Quantitative Characterization of Furin Specificity

ENERGETICS OF SUBSTRATE DISCRIMINATION USING AN INTERNALLY CONSISTENT SET OF HEXAPEPTIDYL METHYLCOUMARINAMIDES*

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Furin, an essential mammalian proprotein processing enzyme of the kexin/furin family of subtilisin-related eukaryotic processing proteases, is implicated in maturation of substrates involved in development, signaling, coagulation, and pathogenesis. We examined the energetics of furin specificity using a series of peptidyl methylcoumarinamide substrates. In contrast to previous reports, we found that furin can cleave such substrates with kinetics comparable to those observed with extended peptides and physiological substrates. With the best of these hexapeptidyl methylcoumarinamides, furin displayed $k_{cat}/K_m$ values greater than $10^5$ m$^{-1}$ s$^{-1}$. Furin exhibited striking substrate inhibition with hexapeptide but not tetrapeptide substrates, an observation of significance to the evaluation of peptide-based furin inhibitors. Quantitative comparison of furin and Kex2 recognition at P$_1$, P$_2$, and P$_4$ demonstrates that whereas interactions at P$_1$ make comparable contributions to catalysis by the two enzymes, furin exhibited a ~10-fold lesser dependence on P$_2$ recognition but a 10–100-fold greater dependence on P$_4$ recognition. Furin has recently been shown to exhibit P$_6$ recognition and we found that this interaction contributes ~1.4 kcal/mol toward catalysis independent of the nature of the P$_4$ residue. We have also shown that favorable residues at P$_2$ and P$_6$ will compensate for less than optimal residues at either P$_1$ or P$_4$. The quantitative analysis of furin and Kex2 specificity sharply distinguish the nature of substrate recognition by the processing and degradative members of subtilisin-related proteases.

The synthesis of bioactive peptides and proteins in eukaryotic cells frequently involves the proteolytic cleavage of precursor proteins as part of the maturation process. In the secretory pathway, the cleavage sites are typically marked by sequences of basic amino acids. Seven distinct proteases that cleave at such sites have been identified in mammals including such neural/neuroendocrine-specific enzymes as PC1 and PC2 and more widely expressed enzymes such as furin, PACE4, and PC7 (1). These enzymes are members of the subtilisin superfamily of serine proteases and are closely related to the yeast proprotein processing protease Kex2 (kex, EC 3.4.21.61) but more distantly related to degradative subtilisins.

Of the mammalian processing proteases, human furin (EC 3.4.21.85) was the first to be identified and has been studied extensively (2, 3). Furin, which is thought to be expressed in essentially all cell types, has been shown to function in the constitutive secretory pathway and to be localized primarily to the trans-Golgi network (3, 4). Although a well defined physiological substrate has yet to be identified conclusively, coexpression studies, studies of furin-deficient cell lines, and studies using purified secreted, soluble furin have implicated furin in processing a wide range of proproteins including precursors of growth factors (5), receptors (6, 7), coagulation factors (8), metalloproteases (9), viral envelope glycoproteins (10, 11), and bacterial toxins (12). These studies, along with a limited number of studies using model peptide substrates, indicate that furin cleavage occurs C-terminal to an Arg that is preceded by one or more basic residues at P$_2$, P$_4$, or P$_6$ (13, 14).

A knockout of the fur gene is an embryonic lethal in mouse, illustrating the essential nature of processing reactions catalyzed by this enzyme (16).

Although the list of proteins that contain this general recognition has expanded in recent years, our understanding of the specific enzyme-substrate interactions responsible for furin specificity has lagged. An important reason for this disparity is the relative lack of specificity data derived from systematic, kinetic studies of structurally informative substrates. Indeed, previous studies have suggested that furin cleaves peptidyl methylcoumarinamide substrates much more poorly than substrates having P residues (17–19) and also much less efficiently than the yeast processing enzyme Kex2 does (20). For example, the 83-kDa anthrax protective antigen was hydrolyzed by furin with a $k_{cat}/K_m$ 5000-fold higher than that for cleavage of a tetrapeptidyl amino/methylcoumarinamide corresponding to the P$_1$–P$_4$ residues of the cleavage site in anthrax protective antigen (13, 17).

The low reactivity of furin reported for fluorogenic peptidyl methylcoumarinamides has led some groups to examine internally quenched substrates designed to more closely mimic biological substrates (17–19). Such substrates reacted much faster than related peptidyl-MCA$^2$ substrates (MCA, methylcoumarinamide) and, based on these data as well as data for the cleavage of anthrax protective antigen, it has been suggested that furin may require specific residues C-terminal to the cleavage site for efficient catalysis, that simple chain extension N-terminal to the P$_4$ site may enhance reactivity or that the bulky coumarin moiety of the peptidyl-MCA substrates may interfere with binding through interactions with the S$_{1'}$ position (17).

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1 Throughout this work we designate the cleavage site as $\cdot$P$_2$–P$_4$.
2 The abbreviations used are: peptidyl-MCA, peptidylmethylcoumarinamide; AMC, 7-amino-4-methylcoumarin; MES, 2-N-morpholinoethanesulfonic acid; HPLC, high performance liquid chromatography.
To examine these possibilities and develop a more detailed understanding of furin specificity, we synthesized a systematic set of hexapeptidyl-MCA substrates that assess enzyme-substrate interactions at all four subsites (P₁, P₂, P₄, and P₆) thought to be involved in furin specificity. Based on a kinetic analysis of the furin-catalyzed cleavage of these substrates, we now report that optimally substituted hexapeptidyl-AMC substrates are hydrolyzed by furin with k₅/Kₘ values comparable to those reported for internally quenched peptide substrates and anthrax protective antigen. These improved peptidyl-MCA substrates have allowed the quantitative characterization of subsite binding preferences for furin and, in particular, the additive contribution by P₆ basic residues to substrate cleavage. We have also found that at high substrate concentrations hexapeptidyl-MCA substrates (but not tetra peptide-MCA substrates) show substrate inhibition with furin. Quantitative analysis of substrate specificity requires determination of k₅/Kₘ values, which can be measured directly under pseudo first-order kinetics (i.e. with [S] ≪ Kₘ). By determining k₅/Kₘ values for furin in this way, substrate concentrations were kept well below those which inhibit furin and therefore substrate inhibition did not affect our characterization of furin specificity. Furin substrate inhibition does, however, have important implications for the interpretation of k₅ and Kₘ values for model substrates and for the development of substrate-based inhibitors of furin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Standard solvents, reagents, and buffers were obtained from Sigma, Aldrich, or Fisher. AMC (7-amino-4-methyl-coumarin) was obtained from Aldrich and was used as received. Acetylated peptides and acetyltetrapeptides were prepared using standard solid phase methods by the University of Michigan Protein and Carbohydrate Synthesis Core Facility. Secreted, soluble furin (hereafter referred to as furin) was purified by a modification of the published method (7). Active-site titration of furin (7) was done by measuring an initial burst of product formation with substrate 5 using a Kinetik RQF-3 rapid quenched-flow apparatus, the enzyme concentration was found to be 1.9 μM.

**Preparation of Peptidyl-MCA Substrates**—Peptidyl-MCA substrates were prepared from the trifluoroacetic acid salts of the acetylhexapeptides and acetyltetrapeptides using a previously described modification of Zimmerman’s chloroformate-based coupling procedure (22, 23). Substrates were also characterized by LC-MS analysis of the furin-catalyzed cleavage of these substrates, we now report that optimally substituted hexapeptidyl-AMC substrates are hydrolyzed by furin with k₅/Kₘ values comparable to those reported for internally quenched peptide substrates and anthrax protective antigen. These improved peptidyl-MCA substrates have allowed the quantitative characterization of subsite binding preferences for furin and, in particular, the additive contribution by P₆ basic residues to substrate cleavage. We have also found that at high substrate concentrations hexapeptidyl-MCA substrates (but not tetrapeptidyl-MCA substrates) show substrate inhibition with furin. Quantitative analysis of substrate specificity requires determination of k₅/Kₘ values, which can be measured directly under pseudo first-order kinetics (i.e. with [S] ≪ Kₘ). By determining k₅/Kₘ values for furin in this way, substrate concentrations were kept well below those which inhibit furin and therefore substrate inhibition did not affect our characterization of furin specificity. Furin substrate inhibition does, however, have important implications for the interpretation of k₅ and Kₘ values for model substrates and for the development of substrate-based inhibitors of furin.

**Preparation of Peptidyl-MCA Substrates**—Peptidyl-MCA substrates were prepared from the trifluoroacetic acid salts of the acetylhexapeptides and acetyltetrapeptides using a previously described modification of Zimmerman’s chloroformate-based coupling procedure (22, 23). Substrates 6, 12, and 13 were solubilized in N-methylpyrrolidinone by addition of LiCl (3 equivalent relative to peptide) prior to activation with isobutyryl anhydride. Peptidyl-MCA substrates were purified by reverse-phase HPLC (Rainin Dynaxam C18 column, 21.4 × 250 mm) using water/acetonitrile gradients containing 0.1% trifluoroacetic acid. All substrates eluted between 15 and 30%. Substrates 2, 7, and 11 were partially (approximately 15–25%) epimerized at P₁ during the coupling reaction as determined by HPLC analysis of the crude reaction products. The diastereomers were separated by HPLC and assignment of the stereochemistry was made by differential tryptic digestion. Substrates were also characterized by UV-visible absorption spectroscopy (scans at 200–450 nm) and mass spectroscopy. Synthetic yields varied with the exact sequence of the peptide (10–30%) with lower yields obtained in the examples with multiple basic residues (e.g. substrates 6, 12, and 13).

**Determination of k₅/Kₘ for Furin Using Pseudo First-order Kinetics**—Standard furin assays were performed at 37 °C in 0.2 M MES (pH 7.0 at 37 °C), 1 mM CaCl₂, 0.1% (v/v) aqueous acetic acid or acetonitrile (depending upon the substrate), and 0.1% (v/v) Triton X-100. Reactions (600 μl) were started by addition of enzyme (final concentration > 12 μM). Samples (100 μl) were taken at times between 2 and 40 min and were quenched with 0.5 M ZnSO₄ (600 μl). Enzyme activity did not decay under assay conditions as shown both by control preincubation experiments and by the linearity of time courses with saturating substrate concentrations (data not shown). Fluorescence was measured using a Perkin-Elmer LS-5B luminescence spectrometer (λₑₓ = 385 nm, λₑᵣᵣ = 465 nm). Raw fluorescence data were fitted to a first-order curve with floating end points (Equation 1) by nonlinear regression using Kaleidagraph v3.0 (Fig. 1B).

\[
\text{Fluorescence} = F_0 e^{-k_{cat} t} + F_{\infty}
\]  
(Eq. 1)

where F₀ is the amplitude of the change in fluorescence, k₅ is the apparent first-order rate constant, and Fₐ is the end point of the reaction. The resulting first-order rate constants were then divided by enzyme concentration to determine k₅/Kₘ. The values of k₅/Kₘ were shown to be independent of substrate concentration or of enzyme concentration for each substrate, confirming that the reactions proceeded under pseudo first-order conditions. At least two substrate concentrations differing by 10-fold or two enzyme concentrations differing by 4-fold were tested for each substrate. Substrate concentrations were all below 1 μM. Only process curves with r > 0.99 (Pearson’s r value) were used in the determination of k₅/Kₘ values. Reported kinetic constants are the mean of at least three trials and errors are reported as standard deviations about the mean.

**Determination of k₅/Kₘ for Furin using Saturation Kinetics**—Reactions (100 μl) were started by addition of enzyme and quenched by addition of 0.5 M ZnSO₄ (600 μl). k₅/Kₘ values were obtained from dividing the slope of the linear portion (low substrate concentrations) of a plot of νᵢ versus [S] by [Eₜᵢᵣᵣ].

\[
u_i = \frac{k_{cat}[E_i][S]}{K_m + S + S'/K_m}
\]  
(Eq. 3)

where Kᵢ values is the binding constant for the second substrate molecule (22, 24). The model did not, however, provide reliable k₅ or Kᵢ values and, hence, all substrate comparisons are based on relative k₅/Kᵢ values. Estimates of k₅ for substrates 2, 3, and 5 were made based on the maximum observed velocities and represent a lower limit for the actual values.

**RESULTS**

**Processing Protease Cleavage of Hexapeptidyl-MCA Substrates**—In order to study the substrate discrimination properties of the four putative furin substrates (P₁, P₂, P₄, and P₆), we prepared a series of hexapeptidyl-AMC substrates based on a core tetrapeptide sequence of Ac-Arg-Tyr-Lys-Arg-MCA. Furin-catalyzed cleavage of the AMC derivative of the core sequence (substrate 1) followed simple Michaelis-Menten kinetics under saturation conditions with k₅ = 1.3 s⁻¹ and Kᵢ = 2.3 μM (k₅/Kᵢ = 5.2 × 10⁵ M⁻¹ s⁻¹; Table I). Extension of the core sequence by two alanine residues gave substrate 2, which was cleaved with a similar k₅/Kᵢ under pseudo first-order conditions (2.3 × 10⁵ M⁻¹ s⁻¹) but which did not display simple saturation kinetics. Indeed, in this case as well as with the other two hexapeptidyl-MCA substrates (3 and 5, Fig. 1A) with which saturation kinetics were attempted, significant substrate inhibition was observed at high substrate concentrations. Attempts to fit these data to a standard kinetic model of substrate inhibition did not provide reliable values for k₅ and Kᵢ (22, 24). Consequently we were not able to determine independent k₅ or Kᵢ values for the hexapeptidyl-MCA substrates. Estimates of the lower limit of k₅ based on the maximum observed velocity indicate that 2 is cleaved with a k₅ (about 1 s⁻¹) similar to the tetrapeptide substrate (k₅ estimate for substrates 3 and 5 were also about 1 s⁻¹). Kex2 cleavage of tetrapeptidyl- and hexapeptidyl-MCAs in this sequence (substrates 1, 3, and 5) was examined as a control. Kex2 exhibited normal saturation kinetics with all of these substrates (Table II), indicating that the substrate inhibition seen with furin is genuine.

**Pseudo First-order Kinetics with Hexapeptidyl-AMC Substrates**—Because the substrate inhibition did not affect data obtained at low substrate concentrations (i.e. concentrations
Values are the mean of at least three independent trials for each substrate, and errors are reported as the standard deviation of those trials. Exceptions are substrates 7 and 9 which were two independent trials. In both of these examples, the reactions were slow enough that cleavage by extraneous, contaminating proteases could not be ruled out. Cleavage sites are indicated by an arrow.

## Table I

| Substrate | Sequence | $k_{cat}/K_m$  | Relative $k_{cat}/K_m$ | Error |
|-----------|----------|----------------|------------------------|-------|
| 1°       | AcRYKR | $5.2 \times 10^5$ | 0.2 | 20 |
| 2°       | AcAARYKR | $2.3 \times 10^5$ | 0.1 | 22 |
| 3°       | AcRARYKR | $2.6 \times 10^4$ | 1.0 | 30 |
| 4°       | AcKARYKR | $2.2 \times 10^4$ | 0.85 | 10 |
| 5°       | AcRYRFKR | $1.3 \times 10^4$ | 0.5 | 34 |
| 6°       | AcRAXYKR | $8.3 \times 10^3$ | 0.03 | 19 |
| 7°       | AcRAXYKR | <1000 | <0.0004 | |
| 8°       | AcAAXYKR | $7.9 \times 10^3$ | 0.002 | 10 |
| 9°       | AcAAXYKR | <100 | <0.00004 | |
| 10°      | AcRA×YKR | $4.4 \times 10^2$ | 0.002 | 20 |
| 11°      | AcAARYKR | $1.7 \times 10^2$ | 0.07 | 3 |
| 12°      | AcRARYKR | $1.9 \times 10^2$ | 0.73 | 7 |
| 13°      | AcRXYKR | $1.6 \times 10^2$ | 0.006 | 5 |

* Values measured under pseudo first-order conditions.
* Values measured from the linear portion of $v_s$ versus $[S]$ plot.
* $\pi$, norvaline.

**Fig. 1. A.** hexapeptidyl-MCA substrates exhibit substrate inhibition when cleaved with furin. Data shown are for substrate 3 and were fit to Equation 3 (18). B. hexapeptidyl-MCA substrates follow normal pseudo first-order kinetics. Data shown are for substrate 4 and are fit to Equation 1.

well below $K_m$, we were able to obtain reliable measurements of $k_{cat}/K_m$ for hexapeptidyl-AMC substrates under pseudo first-order conditions (see example in Fig. 1B) and thereby, compare the specificity of cleavage within an internally consistent set of substrates (Table I). Based on the core hexapeptide substrate 2, substrates with systematic substitutions at $P_6$, $P_4$, $P_2$, and $P_1$ were prepared, and the $k_{cat}/K_m$ for each was determined. The $P_6$ and $P_2$ positions were held constant in the series except in the case of substrate 5, where the $P_6$ Ala was exchanged for a Tyr and the $P_2$ Tyr was converted to a Phe.

**Effect of $P_6$ on Substrate Recognition**—Although numerous reports in the literature have established that substrates with basic residues at $P_6$ are recognized by furin (3), a systematic assessment of the nature of this interaction has not been reported. Toward this end, a series of substrates with either an aliphatic or a basic residue at $P_6$ was prepared. Substitution of the $P_6$ Ala of substrate 2 with either Arg (substitute 3) or Lys (substitute 4) led to an approximately 10-fold increase in $k_{cat}/K_m$. The $k_{cat}/K_m$ value observed for substrate 3 ($2.6 \times 10^6$ M$^{-1}$ s$^{-1}$) was within 2–3-fold of those reported for cleavage of internally quenched substrates and an authentic proprotein (13, 17) indicating that $P_6$ residues do make large contributions to energetics of substrate recognition. In a similar fashion, substitution at $P_6$ of Ala for Arg in substrates 6 and 7 to give substrates 8 and 9 led to 10-fold decreases in $k_{cat}/K_m$, suggesting that the $P_6$ subsite contributes to substrate recognition in an additive manner. Recognition of basic residues at $P_6$ was found to be somewhat sensitive to reaction pH (Table II). Cleavage of $P_6$ Arg substrate 3 at pH 6 was ~5-fold slower than the corresponding reaction at pH 7, whereas cleavage of substrates with Lys (substrate 4) or Ala (substrate 2) at pH 6 was not appreciably altered by lower pH.

**Effect of $P_4$ on Substrate Recognition**—Co-expression studies have shown that biologically relevant furin substrates contain either a basic residue (the majority) or an aliphatic residue at $P_4$. Not surprisingly, replacement of the $P_4$ Arg (substrate 2) with Ala (substrate 9) led to an extremely low $k_{cat}/K_m$ (<100 M$^{-1}$ s$^{-1}$). Likewise, substituting Ala for Arg at $P_4$ within the context of the best substrate (compare substrate 3 and substrate 7) in the series also drastically reduced $k_{cat}/K_m$ from $2.6 \times 10^6$ M$^{-1}$ s$^{-1}$ to $<1 \times 10^6$ M$^{-1}$ s$^{-1}$. Extension of the aliphatic chain of a $P_4$ residue from one carbon (Ala, substrate 7) to three carbons (Nvl, substrate 10) improved reactivity slightly ($k_{cat}/K_m$ of $4.4 \times 10^5$ M$^{-1}$ s$^{-1}$), suggesting that recognition at $P_4$ is partly due to hydrophobic interactions between the substrate and the S$_2$-binding pocket. In contrast to the behavior observed with $P_6$ variations, the identity of the basic residue at $P_4$ did affect reactivity at pH 7 as shown by the fact that replacement of $P_4$ Arg with Lys led to 30-fold reductions in $k_{cat}/K_m$ for substrates 6 and 8.

**Effect of $P_2$ on Substrate Recognition**—The identity of the $P_2$ residue at $P_2$ had little effect on $k_{cat}/K_m$ (substrate 3 versus 12), whereas replacement of a $P_2$ basic residue with Ala led to a decrease in reactivity of 14-fold (substrate 3 versus 11). The conservative replacement of Arg with Lys led to a much larger reduction (150-fold) in reactivity (substrate 3 versus 13). Notably, substrate 13 is one of the few examples of reasonably efficient cleavage by furin at a $P_2$ Lys indicating that, as in the case of Kex2, optimal binding at other subsites

### Table II

| Substrate | Sequence | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-----------|----------|----------|-------|--------------|
| 1°       | AcRYKR | MCA | 193 | 0.8 | $1.2 \times 10^6$ |
| 2°       | AcAARYKR | MCA | ND$^a$ | ND$^a$ | $5.4 \times 10^5$ |
| 3°       | AcBARYKR | MCA | 250$^b$ | 0.9$^c$ | $9.6 \times 10^5$ |
| 4°       | AcRYRFKR | MCA | 200$^d$ | 0.6$^c$ | $1.3 \times 10^4$ |
| 5°       | AcRYKKR | MCA | 50 | 1 | $4.3 \times 10^7$ |
| 15°      | BocRVRKR | MCA | 21 | 19 | $1.1 \times 10^6$ |

* Values determined under pseudo first-order conditions.
* Not determined.
* Preliminary values from single saturation trial.
* Kinetic values from Brenner and Fuller (20).
can partially compensate for a poor at P1 residue (22, 25).

Kex2 Cleavage of Furin Substrates—Selected substrates in this series were also cleaved with the yeast processing protease Kex2 for comparison (Table II). Pre-steady state kinetics of Kex2 cleavage of substrate 3 showed a burst, consistent with previous examinations of Kex2 cleavage of model peptide substrates. Additionally, no substrate inhibition was observed with any of the substrates examined (Table II). Extension of the backbone beyond P4 with Ala-Ala (substrate 2) had very little effect, in keeping with previous observations that tetrapeptidyl- and pentapeptidyl-MCAs were cleaved by Kex2 with comparable efficiency (22, 25). The nature of the P6 side chain had a negligible effect on $k_{cat}/K_m$ for Kex2 (Table II).

Interestingly, higher $k_{cat}$ values were observed for substrates 1, 3, and 5, having Arg at P6, than for substrate 14 having Nle at P4 (Table II; Ref. 25). The observation that substrate 1 had a higher $k_{cat}$ than substrate 15 with Kex2 was surprising, because most known physiological Kex2 cleavage sites contain aliphatic P4 residues.

**DISCUSSION**

We have assessed the substrate specificity of furin at the four positions (P1, P2, P4, and P6) where basic residues are frequently found in peptides processed by furin. Substrates with favorable residues at all four positions (Lys or Arg at P6 and P2 and Arg at P2 and P1) are cleaved at rates consistent to those for internally quenched peptides or authentic proprotein substrates. Prior to this work, the best fluorogenic furin substrates were cleaved with $k_{cat}/K_m$ lower than similarly substituted biopeptides or internally quenched substrates. Several groups explained this rate discrepancy by hypothesizing that specific P′ residues were either required, or excluded, for efficient furin cleavage (14, 17, 19). For example, the bulky aromatic moiety of AMC-derived substrates was suggested to interfere with binding and, thereby, to decrease substrate reactivity (17). Although, recent results certainly indicate that P1′ and P2′ residues may decrease substrate reactivity (19), our data clearly show that the presence of amino-(methyl)-coumarin C-terminal to the cleavage site does not slow furin catalysis appreciably. Indeed, the best substrates in our series closely match $k_{cat}/K_m$ values reported for the best internally quenched substrates (17). Although, the role of P′-S′ interactions in furin specificity remains to be clearly defined, we have demonstrated that peptidyl-MCAs substrates with favorable interactions at P1, P2, P4, and P6 are cleaved with physiologically relevant rates in the absence of P′ residues.

Additionally, simple Ala-Ala extension of the N-terminal portion of a tetrapeptide substrate 1 to give a hexapeptide substrate 2 does not lead to an increase in $k_{cat}/K_m$ with either furin or Kex2. Although backbone contacts between P1′ and P4 are clearly important for cleavage by degradative subtilisins (26, 27), energetically significant interactions between processing proteases and the substrate backbone beyond P4 do not appear to be present.

We have also found that hexapeptidyl-MCA substrates display significant substrate inhibition at relatively low concentrations in saturation experiments (>10 $\mu$M), whereas the corresponding core tetrapeptidyl-MCA (substrate 1) and the commercially available furin substrate Boc-RVRR-MCA did not show any signs of substrate inhibition at substrate concentrations as high as 20-fold above $K_m$ (data not shown). Of the three hexapeptidyl-MCA substrates (2, 3, and 5) examined under saturation conditions, the two substrates with basic residues at P6 had significantly lower apparent $K_s$ (4.6 and 3.3 $\mu$M for 3 and 5, respectively; see Equation 3) than the P6 Ala substrate (93 $\mu$M). Additionally, lowering the pH of the reaction solvent (pH 6) led to a significant increase in apparent $K_s$ for P6 Arg substrate 5 from 4.6 $\mu$M (pH 7) to 28.4 $\mu$M (pH 6). These observations indicate that substrate inhibition depends on the length of the substrate, the number of basic residues present in the substrate, and the protonation state of acidic residues on solvent exposed surfaces of the enzyme. The $S_6$ subsite of furin is thought to be located in a relatively solvent exposed portion of the enzyme (28) and, although we cannot determine if the substrate inhibition is due to specific or nonspecific enzyme-substrate interactions, it is intriguing to note that furin specificity for P$_6$ Arg residues is also modulated by pH.

This substrate inhibition complicates analysis of previous examinations of furin specificity in the literature. For instance, Lazure et al. (19) compared Arg and Gly at P6 in a recent study using internally quenched substrates, reporting that P6 Arg actually decreased the second-order rate constant for otherwise comparable substrates. However, inspection of the saturation curves presented by Lazure and co-workers (Ref. 19, Figs. 4 and 8) indicates the presence of substantial substrate inhibition in those experiments, making the determination of $k_{cat}$ and $K_m$ as independent parameters unreliable. Lazure and co-workers (19) also identified peptides that inhibited furin but not PC1. These peptides were not simple competitive inhibitors, but rather exhibited complex inhibition kinetics involving interactions of the enzyme with at least two molecules of inhibitor. These data are also consistent with a substrate inhibition mechanism. The behavior of these inhibitory peptides along with the substrate inhibition reported here for furin and earlier for Kex2 in cleavage of internally quenched substrates (22), implies the existence of a second, inhibitory site in both furin and Kex2 that is capable of interacting with substrate-like molecules.

Although the $S_6$ subsite is not included in the consensus furin-cleavage site, recent results have shown that it contributes to substrate binding, particularly with peptides that lack optimal residues within the classical furin P1-P4 sequence (3). Our series of substrates has allowed us to examine the effect of P6 aliphatic and basic residues within the context of a variety of substitution patterns at P6. Arg and Lys at P6 both increase the $k_{cat}/K_m$ by 10-fold relative to the core P6 Ala substrate 2. P6 Arg residues act additively to increase the $k_{cat}/K_m$ by 10-fold relative to the corresponding P6 Ala substrate over a range of catalytic efficiencies (compare substrates 2 and 3, 4 and 6, 5 and 8). Exchange of Arg for Ala at P6 led to a 1000-fold increase in reactivity while Lys for Ala increases reactivity 80-fold regardless of the substitution pattern at P6. As depicted in Fig. 2, these data clearly establish that the $S_6$ and $S_5$ subsites of furin interact with the substrate independently and, thereby, repre-
sent the first reported example of subsite independence within the subtilisin family of serine proteases (22, 29).

As mentioned above, modeling studies of furin have suggested that the S_6 subsite is in a relatively solvent-exposed region of the enzyme (28) and, as such, may be sensitive to changes in the pH of the solvent. As shown in Table III, lowering solvent pH from 7 to 6 decreased k_{cat}/K_m for P_6 Arg substrate by 5-fold, whereas the corresponding P_6 Lys and Ala substrates were not affected. This selective decrease in reactivity of the Arg substrate suggests that small changes in the protonation state or hydrogen-bonding pattern of acidic residues in the S_6 subsite weakens interactions with the guanidinium ion of Arg more than with the ammonium ion of Lys. This interpretation is in line with model studies and energy calculations that indicate strong interactions with Arg residues require precise hydrogen bonds or salt-bridges, whereas effective binding of Lys residues is achieved with simple electrostatic interactions (30). Although the effects of lower pH on P_6 specificity are not large, recent work has shown that furin activation requires transport of the enzyme through an acidic compartment prior to secondary cleavage and release of the inhibitory pro-segment (31). Our data support the notion that furin specificity may be modulated by the acid-base properties of the processing compartment.

The S_6 subsite in furin has been well established as an important determinant of substrate specificity. The classical consensus furin motif specifies Arg at P_4 and, consistent with this formulation, replacement of P_4 Arg with Lys results in a 10-fold decrease in k_{cat}/K_m at two different cleavage efficiencies (compare substrates 3 and 6; 2 and 9 and see Fig. 1). Although this is less than the 538-fold defect reported by Lazare and co-workers in a tetrapeptide series (14), it is still quite significant and is consistent with observations that bioproteases with P_4 Arg to Lys exchanges cleave in vitro but at lower levels when compared with the P_4 Arg versions (32). A recent study of Ebola virus glycoprotein processing by furin underscores the biological importance of the Arg to Lys replacement at P_4 (23).

![Table III](image)

| Substrate | Sequence | k_{cat}/K_m (µM^{-1}s^{-1}) | Relative k_{cat}/K_m | Ph 7.0 | Ph 6.0 |
|-----------|----------|----------------------------|---------------------|--------|--------|
| 3         | AcRARYKR | MCA                        | 2.6 x 10^6          | 5.8 x 10^5 | 0.22 |
| 4         | AcRARYKR | MCA                        | 2.2 x 10^6          | 1.5 x 10^6 | 0.58 |
| 2         | AcAARYKR | MCA                        | 2.3 x 10^6          | 1.8 x 10^5 | 0.78 |

As mentioned above, modeling studies of furin have suggested that the S_6 subsite is in a relatively solvent-exposed region of the enzyme (28) and, as such, may be sensitive to changes in the pH of the solvent. As shown in Table III, lowering solvent pH from 7 to 6 decreased k_{cat}/K_m for P_6 Arg substrate by 5-fold, whereas the corresponding P_6 Lys and Ala substrates were not affected. This selective decrease in reactivity of the Arg substrate suggests that small changes in the protonation state or hydrogen-bonding pattern of acidic residues in the S_6 subsite weakens interactions with the guanidinium ion of Arg more than with the ammonium ion of Lys. This interpretation is in line with model studies and energy calculations that indicate strong interactions with Arg residues require precise hydrogen bonds or salt-bridges, whereas effective binding of Lys residues is achieved with simple electrostatic interactions (30). Although the effects of lower pH on P_6 specificity are not large, recent work has shown that furin activation requires transport of the enzyme through an acidic compartment prior to secondary cleavage and release of the inhibitory pro-segment (31). Our data support the notion that furin specificity may be modulated by the acid-base properties of the processing compartment.

The S_6 subsite in furin has been well established as an important determinant of substrate specificity. The classical consensus furin motif specifies Arg at P_4 and, consistent with this formulation, replacement of P_4 Arg with Lys results in a 10-fold decrease in k_{cat}/K_m at two different cleavage efficiencies (compare substrates 3 and 6; 2 and 9 and see Fig. 1). Although this is less than the 538-fold defect reported by Lazare and co-workers in a tetrapeptide series (14), it is still quite significant and is consistent with observations that bioproteases with P_4 Arg to Lys exchanges cleave in vitro but at lower levels when compared with the P_4 Arg versions (32). A recent study of Ebola virus glycoprotein processing by furin underscores the biological importance of the Arg to Lys replacement at P_4 (23).

One of the properties of furin is its specificity for substrates with basic residues at P_2, P_4, and P_6. This specificity is important in the processing of many proprotein substrates, such as the processing of the prosegment of Kex2 (25). Similar observations have been made concerning avian flu viruses (33, 34) and Newcastle disease virus (6, 35) in that viruses to highly virulent, pantropic strains that cause widespread, systemic infections. Based on our findings, a difference in furin substrate recognition of about 2 kcal could mean the difference between a mildly infective or non-pathogenic virus and a virulent, highly pathogenic one.

![Table IV](image)

| Position | Substitution | Furin, ∆ΔG‡ kcal/mol | Kex2, ∆ΔG‡ kcal/mol | Subtilisin, ∆ΔG‡ kcal/mol | Savinase, ∆ΔG‡ kcal/mol |
|----------|--------------|----------------------|---------------------|--------------------------|-------------------------|
| P_4      | R-A          | 1.4                  | 0.4                 | 2.9                      | 1.1                     |
| P_4      | R-A          | >4.8                 | 2.9                 | 3.5                      | 3.5                     |
| P_2      | K-A          | 1.7                  | 3.5                 | -1.2                     | 0.1                     |
| P_2      | R-K          | 3.1                  | 3.4-3.8             | 1.4                      | 2.4                     |

In contrast to Kex2, furin specificity is not crucially dependent on the S_2 subsite. Accordingly, furin does not display a strong preference for substrates with basic residues at P_2. As with S_2 P_6 interactions, inclusion of either Lys or Arg at P_2 improves k_{cat}/K_m by 10-fold relative to the corresponding P_2 Ala substrate. On the other hand, furin, like Kex2 and most other proprotein processing proteases, is highly selective for Arg at P_1 (22). This preference is, however, not absolute as shown by the fact that furin cleaves substrate 13 with a P_1 Lys fairly well. Although the k_{cat}/K_m for substrate 13 is 150-fold lower than that for the corresponding P_1 Arg substrate, this example shows that optimal substitutions at P_2, P_4, and P_6 can compensate for what is normally a poor residue at P_1. Similarly, it has been shown that a favorable P_4 residue can partially compensate for a P_1 Lys for Kex2 (25).

Interestingly, cleavage by Kex2 of P_2 Arg substrates in this series revealed elevated turnover number values relative to substrates based on physiological cleavage sites. These k_{cat} values are significantly higher than that for Kex2 cleavage of one P_2 Arg substrate examined previously (19), a discrepancy which may stem from differences in N-terminal protecting group or from differences in P_2 and P_3 residues.

Based on these systematic studies of furin specificity, it is now possible to make a detailed comparison of the energetics of subsite specificity among furin, Kex2, and two extensively characterized degradative subtilisins, subtilisin BPN' and Savinase (29). In Table IV, we have calculated the maximal values of ∆ΔG‡ for each enzyme at the S_6, S_4, S_2, and S_1 subsites. ∆ΔG‡, as explained in the Table IV legend, reflects the free-energy difference between the free enzyme and substrate and the transition state complex for two substrates. Because the pairs of substrates differ only at a single position, the comparison serves as a quantitative measure of discrimination by these enzymes at each subsite. Not surprisingly, the processing enzymes, furin and Kex2, show much higher levels of specificity. At each subsite, discriminatory energies of furin...
and Kex2 substantially exceed those of the degradative enzymes. Whereas all four enzymes show specificity at P₁, distinctions can be made based on the relative importance of P₂ and P₄. Furin and Savinase cleavage sites are determined primarily by P₁ and P₄ with, in the case of furin, P₂ and P₆ acting as auxiliary, or compensatory, contacts. In contrast, Kex2 and subtilisin BPN’ both exhibit similar degrees of discrimination at P₂ and P₄. Contacts at P₂ and P₄ serve accessory roles in determining cleavage site selection by Kex2 and, to a lesser extent, subtilisin BPN’. Thus, similar specificity patterns can emerge within these divergent families of proteases, illustrative of the modular nature of subsite recognition within the subtilisin superfamily. The increased level of discrimination in the processing enzymes relative to the degradative enzymes may reflect evolution of the processing enzymes from a less specific progenitor to avoid toxic effects of cleaving incorrect sites and substrates. Alternatively, the degradative enzymes may have evolved from the processing enzymes, in which case the residual patterns of subsite discrimination seen in the subtilisins would represent remnants of processing enzyme specificity.

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