of pro-α-factor mutants containing all possible $P_2$ residues in the prodomain cleavage site, followed by a single copy of the mature α-factor sequence.[46] This library was expressed in a haploid *MAT*α yeast strain lacking the chromosomal genes for pro-α-factor. Thus, all production of mature α-factor (and therefore the ability of this strain to mate) relied on Kex2 cleavage of this single site. This allowed specificity to be assessed using a quantitative mating bioassay with a dynamic range of approximately 6 orders of magnitude. The results correlate well with in vitro work in which the appropriate substrates are available (Figure 2). In addition to the

**Table 1. Kex2 Cleavage Sites in Vivo**[a]

| cleavage site | sequence |
|---|---|
| k1 killer toxin (44↓45) | - -LLPR↓EA- - |
| k1 killer toxin (149↓150) | - -VARR↓DI- - |
| k1 killer toxin (188↓189) | - -YVKR↓SD- - |
| k1 killer toxin (233↓234) | - -VAKR↓YV- - |
| MFα1 pro-α-factor (85↓86) | - -LDKR↓EA- - |
| MFα1 pro-α-factor (104↓105)[b] | - -MYKR↓EA- - |
| KEX2 prodomain (109↓110) | - -LFKR↓LP- - |
| exo-1,3-$\beta$-glucanase (40↓41) | - -NKKR↓YY- - |
| MFα2 pro-α-factor (80↓81) | - -LAKR↓EA- - |
| MFα2 pro-α-factor (101↓102) | - -MYKR↓EA- - |
| *S. cerevisiae* HSP150 (71↓72) | - -KAKR↓AA- - |

[a] Eleven known or probable Kex2 cleavage sites from *S. cerevisiae* are listed. Residues surrounding the cleavage site are shown from $P_4$ to $P'_2$. The residue numbers of $P_1$ and $P'_1$ are given, and cleavage sites are indicated by arrows. This compilation is taken from ref 47. [b] The same cleavage site is repeated at 125↓126 and 145↓146.

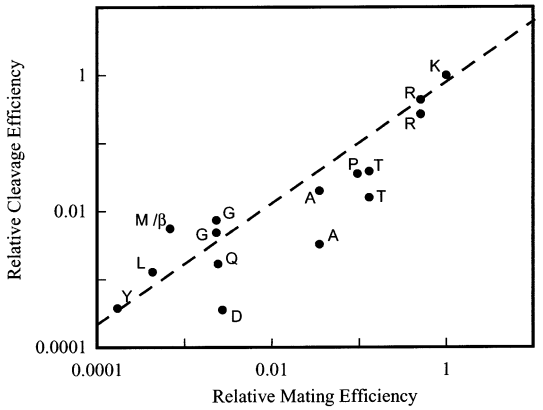
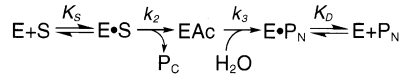



**Figure 2.** Values obtained from in vivo and in vitro studies on the $P_2$ specificity of Kex2 protease[46,52] were plotted against each other for a wide range of substrates containing $P_2$ residues ranging from tyrosine (Y, with $k_{cat}/K_M$ of $1.5 \times 10^4$ $M^{-1}$ $s^{-1}$: ref 52) to lysine (K, with $k_{cat}/K_M$ of $3.9 \times 10^7$ $M^{-1}$ $s^{-1}$: ref 52). The correlation between these data suggests that in vitro studies should give relevant information about Kex2 behavior in vivo. For $P_2$ glycine, threonine, and arginine, multiple substrates were examined in vitro, while the in vivo value for methionine was compared with an in vitro value for norleucine (indicated as M/$\beta$). This figure is modified from Figure 4A in ref 46.

expected preference for basic residues at $P_2$, these data demonstrated the existence of an apparent optimum for side chain volume.[46]

Much in vitro kinetic work has focused on the specificity of Kex2 protease.[47,48,52,58] Both peptidyl-MCA substrates and IQ substrates have been used to derive a picture of the enzyme's steady-state specificity, and this enzyme is unusually efficient at cleaving tripeptidyl-MCA substrates relative to other members of this family.[53,58] Peptidyl-MCA substrates and IQ substrates give essentially equivalent $k_{cat}/K_M$ values for equivalent sequences.[52] This suggests that amino acids C-terminal to the scissile bond (the prime-side in the nomenclature of Schechter and Berger: ref 61) are unimportant for substrate recognition by Kex2, although results obtained with a series of peptides based on an oxytocin cleavage site suggested that large residues were disfavored at the $P'_1$ position.[44] It is thus possible that certain residues will be disfavored on the prime-side, even though enzyme−substrate interactions with this portion of the substrate do not positively contribute to catalysis. Importantly, however, IQ substrates and peptidyl-MCA substrates do not behave the same way under saturating conditions. While peptidyl-MCA substrates exhibit proper Michaelis−Menten kinetics, IQ substrates instead exhibit some form of substrate inhibition.[52] Such behavior has been seen with furin and PC1/3 with IQ and peptidyl-MCA substrates,[48,56,62] and in one case has been ascribed to aggregation because it can be disrupted with detergent or carrier protein (BSA) in the case of PC1/3.[62] However, the substrate inhibition seen with Kex2 does not seem to be due to such a phenomenon, because it is seen in the presence of detergent.[52] Little is known about substrate inhibition with Kex2 in the presence of carrier protein, although in any case the observed disruption of substrate inhibition by BSA with PC1/3 need not indicate that the proposed aggregation model is correct.

**Scheme 1**




Studies on mice lacking functional carboxypeptidase E, responsible for subsequent removal of basic residues from many mammalian cleavage sites, have raised the possibility that PC1/3 and PC2 are susceptible to inhibition by accumulated product,[63] suggesting that these enzymes may suffer from both substrate inhibition and product inhibition, with release of the cleaved product therefore presumably being anomalously slow. However, it is clear that this is not the case for Kex2 (ref 64; for further discussion of this point, see the section on the mechanistic basis of specificity below). Additionally, pre-steady-state studies have demonstrated that PC2 exhibits rate-limiting acylation ($k_2$ in Scheme 1: ref 57), indicating that any inhibition by accumulated product is likely to occur through subsequent rebinding of the cleavage product to the enzyme rather than through intrinsically slow release of the N-terminal cleavage product after the deacylation step ($k_3$ in Scheme 1). Thus, the accumulated cleavage products in these mice are probably acting as competitive inhibitors of further processing without unusually slow off-rates, and the observed inhibition stems from the extremely high concentrations of these products that accumulate in secretory granules (see the section on PC2 specificity below).

The general behavior of Kex2 is roughly similar to other members of the subtilase superfamily, with a pH dependence suggesting a single ionization with optimal activity at neutral pH.[58,65] Earlier reports[14,66] do not correct for differential enzyme stability as a function of pH, which is a major concern.[58,67] Kex2 requires $Ca^{2+}$ ions for activity, and thus is sensitive to chelators such as EDTA.[68] Additionally, Kex2 can be inhibited by thiol-reactive compounds such as DTT and *p*-chloromercuribenzoate,[68] by peptidyl chloromethyl ketones and analogous compounds with appropriate sequences,[59] by derivatives of protein inhibitors such as eglin C,[50] by the serine protease inhibitor DFP,[68] and by heavy metals such as $Cu^{2+}$ and $Zn^{2+}$.[69] Interestingly, Kex2 is activated by $K^+$ and

**Table 2. Steady-State Kinetic Parameters for Kex2 Protease Specificity[a]**

| substrate | $k_{cat}/K_M$ ($M^{-1}$ $s^{-1}$) | relative $k_{cat}/K_M$ |
|---|---|---|
| **Ac*β*YKK↓MCA** | **$9.2 \times 10^4$** | **1** |
| Ac**A**YKK↓MCA | $1.2 \times 10^3$ | 0.013 |
| Ac**R**YKK↓MCA | $1.2 \times 10^5$ | 1.3 |
| Ac**Ç**YKK↓MCA | $4.9 \times 10^3$ | 0.053 |
| Ac**D**YKK↓MCA | <250 | <0.0027 |
| Ac**F**YKK↓MCA | $3.7 \times 10^4$ | 0.40 |
| Ac*χ*YKK↓MCA | $1.3 \times 10^5$ | 1.4 |
| **RJ*β*YKR↓EAEABR** | **$2.5 \times 10^7$** | **1** |
| RJ*β*Y**R**R↓EAEABR | $3.0 \times 10^7$ | 1.2 |
| RJ*β*Y**O**R↓EAEABR | $7.5 \times 10^6$ | 0.3 |
| RJ*β*Y**P**R↓EAEABR | $3.5 \times 10^6$ | 0.14 |
| **AcSLNKR↓MCA** | **$3.9 \times 10^7$** | **1** |
| AcSLN*β*R↓MCA | $2.2 \times 10^5$ | 0.0056 |
| AcSLN**A**R↓MCA | $1.3 \times 10^5$ | 0.0033 |
| AcSLN**D**R↓MCA | $1.4 \times 10^4$ | $3.6 \times 10^{-4}$ |
| AcSLN**Y**R↓MCA | $1.5 \times 10^4$ | $3.8 \times 10^{-4}$ |
| **RJ*β*YKR↓EAEABR** | **$2.5 \times 10^7$** | **1** |
| RJ*β*YK**K**↓EAEABR* | $3.6 \times 10^5$ | 0.014 |
| RJ*β*YK**O**↓EAEABR | $2.2 \times 10^5$ | 0.0088 |
| **AcP*β*YKR↓MCA** | **$3.4 \times 10^7$** | **1** |
| AcP*β*YK**K**↓MCA | $1.5 \times 10^5$ | 0.0044 |
| AcP*β*YK**Ç**↓MCA | <500 | $<1.5 \times 10^{-5}$ |

[a] Selected data for substrates from several different series are presented. For each series, the starting point is indicated in bold, as are the substituted residues. Ac = acetyl; *β* = norleucine; Ç = citrulline; *χ* = *β*-cyclohexylalanine; J = Lys(DABCYL), the IQ substrate chromophore; B = Glu(EDANS), the IQ substrate fluorophore; O = ornithine; MCA, methylcoumarinamide. Data are from refs 47 and 52. All substrates were examined at 37 °C.

other alkali metal cations with certain substrates, probably due to a specific allosteric interaction.[70] This interaction also changes the pre-steady-state behavior of Kex2 in cleavage of physiologically correct sequences substantially (see the section on potassium effects below).

The body of in vitro kinetic characterization performed to date shows that specificity is primarily generated at the $P_1$ position, with energetically significant contacts at $P_2$ and $P_4$ as well.[13,47,52] These subsites are also the principal positions used by subtilisins in subsite recognition, and in both the subtilisins and the Kex2/furin proteases there is considerable interaction between subsites (refs 47 and 71; Rozan, L., Krysan, D. J., Rockwell, N. C., and Fuller, R. S., unpublished results). Intriguingly, both subtilisins and Kex2/furin proteases tend to have one of two patterns, either relying extensively on $P_1$ and $P_4$ (e.g., furin and Savinase: refs 39 and 48) or else utilizing $P_1$ with accessory contacts at $P_2$ and $P_4$ (e.g., Kex2 and subtilisin BPN′: refs 39, 47, and 52), though the energetic contribution of the individual subsites to catalysis by the processing proteases is larger, as befits their higher substrate specificity.[48] However, it is clear that this pattern is not obligate for all members of the subtilase superfamily, because the mammalian cell-surface processing protease SKI-1 relies on the $P_4$ and $P_2$ positions for cleavage site selection, with little apparent specificity at $P_1$.[72]

## A. $P_4$ Specificity

Kex2 exhibits dual recognition of the $P_4$ residue,[47] with either aliphatic or basic side chains giving efficient cleavage (Table 2). In contrast, small side chains are disfavored, and the acidic side chain of Asp results in extremely poor cleavage. A substrate containing the aromatic Phe at $P_4$ is cleaved with a $k_{cat}/K_M$ value approximately 3-fold below that of the saturated analogue *β*-cyclohexylalanine, suggesting that the flexibility of an aliphatic side chain is slightly advantageous at this subsite. On the other hand, recognition of basic residues at $P_4$ occurs via an electrostatic interaction, because the substitution of citrulline for Arg at this position in otherwise identical substrates results in a defect of approximately 24-fold, corresponding to 2.0 kcal/mol of energy. Thus, either positive charge or a large aliphatic side chain is sufficient to satisfy the enzyme−substrate interaction at $P_4$.

## B. $P_2$ Specificity

Kex2 recognizes basic residues at $P_2$[52,58] with Arg, Lys, and ornithine giving approximately equal $k_{cat}/K_M$ values in otherwise identical IQ substrates (Table 2). This recognition is purely electrostatic, because a substrate containing a norleucine residue at this position (equivalent to the Lys side chain without the *ϵ*-amino group) exhibits a $k_{cat}/K_M$ equal to that of an otherwise identical substrate containing a $P_2$ Ala (Table 2). Thus, the contribution of the aliphatic portion of the Lys or Arg side chains to catalysis is negligible at this position, and basic side chains are recognized by purely electrostatic interactions. However, acidic or aromatic residues result in a larger defect than can be explained by the loss of these interactions, indicating that such residues are excluded from this position. It is possible that such side chains result in a deformation of the enzyme−substrate complex which disrupts additional interactions, either with the amide backbone of the substrate or with other side chains. Interestingly, Pro is well tolerated at $P_2$, and at least one Pro−Arg sequence is known to be cleaved by Kex2 in vivo (Table 1).

## C. $P_1$ Specificity

At $P_1$, Kex2 exhibits extremely stringent specificity for Arg. Neither Lys nor ornithine can substitute at this position despite the conservation of positive charge with both residues, with either residue introducing a $k_{cat}/K_M$ defect of at least 70-fold in an otherwise identical context (the energetic consequences of this substitution vary depending on the context, ranging from 2.6 to 3.8 kcal/mol depending on the leaving group and the nature of the $P_4$ residue: refs 47 and 52). Moreover, an otherwise identical substrate containing citrulline at this position (isosteric to Arg but uncharged) results in a much larger defect in $k_{cat}/K_M$ ($>10^5$-fold), indicating that the positive charge on the Arg side chain at $P_1$ contributes ≥6.8 kcal/mol to catalysis by Kex2 protease. This energetic contribution is substantially larger than those seen at $P_4$ (2.9 kcal/mol: refs 47 and 48) or $P_2$ (3.5 kcal/mol: refs 48 and 52), demonstrating the primary importance of electrostatic interaction with Arg at the $P_1$ position as a determinant of Kex2 specificity.

It is known that Kex2 exhibits other enzyme−substrate interactions, but none of these contribute

as much to catalysis. For example, Kex2 exhibits very weak preferences for basic residues at $P_6$ (approximately 2-fold: ref 48), and it is known that Asp is disfavored at the $P_3$ position by approximately 10-fold.[52] Further study is likely to focus on the prime-side, the energetic importance of interactions with the peptide backbone, and subsite interdependence.

## V. The Specificity of Furin

Furin was the first mammalian proprotein processing protease identified and remains the most extensively studied member of the seven known mammalian processing proteases. *fur* was identified as a Kex2 homologue in 1989 through a search of the human genome database.[73] *fur* had previously been identified by Roebroek, Van de Ven, and colleagues as a gene closely associated with the proto-oncogene c-*fes*/*feps* (*fes*/*fep* *u*pstream *r*egion).[74] Subsequent cloning of the full-length cDNA and expression confirmed the homology of furin with Kex2.[75−77]

Like other processing proteases of the constitutive secretory pathway, furin is a type I transmembrane protein, is a calcium-dependent serine protease with a subtilisin-like catalytic fold, and is a protease that cleaves on the C-terminal side of polybasic sequence motifs.[43] Furin is ubiquitously expressed in human tissue, and no tissue yet tested has failed to show evidence of furin expression.[78] Immortalized cell lines deficient in furin have been successfully established (LoVo and CHO RPE.40), although their viability may be because other members of this family (such as PACE4) continue to be expressed and may have overlapping substrate processing capability.[79,80] In contrast, −/− *fur* mouse embryos die early in development with defects in heart tube fusion and looping as well as failure of the embryo to undergo global axial rotation.[81]

Furin functions mainly in the constitutive secretory pathway and is predominantly, but not exclusively, localized to the TGN.[82] Furin is distributed through several processing compartments through an intricate series of intracellular trafficking steps that are dependent on signals and motifs present in the cytosolic tail.[83−85] These compartments are the trans Golgi network (TGN), the endosome, the plasma membrane, and the extracellular medium (as a result of C-terminal proteolysis releasing secreted or "shed" furin). Potential substrates have been identified for each of these compartments, and many of the molecular details of furin trafficking have been delineated. These topics have been recently reviewed in detail.[4,86]

Since furin is ubiquitously expressed, it is perhaps not surprising that a large number of potential substrates have been identified. To date, candidate substrates from the following general categories of propeptides have been identified: growth factors and hormones, cell surface receptors, coagulation factors, matrix metalloproteases, extracellular matrix proteins, secretases, bacterial toxins, and viral glycopeptides.[43,86] The number of putative substrates is growing rapidly, and a list of selected sites is provided in Table 3. It is important to note that many proteins have polybasic cleavage sites at the propeptide junction and, therefore, are cleaved by furin in vitro. However, such observations do not prove that a given protein is a physiological furin substrate. As mentioned earlier, other processing proteases are active in the constitutive secretory pathway, and the different roles of these processing proteases in protein processing have yet to be clearly defined.

**Table 3. Selected Furin Cleavage Sites in Vivo**

| proprotein | cleavage site | ref |
|---|---|---|
| **Viral Glycoproteins** | | |
| avian influenza HA (H5N1) | RRRKKR- | 108 |
| Borna disease virus | LKRRRR- | 167 |
| cytomegalovirus gB | HNRTKS- | 168 |
| Ebola Zaire GP | GRRTRR- | 94 |
| HIV gp160 | VQREKR- | 169 |
| human parainfluenza virus type 3 $F_o$ | DPRTKR- | 170 |
| measles virus $F_o$ | SRRHKR- | 171 |
| Newcastle disease virus $F_o$ | GRRQRR- | 95 |
| Sindbis virus gpE2 | SGRSKR- | 95 |
| respiratory synctial virus $F_o$ | KKRKRR-, NNRARR- | 95 |
| **Bacterial Toxins** | | |
| anthrax protective antigen | NSRKKR- | 88 |
| *Clostridium septicum* α-toxin | KRRGKR- | 172 |
| diptheria toxin | GNRVRR- | 173 |
| proaerolysin | KVRRAR- | 174 |
| *Pseudomonas aeruginosa* exotoxin A | RHRQPR- | 95 |
| Shiga toxin | ASRVAR- | 98 |
| **Serum Proteins and Coagulation Factors** | | |
| proalbumin | RGVFRR- | 86 |
| pro-factor IX | LNRPKR- | 175 |
| pro-factor X | LERRKR- | 176 |
| pro-von Willebrand factor | SHRSKR- | 75 |
| pro-protein C | RSHLKR- | 177 |
| **Growth Factors and Hormones** | | |
| bone morphogenic factor-4 | RRAKR- | 178 |
| pro-β-nerve growth factor | THRSKR- | 82 |
| pro-endothelin | LRRSKR- | 179 |
| pro-insulin-like growth factor | SVRAQR- | 91 |
| pro-parathyroid hormone | KSVKKR- | 180 |
| pro-parathyroid-related peptide | LRRLKR- | 181 |
| pro-transforming growth factor β1 | SSRHRR- | 182 |
| **Cell Surface Receptors** | | |
| hepatocyte growth factor proreceptor | EKRKKR- | 183 |
| insulin proreceptor | PSRKKR- | 5 |
| insulin-like growth factor proreceptor | PERKRR- | 184 |
| notch-1 proreceptor | PSRKRR- | 185 |
| **Extracellular Matrix Proteins** | | |
| bone morphogenic factor-1 | RSRSRR- | 186 |
| integrin α3 | PQRRRR- | 187 |
| integrin α6 | NSRKKR- | 187 |
| pro-fibrillin | RGRKRR- | 188 |
| MT-MPP1 | NVRRKR_ | 189 |
| stromelysin-3 | RNRQKR- | 190 |
| ZP1 | IARRRR- | 191 |
| ZP2 | SLRSKR- | 192 |
| **Other Proteins** | | |
| beta-site APP cleaving enzyme (BACE) | RLPR- | 193, 194 |
| ectodysplasin-A | RVRREKR- | 195 |

The substrate specificity of furin has been investigated by a number of groups using both in vivo and in vitro methods.[43] In vivo methods have focused on coexpression studies where, for example, a candidate

proprotein is introduced into a furin-deficient cell line (e.g., LoVo cells) and then shown to be expressed in unprocessed form. Recombinant furin is then coexpressed in the cell line to restore cleavage to the mature peptide. Site-directed mutagenesis of the putative furin recognition sites in the protein can then be used to define the sequence requirements for processing. The in vitro cleavage of purified wild type and variant proteins such as pro-renin,[87] pro-albumin,[88,89] anthrax protective antigen,[90] and pro-insulin-like growth factor I[91] has also been examined in a manner similar to that used in the in vivo studies. Kinetic studies have also been performed using fluorogenic peptidyl substrates derived from 7-amino-4-methylcoumarin (peptidyl-MCA substrates: Figure 1; refs 5, 48, 53, and 92) or containing functionality that generates internally quenched fluorescence.[55,56]

On the basis of in vivo studies of substrates such as pro-von Willebrand factor[75] and $\beta$-nerve growth factor,[82] furin was shown to cleave prosegments C-terminal to polybasic motifs. Based mainly on studies of pro-renin[87] and pro-albumin variants[88,89] as well as inspection of proposed physiological furin substrates, Nakayama proposed that furin preferentially recognized Arg−X−(Arg/Lys)−Arg;[93] this sequence is now frequently referred to as the "furin consensus cleave site". Thomas and co-workers also proposed that the minimal furin cleavage site is of the form Arg−X−X−Arg based on in vitro studies of furin cleavage of anthrax protective antigen variants.[90] In a 1997 review article, Nakayama further refined the consensus sequence and developed the following set of general rules for furin specificity, albeit with known exceptions:[43]

(i) At the $P_1$ position an Arg is essential.

(ii) In addition to the $P_1$ Arg, at least two out of the three residues at $P_2$, $P_4$, and $P_6$ are required to be basic for efficient cleavage.

(iii) At the $P_1'$ position, an amino acid with a hydrophobic side chain is not suitable.

In the sections below, we will summarize and discuss the specificity and kinetic studies that contributed to the development of these general rules as well as to subsequent advances in the understanding of furin specificity.

## A. $P_1$ Specificity

As indicated in the previous section, the consensus description of furin specificity stipulates that $P_1$ Arg is required for cleavage. Indeed, all biologically active substrates that are cleaved by furin have Arg at $P_1$ (see Table 3). A number of in vivo and in vitro studies of such substrates have also shown that replacement of $P_1$ Arg by site-directed mutagenesis results in essentially undetectable cleavage.[5,90,94,95] Similarly, mutation of the $P_1$ Arg at the intramolecular cleavage site of pro-furin prevents autocatalytic removal of the prodomain and results in an inactive enzyme.[96] However, the requirement for Arg at $P_1$ is not absolute, and one study has documented low levels of cleavage at a $P_1$ Lys site.[91] Consistent with these findings, a fluorogenic hexapeptidyl-MCA substrate containing a $P_1$ Lys and favorable contacts at $P_2$, $P_4$, and $P_6$ was cleaved by furin with a $k_{cat}/K_M$ defect of only ~160-fold relative to the equivalent substrate with Arg at $P_1$ (ref 48; Table 4). The fact that the substrate with Lys at $P_1$ can be cleaved with reasonable efficiency underscores the ability of basic residues at $P_2$, $P_4$, and $P_6$ to compensate for a poor contact at $P_1$. This behavior has also been observed with Kex2 cleavage, in which favorable $P_4$ contacts will compensate for unfavorable replacement of $P_1$ Arg with Lys (see the above section on Kex2 specificity).

**Table 4. Steady-State Kinetic Parameters for Furin Specificity[a]**

| substrate | $k_{cat}/K_M$ ($M^{-1}$ $s^{-1}$) | relative $k_{cat}/K_M$ |
|---|---|---|
| Hexapeptide Substrates | | |
| **AcRARYKR↓MCA** | **$2.6 \times 10^6$** | **1.00** |
| AcRARYK**K**↓MCA | $1.6 \times 10^4$ | 0.0062 |
| AcRARY**R**R↓MCA | $1.9 \times 10^6$ | 0.73 |
| AcRARY**A**R↓MCA | $1.7 \times 10^5$ | 0.065 |
| AcRA**K**YKR↓MCA | $8.3 \times 10^4$ | 0.032 |
| AcRA**A**YKR↓MCA | <1000 | <0.0004 |
| AcRA$\pi$YKR↓MCA | $4.4 \times 10^3$ | 0.0017 |
| Ac**K**ARYKR↓MCA | $2.2 \times 10^6$ | 0.85 |
| Ac**A**ARYKR↓MCA | $2.3 \times 10^5$ | 0.088 |
| Ac**A**A**K**YKR↓MCA | $7.9 \times 10^3$ | 0.0030 |
| Tetrapeptide Substrates | | |
| **AcRSKR↓MCA** | **$8.1 \times 10^3$** | **1.00** |
| Ac**K**SKR↓MCA | 15 | 0.0019 |
| Ac**O**SKR↓MCA | 29 | 0.0036 |

[a] All values are from refs 48 and 53. For each series, the starting point is indicated in bold, as are the substituted residues. Ac = acetyl; $\pi$ = norvaline; O = ornithine; MCA = methylcoumarinamide.

## B. $P_2$ Specificity

In contrast to Kex2 and mammalian enzymes such as PC2, furin specificity is not heavily reliant on contacts at $P_2$. As discussed above, the minimal furin cleavage site, Arg−X−X−Arg, does not contain a specific $P_2$ residue. In fact, several biologically active substrate candidates lack basic residues at $P_2$ (e.g., proaerolysin, *Pseudomonas aeruginosa* exotoxin, and Shiga toxin: refs 43, 97, and 98). It should be noted, however, that the vast majority of biologically active substrates shown to be cleaved by furin do, indeed, have $P_2$ Lys or Arg. Interestingly, site-directed mutagenesis of $P_2$ Arg to Ala in pro-albumin,[89] pro-insulin receptor,[5] and anthrax protective antigen[90] had little effect on cleavage efficiency. The relative unimportance of $P_2$ basic residues has been quantified by kinetic analysis of peptidyl-MCA substrates. Substitution of Lys with Ala at $P_2$ resulted in only a 10-fold decrease in $k_{cat}/K_M$ in the context of favorable contacts at $P_1$, $P_4$, and $P_6$, while the identity of the basic residue had no effect (ref 48; Table 4). One of the biological consequences related to the weak contribution of $P_2$ to furin specificity is that furin does not often cleave after paired basic sites without additional upstream basic residues. This feature distinguishes furin from processing proteases of the regulated secretory pathway, such as PC1 and PC2, that readily cleave neuroendocrine precursors at such motifs.

## C. $P_4$ Specificity

The minimal furin recognition site has been proposed to consist of Arg at $P_4$ and $P_1$ (see the general

discussion of furin specificity above). As with $P_1$, replacement of Arg in biological substrates has been shown to essentially abolish furin cleavage in examples such as pro-anthrax protective antigen[90] and insulin proreceptor.[5] Recognition at $P_4$ is also like that at $P_1$, and in contrast to that at $P_2$, because furin is able to discriminate between Arg and Lys at $P_4$. However, the preference for Arg at $P_4$ is not as stringent as that at $P_1$. An elegant demonstration of this is the series of experiments reported by Duguay et al., in which furin processing of pro-insulin-like growth factor IA (IGF-IA) mutants was examined.[91] IFG-IA contains a series of basic amino acids at four-residue intervals in the following pattern: $Lys_{65}$, $Lys_{68}$, $Arg_{71}$, $Arg_{74}$, and $Arg_{77}$. By manipulating these residues and determining the relative amount of cleavage at each position, the relative sequence preferences could be determined by a type of internal competition experiment. For example, 90% of wt protein was cleaved at $Arg_{71}$ while only 1% was cleaved at $Lys_{68}$. The $Arg_{71}$ cleavage site has a Lys at $P_4$ and shows that Lys can be tolerated at this position. Substitution of Arg for Lys at position 68 results in an IGF-IA mutant with two possible $P_1$ Arg positions, $Arg_{71}$ and $Arg_{68}$. $Arg_{71}$ has a $P_4$ Arg, while $Arg_{68}$ has a $P_4$ Lys. In this case, 54% of cleavage occurs at $Arg_{71}$ and 34% at $Arg_{68}$, and this demonstrates that Arg is favored over Lys at $P_4$.

Experiments with full-length proteins such as those just discussed are complicated by the local and global structural features of the protein and do not always lend themselves to clearly defined conclusions. Studies of fluorescent peptidyl substrates have allowed the quantification of the energetics of furin specificity at $P_4$. In a series of tetrapeptidyl-MCA substrates, replacement of $P_4$ Arg in the core sequence resulted in a 540-fold decrease in $k_{cat}/K_M$; this corresponds to a $\Delta\Delta G$ of 2.5 kcal/mol (ref 53; Table 4). A significant, if less dramatic, decrease in $k_{cat}/K_M$ was also observed in the context of hexapeptidyl-MCA substrates.[48] Substitution of Lys for Arg in this series resulted in a 30-fold reduction in $k_{cat}/K_M$ (Table 4). The smaller magnitude of the reduction in the hexapeptidyl series appears more consistent with the IGF-IA results than the reduction observed with the tetrapeptidyl series, but it should be noted that none of the substrates in Table 3 has a Lys at $P_4$.

Introduction of nonbasic residues at $P_4$ in peptidyl-MCA substrates has confirmed that, for substrates with $P_4$ Phe and Ala residues, little or no cleavage occurs with furin. In the tetrapeptidyl-MCA series, substitution of ornithine for Arg at $P_4$ was shown to result in a 280-fold reduction in $k_{cat}/K_M$ (ref 53, Table 4). Although there is a large decrease in reactivity, this suggests that at least some of the recognition of the $P_4$ Arg side chain involves the $\delta$-amino group, as it is slightly better than Lys at this position. The contribution of hydrophobic interactions with the aliphatic portion of the Arg side chain is suggested by the fact that in the hexapeptidyl-MCA series extension of $P_4$ Ala by two carbons to norvaline increases the $k_{cat}/K_M$ by at least 5-fold (Table 4).

The ability of furin to recognize aliphatic residues at $P_4$ has also been addressed with internally quenched (IQ) fluorogenic peptide substrates based on human parathyroid hormone (hPTH: ref 56), although substrate inhibition was not taken into consideration in this case. The pro-hPTH cleavage site lacks a $P_4$ Arg and differs substantially from the canonical consensus motif (Table 3). An IQ substrate based on this sequence is efficiently cleaved with a $k_{cat}/K_M$ that is essentially identical to an otherwise equivalent substrate containing $P_4$ Arg, suggesting that Val is able to substitute for Arg at $P_4$ in this context.[56] Although no comparison of Val with norvaline is available within consistent sequence contexts, it seems that the $\beta$-branch in the Val side chain may play an important role in hydrophobic binding at $S_4$. $P_4$ Val residues are present in hPTH as well as in albumin and the second autocatalytic furin cleavage site, and these sites occur with basic residues at $P_1$, $P_2$, and $P_6$ (Table 3), although in the context of pro-albumin the Arg at $P_6$ was shown to contribute only 5-fold toward furin cleavage of a site with Val at $P_4$.[89] Clearly, Val is a suitable $P_4$ residue for furin, and this fact serves to underscore the similarity between the $P_4$ specificity of furin and the dual basic/aliphatic $P_4$ specificity of Kex2. However, Kex2 seems to prefer aliphatic residues without a $\beta$-branch (such as Leu or Met: Table 1; ref 47), whereas furin seems to prefer $\beta$-branched side chain of Val and is less generally tolerant of hydrophobic residues at $P_4$.

## D. $P_6$ Specificity

One feature of furin specificity that distinguishes it from some other processing proteases is its ability to recognize basic residues at $P_6$. $P_6$ was first identified by Nakayama and co-workers as a position contributing to furin specificity as part of in vivo studies examining the sequence requirements for constitutive processing of pro-renin mutants in CHO cells.[87] They found that introduction of a $P_6$ Arg into an otherwise nonprocessed pro-renin led to 30% cleavage, a level that was one-third that observed with the corresponding $P_4$ Arg variant. Indeed, inspection of the furin substrates in Table 3 clearly shows that many contain $P_6$ basic residues and both Lys and Arg appear with nearly equal frequency.

The contribution of $P_6$ binding to furin specificity has been quantified using hexapeptidyl-MCA substrates.[48] As shown in Table 4, the presence of either Arg or Lys at $P_6$ increases $k_{cat}/K_M$ by 10-fold relative to the otherwise identical substrate with Ala at $P_6$ regardless of the nature of the $P_4$ residue.[48] The fact that $P_6$ Arg increases $k_{cat}/K_M$ for substrates with both favorable and unfavorable $P_4$ contacts is in contrast to the interdependence of the $S_4$ and $S_1$ subsites reported in the case of Kex2,[47] as well as the subsite interdependence observed with the subtilisins.[71] Prior to these experiments, subsite independence had not been observed within the subtilisin family of serine proteases. $P_6$ interactions with furin also have effects on the binding of several protein inhibitors to furin, as discussed in detail in the section on inhibition. These data are also in good agreement with the 5-fold increase in cleavage seen with pro-albumin upon introduction of an Arg at $P_6$.[89]

## E. P′ Specificity

Peptidyl-MCA substrates necessarily lack P′ contacts, and some studies found that such substrates were much less reactive with furin than either full-length protein substrates or IQ substrates. For example, Thomas and co-workers found that anthrax protective antigen was cleaved by furin with a $k_{cat}/K_M$ 5000-fold higher than the tetrapeptidyl-MCA substrate corresponding to the $P_1-P_4$ residues of the protective antigen cleavage site. On the other hand, the IQ substrate containing the same $P_1-P_4$ residues and P′ residues was only 2-fold less reactive.[55] These authors, as well as others, speculated that furin may require prime-side contacts for optimal efficiency and that one of the reasons for this reactivity difference was the lack of crucial P′ contacts in the peptidyl-MCA. However, more recent results with hexapeptidyl-MCA substrates with good contacts at $P_1$, $P_2$, $P_4$, and $P_6$ have shown that $k_{cat}/K_M$ values within 2-fold of those for anthrax protective antigen are observed.[48]

Although these results indicate that contacts with residues C-terminal to the scissile bond are not required for efficient furin cleavage, it appears that some residues are more commonly encountered in putative biological substrates and that some P′ residues are unfavorable. For example, one-third of the substrates listed in Table 3 have serine at $P_1'$, and Tyr and Phe are also well represented. In contrast, bulky aliphatic residues such as Leu and Val are absent from this list, as are basic residues at either $P_1'$ or $P_2'$. Acidic residues (Asp and Glu) seem to be tolerated at $P_1'$, as is His.

In vitro studies have confirmed that certain prime-side residues interfere with furin processing. Brennan and Nakayama have shown that replacement of $P_1'$ Asp with Lys in proalbumin abolishes furin cleavage.[89] Another report examined some variations in P′ residues with IQ substrates and found that replacement of $P_1'$ Ser with Val generates a non-cleaved substrate, while introduction of Gly at the same position decreases $k_{cat}/K_M$ by 10-fold.[56] On the basis of these results, Lazure and Nakayama have independently proposed that efficiently cleaved furin substrates cannot contain bulky hydrophobic residues at both $P_1'$ and $P_2'$, nor can there be a Lys at either position.[43,56] Indeed, two IQ sequences reported by Lazure and co-workers actually behaved as specific furin inhibitors, although the exact nature of this inhibition is difficult to interpret because their data was complicated by substrate inhibition (see the discussion of furin substrate inhibition below). Still, it is clear that certain residues are excluded by furin on the C-terminal side of the scissile bond.

## F. Substrate Inhibition

As alluded to above, furin can be inhibited by some substrates at high substrate concentration. Inspection of the saturation curves reported by Lazure and by our laboratory[48,56] indicate that velocity decreases after the substrate concentration reaches a certain threshold which is dependent on the substrate and can be as low as 3−5 $\mu$M,[48] consistent with the binding of a second substrate molecule which inhibits the enzyme.[99] This behavior could be influenced by the $P_6$ residue, because substitution of Ala for Arg at $P_6$ resulted in an apparent affinity for the second, inhibitory site approximately 30-fold lower than the equivalent value for the $P_6$ Ala substrate.[48] Substrate inhibition was also somewhat sensitive to pH, and it was not observed with tetrapeptidyl-MCA substrates.[5,48,53] Therefore, in the case of furin, substrate inhibition seems to be dependent on substrate length and, possibly, on the number of basic residues. Substrate inhibition has also been observed in the case of Kex2 under saturating conditions with IQ substrates,[52] but no substrate inhibition has been observed with Kex2 with either tetrapeptidyl-MCA or hexapeptidyl-MCA substrates.[48,52,58] A possible explanation for this behavior is that furin and Kex2 may have a second, inhibitory site that interacts with a second substrate molecule at high concentrations, with the Kex2 site unable to accommodate the coumarin leaving group of peptidyl-MCA substrates. Whether this hypothetical site would be accessible or relevant for a second proprotein substrate in vivo remains unclear.

## G. Homology Models and Mutational Studies Toward a Structural Basis of Furin Specificity

Since no X-ray crystallographic data are available for furin or any other subtilisin-like processing protease, any structural basis for furin specificity is necessarily based on indirect information obtained by analysis of homology models, sequence alignments, and furin variants generated by site-directed mutagenesis. Siezen has proposed a model for the catalytic domain of furin that is based on the prokaryotic subtilisins thermitase, subtilisin Carlsberg and subtilisin BPN′,[100] and a similar study has been reported for PC1/3 and PC2.[101] Inspection of these models as well as of sequence alignments[34] reveal a striking abundance of negatively charged, acidic residues in the regions of furin predicted to interact with substrate. Additionally, there appears to be an insertion loop in furin that also contains numerous negatively charged residues and that may contribute to the $S_6$ site (ref 34; degradative subtilisins have no known $P_6$ specificity).

Creemers, van de Wen, and co-workers have reported the only series of furin variants with site-directed mutations designed to probe the contributions of specific residues to substrate binding.[49] Furin variants were coexpressed in COS-1 cells with both wildtype and variant pro-von Willebrand factor (pro-vWF), and the extent of processing was compared. On the basis of the homology model, $Asp_{199}$ was predicted to be at the bottom of the $S_1$ binding pocket and, consistent with this prediction, $D_{199}$N-furin was devoid of detectable pro-vWF processing activity. Similarly, $Asp_{47}$ was predicted to be crucial to $P_2$ basic residue binding and substitution of Thr for Asp at this position led to severely impaired processing of wt pro-vWF. The magnitude of the effect of $D_{47}$T on processing is surprising given the fact that kinetic data have shown that $P_2$ is relatively unimportant to furin specificity. Again, it is difficult to interpret

this result with confidence because of the lack of structural data, but one cannot exclude the possibility that $Asp_{47}$ may actually contribute to binding at one of the more important furin subsites such as $S_1$ or $S_4$ instead of to $S_2$, or it may result in substantial changes in the environment of the active site which are generally unfavorable for catalysis.

Two acidic residues predicted to be involved in $P_4$ binding were also mutated to nonacidic residues ($E_{129}V$ and $D_{126}N$) and both variants showed decreased activity toward pro-vWF as well as decreased autoprocessing. Finally, a number of mutations were made in acidic residues predicted to be remote to the substrate binding sites and these, too, led to variants with decreased activity. These results further emphasize the need for cautious interpretation in the absence of structural data, because mutation of acidic residues may affect protein folding, confirmation and stability by disruption of crucial salt bridges or hydrogen bonds in addition to, or in place of, direct effects on substrate binding.

Another protein engineering-based approach to obtaining information about the structural basis of furin specificity is to introduce acidic residues at key positions in bacterial subtilisins that do not normally cleave at basic residues. Wells and co-workers engineered a subtilisin BPN′ with $Gly_{166}$ replaced by Asp (this position is equivalent to $Asp_{199}$ in furin) and showed that it cleaved substrates with Arg or Lys at $P_1$ at increased rates compared to wt enzyme.[102] Later, this work was extended and a variant termed "furilisin" was engineered which showed specificity for basic residues at both $P_1$ and $P_4$.[103] These studies provide further support for the association of $Asp_{199}$ with $P_1$ binding and $Glu_{129}/Asp_{126}$ with $P_4$ binding in furin. However, both of these subtilisin variants retained substantial activity against hydrophobic sequences and were unable to discriminate between Lys and Arg at $P_1$, in contrast to furin and Kex2.

## H. pH Dependence of Furin Processing

Like bacterial subtilisins, furin is synthesized as a zymogen that undergoes proteolytic removal of an N-terminal, proregion to generate the active protease. The proregions of bacterial subtilisins remain associated with the enzyme after autocatalytic cleavage and act as inhibitors that are finally degraded to release free protease. Furin is activated through a similar series of events. Thomas and co-workers have shown that the furin propeptide undergoes two specific cleavages that are dependent on pH (refs 96, 104; reviewed in ref 4). In the ER, pro-furin autocatalytically cleaves the proregion at the sequence $-RTKR_{107}-$ and, after processing, furin is transported to the TGN with the proregion still associated as a stable complex. This initial cleavage site is a consensus furin site and occurs in the neutral pH of the ER. Once in the TGN, the associated proregion is cleaved at $-RGVTKR_{75}-$ and the proregion is released to give active furin that is able to process proteins in trans. The TGN is an acidic compartment with pH 6, and the second cleavage requires such acidic conditions to occur; furin will not cleave the associated proregion at neutral pH, and in vitro cleavage of peptide substrates based on these sequences demonstrates that the peptide substrate mimicking the second site is indeed more efficiently cleaved at low pH, although structural changes in the furin−propeptide complex may also be involved.[105] Limited digestion of the furin/propeptide complex with trypsin will also generate active furin at either pH 6 or pH 7.5, showing that removal of propeptide is required for activation and that pH-dependent conformational changes in the structure of the catalytic domain are unlikely to be solely responsible for activation.

The second cleavage site is not a canonical furin sequence in that it does not have a $P_4$ Arg but rather has a $P_4$ Val.[96,105] This site does, however, contain $P_6$ Arg as well as $P_2$ and $P_1$ basic residues, compensating for the lack of the usual $P_4$ Arg. Indeed, a proregion variant with the $P_6$ Arg replaced by Ala was less active than wildtype; the activity was, however, restored to wild-type levels after trypsin cleavage. Thomas and co-workers have asserted that $P_6$ Arg is, therefore, essential for pH-dependent cleavage of the proregion. Interestingly, introduction of Arg at $P_4$ in this site results in an inactive enzyme that is unable to exit the ER,[105] suggesting that a pH-insensitive cleavage site at this position interferes with the intramolecular chaperone function of the propeptide.

Although little in vitro kinetic data are available regarding the effects of pH on proprotein processing by mature furin, it is known that furin tolerates slightly acidic pH. The effects of pH were assayed with a small set of hexapeptidyl-MCA substrates[48] and, curiously, lower pH was found to decrease $k_{cat}/K_M$ by 5-fold for AcRARYKR-MCA relative to neutral pH, although $k_{cat}/K_M$ for the corresponding $P_6$ Lys and Ala substrates was little affected by the lower pH. As discussed earlier, acidic reaction media also reduced the severity of the substrate inhibition observed with the hexapeptidyl-MCA substrates. Clearly, the pH of the reaction media or the intracellular compartment has an important effect on furin processing, but general conclusions about the nature of these effects await more detailed kinetic studies as well as structural information.

## I. Biological Consequences of Furin Cleavage Sites: Viral Glycopeptide Processing and Viral Pathogenicity

As is evident from Table 3, furin has been implicated in the processing of a number of viral glycopeptides. Since furin is ubiquitously expressed, such processing could, in principle, occur in any tissue that could also support viral replication. Furin is not the only protease that is involved in viral glycopeptide processing. For example, most avian and mammalian influenza viruses have hemagglutinin (HA) glycopeptides that contain cleavage sites at single Arg residues.[106] Clearly, these cannot undergo furin-mediated processing and are instead processed by proteases expressed by specific cell types. Of the 14 subtypes of avian influenza, 12 have HA molecules that are cleaved at single Arg residues.[107] These subtypes cause only a localized respiratory infection. This is in contrast to two subtypes, H5 and H7, that

cause systemic, fatal infection resulting from viral production in all organs (these two subtypes are also known as fowl plague). The virulent subtypes both contain polybasic cleavage sites suitable for cleavage by furin.[108,109] In this example, viral tropism is strongly associated with the introduction of a furin cleavage site that allows processing in a wide range of host tissue. More recently, Hong Kong experienced a human outbreak of an exceptionally virulent influenza that was shown to be H5N1, a highly pathogenic fowl plague virus.[110] Sequencing of the HA gene of this subtype showed that it contained an insertion immediately upstream of the cleavage site resulting in a polybasic tract (-RERRRKKR-: ref 110) that may very well have contributed to its high lethality (30% case-fatality) through more efficient cleavage by furin or other members of this family, such as PC6. Indeed, in vitro cleavage of a series of internally quenched peptide substrates demonstrated that this insertion was able to confer ~5-fold increase in cleavage by furin relative to the sequence lacking the insertion.[111]

An additional example where the pathogenicity of a virus may be altered by the mutation of its glycopeptide processing site is that of the notorious hemorrhagic fever virus, Ebola.[94] Klenk and coworkers have shown that Ebola virus glycopeptide is processed at the furin cleavage site -RVRR- and that mutation of $P_1$ from Arg to Ser, or treatment with a furin inhibitor, blocks glycopeptide cleavage. Additionally, expression of Ebola glycopeptide in furin-deficient LoVo cells led to no cleavage unless a vector with recombinant furin was coexpressed. Interestingly, the strains of Ebola that are human pathogens, Ebola Zaire, Sudan, and Ivory Coast, all have glycopeptide cleavage sites that are furin consensus sequences (-RXR/KR-) whereas Ebola Reston, which is not a human pathogen, instead has a cleavage site that has Lys at $P_4$. Consistent with expectations, Ebola Reston glycopeptide is less efficiently cleaved in HeLa cells than are glycopeptides from the other strains of Ebola. Although it is not yet clear if cleavage of Ebola glycopeptide is required for pathogenicity, it is intriguing to consider that the decreased pathogenicity of Ebola Reston is because its glycopeptide contains a suboptimal furin cleavage site.

## *VI. The Specificity of PC1/3 and PC2*

PC2 and PC1/3 were originally isolated as the enzymes responsible for cleaving the dibasic sites found in proinsulin,[16−19] which have basic residues at $P_1$ and $P_2$ but not at $P_4$, although subsequent work on POMC demonstrated that both enzymes were also capable of cleaving at sites with basic $P_4$ residues.[112] Thus, one might expect that these enzymes would display specificity patterns similar to that of Kex2 rather than that of furin, with the ability to cleave at sites with or without basic $P_4$ residues. Like Kex2 and furin, both of these enzymes have now been purified to near homogeneity,[113,114] permitting examination of their specificity without concerns about contaminating activities. The biogenesis of active enzyme is more complicated with both of these enzymes than is the case for either Kex2 or furin, with PC1/3 undergoing C-terminal proteolysis which may result in more active (but less stable) enzyme[113] and PC2 undergoing intramolecular activation late in the secretory pathway as a consequence of the action of its intramolecular chaperone, 7B2.[40,115] The specificity of both of these enzymes has been the subject of a recent review,[45] and we therefore present only a brief summary of the major results obtained to date.

### A. The Specificity of PC1/3

The initial purification of PC1/3 reported by Lindberg and co-workers yielded an enzyme with much lower specific activity than is seen with Kex2 or furin,[113] and subsequent work has borne out this early finding.[56] Although the published pH dependence for PC1/3 does not correct for potential effects on stability rather than catalysis, it is clear that PC1/3 exhibits optimal activity over a range from pH 5−7, permitting it to be active both in the trans Golgi network and in the more acidic environment of the mature secretory granule.[113] PC1/3 undergoes C-terminal aotoproteolytic processing resulting in several forms of the enzyme,[113,116,117] and some reports indicate that these forms may exhibit slight changes in kinetic behavior and pH optimum.[116,117] Further work on this enzyme[53] has demonstrated that it cleaves tripeptide substrates very poorly, implying the need for a $P_4$ residue, but it is clear that that $P_4$ residue need not be a basic residue.[56,118] PC1/3 does not seem to rely on residues beyond $P_4$,[56] and $P_3$ substitutions produce only minor effects.[53,56,118] In a study of several peptidyl-MCA substrates, substitution of Ala for Lys at $P_2$ produced only a 5-fold defect,[53] and a study of unmodified peptides showed a 12-fold defect for the same substitution with Val at $P_4$ and a 3-fold defect with Arg at $P_4$.[118] However, a $P_2$ His reduced cleavage much more drastically.[118] In vivo, processing of mouse proinsulin isoforms in mice lacking functional PC2 demonstrates that basic residues at $P_4$ (found in proinsulin I) confer an ~3-fold advantage over sites lacking such residues (found in proinsulin II: ref 119). Thus, PC1/3 seems to prefer basic residues at $P_4$ and $P_2$ for efficient cleavage, although it has been proposed that basic residues at $P_6$ or $P_8$ may be able to compensate if none are present at $P_4$ and/or $P_2$ (for further discussion of this point, see ref 45). It is unclear whether such contacts indicate the presence of a true $S_6$ or $S_8$ in PC1/3; it is also possible that these substrate residues are binding to the normal $S_4$, with concomitant distortion of the substrate backbone.

The energetic consequences of $P_1$ residues other than Arg are not yet known for PC1/3. There are a few examples of proproteins with $P_1$ Lys which seem to be cleaved by PC1/3, but these are rare, and it is not yet known whether other elements in these sequences are required to permit cleavage. It is more clear that PC1/3 cannot tolerate certain residues C-terminal to the scissile bond.[45] In the $P_1'$ position, introduction of Lys or Gly residues was sufficient to reduce cleavage over 40-fold.[56,118] Moreover, basic residues are clearly disfavored at the $P_2'$ posi-

**Table 5. Steady-State Kinetic Parameters for PC2[a]**

| substrate | $k_{cat}/K_M$ ($M^{-1}$ $s^{-1}$) | relative $k_{cat}/K_M$ |
|---|---|---|
| **VPEMEKR↓YGGFM** | **$4.6 \times 10^4$** | **1.0** |
| **A**PEMEKR↓YGGFM | $3.1 \times 10^4$ | 0.67 |
| V**A**EMEKR↓YGGFM | $3.1 \times 10^4$ | 0.67 |
| VP**A**MEKR↓YGGFM | $4.5 \times 10^4$ | 0.98 |
| VPE**A**EKR↓YGGFM | $3.8 \times 10^3$ | 0.083 |
| VPEM**A**KR↓YGGFM | $3.3 \times 10^4$ | 0.72 |
| **R**PEMEKR↓YGGFM | $5.4 \times 10^4$ | 1.2 |
| V**R**EMEKR↓YGGFM | $4.5 \times 10^4$ | 0.98 |
| VP**R**MEKR↓YGGFM | $9.4 \times 10^4$ | 2.0 |
| VPE**R**EKR↓YGGFM | $6.6 \times 10^4$ | 1.4 |
| VPEM**R**KR↓YGGFM | $2.5 \times 10^4$ | 0.54 |

[a] The canonical sequence is indicated in bold, as are the substituted residues. Data are from ref 121. All substrates were examined at 37 °C and consisted of the indicated sequence placed between an anthranilic acid (on the N-terminus) and a nitrotyrosine residue (on the C-terminus), generating internally quenched substrates (ref 196).

tion[56,118] These in vitro results are borne out by examination of the known data on in vivo cleavage sites.[45]

## B. The Specificity of PC2

Lindberg and co-workers have also reported the purification of PC2.[114] The maturation of PC2 is a complicated process, as PC2 requires either coexpression with the neuroendocrine protein 7B2 or incubation with 7B2 in the presence of Golgi membranes to achieve a catalytically active conformation.[120] Thus, PC2 maturation finishes much later in the secretory pathway than is seen with other members of this family, and this enzyme displays a narrower pH optimum near pH 5.[114] 7B2 contains two domains, an N-terminal domain that stimulates PC2 exit from the ER and permits its maturation late in the secretory pathway[120] and a C-terminal domain containing a basic motif that acts as a potent inhibitor of PC2. PC2 has been successfully active-site titrated both with a peptide derived from this inhibitory 7B2 sequence and by initial burst titration with a peptidyl-MCE substrate.[57,60]

The specificity of PC2 over the residues from $P_7$ to $P_3$ has been investigated by examining substrates containing Ala or Arg substitutions at each of these positions within a series of internally quenched peptides based on a PC2 cleavage site found in proenkephalin (ref 121; Table 5). As can be seen, substitutions at $P_7$, $P_6$, $P_5$, and $P_3$ had little effect. However, Ala was not tolerated at $P_4$, which was also seen in a study of prodynorphin cleavage.[63] In contrast, either Arg or the physiological Met were well tolerated at the proenkephalin site,[121] indicating that the $P_4$ specificity of PC2 is similar to that of Kex2, with dual recognition of either basic or aliphatic residues (see the section on Kex2 specificity above).

A similarly rigorous study of substrate recognition at $P_2$ and $P_1$ is unavailable for PC2. It is known that the enzyme has a clear preference for basic residues at $P_2$,[45] and a peptidyl-MCA substrate with Ala at $P_2$ was cleaved very poorly by PC2 (~3.8 kcal/mol defect: ref 121), confirming a need for basic residues at $P_2$, but this substrate was not totally consistent with other peptidyl-MCA substrates examined in this study, so it is possible that the observed defect includes other effects as well as the $P_2$ preference.

$P_1$ specificity is somewhat relaxed in PC2, with little discrimination between Arg and Lys in some cases.[121] However, it is also established that $P_1$ Lys can substantially reduce cleavage in other sequences (relative to $P_1$ Arg: ref 63), so there are likely to be additional factors (such as the precise nature of enzyme−substrate contacts at $P_2$ and $P_4$) which have yet to be fully elucidated. It is known that PC2 is able to cleave sites with Pro in the $P_1'$ position (in contrast to subtilisins: refs 39 and 63), and there is also an example of PC2 cleavage of a site containing Arg residues at $P_1$ and $P_8$.[63] It is possible that this last site is recognized by an aliphatic residue at $P_4$ (Val), and the $P_2$ residue in this case (Thr) may not be terribly disfavored (by analogy to $P_2$ specificity in Kex2: Figure 2).

The kinetic behavior of PC2 has also attracted attention recently due to studies of mice lacking functional carboxypeptidase E,[122] the enzyme responsible for removing basic residues from the N-terminal cleavage product after endoproteolytic cleavage by Kex2/furin family members.[41] The accumulation of peptides containing basic residues in these mice is associated with lower endoproteolytic activity and the accumulation of precursors awaiting endoproteolytic cleavage (reviewed in ref 41), leading to the suggestion that the enzymes of the secretory granule, such as PC1/3 and PC2, exhibited product inhibition.[63] Such product inhibition could arise either due to very tight binding of the N-terminal cleavage product ($P_N$ in Scheme 1) to the enzyme, such that product release becomes rate-limiting, or due to the presence of very high concentrations of product in vivo. However, as discussed below (see the section on mechanistic basis for specificity below), PC2 is now known to exhibit rate-limiting acylation.[57] Therefore, product release cannot be rate-limiting. It therefore seems likely that the apparent product inhibition stems from the accumulation of extremely high concentrations of peptide in the secretory granule, such that the enzyme is inhibited by these molecules after cleavage rather than during the course of the catalytic cycle.

## *VII. The Specificity of Other Family Members*

The remaining mammalian enzymes have not been characterized in similar detail, partly because these enzymes frequently have rather low specific activity compared to Kex2 and furin. Preliminary characterization of crude PC4 has been reported,[123] and this enzyme was found to have optimal activity at neutral pH, with the ability to cleave several substrates containing different residues at $P_4$ (Arg, Lys, or Leu) and $P_2$ (Arg, Lys, or Glu, with Ala resulting in less efficient cleavage). PC4 was unable to cleave a tripeptide substrate in this study, indicating a requirement either for an aliphatic/basic $P_4$ side chain (similar to Kex2 and PC2) or the presence of enzyme−substrate contacts with the peptide backbone of the $P_4$ residue.

Preliminary characterization has also been reported for PC7.[124] This enzyme has a slightly broader

pH optimum (pH 6−7), more reminiscent of furin. PC7 was unable to cleave substrates lacking Arg at $P_4$[123,124] but was able to cleave a substrate with $P_2$ Ala with only a 5-fold defect,[124] suggesting a specificity pattern similar to that of furin. Analysis of several tetrapeptidyl-MCA substrates and peptide substrates indicated that the $K_M$ values for the peptidyl-MCA substrates were substantially higher than those for the peptide substrates, indicating either recognition of the $P_1'$ residue or additional factors that have not yet been elucidated.[124] The peptide substrates examined in this study both had basic $P_6$ residues, which the tetrapeptidyl-MCA substrates obviously lacked, so it is possible that PC7 may recognize the $P_6$ residue.

Preliminary characterization of partially purified PC5/6 has also recently been reported.[125] Although the presence of other proteases in the final fraction makes conclusions about specificity somewhat tenuous, it is clear that this enzyme is able to cleave after dibasic sites[125] as well as at cleavage sites modeled on viral glycopeptide cleavage motifs.[111] There may not be a strict requirement for a $P_4$ residue, and the enzyme may not discriminate between Arg and Lys at $P_1$,[125] but firm conclusions on these points await characterization of homogeneous enzyme. Even less has been reported about the behavior of PACE4. The maturation of PACE4 is quite slow compared to other members of this family, and recent work indicates that a C-terminal sequence may actually slow the exit of PACE4 from the ER.[126] This enzyme seems to require Arg at $P_4$ and $P_1$, like furin,[127−129] but a quantitative study of enzyme−substrate recognition for this enzyme has not been reported.

The characterization of Kex2/furin processing proteases from lower eukaryotes has also been neglected to date, with Kex2 being the only enzyme to be purified and subjected to detailed characterization. Preliminary characterization of krp1 from *Sch. pombe* has been reported, and this enzyme is known to cleave after basic motifs,[27] but little else is known about other fungal members of this family. Finally, it is also possible that some degradative subtilases may exhibit similar specificity patterns[130] and could thus provide interesting comparative data as to the roles of the P-domain (which is absent in such enzymes) and of the pre-steady-state behavior of the processing proteases (see the section on mechanistic basis of specificity below).

## *VIII. Development of Inhibitors for Kex2/Furin Proteases*

Many proteases localized to degradative compartments such as the yeast vacuole or the mammalian lysosome can be potently inhibited by cytosolic proteins, presumably as insurance against wholesale proteolysis in the case of transient leakage from such compartments. Due to the low expression, restricted localization, and stringent substrate specificity of the kex2/furin proteases, it is unclear whether such inhibitors will be found for all of these proteases, although the cytosolic serpin PI8 can act as a furin inhibitor.[131,132] However, several naturally occurring sequences are known to inhibit processing proteases, including the prodomains of the proteases themselves and the neuroendocrine proteins 7B2 and proSAAS.

The prodomains are known to be critical for folding of Kex2, furin, degradative subtilisins, and the unrelated α-lytic protease.[4,13,96,105,133−135] In the case of the Kex2/furin family, these sequences contain multiple cleavage sites and are potent inhibitors until the second site is cleaved (as discussed above for the case of furin). Peptides based on the cleavage sites found at the C-terminus of processing protease prodomains are frequently potent inhibitors of these enzymes. For example, a synthetic 24-mer containing the C-terminus of the rat PC7 prodomain inhibited rat PC7 with a $K_i$ of 7 nM.[136] Two other regions of the PC7 prosegment lacking the C-terminal sequence did not exhibit significant effects on PC7 activity. Prodomain inhibitors may act as slow-binding inhibitors,[136,137] but this is not always the case.[138]

As discussed above (under the specificity of PC2), 7B2 contains both an N-terminal region required for PC2 maturation and a C-terminal region which is a potent PC2 inhibitor. The interactions between 7B2 and PC2 have been the subject of detailed recent reviews,[40,45] and a comprehensive review of 7B2 itself has also recently appeared,[139] so comparable detail will therefore not be offered here. At the risk of redundancy, however, some information will be repeated to provide a basis for comparison with the interaction between proSAAS and PC1/3.

7B2 is known to bind proPC2 in the ER.[140] Pulse-chase studies have indicated that, whereas newly synthesized 7B2 rapidly acquires the capacity to bind proPC2 (i.e., PC2 with the prodomain still covalently attached), newly synthesized proPC2 becomes competent to bind 7B2 more slowly.[120] Additionally, mutation of PC2 glycosylation sites or perturbation of disulfide bonding blocked proPC2 binding to 7B2 as well as PC2 maturation.[120] It is thus likely that proPC2 folding is independent of 7B2. However, studies of glycosylation indicated that 7B2 greatly accelerates the transport of proPC2 from the ER to the Golgi and the Golgi-dependent conversion of proPC2 to mature PC2 by autocatalytic removal of the prodomain.[120] Thus, there is some debate whether 7B2 constitutes an authentic chaperone; while it is able to protect PC2 and/or proPC2 from inactivation by heat or low pH,[114,120] it has no effect on aggregation of either wildtype or glycosylation-deficient proPC2.[120] Additionally, expression of 7B2 is more widespread than expression of PC2,[141] and mice lacking 7B2 exhibit a more severe phenotype than mice lacking PC2,[142] demonstrating that 7B2 likely exhibits functions in addition to those involved in PC2 maturation. 7B2 is itself processed in the TGN from a 27-kDa form into a 21-kDa form (probably by furin: ref 139), and the C-terminal peptide derived from 7B2 is a potent PC2 inhibitor that has been used to active-site titrate the enzyme.[60] The precise role of this inhibition in vivo remains unclear, as coexpression of 7B2 with PC2 results in a drastic increase in the amount of active, mature PC2 present within the cell.[45,139]

Like the 7B2−proPC2 interaction, the interaction between proSAAS and PC1/3 is high-affinity and

**Table 6. $K_I$ Values for Inhibition of Kex2 and Furin[a]**

| type of inhibitor | sequence | Kex2 | furin | ref |
|---|---|---|---|---|
| peptidyl-chloromethyl ketone | P$\pi$YKR-cmk[b] | $3.7 \times 10^{-9}$ | N/D | 59 |
| peptidyl-ketomethlyene | dec-RVKR$\phi$AVG[b] | N/D | $3.4 \times 10^{-9}$ | 147 |
| polyarginine | AcRRRRRRRRR-$NH_2$[c] | N/D | $4.0 \times 10^{-8}$ | 128 |
| polyarginine | AcRRKRRR-$NH_2$[d] | N/D | $1.3 \times 10^{-6}$ | 128 |
| polyarginine | AcWRRRRR-$NH_2$[e] | N/D | $2.4 \times 10^{-6}$ | 128 |
| ovomucoid | RCKR | $1 \times 10^{-6}$ | $9.1 \times 10^{-8}$ | 149, 150 |
| kexstatin | LCTK | $4.4 \times 10^{-8}$ | none[f] | 156 |
| kexstatin | LCKR | $3.2 \times 10^{-10}$ | none[f] | 156, 157 |
| $\alpha_1$-PDX[b] | RIPR | N/D | $6.0 \times 10^{-10}$ | 152 |
| ovalbumin serpin (PI8) | RNSR | N/D | $5.4 \times 10^{-11}$ | 131, 132 |
| eglin C (wild type) | PVTL | $\sim 5 \times 10^{-4}$ | $1.1 \times 10^{-4}$ | 50 |
| $M_4R_1$ eglin C | MVTR | $3.4 \times 10^{-10}$ | $3.6 \times 10^{-6}$ | 50 |
| $R_4R_1$ eglin C | RVTR | $9.1 \times 10^{-10}$ | $2.5 \times 10^{-9}$ | 50 |
| $R_4K_1$ eglin C | RVTK | $2.5 \times 10^{-9}$ | $3.8 \times 10^{-8}$ | 50 |

[a] $K_I$ values are listed for inhibition of the indicated proteases. Values are in M. For peptide inhibitors, the entire sequence is given. For protein inhibitors, only the sequence directed against the active site is listed. [b] $\pi$ = norvaline; cmk = chloromethyl ketone; $\phi$ = ketomethylene; $\alpha_1$-PDX = $\alpha_1$-antitrypsin Portland. [c] This inhibitor also inhibited PACE4 with a $K_I$ of $1.1 \times 10^{-7}$ M. [d] This inhibitor also inhibited PC2 with a $K_I$ of $4.6 \times 10^{-4}$ M. [e] This inhibitor contained all D-amino acids and also inhibited PC2 with a $K_I$ of $3.4 \times 10^{-4}$ M. [f] No inhibition was detected.

specific.[143−145] Binding of proSAAS to PC1/3 is inhibitory, with inhibition dependent upon a C-terminal heptapeptide sequence containing a basic motif analagous to that found in 7B2.[143,144] However, the N-terminal domain of proSAAS acts as an inhibitor of PC1/3 and additionally blocks its autocatalytic C-terminal processing, even though higher levels of PC1/3 are produced in coexpressing cells.[145] Thus, while proSAAS coexpression facilitates the production of PC1/3, it also seems to act as an inhibitor of this enzyme and therefore may facilitate the production of a conformation with lower specific activity or perhaps optimize the activity of PC1/3 for a particular pH or a slightly different specificity, functions thought to be regulated by the C-terminal autocatalytic processing event proSAAS inhibits.[116,117,145]

In addition to naturally occurring inhibitors such as 7B2 and proSAAS, a number of inhibitors have been engineered for members of the Kex2/furin family. Proteases of this family cleave not only neuropeptide hormones but also key molecules necessary for pathogenesis, such as the envelope glycoproteins of HIV, avian influenza virus, and Ebola virus, as well as *Pseudomonas* exotoxin and anthrax protective antigen (see the above discussion of furin specificity). This makes the development of inhibitors or regulators of proprotein processing proteases a potentially attractive therapeutic route. To date, a number of different types of inhibitors have been developed, particularly for furin. This work has increased in recent years, as more information about the specificity of different family members has become available. Table 6 summarizes the inhibition constants ($K_I$) for a number of inhibitors of Kex2 and/or furin.

A number of moieties have been developed that can reversibly or irreversibly inhibit the active sites of serine proteases. Such moieties are typically then attached to a short peptide which increases the affinity of the inhibitor for the active site and confers specificity for a given protease or protease specificity pattern (for example, an inhibitor directed against furin would likely also inhibit trypsin, because a furin cleavage site also contains a trypsin cleavage site). The first such inhibitors to be synthesized for the processing proteases were peptidyl chloromethyl ketones. The peptidyl chloromethyl ketone Phe−Ala−Lys−Arg−$CH_2$Cl blocked maturation of fowl plague virus hemagglutinin when added to infected chicken embryo cell culture at 10 mM.[146] Capping the N-terminus of this inhibitor with a palmitoyl group increased inhibitory activity by 100- to 200-fold, presumably due to improved internalization.[146]

A systematic series of peptidyl chloromethyl ketones containing multiple Arg and Lys residues were synthesized to study inhibition of Kex2 in vitro.[59] The most potent inhibitor obtained contained a $P_4$ norvaline, $P_2$ Lys, and $P_1$ Arg (Table 6; ref 59). In this study, the N-terminus was blocked with a decanoyl moiety to improve cell permeability while reducing lytic activity relative to a palmitoyl group. This study also highlights the racemization problem associated with peptidyl chloromethyl ketones, because active-site titration of Kex2 with these compounds gave an enzyme concentration differing from that obtained by initial burst titration by a factor of 2, indicative of racemization at $P_1$.[59] To avoid the toxicity associated with peptidyl chloromethyl ketones, the peptidyl ketomethylene inhibitor dec-Arg−Val−Lys−Arg−$CH_2$−Ala−Val−Gly-$NH_2$ was synthesized and shown to inhibit furin with a $K_i$ of 3.4 nM (Table 6; ref 147). *N*-decanoyl peptidyl chloromethyl ketone inhibitors are now commercially available and can be used for active-site titration of proprotein processing proteases[53,59] as well as for inhibition studies in cell culture.[146] However, the nanomolar affinities and high toxicity of the available compounds limit therapeutic applications, and racemization of the $S_1$-directed moiety can result in substantial errors in active-site titration if the inhibitor is not calibrated with an enzyme preparation of known concentration.[59] As an alternative, peptidyl phosphonate inhibitors were tested on Kex2 in vivo.[148] These compounds were able to disrupt Kex2 processing without substantially affecting Kex2 localization or endocytosis in general.[148]

To identify selective potent, yet reversible inhibitor sequences for Kex2/furin family processing proteases,

positional scanning hexapeptide combinatorial libraries were developed.[128] The most potent furin inhibitor reported, nona-L-arginine, inhibited furin with a $K_i$ of 40 nM (Table 6). The same peptide inhibited PACE4 with a $K_i$ of 110 nM and PC1 with a $K_i$ of 2.5 $\mu$M, indicating that it may be possible to generate sequences that discriminate among the different processing proteases.[128] However, these authors did not see significant differences in inhibition between L-peptides and D-peptides (Table 6). This indicates that the interaction does not rely on the stereochemistry or on contacts with the peptide backbone of the inhibitor, so it is possible that this inhibition is occurring via nonspecific electrostatic interactions or via the second site postulated to account for substrate inhibition.[48,56] Moreover, the nona-Arg-peptide was slowly cleaved, as might be expected.[128] Thus, small peptide inhibitors are able to inhibit the enzymes of this family with nanomolar affinities, but to date problems remain with turnover of inhibitors (for unmodified peptides) and cytotoxicity (for chloromethyl ketones). Protein inhibitors may provide a means around these concerns.

The first protein inhibitors developed for Kex2/furin family proteases were mutated derivatives of turkey ovomucoid third domain, which directs the sequence -KPACTL↓EY- to the active site (where the arrow indicates the bond positioned adjacent to the active-site serine). The best furin inhibitor derived from the turkey ovomucoid third domain inhibited furin with a $K_i$ of $9.1 \times 10^{-8}$ M and inhibited Kex2 with a $K_i$ of $10^{-6}$ M (Table 6; refs 149 and 150).

$\alpha_1$-Antitrypsin Portland ($\alpha_1$-PDX) was also developed as a potent furin inhibitor.[151] Having the minimal furin recognition sequence of Arg at $P_4$ and $P_1$, $\alpha_1$-PDX irreversibly inhibits furin with a $K_i$ of 600 pM.[152] Following the unique serpin inhibitory mechanism, $\alpha_1$-PDX reacts with furin in a branched reaction pathway partitioning between cleaved (substrate) and stably inhibited paths. The partition ratio (total number of the inhibitor required to inhibit one molecule of the enzyme, indicating the relative efficiency of these two branches) was 2 for this interaction. This inhibitor exhibits intracellular activity when added to the cell culture medium at 10 $\mu$M.[152] More recently, $\alpha_1$-antitrypsin variants with multiple Arg residues in the $P_6$ to $P_1$ region (but maintaining Glu at $P_5$) were expressed and tested for furin inhibition.[153] All $\alpha_1$-PDX variants behaved in a manner similar to $\alpha_1$-PDX, but with higher partition ratios (2.9 to >29: ref 153). However, serpin family inhibitors generally exhibit high partition ratio for noncognate interactions (that is, the physiologically relevant interaction exhibits substantially less turnover). For example, $\alpha_1$-antitrypsin inhibits human neutrophil elastase extremely efficiently, forming a 1:1 stoichiometric covalent complex (a partition ratio of 1: ref 154), while it inhibits subtilisin Carlsberg with a partition ratio of 5 and proteinase K with a partition ratio of 8.[155]

A natural inhibitor of Kex2, kexstatin, has also been reported (Table 6; ref 156). Kexstatin belongs to the Streptomyces subtilisin inhibitor (SSI) family. It inhibited Kex2 with a $K_i$ of $4.4 \times 10^{-8}$ M, and an engineered variant with a $P_1$ Arg exhibited a $K_i$ of $3.2 \times 10^{-10}$ M (Table 6; ref 157). Like the ovomucoid third domain, kexstatin has a $P_3$ Cys and a conserved hydrogen bond interaction between Thr at $P_2$ and Glu at $P'_1$. No kexstatin variant inhibited furin,[157] although similar mutation of the ovomucoid third domain resulted in better inhibition of furin than Kex2.[149,150]

It has also proven possible to engineer a potent subtilisin inhibitor, eglin c, for efficient inhibition of the processing proteases.[50] An eglin c variant with Arg at $P_4$ and $P_1$ inhibited Kex2 with $K_i$ of $8.3 \times 10^{-10}$ M and furin with a $K_i$ of $2.5 \times 10^{-9}$ M (Table 6). These inhibitors form 1:1 stoichiometric stable complexes with the proteases with a $k_{ass}$ of $\sim 10^5$ $s^{-1}$ $M^{-1}$. To improve affinity further, introduction of basic residues at $P_6$ and $P_2$ was performed. The inhibitor with Arg at $P_6$, $P_4$, and $P_1$ was a temporary inhibitor of furin, with cleavage occurring at the $P_1-P'_1$ bond during long incubation. However, the same molecule inhibited Kex2 with $K_i$ of $2.9 \times 10^{-10}$ M, forming a stable complex (Table 6). The second variant with Lys at $P_2$ was cleaved by both proteases. This approach is currently being extended by optimizing additional contact sites outside the active-site loop to improve specificity for individual members of the Kex2/furin family proprotein processing proteases.

The peptide and protein inhibitors discussed above are all ultimately substrate mimics in that they are peptides or peptide derivatives that bind to the active site. This explains why apparent optimization of the sequences of protein inhibitors can readily result in turnover,[50] as such optimization provides sufficient free energy to permit the active-site directed sequence to bind not in the inhibitory conformation but in a slightly different configuration which now permits cleavage. Recently, a diterpine compound from *Andrographis paniculata* has been shown to be an inhibitor of several processing proteases, including furin.[158] This natural product, andrographolide, was able to inhibit furin and PC1/3 with an affinity near 1 mM, but derivatization of the compound with succinic acid resulted in several much more potent inhibitors, with $K_I$ values as low as 2.6 $\mu$M for furin and 10 $\mu$M for PC1/3.[158] Even more promising, some of these derivatives were able to discriminate among the tested proteases, with the most effective furin inhibitor exhibiting $K_I$ values an order of magnitude higher for the other enzymes. These compounds may thus provide an alternative to the peptide and protein approaches discussed earlier.

## *IX. The Mechanistic Basis for Specificity in the Kex2/Furin Family of Proteases*

The exceptionally high substrate specificity seen with Kex2 protease and related enzymes is in marked contrast to the behavior of the related enzymes of the subtilisin family, which exhibit comparatively low substrate specificity.[38,39] These two families of enzymes thus provide an interesting contrast, in that they use the same basic fold and catalytic mechanism to generate distinct specificity patterns and biological functions. This begs the question of how this specificity is generated. In the case of subtilisins, it seems

that a number of substrate side chains are able to contribute to both $k_{cat}$ and $k_{cat}/K_M$.[38] Preliminary steady-state characterization of Kex2 specificity suggested this was not the case, because a substitution at $P_1$ resulted in a slight defect in $K_M$ with a much more substantial defect in $k_{cat}$.[58] This was in contrast to substitutions at $P_2$, which produced little effect on $k_{cat}$ but large changes in $K_M$.[58] Later work with internally consistent sequences confirmed these findings (refs 47 and 52; Rockwell, N. C., and Fuller, R. S., unpublished data), suggesting that Kex2 utilizes different enzyme−substrate interactions at different points in the catalytic cycle. Therefore, an examination of the pre-steady-state parameters for multiple substrates seemed worthwhile.

Like all subtilases, Kex2 utilizes the serine protease mechanism, in which a covalent acylenzyme intermediate is formed and then broken down during a single catalytic cycle (Scheme 1; refs 35 and 36). Thus, there are a minimum of four steps that could be kinetically significant: substrate binding, acylation, deacylation, and release of the N-terminal product ($P_N$ in Scheme 1). Additionally, release of the C-terminal product must happen before deacylation can proceed, but this is often unmeasurably rapid. For serine proteases, $k_{cat}$ is typically determined by either the acylation rate ($k_2$ in Scheme 1) or the deacylation rate ($k_3$), depending on the enzyme and the type of substrate.[35,36,38,159,160] These possibilities can be distinguished in certain cases by examining different leaving groups. For instance, in the case of subtilisins, such a leaving-group effect can be used to show that acylation is rate-limiting in cleavage of amide substrates. Equivalent ester substrates exhibit substantial increases in $k_{cat}$, indicating that the leaving group of the scissile bond is involved at the rate-determining step.[38] Therefore, it must be involved at the acylation step and not at the deacylation step, because the C-terminal cleavage product $P_C$ has been released prior to the deacylation step (Scheme 1).

The formation of $P_C$ as a function of time can also provide information about the nature of the rate-limiting step for serine proteases. If acylation is rate-limiting, formation of $P_C$ will be linear with time, since this product is generated at the rate-limiting acylation step. However, in the event of rate-limiting deacylation, the first turnover will generate $P_C$ more rapidly than subsequent steady-state turnovers, because acylation will proceed faster than deacylation. This will generate a burst of product stoichiometric with enzyme before the establishment of the steady state. Of course, such a burst will also occur in the event of rate-limiting product release or any other rate-determining step after acylation. Thus, an initial burst of C-terminal cleavage product indicates that acylation is not rate-limiting. In contrast to subtilisin, which exhibits rate-limiting acylation,[38] Kex2 exhibits such an initial burst in cleavage of a peptidyl-MCA substrate (Figure 3; ref 58). This behavior also is seen in cleavage of an IQ substrate (Figure 3; ref 64), demonstrating that it is not an artifact of the activated coumarin leaving group.

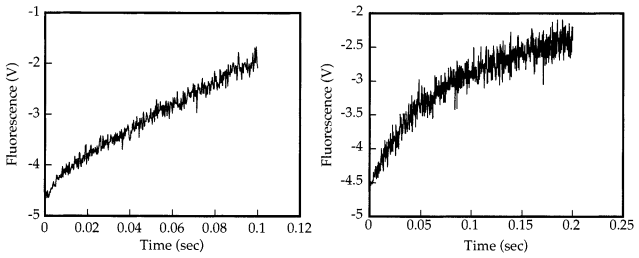



**Figure 3.** Kex2 cleavage of both peptidyl-MCA substrates (Z$\beta$YKR↓MCA, left) and IQ substrates (RJ$\beta$YKR↓EAEABR, right) exhibits burst kinetics in formation of the acylation product ($P_C$ in Scheme 1). In both cases, Kex2 protease was reacted with substrate in an Applied Photophysics stopped-flow fluorimeter at 21 °C as described in refs 64 and 161. The initial bursts of product indicate that acylation is not rate-limiting in cleavage of either substrate.

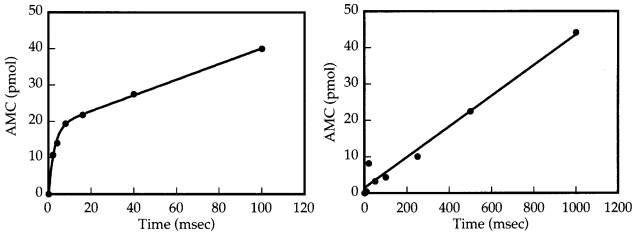



**Figure 4.** $P_1$ substitutions result in a change in rate-limiting step for Kex2 protease. Cleavage of Ac$\beta$YKR↓MCA proceeds with burst kinetics (left). However, cleavage of Ac$\beta$YKK↓MCA does not display such a burst (right), indicating that this substrate has a different rate-determining step. Both substrates were examined by rapid-quenched-flow at 21 °C as described in ref 47.

This result demonstrates that Kex2 is not limited by acylation in cleavage of peptide bonds when cleaving correct substrates. Substitution of incorrect residues at $P_2$ or $P_4$ does not change this behavior.[161] However, the conservative substitution of Lys for Arg at $P_1$ results in a loss of this behavior (ref 47; Figure 4), indicating that this substitution results in a change in rate-limiting step and confirming the key role of $P_1$ in Kex2 specificity. An equivalent ester substrate with Lys at $P_1$ exhibited burst kinetics and a substantial increase in $k_{cat}$,[57,161] indicating that the introduction of $P_1$ Lys in the amide substrate produced a specific defect in acylation such that it became rate-limiting. The nature of the rate-determining step for correct sequences with Arg at $P_1$ was addressed by applying similar reasoning. The two likely candidates for this step were product release and deacylation. These two steps can be distinguished by monitoring the formation of the N-terminal product $P_N$, because rate-limiting product release will confer burst kinetics on this product while rate-limiting deacylation will not. The formation of this product was monitored directly by incorporating a stable oxygen label at the new C-terminus of $P_N$ and then quantitating product by mass spectrometry.[64] Burst kinetics were observed with the C-terminal cleavage product, but not the N-terminal cleavage product (Figure 5). Therefore, this experiment conclusively demonstrated that product release could not be rate-determining for cleavage of correct sequences, implicating deacylation as the rate-limiting step for such sites.[64] Additional experiments demonstrated that no product inhibition was ob-

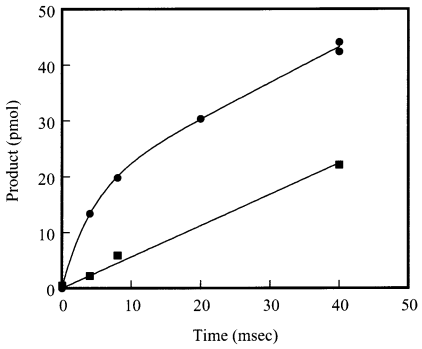



**Figure 5.** Deacylation is rate-limiting for physiologically correct cleavage sites. $d_3$-Ac$\beta$YKR↓MCA was cleaved with Kex2 at 21 °C in a Kintek RQF-3 rapid-quenched-flow mixer. Duplicate timepoints were assayed by fluorescence (circles) to measure the acylation step and by mass spectrometry (squares) to measure the deacylation step. These data demonstrate that deacylation is at or after the rate-determining step in cleavage of this substrate. Data are from ref 64.

served even at 1 mM peptide product,[64] allowing an estimate of the rate of product release as $\geq 10^4$ $s^{-1}$ by assuming a one-step binding model with conservative estimates of the affinity for product as 1 mM and the on-rate as approximately $10^7$ $M^{-1}$ $s^{-1}$ (derived from the highest concentration used and the $k_{cat}/K_M$ for the equivalent substrate).

These results indicated that product release was likely to be rapid, leaving three microscopic rate constants to be determined: substrate binding, acylation, and deacylation. Microscopic rate constants for the chemical steps could be determined through simple saturation kinetics for substrates with Lys at $P_1$, because in this case acylation is limiting for the amide and deacylation is limiting for the ester.[161] Additionally, the affinity constant for the substrate under these conditions is simply given by $K_M$ for the amide substrate, allowing the determination of all three parameters in this context.

The situation was more complicated for substrates with the correct Arg at $P_1$. In this context, deacylation is rate-limiting for the amide substrate, so the deacylation rate for such sequences is determined by $k_{cat}$. However, the acylation rate and affinity constant are harder to measure. In practice, this was addressed by examining the saturation of the burst phase as a function of substrate concentration using stopped-flow fluorimetry, but the resulting data suggested that the actual rate was very fast and that limitations of the equipment prevented proper determination of these parameters.[161] This approach did permit the estimation of lower bounds on acylation and substrate binding, and even these limited data provide a uniquely detailed view of how Kex2 specificity is generated at $P_1$ and $P_4$ (ref 161; Table 7).

Interaction between Kex2 and the $P_1$ side chain is almost entirely manifested at the acylation transition state (ref 161; Table 7); the introduction of Lys instead of Arg at this position results in a reduction in $k_2$ of >200-fold. This defect corresponds to a loss of ≥3.1 kcal/mol of transition state stabilization with the Lys substitution, which is sufficient to account for the observed difference in $k_{cat}/K_M$ between these two sequences. In contrast, the maximal improvement in binding for Arg relative to Lys at $P_1$ is only 4-fold, and the deacylation rate is actually slightly faster with the incorrect Lys at $P_1$. Thus, the importance of $P_1$ for Kex2 specificity is not due to its importance in substrate binding, which is minimal, but rather because it is vitally important for driving the acylation step.

**Table 7. Pre-Steady-State Parameters for Kex2 Protease[a]**

| $P_4$ residue | $P_1$ residue | $K_S$ ($\mu$M) | $k_2$ ($s^{-1}$) | $k_3$ ($s^{-1}$) |
|---|---|---|---|---|
| norleucine | Arg | ≥25 | ≥550 | 27 |
| norleucine | Lys | 28 | 2.6 | 38 |
| Ala | Arg | ≥30 | ≥550 | 73 |
| Ala | Lys | 330 | 0.12 | 44 |

[a] Data are from ref 161. $K_S$ and $k_2$ were measured at pH 7.26 and 21 °C. $k_3$ was measured at pH 6.0 and 21 °C.

The contact at $P_4$ makes a less dramatic contribution to the acylation step, but it also significantly aids substrate binding.[161] At this position, substitution of the small Ala for the aliphatic norleucine results in a 22-fold drop in the acylation rate and a 7-fold drop in affinity for the substrate (Table 7), equivalent to 1.1 kcal/mol for binding and 1.8 kcal/mol for catalysis. Strikingly, the $P_4$ contact is again unimportant for the deacylation step, with the incorrect Ala actually resulting in a slight increase in deacylation rate (≤3-fold: ref 161). Thus, this accessory contact plays a role in both binding the substrate and aiding catalysis.

To date, it has not been possible to carry out a similar analysis of substitutions at the $P_2$ position, because substitutions at this position result in very high $K_M$ values (≥100-fold increase: refs 47 and 58). $P_2$ substitutions do not cause a loss of burst kinetics in formation of the C-terminal cleavage product (ref 47; Rockwell, N. C., and Fuller, R. S., unpublished data). This indicates that the enzyme−substrate binding constant $K_S$ (Scheme 1) will be very high for such substrates, and in practice the limited solubility of peptidyl-MCA substrates precludes the necessary experiments. However, the preliminary data make it clear that, relative to correct sequences, substitutions at $P_2$ produce no change in rate-limiting step, little effect on deacylation rate (≤4-fold), and a large decrease in affinity. Therefore, this position is likely to play an important role in substrate binding but little role in stabilizing the transition state for either chemical step. Moreover, the absence of an effect on deacylation rate with $P_2$ substitutions confirms the observation that the Kex2 deacylation rate is largely insensitive to the substrate sequence (Table 7).

This work has been done with peptidyl-MCA and peptidyl-MCE substrates, so it is important to consider how relevant it will prove for authentic precursors in which processing occurs at a peptide bond. The activated nature of the coumarin leaving group makes it likely that acylation rates will be significantly faster for peptidyl-MCA substrates than for IQ substrates or authentic precursors, and preliminary experiments with IQ substrates suggested that this was likely to be the case (Rockwell, N. C., and Fuller, R. S., unpublished experiments). However, the substrate inhibition and lower solubility seen with

such substrates would have complicated the pre-steady-state analysis considerably, and the fact that $k_{cat}/K_M$ values seen with these substrates are approximately equal to those observed with equivalent peptidyl-MCA substrates[52] allows a number of conclusions to be drawn about how such substrates behave. For serine proteases, $k_{cat}/K_M$ is equivalent to $k_2/K_S$ regardless of the nature of the rate-limiting step.[35] Therefore, if $k_2$ is lower for peptide bonds than for peptidyl-MCA substrates, it must be offset by a similar reduction in $K_S$. In other words, slower acylation with actual precursors must be accompanied by tighter binding, indicating that any enzyme−substrate interactions involving the leaving group are specifically affecting the ground state and not the transition state (i.e., they are used to improve binding not catalysis: ref 162). In addition, it is known that an IQ substrate with a $P_1$ Arg exhibits burst kinetics (Figure 3), while substitution of Lys at $P_1$ again results in a loss of burst kinetics as for peptidyl-MCA substrates.[64] This indicates that $P_1$ does play a key role in determining acylation rate for authentic peptide bonds. Moreover, the nature of the leaving group will not affect the deacylation step, because the leaving group is the new N-terminus of the C-terminal cleavage product $P_C$ and is therefore released prior to deacylation (Scheme 1). Thus, the leaving group may act to stabilize the enzyme−substrate complex E·S (Scheme 1), but it does not alter the insensitivity of the Kex2 deacylation rate to the substrate sequence, nor does it alter the pivotal role played by $P_1$ in determining the rate of acylation, which is the first irreversible step in the reaction.

The evolutionary advantages of this behavior remain grounds for speculation. The slow deacylation seen with Kex2 cannot alter specificity (formally defined as the *ratio* of $k_{cat}/K_M$ values for two competing substrates: ref 37), because the deacylation step is after the first irreversible step and its steady-state consequence is to reduce the concentration of free enzyme, which affects all substrates equally. It may be that this reduction in free enzyme concentration serves to keep the actual *rate* of cleavage at incorrect sites below some toxic threshold,[64] but this remains unclear to date, as do the structural factors that contribute to the slow acylation and specific $P_1$ effect seen with Kex2.

The Kex2 paradigm for the pre-steady-state behavior of these enzymes has been tested with preliminary studies of two related enzymes, furin and PC.[25,48,57] Like Kex2, furin exhibits burst kinetics in the cleavage of peptidyl-MCA substrates with Arg at $P_1$.[5,48] This indicates that acylation is not rate-limiting for furin cleavage of such sites, and it is known that substitution of Lys for Arg at $P_1$ results in a substantial defect in $k_{cat}/K_M$ which is quite comparable to that seen with Kex2 for the same substitution (corresponding to 3.1 kcal/mol in furin and 3.4−3.8 kcal/mol in Kex2: refs 47 and 48). However, it is not yet known whether deacylation or product release will prove rate-limiting for furin, nor is it known whether substitutions at $P_1$ alter the rate-limiting step for this enzyme. Inhibition of both furin and PC1/3 with a peptidyl-chloromethyl ketone[53] proceeded with high second-order rate constants which were interpreted as being indicative of rate-limiting deacylation, though the reaction of such compounds need not mirror the reaction pathway for cleavage of actual substrates.

A somewhat different picture emerges from the characterization of PC2. As discussed previously, PC2 operates in a substantially different intracellular environment than Kex2 and furin, and it has an aspartate in place of the conserved oxyanion hole Asn (see the introduction to the Kex2/furin family above). It is clear that acylation is rate-determining for PC2 cleavage of amide substrates, because burst kinetics are not seen in formation of the C-terminal cleavage product with amide substrates but are seen in formation of this product with ester substrates.[57] While the contributions of individual steps to the catalytic mechanism have not been assessed, the ability of this enzyme to cleave substrates with Lys or Arg at $P_1$ with very comparable $k_{cat}/K_M$ values[121] indicates that PC2 will not behave like Kex2 on a pre-steady-state level. On the other hand, PC2 operates in a very different milieu from Kex2 and furin, so it is perhaps unsurprising that it exhibits substantially different behavior. The substantial reduction in acylation rate seen with this enzyme relative to other members of this family suggests that this enzyme may be intrinsically crippled, and the observed acylation rate constant $k_2$ for this enzyme is comparable to those for mutant forms of Kex2[65] and subtilisin BPN′[163] in which the oxyanion hole Asn was mutated.

Further support for the idea that PC2 is intrinsically crippled comes from comparison of turnover numbers for PC2 with Kex2 and furin: PC2 routinely exhibits $k_{cat}$ values of $\leq 2\ s^{-1}$, while Kex2 exhibits $k_{cat}$ values of $40-50\ s^{-1}$ or higher with similar sequences. Comparison with furin is somewhat problematic due to the substrate inhibition frequently seen with this enzyme, but Thomas and co-workers have reported several substrates that are free of this problem,[55,105] and these substrates exhibit turnover numbers as high as $40\ s^{-1}$.[105] However, it is important to note that this comparison underestimates the differences between PC2 and the other enzymes, because $k_{cat}$ measures the acylation step for PC2 but a slower step than acylation for the other enzymes. In the case of Kex2, the acylation rate has been conservatively estimated at $\geq 550\ s^{-1}$ (Table 7), showing that the ability of PC2 to carry out the same microscopic step in the mechanism is actually reduced by at least 2 orders of magnitude.

## X. A Potential Role for Potassium as an Allosteric Modulator of Kex2 and Furin

It has recently been reported that Kex2 and furin are activated by potassium and other monovalent cations.[70] In the case of Kex2, this activation takes place with an apparent affinity of approximately 22 mM and results in a 3-fold increase in $k_{cat}$ for substrates with Arg at $P_1$.[70] However, Kex2 cleavage of a substrate with Lys at $P_1$ exhibited a reduction in $k_{cat}$ in the presence of potassium. Similarly, furin cleavage of a good hexapeptidyl-MCA substrate was

stimulated, while cleavage of a poorer tetrapeptidyl-MCA substrate was inhibited. In contrast, PC2 cleavage of three substrates differing in length and $P_4$ side chain was inhibited in all cases, again distinguishing this enzyme from Kex2 and furin.

Potassium effects on the pre-steady-state behavior of Kex2 were also examined. Surprisingly, it was found that Kex2 cleavage of peptidyl-MCA substrates no longer exhibits burst kinetics in the presence of potassium. However, burst kinetics are still seen in cleavage of a peptidyl-MCE substrate, indicating that acylation is likely to be rate-determining for the cleavage of amide substrates by potassium-bound Kex2. This implies that potassium binding results in slower acylation, because the value of $k_{cat}$ in the presence of potassium is well below the lower bound for the acylation rate determined in the absence of potassium by stopped-flow fluorimetry (approximately 150 versus $\geq$550 $s^{-1}$: refs 70 and 161). Moreover, the value of $k_{cat}$ for Kex2 cleavage of correct amide substrates in the presence of potassium is higher than the known deacylation rate for the same reaction in the absence of potassium, indicating that potassium binding results in faster deacylation (150 versus 50 $s^{-1}$: refs 70 and 161). Thus, potassium binding to Kex2 retards acylation but speeds deacylation in cleavage of correct sequences, resulting in a change in rate-limiting step from deacylation to acylation. Essentially, potassium binding switches the enzyme from a conformation with fast acylation and slow deacylation to a conformation with slower acylation and faster deacylation.

There are several lines of evidence that the interaction between Kex2 and potassium is a direct allosteric effect and not due to changes in the nature of bulk solvent.[70] First, the effect is specific for a monovalent cation of a certain radius, suggesting a binding site. Furthermore, counter-titration of sodium and potassium at constant ionic strength gave a nonlinear response, suggesting that the two ions were competing for a single site. Additionally, the apparent Kex2 affinity for potassium is substrate-dependent, suggesting cooperativity between binding potassium and binding substrate. Finally, single-turnover experiments with or without potassium demonstrated that the apparent first-order rate constant for cleavage *decreased* with increasing substrate in the presence of potassium, while it was independent of substrate concentration (as expected) in the absence of potassium. This counterintuitive result is thought to arise because of cooperativity between the binding of substrate and ion together with the aforementioned effects of potassium binding on the acylation and deacylation steps, such that increasing substrate concentration results in an increasing contribution of the potassium-bound species to the observed rate constant, and hence results in a drop in the apparent acylation rate because the potassium-bound population of enzyme carries this step out more slowly.

To date, the potential implications of these results for processing protease function in vivo have not been experimentally addressed. Recent results suggest that a family of cation−proton antiporters localized to late Golgi or TGN compartments in eukaryotic cells[164−166] may in fact be $K^+/H^+$ antiporters in vivo.[166] This raises the possibility that enzymes such as Kex2 and furin may cycle between compartments with substantially different potassium concentrations, resulting in different kinetic behavior in different compartments. A detailed characterization of the specificity of Kex2 and furin in the presence of potassium with both peptidyl-MCA substrates and IQ substrates will improve our understanding of the possible implications of this behavior for processing in vivo.

## XI. Concluding Remarks

This review has primarily focused on specificity in the processing proteases of the Kex2/furin family. Enzymes such as Kex2 and furin have been well characterized at the steady-state level, and pre-steady-state studies of Kex2 have established a model that seems likely to hold true for furin but which will not describe PC2. Future studies of these enzymes will vary depending on the enzyme; for Kex2, further studies will probably focus on enzyme−substrate interactions that are not yet fully understood, such as subsite interactions, the energetics of interactions with the substrate backbone, and acylation rates for substrates with peptide leaving groups. Furin and PC2 are now ripe for pre-steady-state characterization, further expanding our understanding of these proteases. PC1/3 has been characterized in some detail, but our understanding of this enzyme at the steady-state level is still incomplete. Other enzymes such as PACE4 or PC7 have not yet been characterized in detail at the steady-state level, so such work will be necessary before pre-steady-state work will prove fruitful. However, basic pre-steady-state characterization of a number of these enzymes will help us understand how fundamental the distinction between the enzymes of the constitutive and regulated secretory pathway is and will also aid in understanding how these enzymes function together in mammalian cells to carry out proper processing.

The recent finding that potassium can act as a modulator for Kex2 and furin may also be an area of future study. A reevaluation of the specificity of these enzymes in the presence of potassium is necessary. It will also be interesting to characterize other enzymes to learn whether this is a general feature of the processing proteases of the constitutive secretory pathway. Should crystal structures become available soon, they will provide the basis for further study of these proteases, both through identification of possible potassium sites and by providing structural information to allow the further characterization of specificity through mutagenesis of the proteases themselves.

Additional study is likely to focus on topics that are beyond the scope of the current review, such as the sorting of both substrates and proteases, the regulation of expression of these molecules, and the phenotypic consequences of defects in members of this family. Finally, a number of inhibitors for these enzymes are available and are being exploited for research purposes, raising the possibility that such

inhibitors will soon make the transition from research reagents to therapeutic tools.

## XII. References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