Yeast Kex2 and human furin are subtilisin-related proprotein convertases that function in the late secretory pathway and exhibit similar though distinguishable patterns of substrate recognition. Although both enzymes prefer Arg at P1 and basic residues at P2, the two differ in recognition of P4 and P6 residues. To probe P4 and P6 recognition by Kex2p, furin-like substitutions were made in the putative S4 and S6 subsites of Kex2. T252D and Q283E mutations were introduced to increase the preference for Arg at P4 and P6, respectively. Glu255 was replaced with Ile to limit recognition of P4 Arg. The effects of putative S4 and S6 mutations were determined by examining the cleavage by purified mutant enzymes of a series of fluorogenic substrates with systematic changes in P4 and/or P6. Whereas wild type Kex2 exhibited little preference for Arg at P4, the T252D mutant and T252D/Q283E double mutant exhibited clear interactions with P6 Arg. Moreover, the T252D and T252D/Q283E substitutions altered the influence of the P4 residue on P1Arg. We infer that cross-talk between S4 and S6, not seen in furin, allows wild type and mutant forms of Kex2 to adapt their subsites for altered modes of recognition. This apparent plasticity may allow the subsites to rearrange their local environment to interact with different substrates in a productive manner. E255I-Kex2 exhibited significantly decreased recognition of P4 Arg in a tetrapeptide substrate with Lys at P1, although the general pattern of selectivity for aliphatic residues at P4 remained unchanged.

The subtilisin superfamily includes a subfamily of related processing proteases, the proprotein convertases that function in the late secretory pathway of diverse eukaryotic organisms including Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster, and mammals (1–4). Unlike the degradative subtilisins, which display a broad substrate specificity for hydrophobic residues (5), the proprotein convertases are post-translational modifying enzymes that process secretory proteins in a sequence-specific manner. In general, these proteases cleave C-terminal to clusters of basic residues, but their exact sequence specificity differs among the members of this family, even though they are >45% identical within their subtilisin-related domains. Similarities and differences in substrate recognition were illustrated by the enzymatic characterization of two members of this family, the S. cerevisiae protease, Kex2, and the human homologue, furin.

A detailed understanding of substrate recognition by Kex2 and furin has emerged from extensive analysis of the purified secreted, soluble enzymes using model peptide substrates. Based on these studies, the consensus cleavage site for Kex2 was determined to be (Ali/Arg)-Xaa-(Lys/Arg)-Arg (where Ali indicates an aliphatic amino acid), with the principal determinants being a basic residue at P2 and Arg at P1 (6–9).1 A conservative substitution of Lys for Arg at P1 reduced $k_{cat}/K_m$ of Kex2 1000-fold (10) and resulted in a change in the rate-limiting step from deacylation to acylation (10–12). Kex2 exhibits a less stringent preference at P4, with a dual specificity for either a basic or an aliphatic residue (10). Furin also exhibits a strict requirement for Arg at P1, but, unlike Kex2, it has reduced selectivity for P4 and increased dependence on P6 recognition (13–15). For example, substitution of Ala for Arg at P4 resulted in a 2500-fold decrease in $k_{cat}/K_m$ (14). Kex2 and furin also differ in P6 recognition. Furin exhibited a 10-fold preference for Arg versus Ala at P6 (14). Furthermore, the presence of a basic residue at P6 can partially compensate for the lack of Arg at P4 (14, 16–18). An examination of physiological Kex2 substrates does not indicate any obvious P6 selectivity, and in experiments with peptide substrates, Kex2 exhibits only a 2-fold preference for Arg at P6 (14). This difference in P6 recognition was also observed in interactions with derivatives of eglin-c that had been engineered to be potent inhibitors of Kex2 and furin (19). Kex2 exhibited only a slightly higher (~3-fold) affinity for Arg (as opposed to Gly) at P6 in an eglin-c variant having Arg at P1 and P4 (19). However, this same substitution of Arg for Gly at P6 had a striking qualitative effect on the interaction of the inhibitor with furin, in that it caused the eglin-c variant to be cleaved. This result implies that the mode of P6 recognition also is fundamentally different between Kex2 and furin, suggesting that, unlike Kex2, furin has a well defined S6 subsite.

Crystal structures of subtilisin-inhibitor complexes, such as that of subtilisin bound to Streptomyces subtilisin inhibitor, illustrate that the principal residues in subtilisin BPN’ that contact P3 are Tyr104 and Ile107 (20). Based on these structural

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1 Substrate residues near the cleavage site, N- to C-terminal, are designated Pn, Pn+1, Pn+2, ... P1, P0, where the arrow represents the scissile bond. The complementary enzyme subsites are designated Sn, Sn+1, Sn+2, ... S1 (6).
studies, several groups have mutated the S₄ pocket in an attempt to alter the P₄ substrate specificity of subtilisin (21–26). Wells and co-workers (27, 28) found that substitutions of Asp for Tyr104 in subtilisin BPN’ increased cleavage of substrates containing a P₄ Arg, but the resulting mutant protease did not discriminate between Arg and Phe at this position. In another study, acidic residues in furin predicted to interact with P₄ were mutated, and the mutant furin enzymes were co-transfected with a furin substrate, pro-von Willebrand factor. Substitution of Val for Asp in subtilisin, resulted in an enzyme that cleaved pro-von Willebrand factor with Ala at P₄ better than the wild type (WT) substrate (29).

In this work, the differences in substrate recognition by the S₄ and S₆ subsites of Kex2 and furin were explored by mutagenesis. Residues predicted to contribute to the specificity of P₄ and P₆ binding and that were different in Kex2 and furin were mutated in the yeast enzyme, and the substrate specificity of the mutants was analyzed. Substitutions in Kex2 were chosen prior to the availability of crystallographic data for Kex2 or furin and thus were based on examination of three-dimensional structures of subtilisins and the amino acid sequences and structural models of Kex2 and furin (20, 30–33). One group of mutations was generated with the goal of making Kex2 specificity more furin-like, by increasing selectivity for basic residues at P₄ and for Arg at P₆. In addition, the model for the dual specificity of the S₆ subsite was tested by making a Kex2 mutant that was predicted to exhibit reduced recognition for basic residues at P₄ while maintaining selectivity for aliphatic P₆ residues. Recently, the x-ray crystal structures were solved of the Kex2 catalytic domain complexed with tripeptidyl and tetrapeptidyl boronic acid inhibitors and of the furin catalytic domain complexed with a tetrapeptidyl chloromethylketone (34–36). Through the comparison of the P₄-S₄ interactions in Kex2 and furin, the structures have allowed us to interpret the results of these mutagenesis experiments with greater clarity. The biochemical data presented here will be discussed in light of the crystallographic structures.

EXPERIMENTAL PROCEDURES

Expression Strain—The genotype of the S. cerevisiae strain CB017 was MATa kex2:TRP1 pep4::HIS3 prb1::hisG pr1::hisG can1 ade2 leu2 ura3.

Materials—DNA restriction enzymes, T4 DNA ligase, and oligonucleotides were from Invitrogen, and Pfu turbo polymerase was from Stratagene. Peptide substrates Boc-LKR, MCA, and Pyr-RTKR were from Bachem, and all other peptide substrates were synthesized as described previously (7, 10, 14). General laboratory reagents were from Sigma and Fisher.

Site-directed Mutagenesis—All of the mutations were made by overlap extension (37). The following primers were used to make point mutations: (i) T252D, GGTGATATTACTCGTCTGCTGCTAGCTTGATTTA (sense) and GTCGAATATCACC (antisense); (ii) E255I, CGGAACATGTTGCTAGCTGCTGATTTA (sense) and TAAATGTCTTCC (antisense); (iii) Q283E, GGGAGACATTTAAGGGCCCTAGT and CTAGGCTTCCTAATGCTGCC (antisense); and (iv) V289A, GTACAGCTGCTGCTGCTGCTGCT and GCTCTTCTCTCTCCGCTGCC (antisense). The template for all single mutants was pAL7, which was used to create a PCR template vector encoding the full-length KEX2 gene with an additional XhoI site downstream of the P-domain (39), and its expression was regulated by its WT promoter. Q283E-Kex2 served as a PCR template for creation of the T252D/Q283E-Kex2 double mutant. PCR products were subcloned into pAL7α HindIII to BglII fragments, and the incorporation of each mutation was confirmed by DNA sequencing (University of Michigan DNA sequencing core).

Expression and Purification of Mutants—The substituted Kex2 DNAs were recombined with a linearized expression vector for the production of secreted, soluble Kex2 mutants. The general method was described in Ref. 40. Briefly, the expression vector pAL10 was a derivative of the secreted, soluble Kex2 expression plasmid, pG5KEX2A613 (9), in which a XhoI site was inserted in place of internal KEX2 sequences from a point 370 nucleotides downstream from the start codon to a point 1788 nucleotides downstream from the start codon, just 3′ to sequences encoding the P-domain. pAL7 vectors encoding the Kex2 mutants were linearized with BamHI and co-transformed into CB017 with XhoI-digested pAL10. Transformants containing recombinant plasmids and thus encoding mutant-secreted, soluble Kex2 were selected on synthetic dextrose complete-Ura plates. Individual colonies were grown overnight in synthetic dextrose complete-Ura liquid medium and then inoculated into 1040 expression medium (41). After growth at 30 °C for 24 h, the medium was checked for activity. Equal amounts of medium and substrate solution (140 μM BecQR, 1 MCA, 400 mM BisTris, 2 mM CaCl₂) were mixed in wells of a 96-well plate and release of the fluorogenic reporter was determined using a Molecular Devices fluoroscan fluorescence plate reader. The cells secreting active enzyme were reincultured into fresh medium and incubated for 24 h at 30 °C. The enzymes were purified as described (9). The purified proteins were active site-titrated as described (7, 9).

Kinetic Characterization of Wild Type and Mutant Enzymes—Pseudo first order and saturation measurements were carried out at 37 °C in 0.2 mM BisTris, 1 mM CaCl₂, 0.1% Triton X-100 as described (7, 10, 14).

Error Analysis—The error for all experiments is listed as S.D. in the form of the percentage of deviation of the average value for each data point. These values were calculated using Microsoft Excel.

RESULTS

Mutation of Kex2 to Alter Recognition of Basic Residues at P₄ and P₆—To increase recognition of basic side chains at P₄ and P₆, putative S₄ and S₆ residues were selected by comparison of Kex2 and furin sequence alignments and model structures as well as on results of previous mutagenesis experiments (24, 28, 29). Again, these residues were chosen prior to the availability of any crystallographic data. Substitution of Asp for Tyr104 in Kex2, a position equivalent to S₄ residue Tyr104 in subtilisin BPN’ and Asp233 in furin, was originally introduced to increase recognition of basic versus aliphatic residues at P₄, but Seizen et al. (32) tentatively assigned an insertion loop, with respect to subtilisin, in furin to form the S₄ subsite. Only very recently, the crystallographic data for Kex2 clarified the structure of this insertion (see “Discussion”). Within this region, furin has Asp at amino acid 264, equivalent to Glu263 in Kex2 (Figs. 1 and 2). Because substitutions at this position in both subtilisin and furin exhibited significant alterations in their P₄ specificities, this residue was also considered a good candidate for tuning the P₄ specificity of Kex2 (24, 28, 29). The S₆ subsite was more difficult to model because the degradative subtilisin described to date do not have a distinct binding pocket for P₆, but Seizen et al. (32) tentatively assigned an insertion loop, with respect to subtilisin, in furin to form the S₆ subsite. Only very recently, the crystallographic data for Kex2 clarified the structure of this insertion (see “Discussion”). Within this region, furin has Asp at amino acid 264, equivalent to Glu263 in Kex2 (Figs. 1 and 2). Glu263 was mutated to a Glu to mimic the charge at that position in furin while minimizing the change in geometry in the binding site. Glu at this position was expected to be well tolerated as it is also found in PC1/3 (33). The T252D/Q283E double mutant was constructed to determine whether this would result in a Kex2 mutant with furin-like specificity at P₄ and P₆. Finally, to decrease recognition of basic residues at P₄ while maintaining interactions with aliphatic side chains, Ile was substituted for Glu255, a potential site of interaction with basic residues equivalent to Ile167 in subtilisin and Glu256 in furin.

Effects of the T252D and Q283E Substitutions on Specificity for Basic and Aliphatic Amino Acids at P₄ in the Context of Tetrapeptide Substrates—Although the majority of known
physiological Kex2 substrates have an aliphatic residue at P₄, purified Kex2 can also cleave substrates with Arg at P₄. In fact, using the model substrates Ac-RYKR \( \text{MCA} \) and Ac-RYKR \( \text{MCA} \), Kex2 exhibited a 3-fold preference for the P₄ Arg substrate (see Table I) (10). Further investigation indicated that the positive charge of the guanidinium group of Arg, and not the aliphatic portion, was the critical determinant for the specificity have been performed with substrates lacking a P₆ residue. To evaluate the contribution of the S₆-P₆ interaction toward the processing of hexapeptide substrates, the proteolysis of a series of hexapeptide substrates was analyzed using pseudo first order kinetics (Fig. 3). However, in addition to specific interactions, P₅ and P₆ residues in hexapeptide substrates could conceivably provide nonspecific backbone contacts that could reduce the relative importance of P₄ binding. This possibility was tested for WT and mutant enzymes by comparing the \( k_{\text{cat}}/K_m \) ratio for a pair of hexapeptide substrates with Ala at P₅ and P₆ (AcAARYKR-MCA and AcAARYKR-MCA) to the \( k_{\text{cat}}/K_m \) ratio for the analogous tetrapeptide substrates (AcRYKR-MCA and AcAYKR-MCA; Fig. 4, Table I). In the case of WT Kex2, the specificity for Arg versus Ala at P₄ decreased from 28-fold in the tetrapeptide context to 3.4-fold in the hexapeptide context. Moreover, the presence of nonspecific contacts at P₅ and P₆ resulted in a 3.7-fold increase in \( k_{\text{cat}}/K_m \) of WT Kex2 for AcAARYKR-MCA as compared with AcAYKR-MCA, but no such increase was observed with any of the mutants (Table I). Furthermore, the mutants were markedly more specific than WT Kex2 for Arg versus Ala at P₄ in the P₅, P₆ Ala substrates. T252D/Kex2 displayed a 6.6-fold and Q283E-Kex2 displayed a 13-fold preference for P₄ Arg in the context of the hexapeptides. Even more strikingly, T252D/Q283E-Kex2 exhibited a 32-fold higher \( k_{\text{cat}}/K_m \) values with Arg versus Ala at P₄ with both the tetrapeptide and hexapeptide substrates (Table I and Fig. 4). Unlike wild type, the additional potential nonspecific contacts at P₅ and P₆ did not result in a decrease in P₄ specificity. T252D/Q283E-Kex2 displayed a 10-fold higher specificity than did WT-Kex2 for P₄ Arg in the context of the nonspecifically extended substrates. T252D Affects P₆ Recognition—Although the presence of Ala at P₅ and P₆ did not affect the P₄ specificity of the Kex2 mutants, this fact did not rule out P₆ recognition by these enzymes. A preference for Arg at P₆ was exhibited to some degree by both WT and the mutant Kex2 proteins (Table I and Fig. 5). In terms of \( k_{\text{cat}}/K_m \) for cleavage of AcRAAYKR-MCA and AcAARYKR-MCA, both WT and Q283E-Kex2 exhibited relatively modest preferences for Arg (4- and 2.4-fold, respectively). Surprisingly, however, the T252D mutation in the pu-
The specificity profile of the mutant Kex2 proteins. The filled bars represent the $k_{cat}/K_m$ ratio of AcAAARYKR $\mid$ MCA/AcAAARYKR $\mid$ MCA, and the open bars are the ratio of AcAYKR $\mid$ MCA/AcAYKR $\mid$ MCA. All of the $k_{cat}/K_m$ data are listed in Table I.

In the case of furin, the P$_3$ side chain can clearly make a significant contribution to substrate recognition (14, 16, 42). A possible manifestation of this P$_6$ recognition was the observation that purified, soluble furin was inhibited by high concentrations ($>5\ \mu$M) of hexapeptide, but not tetrapeptide, substrates (14). In contrast, high concentrations of hexapeptide substrates did not inhibit WT Kex2 (14). Because T252D/Q283E-Kex2 showed improved P$_6$ recognition relative to WT Kex2, saturation kinetics were performed using AcRKYKR $\mid$ MCA/AcRKYKR $\mid$ MCA. However, the T252D/Q283E-Kex2 exhibited saturation kinetics indicating that substrate inhibition did not occur (Fig. 6). In the cleavage of AcRKYKR $\mid$ MCA, T252D/Q283E-Kex2 had a lower $k_{cat}$ than WT Kex2 (40 s$^{-1}$ for T252D/Q283E-Kex2 and 200–250 s$^{-1}$ for WT) but also exhibited a lower $K_m$ (0.2 $\mu$M T252D/Q283E-Kex2 and 0.8 $\mu$M for WT) (14).

Cross-talk between S$_4$ and S$_6$ Subsites of Kex2—The $k_{cat}/K_m$ ratios for cleavage of AcRKYKR $\mid$ MCA and AcAAAYKR $\mid$ MCA were compared with $k_{cat}/K_m$ values for cleavage of AcRKYKR $\mid$ MCA and AcAAAYKR $\mid$ MCA to reveal whether the presence of a favorable residue (i.e. Arg) at P$_6$ altered the P$_4$ specificity profile of WT or mutant forms of Kex2 (Fig. 7). Whereas P$_4$ specificity of WT and Q283E-Kex2 was only slightly decreased when Arg was present at P$_6$, specificity for Arg at P$_4$ was substantially reduced with Arg at P$_6$ in the case of T252D-Kex2 and T252D/Q283E-Kex2 (Table I and Fig. 7). The relative preference of T252D-Kex2 for P$_4$ Arg versus Ala decreased from 6-fold with Ala at P$_6$ to 1.2-fold with Arg at P$_6$. The effect of P$_6$ on P$_4$ specificity was even more pronounced with T252D/Q283E-Kex2. In the context of P$_6$ Ala, the double mutant had a 32-fold preference for P$_4$ Arg versus Ala. The inclusion of Arg at P$_6$ decreased P$_4$ specificity nearly 10-fold, resulting in only a 3.5-fold preference for P$_4$ Arg.

The E255I Substitution Reduces Recognition of Basic Residues at P$_6$—The effects of the E255I, predicted to lessen the recognition of basic residues at P$_6$, were clearest in the context of a P$_4$ Lys residue. E255I-Kex2 showed a marked decrease in preferential processing of a substrate with a P$_4$ Arg relative to one with a P$_4$ Ala when Lys was present at P$_6$ (Table II and Fig. 8). Whereas WT Kex2 exhibits a ~100-fold higher $k_{cat}/K_m$ for AcRYKK $\mid$ MCA than for AcAYKK $\mid$ MCA, E255I-Kex2 exhibited only a 6-fold preference for the P$_4$ Arg substrate (Table II and Fig. 8). This was the result of a 4-fold decreased $k_{cat}/K_m$ for cleavage of AcRYKK $\mid$ MCA by the mutant enzyme as compared with the WT combined with a 4-fold increase in $k_{cat}/K_m$ for cleavage of AcAYKK $\mid$ MCA by the mutant enzyme as compared with the WT.

In contrast, E255I-Kex2 exhibited little or no decrease in the recognition of aliphatic residues at P$_6$ in the context of Lys at P$_4$. Relative to WT Kex2, E255I-Kex2 exhibited 2–4-fold higher $k_{cat}/K_m$ values for cleavage of P$_4$ Lys substrates having an aliphatic residue, Ala, Nle, Val, or cyclohexylalanine, at P$_6$. E255I-Kex2 cleaved AcQYKK $\mid$ MCA, with norleucine at P$_4$, with a $k_{cat}/K_m$ increased ~2-fold relative to the WT enzyme. As a result, E255I-Kex2 maintained a 30-fold preference for Nle versus Ala at P$_4$ (WT Kex2 exhibits a 77-fold preference). The ratios of catalytic efficiencies for processing of AcVYKK $\mid$ MCA versus AcAYKK $\mid$ MCA and AcAYKK $\mid$ MCA versus AcAYKK $\mid$ MCA were not significantly altered by the E255I substitution (Fig. 9). Thus, E255I Kex2 exhibited reduced recognition of a basic residue at P$_6$ without a concomitant loss of recognition of aliphatic residues. As a result, whereas WT Kex2 exhibits similar $k_{cat}/K_m$ values for P$_6$ Arg and aliphatic substrates having Lys at P$_1$, the mutant enzyme exhibited 5-fold preference for Nle versus P$_1$ Arg and a 13-fold preference for cyclohexylalanine versus P$_1$ Arg.

The preferences shown by E255I-Kex2 for P$_4$ aliphatic versus basic residues largely disappeared with Arg at P$_4$. Relative to WT Kex2, E255I-Kex2 exhibited slightly diminished activity and slightly relaxed P$_4$ recognition in cleavage of the hexapeptide and tetrapeptide substrates having a P$_1$ Arg (Table II). Pairwise comparison of AcRKYKR $\mid$ MCA and AcAAAYKR $\mid$ MCA reveals that WT Kex2 exhibited a 3.4-fold preference of WT Kex2 for P$_6$ Arg versus Ala. In contrast, E255I-Kex2 did not discriminate between the two substrates. The ratio of $k_{cat}/K_m$ values for cleavage of AcRKYKR $\mid$ MCA and AcAAAYKR $\mid$ MCA was reduced from 10-fold for WT Kex2 to 3-fold for E255I-Kex2. Similarly, the ratio of $k_{cat}/K_m$ values for cleavage of AcQYKK $\mid$ MCA and AcAYKK $\mid$ MCA was reduced from 10-fold for WT Kex2 to 4-fold for E255I-Kex2. P$_6$ recognition was not significantly affected, however. Comparison of the ratios of $k_{cat}/K_m$ values for cleavage of AcRKYKR $\mid$ MCA and AcAAAYKR $\mid$ MCA revealed that WT Kex2 exhibited a 4.3-fold and E255I-Kex2 exhibited a 3.3-fold preference for P$_6$ Arg versus Ala.

**DISCUSSION**

In this work, we performed site-directed mutagenesis of residues initially predicted to be key elements of the S$_4$ and S$_6$ subsites of Kex2 and examined their effects on P$_4$ and P$_6$ recognition. The specificity of the Kex2 mutants was tested by measuring $k_{cat}/K_m$ for cleavage of a series of substrates with systematic substitutions at P$_4$ and P$_6$, and none of the substitutions significantly affected the stability or activity of the mutant enzymes. Although, in general, recognition of the tetrapeptide substrates by the mutant enzymes only differed slightly from that by wild type Kex2, experiments with...
Mutational Analysis of Kex2 Protease P4 and P6 Specificity

In a complementary set of experiments, an attempt to further investigate the limit the recognition of P4 basic substrates, the wild type Glu at mutants. In a complementary set of experiments, an attempt to solvent-exposed groove just beyond the P3 residue, consistent presence of a favorable, basic P6 significantly affected the en -

AcAAKYKR
AcARYKR
AcAARYKR
AcRAKYKR
AcRARYKR
AcRaαYKR
AcAYKR
AcRYKR
AcBFYKR
AcAYKK
AcRYYKK
AcβYKK

These data were previously published (14).

The standard deviations were ≤ ±15% of each kcat/Km value.

| Substrate       | Wild type | T252D | Q283E | T252D/Q283E |
|-----------------|-----------|-------|-------|-------------|
| AcAAAYKR        | 1.6 × 10⁸ | 8.3 × 10⁶ | 1.6 × 10⁷ | 3.7 × 10⁸ |
| AcAARYKR        | 5.4 × 10⁷ | 5.3 × 10⁷ | 2.1 × 10⁷ | 1.2 × 10⁸ |
| AcRAKYKR        | 7.0 × 10⁸ | 1.2 × 10⁸ | 3.9 × 10⁷ | 5.5 × 10⁸ |
| AcRARYKR        | 9.6 × 10⁷ | 1.4 × 10⁸ | 8.0 × 10⁷ | 2.0 × 10⁹ |
| AcRaαYKR        | 1.4 × 10⁹ | 1.2 × 10⁶ | 3.9 × 10⁶ | 1.9 × 10⁸ |
| AcAYKR          | 1.5 × 10³ | 5.6 × 10³ | 2.5 × 10⁶ | 3.2 × 10⁹ |
| AcRYKR          | 4.3 × 10³ | 8.0 × 10³ | 1.0 × 10⁷ | 3.6 × 10⁹ |
| AcβYKR          | 1.5 × 10⁶ | 6.5 × 10⁶ | 1.5 × 10⁷ | 1.1 × 10⁹ |
| AcAYKK          | 1.5 × 10⁶ | 1.2 × 10⁴ | 5.0 × 10² | 1.1 × 10³ |
| AcRYYKK         | 1.2 × 10⁸ | 8.3 × 10³ | 1.5 × 10⁷ | 2.9 × 10⁹ |
| AcβYKK          | 9.2 × 10⁸ | 2.8 × 10⁵ | 3.8 × 10⁸ | 4.8 × 10⁹ |

Fig. 5. T252D-Kex2 can distinguish between Arg and Ala at P4. The bars represent the kcat/Km ratio of AcRAAYKR | MCA/ AcAAAYKR | MCA. The kcat/Km ratios with Arg or Lys at P6 (AcRARYKR | MCA/AcAARYKR | MCA or AcAARYKR | MCA/ AcAAAYKR | MCA) both demonstrate a 10-fold preference for P6 Arg (14). All of the kcat/Km data are listed in Table I.

hexapeptide substrates revealed specific recognition of P6 Arg by T252D substituted Kex2 mutants. To further investigate the nature of this specific effect on P6 Arg substrates, P4 recognition in the context of different P6 residues was examined. The presence of a favorable, basic P6 significantly affected the enzyme-substrate interaction at P4 by T252D substituted Kex2 mutants. In a complementary set of experiments, an attempt to limit the recognition of P4 basic substrates, the wild type Glu at 255 was substituted with Ile. Indeed, E255I diminished recognition of P4 Arg in substrates having Lys at P1.

After these experiments were completed, crystal structures of Kex2 and furin became available (34–36). The recent crystallographic data consist of the Kex2 subtilisin and P-domains complexed with a tripeptidyl and tetrapeptidyl boronic acid inhibitors, acetyl-Ala-Lys-Arg-Boro and acetyl-Arg-Glu-Lys-Arg-Boro, and furin inhibited by decanoyl-Arg-Val-Lys-Arg-chloromethylketone. All of the residues mutated in this study, Thr232, Glu285, and Gin283, reside on the surface of a shallow, solvent-exposed groove just beyond the P4 residue, consistent with these residues contributing to extended substrate selec-

Fig. 6. T252D/Q283E-Kex2 does not exhibit substrate inhibition with AcARAYKR | MCA. As demonstrated previously (14), furin exhibits substrate inhibition with hexapeptide substrates. Our most furin-like enzyme, T252D/Q283E-Kex2, was tested for substrate inhibition, and none was observed. (r = 0.96.)

Table II
Comparison of steady state kinetics for wild type and E255I Kex2
The standard deviations were ≤ ±15% of each kcat/Km value.

| Substrate       | Wild type | E255I |
|-----------------|-----------|-------|
| AcAAAYKR        | 1.6 × 10⁷ | 5.5 × 10⁶ |
| AcAARYKR        | 5.4 × 10⁷ | 5.7 × 10⁶ |
| AcRAKYKR        | 7.0 × 10⁷ | 1.8 × 10⁷ |
| AcRARYKR        | 9.6 × 10⁷ | 2.1 × 10⁷ |
| AcRaαYKR        | 1.5 × 10⁷ | 6.8 × 10⁶ |
| AcAYKR          | 4.3 × 10⁷ | 5.4 × 10⁶ |
| AcRYYKK         | 1.2 × 10⁸ | 1.7 × 10⁷ |
| AcβYKK          | 4.3 × 10⁷ | 2.1 × 10³ |
| AcAYKK          | 1.2 × 10⁸ | 5.0 × 10³ |
| AcRYYKK         | 1.2 × 10⁸ | 3.3 × 10³ |
| AcβYKK          | 9.2 × 10⁸ | 1.7 × 10³ |
| AcAYKK          | 1.4 × 10⁸ | 5.5 × 10⁴ |
| AcβYKK          | 1.3 × 10⁸ | 4.3 × 10³ |

a These data were previously published (14).
b These data were previously published (10).
direct interaction between P$_4$ and either Thr$_{252}$ or Gln$_{283}$ is not observed in the Kex2 structure.

Although these structures provide information about the S$_4$-P$_4$ interaction, there is no direct structural data in regards to the S$_6$-P$_6$ interaction. However, some general aspects of P$_6$ recognition have been postulated (34, 35). Because Thr$_{252}$ and Gln$_{283}$ are located on the protein surface and are solvent-accessible, it is possible that either substitution at these positions or binding of an extended substrate could facilitate reorientation of the side chains within the groove to maximize contact with the substrate. On the other hand, although the putative binding site for P$_6$ is not clear from this structure, Thr$_{252}$ is oriented in a groove distal to the active site and could potentially interact with a P$_6$ residue. Further analysis will require a structure of Kex2 in complex with a hexapeptidyl adduct.

The furin crystal structure revealed structural evidence for the critical requirement for P$_1$ and P$_4$ Arg substrate residues (34). In this structure, residues equivalent to Glu$_{255}$ and Gln$_{283}$ in Kex2 interact specifically with the P$_4$ Arg (Glu$_{236}$ and Asp$_{264}$ in furin). The analogous residue to Thr$_{252}$ in furin is Asp$_{233}$ and appears to orient the Glu$_{236}$ toward the P$_4$ Arg. Because this structure consists of furin complexed with a tetrapeptide chloromethylketone inhibitor, no direct information is provided as to the P$_6$-S$_6$ interaction. Furthermore, the location of a S$_6$ binding pocket is not obvious, even though biochemical evidence suggests a separate binding site for P$_6$ Arg residues in the context of a P$_4$ Arg substrate (14). Henrich et al. (34) predict that Glu$_{230}$ and the furin residue that is equivalent to T$_{252}$D in Kex2, Asp$_{233}$, may interact with the P$_6$ Arg directly.

This study revealed that wild type Kex2 does exhibit a modest preference for Arg at P$_6$, a fact that was previously underappreciated (14). However, T$_{252}$D-Kex2 and T$_{252}$D/Q$_{283}$E-Kex2 both displayed much stronger recognition of P$_6$ Arg. Moreover, the P$_6$ specificities of T$_{252}$D-Kex2 and the double mutant were similar to furin in their preference for Arg versus Ala at P$_6$ (14). Thus, the T$_{252}$D substitution was able to discern a favorable P$_6$ substrate to the same degree as furin. Although T$_{252}$D was initially predicted to interact primarily with P$_4$ and not P$_6$ in the previous models of Kex2, the crystal structure of furin suggested that the analogous residue in furin, Asp$_{233}$ may form the S$_6$ subsite (34). Our biochemical data indicate that T$_{252}$D imparts discrimination at P$_6$ and support that this residue may be involved in identification of P$_6$ residues. In an effort to further compare the mode of recognition for hexapeptide substrates by furin and the Kex2 mutants, saturation kinetics were performed with T$_{252}$D/Q$_{283}$E-Kex2 and a hexapeptidyl adduct. In previous experiments, furin displayed substrate inhibition at high concentrations of substrate.

**FIG. 7.** The substrate residue at P$_4$ can affect the P$_6$ specificity of T$_{252}$D-Kex2 substituted Kex2, and Q$_{283}$E-Kex2 exhibits P$_4$ specificity that was relatively independent of the context of P$_6$. The filled bars represent the $k_{cat}/K_m$ ratio of AcAARYKR | MCA/ AcAAAYKR | MCA, and the open bars are the ratio of AcRARYKR | MCA/AcRAAYKR | MCA. All of the $k_{cat}/K_m$ data are listed in Table I.

**FIG. 8.** Comparison of relative processing of Ac-RYKK | MCA versus Ac-AYKK | MCA by wild type and E$_{255}$I Kex2. WT Kex2 exhibits 100-fold preference for Arg versus Ala at P$_4$ in the context of a P$_1$ Lys tetrapeptide substrates. In contrast, E$_{255}$I-Kex2 preference for Arg versus Ala in the same context is only 6-fold. E$_{255}$I-Kex2 has a greatly diminished recognition for Arg, which is consistent with the model that Glu$_{255}$ is involved in P$_4$ Arg substrate recognition. All of the $k_{cat}/K_m$ data are listed in Table II.

**FIG. 9.** The pattern of recognition of aliphatic P$_2$ residues by E$_{255}$I-Kex2 is relatively unchanged as compared with wild type. The catalytic efficiency of processing either AcVYKK | MCA (filled bars), AcVYKK | MCA (open bars), or AcFYKK | MCA (hatched bars) relative to AcAYKK | MCA is shown. The ratios were derived from the data in Table II.
This inhibition was potentially due either to an inhibitory binding mode of the substrate within the active site or to substrate binding at a second site that was outside of the active site and inhibited processing via a putative allosteric mechanism (14). Although no substrate inhibition was observed with T252D/Q283E-Kex2 under saturating conditions, the hexapeptide substrate may not bind to the active site of T252D/Q283E-Kex2 in an inhibitory fashion, or Kex2 may not possess the second, inhibitory binding site. However, this finding did not necessarily indicate that T252D/Q283E-Kex2 recognized P_6 Arg in a different manner than furin because the substrate identification by furin and its inhibition by substrate may be two unrelated, independent events.

Because T252D-substituted Kex2 enzymes specifically recognized P_6 Arg, the effect of P_6 identity on P_4 recognition was investigated. When substrates were extended nonspecifically with Ala at P_5 and P_6, all of the mutants, but not wild type Kex2, maintained their P_4 specificity for Arg versus Ala with both the tetrapeptide and hexapeptide substrates. This suggested that, unlike wild type, interaction of the mutant Kex2 enzymes with neither the Ala side chain nor the peptide backbone contributed significantly to substrate discrimination. Although in the case of the tetrapeptide substrates, T252D-Kex2 and Q283E-Kex2 displayed slightly less specificity for P_4 Arg than did wild type enzyme, the mutant enzymes exhibited a greater preference for P_4 Arg in the context of hexapeptide substrates than did wild type. The specificity for P_4 by T252D/Q283E-Kex2 was not only unaltered by extending the substrate nonspecifically, but the degree of specificity was similar to that of wild type Kex2 with the tetrapeptide substrates. These data indicated that the recognition of both P_4 and P_6 by the mutant Kex2 enzymes differed from wild type and that the structures of their S_4 and S_6 subsites were distinct from those of wild type Kex2.

The presence of an Arg residue at P_6 did not influence the specificity at P_4 for Arg versus Ala in Q283E-Kex2. The relatively small increase in kcat/Km for the P_6 Arg substrates as compared with those having Ala at P_6 suggested that Glu_{252} may not interact directly with P_6 as was initially predicted by our model. Instead, Glu_{252} may function in the S_4-P_4 interaction, making direct contact with P_4 similarly to furin (34). Alternatively, the interface of the S_4-S_6 subsite may be rearranged in Q283E-Kex2 such that S_4 is shielded from the consequences of the S_6 interaction with substrate. This explanation is supported by the fact that Q283E-Kex2 did not exhibit a strong preference for either Ala or Arg at P_6. Moreover, the crystal structure of Kex2 indicated that Glu_{252} may play an indirect role in substrate recognition by altering the structure of the pocket and orienting the substrate toward Glu_{252} and Glu_{499} of the S_4 subsite (35).

In contrast to Q283E-Kex2 and furin, the P_4 specificities of wild type Kex2, T252D-Kex2, and T252D/Q283E-Kex2 were affected by the presence of Arg at P_6. The energetically favorable contact provided by P_6 Arg seemed to diminish P_4 recognition by the T252D-containing enzymes. In effect, cross-talk between S_5 and S_6, which was seen at a low level with wild type Kex2, was amplified by Asp at 252. The addition of Q283E further increased the degree of cross-talk between the subsites. This interdependence between the S_5 and S_6 subsites was characterized by the ability of the P_6-S_6 interaction to influence the S_5 subsite interaction with its corresponding P_4 residue. These data suggest a fundamental difference between Kex2 and furin, which, from biochemical data, clearly displays independent recognition for P_4 and P_6 (14).

The interplay between the S_4 and S_6 subsites in T252D-Kex2 and T252D/Q283E-Kex2 and, to a lesser degree, in wild type Kex2 suggests that a degree of plasticity exists between the S_4 and S_6 subsites. When a P_6 Arg is present, S_4 and S_6 may reorganize to form the largest possible number of electrostatic and hydrophobic contacts to drive catalysis (Fig. 10). Moreover, the T252D substitution in Kex2 enhanced the ability for the S_4 and S_6 subsites to adapt to substrates with various P_4 and P_6 residues, possibly by increasing the number of possible local conformations of the enzyme. Because residue 252 was modeled to be at the interface of S_4 and the putative S_6, it may contact directly or enhance other interactions with substrate side chains in either the S_5 or S_6 subsite. Also, the loop formed by residues 249–252 in Kex2 is in an alternate conformation than is observed for furin, and this repositioning of the loop has been hypothesized to contribute to the differences in P_4 substrate recognition between Kex2 and furin (34, 35).

Multiple modes of substrate recognition may result from these malleable, extended subsites. Similar flexible binding sites were also observed when mutations were made in the α-lytic protease S_1 subsite. Crystallographic data of M192A and M213A α-lytic protease mutants, with and without substrate analogues bound, demonstrated that there were local changes in conformation to accommodate the various P_1 residues; consequently, the mutant appeared to have relaxed specificity (43). Furthermore, crystal structures of Savinase with a Gly substitution residue, Ile_{107}, which is analogous to Glu_{252} in Kex2, demonstrated that the Savinase S_6 subsite was structurally flexible. Other residues in the binding pocket, as well as the protein backbone, rearranged to adapt to the mutation of Ile_{107} (24). These examples demonstrate that the crystal structures of the native enzyme can only serve as a tool when analyzing mutagenesis data because substitutions may alter the structure of an enzyme.

The other Kex2 mutant with an altered P_6 specificity profile was E225I. Previously, a dual mode of binding aliphatic and basic P_6 residues was observed with wild type Kex2 (10), and the E225I substitution in Kex2 affected each binding mode differentially. Recognition of substrates with Arg at P_6 was greatly decreased by E225I, which suggests that this residue is involved in recognition of P_6 basic residues. Indeed, Glu_{252} in the crystal structure of Kex2 also exhibits a direct contact with the P_4 Arg substrate (35). However, instead of a dramatic increase in specificity for substrates with aliphatic amino acids at P_4, E225I displayed a relaxed preference for Ala, Nle, Val, and cyclohexylalanine at P_4. These results support the hypothesis that recognition of P_4 basic and aliphatic residues may involve independent modes of binding. The ability of E225I-
Kex2 to accommodate different P₄ residues also indicates that the P₄ pocket may be flexible. Although members of the Kex2/furin family of proteases are highly homologous and many of the mammalian members have overlapping tissue expression profiles, genetic studies with mice have indicated that the function of the mammalian proteases are quite distinct from one another (44–46). Although all of these proteases are believed to cleave preferentially C-terminal to a P₁ Arg, not much is known about their extended specificity except in the case of Kex2 and furin. However, preliminary data indicate that the substrate recognition at a tetrapeptide substrates tested, suggesting that PC7 may have the overall activity of Kex2 protease. Binding site distal to the active site residues without sacrificing and that amino acid substitutions can be made in the substrate recognition for residues beyond P₄ (47). Modulation of extended substrate recognition might enable the evolution of an expanded repertoire of protease specificities without impacting either the catalytic mechanism or the proximal elements of substrate recognition. Indeed the present work demonstrates that the P₄ pocket may be flexible.

Acknowledgments—We thank Todd Holyoak and Dagmar Ringe for communication of results prior to publication.

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