One-step site-directed mutagenesis of the Kex2 protease oxyanion hole

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Background: Members of the subtilisin family of serine proteases usually have a conserved asparagine residue that stabilizes the oxyanion transition state of peptide-bond hydrolysis. Yeast Kex2 protease is a member of the subtilisin family that differs from the degradative subtilisin proteases in its high substrate specificity; it processes pro-α-factor, the precursor of the α-factor mating pheromone of yeast, and also removes the propeptide from its own precursor by an intramolecular cleavage reaction. Curiously, the mammalian protease PC2, a Kex2 homolog that is likely to be required for pro-insulin processing, has an aspartate in place of asparagine at the 'oxyanion hole'.

Results: We have tested the effect of making substitutions of the conserved oxyanion-hole asparagine (Asn 314) of the Kex2 protease. To do this, we have developed a rapid method of site-directed mutagenesis, involving homologous recombination of a polymerase chain reaction product in yeast. Using this method, we have substituted alanine or aspartate for Asn 314 in a form of Kex2 engineered for secretion. Transformants expressing the two mutant enzymes could be identified by failure either to produce mature α-factor or to mate. The Ala 314 enzyme was unstable but the Asp 314 enzyme accumulated to a high level, so that it could be purified and its activity towards various substrates tested *in vitro*. We found that, with three peptides that are good substrates of wild-type Kex2, the k_{cat} of the Asp 314 enzyme was reduced ~4500-fold and its K_M ~4-fold, relative to the wild-type enzyme. For the peptide substrate corresponding to the cleavage site of pro-α-factor, however, k_{cat} of the Asp 314 enzyme was reduced only 125-fold, while the K_M was increased 3-fold. Despite its reduced catalytic activity, however, processing of the mutant enzyme *in vivo* — by the intramolecular cleavage that removes its amino-terminal pro-domain — occurs at an unchanged rate.

Conclusions: The effects of the Asn 314–Asp substitution reveal contributions to the reaction specificity of the Kex2 protease of substrate residues amino-terminal to the pair of basic residues at the cleavage site. Aspartate at the oxyanion hole appears to confer k_{cat} discrimination between substrates by raising the energy barrier for productive substrate binding: this may have implications for pro-insulin processing by the PC2 protease, which has an aspartate at the equivalent position. The rate of intramolecular cleavage of pro-Kex2 may be limited by a step other than catalysis, presumably protein folding.

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Background

Serine proteases are among the most extensively characterized enzymes (for review, see [1]). The hydrolytic mechanism involves the catalytic triad of residues, Asp, His and Ser, arranged in a conserved geometry. Remarkably, this mechanism appears to have evolved at least twice: once with the chymotrypsin fold and another time with the subtilisin fold. Crystal structures of serine proteases in complexes with transition state analogs have identified key hydrogen bonds that stabilize the oxyanion reaction intermediate. In chymotrypsin, backbone amide hydrogens are positioned to interact with the carbonyl oxygen when it develops a negative charge [2]. In subtilisin, the side chain of Asn 155 forms part of the 'oxyanion hole', contributing a key hydrogen bond to the carbonyl oxygen, in addition to the hydrogen bond donated by the amide proton of the nucleophilic Ser 221 [3]. The oxyanion-hole asparagine is a conserved feature of the subtilisin family, but its role has been evaluated by mutagenesis only in subtilisin itself [4,5].

Kex2 protease (*Saccharomyces cerevisiae* Kexin, E.C. 3.4.21.61) is the prototype of a 'Kex2 subfamily' of subtilisin-related enzymes, which are thought to possess high substrate specificity and to function within Golgi or post-Golgi organelles to process precursor proteins by cleavage carboxy-terminal to pairs or multiples of basic residues [6,7]. The biosynthesis [8], intracellular localization [9,10], and kinetic properties of Kex2 protease have been studied extensively. By truncating the Kex2 coding sequence before the region encoding the protein's transmembrane anchor, we have been able to purify and characterize a secreted, soluble form of the enzyme — ss-Kex2 [11].

Kex2 is synthesized as a pro-enzyme that is processed in the endoplasmic reticulum [8] by cleavage at the peptide bond carboxy-terminal to a Lys–Arg dipeptide, removing an amino-terminal pro-peptide. The cleavage reflects the intrinsic specificity of the enzyme [11] and occurs by an intramolecular reaction ([12] and P Gluschankof and RS Fuller, unpublished data). The pro-domain of an active-site His 213–Ala mutant of Kex2 protease was not cleaved, even when expressed in cells containing wild-type Kex2 protease (P Gluschankof and RS Fuller, unpublished data). In *Bacillus subtilis*, however, active-

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site mutants of subtilisin can be made and obtained as mature, though sparingly active, proteases by co-cultivation with bacteria expressing wild-type subtilisin [13,14].

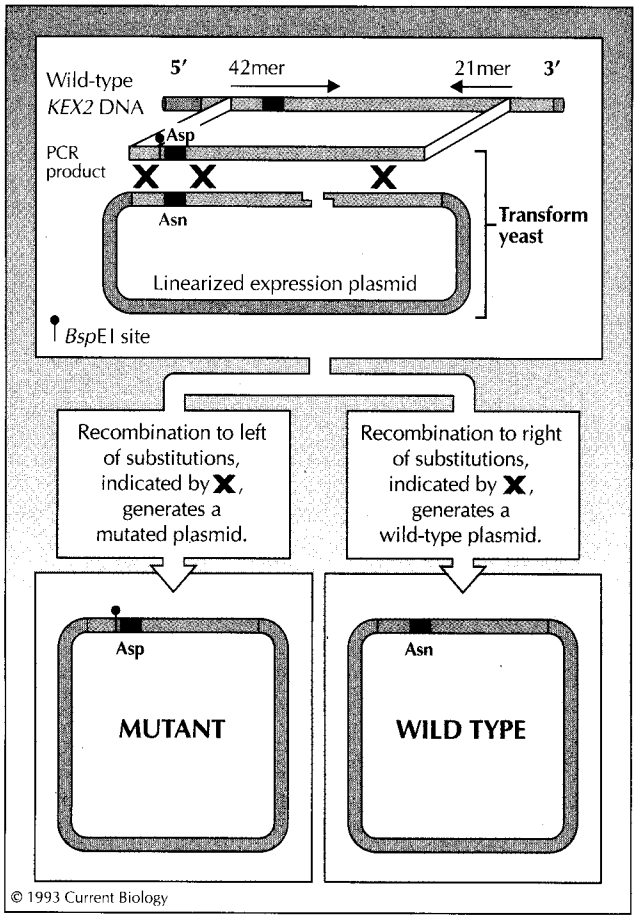

Fig. 1. One-step site-directed mutagenesis strategy. A 153 bp fragment encoding an amino-acid substitution at codon 314 and silent substitutions at codon 312 that introduce a site for *Bsp*EI was generated by PCR using the indicated primers. Yeast cells were co-transformed with this fragment and *Bgl*II-digested yeast expression plasmid pG5-*KEX2Δ613*. This plasmid is identical to pG5-*KEX2ΔC3* [11]. The allele number '*Δ613*' now indicates truncation of the *KEX2* coding sequence after codon 613, deleting the Ser/Thr-rich domain, the transmembrane domain and cytosolic tail. In addition to *KEX2* sequences, the plasmid contains pBR322 with an intact β-lactamase gene and the *URA3* gene and 2μ circle of *S. cerevisiae*. Ligation of the linear plasmid or recombination on both sides of the *Bgl*II site is required for the plasmid to be replicated in yeast. When the plasmid is regenerated by recombination distal to the sites of mutation, the mutation is incorporated into the plasmid and the resulting yeast transformant produces the mutant protein.

In the present work, substitution of Asn314 by Ala or Asp was accomplished by recombination *in vivo* of a polymerase chain reaction (PCR) [15] product and a linearized yeast expression plasmid. Changing Asn314 to Ala reduced both the activity and the stability of the protein. Surprisingly, however, changing Asn314 to Asp, which is present at the equivalent position in the mammalian Kex2 homolog PC2 [16], had little effect on the rate of intramolecular pro-domain cleavage or the pH-dependence of catalysis, despite reducing k_{cat}/K_M for the cleavage of peptide substrates 1000-fold. Although three good Kex2 substrates were cleaved by ss-Kex2 Asp314 with ~4500-fold lower k_{cat}, the best substrate, derived from the site of cleavage in the physiological Kex2 substrate pro-α-factor, was cleaved with only a 125-fold lower k_{cat}. This k_{cat} discrimination, not observed in cleavage of the same substrates by wild-type Kex2 protease, may be relevant in understanding the specificity of pro-insulin processing by PC2 [17,18].

Results

One-step site-directed mutagenesis

Mutations were introduced in one step into the gene encoding ss-Kex2 on an expression plasmid. Yeast cells were co-transformed with the linearized plasmid and PCR products carrying sequence changes introduced by the primers used in their synthesis (Fig. 1). The yeast expression plasmid for ss-Kex2, pG5-*KEX2Δ613* (formerly called pG5-*KEX2ΔC3*) [11], was linearized at its unique *Bgl*II site, 87 nucleotides 3′ to the Asn314 codon. A 153 base pair (bp) mutant PCR product was generated from a wild-type *KEX2* subclone (Fig. 2a). Mismatches were incorporated at nucleotides 25–32 of the 42 bp 5′ PCR primers (Fig. 2b, primers NA and ND), allowing efficient priming by the 3′ ends of the primers, but permitting recombination distal (5′) to the altered sites. The 3′ primer, 21mer Nrev, contained only wild-type sequences and was used in both mutagenic PCRs.

Table 1. Ura$^+$ colonies per pG5-*KEX2Δ613* transformation.

Bgl II treatment		–	+	+
Inclusion of PCR product		–	–	+
Strain	5′ primer			
ABY01	ND	244	300	792
CB017	NA	92	219	636

Plasmid pG5-*KEX2Δ613* carries the *URA3* gene and confers growth on uracil-lacking (–ura) media. Yeast strains were transformed as described in [35] with 60 ng of plasmid and, where indicated, 60 ng of PCR product (100-fold excess).

The results of two sets of yeast transformations are shown in Table 1. Addition of PCR products to linearized DNA increased transformation efficiency threefold, suggesting that roughly two out of three transformants arose from recombination between plasmid and PCR product sequences. Recombination between the plasmid and the PCR product could regenerate either a wild-type or mutant plasmid (Fig. 1). Recombination at the 5′ side of the *Bgl*II site could occur either proximal (3′) or distal (5′) to the sites of mutation. Proximal events would regenerate the wild-type plasmid, distal events would introduce the mutations.

Fig. 2. (a) DNA sequence of a portion of the *KEX2* gene, showing the encoded protein sequence [36]. The *Bgl*II site is indicated. Note that 72 bp in the middle are not shown. **(b)** DNA sequences of the PCR primers used in this study. The *Bsp*EI site is indicated. Bases that differ from wild type are shown are in bold. Primers NA and ND are mutagenic. Primer NX is used for diagnosing whether a plasmid has incorporated the *Bsp*EI site. Primer Nrev is the common 3′ primer. Oligonucleotides (PAN Facility, Stanford University) were used without purification. **(c)** Sequences of mutant isolates. NA3, *KEX2Δ613-Ala 314* isolate 3; NA6, *KEX2Δ613-Ala 314* isolate 6; ND2, *KEX2Δ613-Asp 314* isolate 2; ND13, *KEX2Δ613-Asp 314* isolate 13. Site-directed mutations are in bold.

To screen yeast transformants for the presence of site-directed mutations, DNA was amplified from ABY01 transformants (*MATa, ura3*) derived from the Asn 314–Asp mutagenesis. The mutagenic primers incorporated three mismatches distal to the Asn 314 codon that changed the Ser 312 codon AGT to TCC. Because the following codon was GGA (Gly 313), recombination of these sequences into the plasmid would generate a *Bsp*EI site (5′TCCGGA). Amplification of transformant DNA with primers NX and Nrev (Fig. 2b) showed whether the resulting PCR products contained the *Bsp*EI site. Six of 48 transformants produced PCR products that were partially digestible with *Bsp*EI (data not shown), indicating that the mutagenic PCR product had been introduced into the genome or the plasmid, but not both. Because the *Bsp*EI site was introduced distal to the Asn codon, all recombinantscontaining the *Bsp*EI site would be expected to contain the Asn 314–Asp substitution. Conversely, few recombinants lacking the *Bsp*EI site were expected to contain mutations at codon 314 (see below).

Transformants were grown to stationary phase in 1040 medium [11] and assayed for secreted, soluble Kex2 activity. Colonies without the introduced *Bsp*EI site displayed high levels of ss-Kex2 activity, characteristic of wild-type overexpressers (~400 u/μl in ABY01). Those with the introduced site secreted ~0.1 % the activity of wild-type. The fact that the *Bsp*EI site correlated with an effect on secreted activity ruled out the possibility that the genomic copy of *KEX2* had been modified.

The low activity of putative Asn 314–Asp substitution mutants of ss-Kex2 prompted us to test whether the yeast transformants could be assayed biologically for the presence of mutations. Kex2 protease activity is required for mating by cells of the α mating type [19]. Strain CB017 (*MATα kex2Δ*) was transformed with *Bgl*II-linearized pG5-*KEX2Δ613* plus PCR products from a reaction with NA and Nrev primers (Fig. 2) and Ura$^+$ prototrophs were selected (Table 1). Six of 34 transformants tested for mating proficiency failed to mate. The sterile transformants secreted ~1000-fold less ss-Kex2 activity into culture medium than mating-proficient transformants (data not shown).

For each mutagenesis experiment, plasmids that conferred ampicillin resistance in *Escherichia coli* were recovered from two independent transformants (candidate Asp 314 mutants were screened by PCR and activity assays, and Ala 314 mutants by mating and activity assays). Both strands of each purified plasmid were sequenced throughout and beyond the region potentially replaced by the PCR product (Fig. 2c). Each plasmid had the site-directed mutation at codon 314 and no additional mutations that might have been derived from unfaithful DNA replication, recombination or repair. One isolate, NA3, that was picked on the basis of sterility and low activity, encoded Asn at codon 314 but did not incorporate the changes at codon 312 that would generate the *Bsp*EI site (Fig. 2c). This isolate must have arisen from recombination between codons 312 and 314. PCR products amplified from each purified plasmid had the expected sensitivity or resistance to *Bsp*EI (data not shown). The apparent frequency of

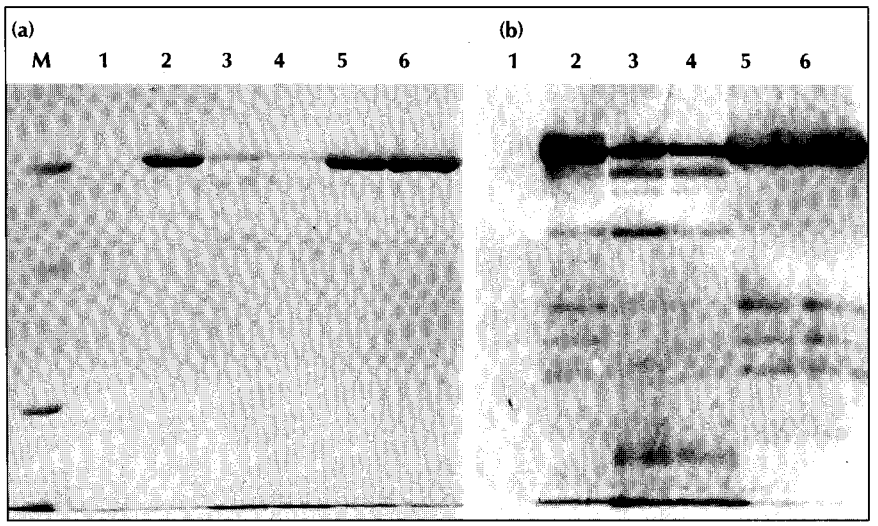

Fig. 3. Protein secreted by ABY01 transformants. **(a)** Protein was precipitated from 1 ml of chilled, cell-free medium from saturated cultures of ABY01 transformants by the addition of Na deoxycholate to 0.015 % and trichloroacetic acid to 6 %. Samples were run on SDS-10 % PAGE and stained with Coomassie brilliant blue. Lanes are as follows: M, marker proteins of 97, 68, 43, 29, 20 and 14 kD; 1, ABY01 [pG5]; 2, ABY01 [pG5-*KEX2Δ613*]; 3, ABY01 [pG5-*KEX2Δ613-Ala 314*] isolate 3; 4, ABY01 [pG5-*KEX2Δ613-Ala 314*] isolate 6; 5, ABY01 [pG5-*KEX2Δ613-Asp 314*] isolate 7; 6, ABY01 [pG5-*KEX2Δ613-Asp 314*] isolate 9. **(b)**. The stained gel shown in (a) was soaked 20 minutes in SDS–PAGE running buffer and electroblotted to nitrocellulose (Millipore). The blot was probed with a polyclonal antiserum raised against purified ss-Kex2 protease that had been blocked against non-specific yeast antigens, washed, probed with a goat anti-rabbit IgG conjugated to horseradish peroxidase, and developed with an enhanced chemiluminescence kit (Amersham). Lanes were the same as in (a).

introduction of site-directed mutations into the yeast expression system was 15 % (12 out of 82 transformants tested). Because one-fifth of the length of each PCR product from the 5′ terminus to the *BglII* site is distal to the sites of mutation and two-thirds of the colonies arose because of recombination, the 15 % frequency of introduced mutations was expected.

To prove that the mating defects and low activities of Asn 314 mutants were linked to the mutations, each strain was re-transformed with two independent isolates of each mutant plasmid. CB017 transformants of each mutant plasmid mated at 10^{-5} times the frequency of CB017 carrying pG5-*KEX2Δ613* (data not shown). Thus, *kex2Δ613-Ala 314* and *kex2Δ613-Asp 314* alleles were nearly as sterile as *kex2Δ* strains, the mating efficiency of which is indistinguishable from the background levels of auxotrophic reversion events (10^{-6}).

Biosynthesis of Asn 314 substitution mutants

The biosynthesis of three *kex2* loss-of-function mutants has been analysed previously. Mutation of the active site His or truncation of the P domain, an essential 155 amino-acid domain carboxy-terminal to the subtilisin domain, blocked pro-domain cleavage and resulted in the accumulation of inactive proteins, even when expressed in a Kex2$^+$ background (P Gluschankof and RS Fuller, unpublished data). Similarly, the active site Ser was shown to be essential for the production of active, mature-sized ss-Kex2 in baculovirus-infected insect cells [12].

Culture media from pG5-*KEX2Δ613-Ala 314* and pG5-*KEX2Δ613-Asp 314* transformants of ABY01 were collected and total protein samples were subjected to SDS-PAGE (Fig. 3a). Surprisingly, despite the barely detectable levels of secreted activity and biological activity, Asp 314 ss-Kex2 accumulated to the same high level as wild-type ss-Kex2, accounting for greater than 50 % of total protein in culture medium. Ala 314 ss-Kex2 accumulated to approximately 10 % the level of wild-type ss-Kex2. Increased protein turnover may account for the decreased accumulation of Ala 314 ss-Kex2. A western blot of the gel shown in Figure 3a shows that Ala 314 ss-Kex2 was substantially degraded in culture medium. In contrast, the Asp 314 ss-Kex2 was as stable as wild-type ss-Kex2 (Fig. 3b). Because of the instability of Ala 314 ss-Kex2, kinetic analyses were performed only on wild-type and the Asp 314 enzymes.

Table 2. I_1 to I_2 conversion of wild-type and mutant ss-Kex2 proteases.

Enzyme	t=0 minutes		t=3 minutes		I_2 formation	
	I_1	I_2	I_1	I_2	k	$t_{1/2}$
	(counts x 10^{-3})				(min^{-1})	(s)
wt	11.4	9.7	8.6	21.0	0.49	85
Asp314	26.8	12.0	37.2	51.5	0.45	92

A Molecular Dynamics PhosphorImager was used to quantify the immunoprecipitation shown in Figure 4. Phosphorescent pixel counts in I_2 (intracellular plus extracellular) at 0 and 3 minutes were used to solve the equation: Counts = limit $(1 - e^{-k\,(t\,-\,lag)})$. Lag times were −0.5 to −1 minutes, indicating that, on average, I_1 was created (after translation, translocation and glycosylation) early in the latter half of the 2 minutes labeling period. Because different cultures can have different kinetics and extents of amino-acid uptake, the rates of formation and consumption of I_1 were considered less reliable than the rates of I_2 formation.

The rates of pro-domain cleavage of wild-type and Asp 314 forms of ss-Kex2 were measured by pulse–chase/ immunoprecipitation [8]. Cells pulse-labeled with ^{35}S-amino acids for 2 minutes were subjected

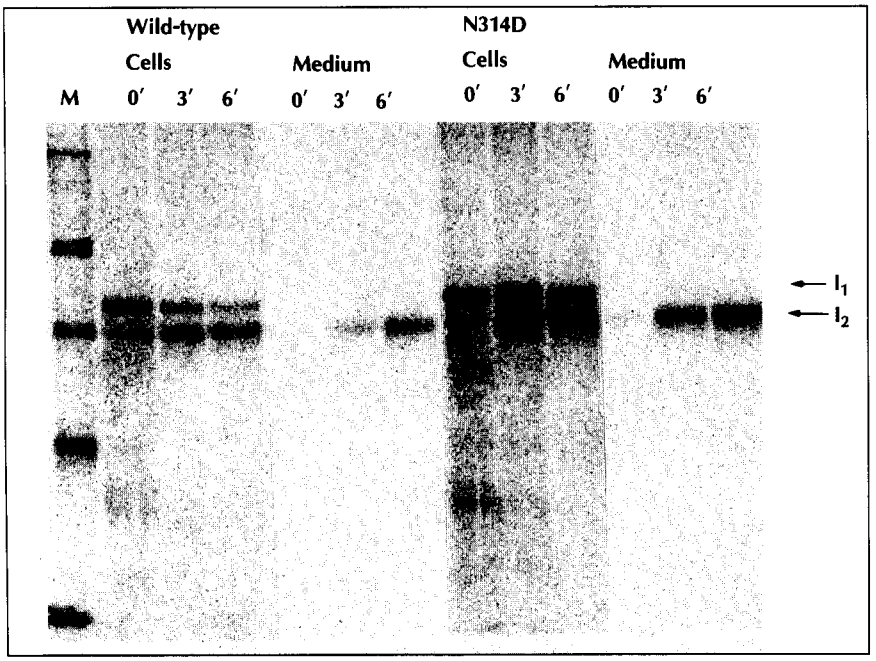

Fig. 4. Pulse–chase immunoprecipitation of Kex2 biosynthetic intermediates. Indicated samples were pulse-labeled for 2 minutes and harvested after the indicated times of chase. Arrows mark the positions of I_1 (pro-ss-Kex2) and I_2 (mature ss-Kex2).

to a cold chase and harvested after 0, 3 and 6 minutes of chase. Analysis of immunoprecipitated Kex2 protein by SDS–PAGE permits quantification of conversion of the intermediate 'I_1' (core-glycosylated pro-Kex2) to intermediate 'I_2' (core-glycosylated enzyme with pro-domain removed) [8]. I_1 to I_2 conversion has a half-time of ~1 minute for wild-type Kex2 protease [8] and similar kinetics for the secreted, soluble form (P Gluschankof and RS Fuller, unpublished data). Remarkably, I_2 appeared almost as rapidly in the cells carrying Asp 314 ss-Kex2 as it did in the cells carrying wild-type ss-Kex2 protease (Fig. 4; Table 2). In the case of wild-type ss-Kex2 protease, at 0 and 3 minutes of chase, I_2 constituted 46 % and 71 % of the radioactivity in the two bands, respectively. With Asp 314 ss-Kex2, I_2 constituted 31 % and 58 % of the radioactivity at the same time points. The half-times for appearance of I_2 were calculated to be 85 and 92 seconds, respectively.

Enzymatic characterization of Asp 314 ss-Kex2

Greatly reduced activity of Asp 314 ss-Kex2 could have resulted from the inactivation of the majority of the secreted enzyme. Figure 5a shows a titration of active sites of concentrated and desalted Asp 314 ss-Kex2 with Pro–Nvl–Tyr–Lys–Arg–CH_2Cl, a high-affinity peptidyl chloromethylketone [20]. The quantity of inhibitor that is sufficient to inactivate all of the enzyme was determined by extrapolating inhibition by substoichiometric inhibitor to zero activity. This indicated that the concentration of active sites in the Asp 314 ss-Kex2 preparation was 18 μM, equivalent to a concentration of 1 mg/ml of active ss-Kex2. Total protein was 1.17 mg/ml, indicating that 85 % of the protein in the preparation was active Asp 314 ss-Kex2. On SDS–PAGE (Fig. 5b), the protein appeared to be 85–90 % pure, indicating that nearly all of the enzyme was active.

Table 3. Kinetic constants in hydrolysis of synthetic substrates.

	Substrate $P_5P_4P_3P_2P_1\ P_1'$	Enzyme	k_{cat} s^{-1}	k_{cat} rel.	K_M μM	K_M rel.	k_{cat}/K_M $s^{-1}M^{-1}$	k_{cat}/K_M rel.
(1)	Ac-P-M-Y-K-R-MCA	wt	25.0	1.0	2.2	1.0	11 000 000	1.0
(1)	Ac-P-M-Y-K-R-MCA	N314D	0.2000	1.0	6.1	1.0	33 000	1.0
(2)	Boc-L-K-R-MCA	wt	23.0	0.92	3.9	1.8	5 900 000	0.54
(2)	Boc-L-K-R-MCA	N314D	0.0055	0.028	1.2	0.20	4 700	0.14
(3)	Boc-L-R-R-MCA	wt	45.0	1.8	17.0	7.7	2 600 000	0.24
(3)	Boc-L-R-R-MCA	N314D	0.0085	0.043	4.0	0.66	2 100	0.064
(4)	Boc-Q-R-R-MCA	wt	21.0	0.84	13.0	5.9	1 600 000	0.15
(4)	Boc-Q-R-R-MCA	N314D	0.0050	0.025	2.7	0.44	1 800	0.055

Wild-type k_{cat}, K_M, and k_{cat}/K_M values are from [11]. k_{cat}, K_M and k_{cat}/K_M rel. values are k_{cat}, K_M and k_{cat}/K_M values for a given substrate divided by the corresponding value for substrate 1. Ac, acetyl; Boc, tertbutoxycarbonyl; P, Pro; M, Met; Y, Tyr; K, Lys; R, Arg; L, Leu; Q, Gln; wt, wild-type form of ss-Kex2 protease; N314D, Asp314 form of ss-Kex2 protease.

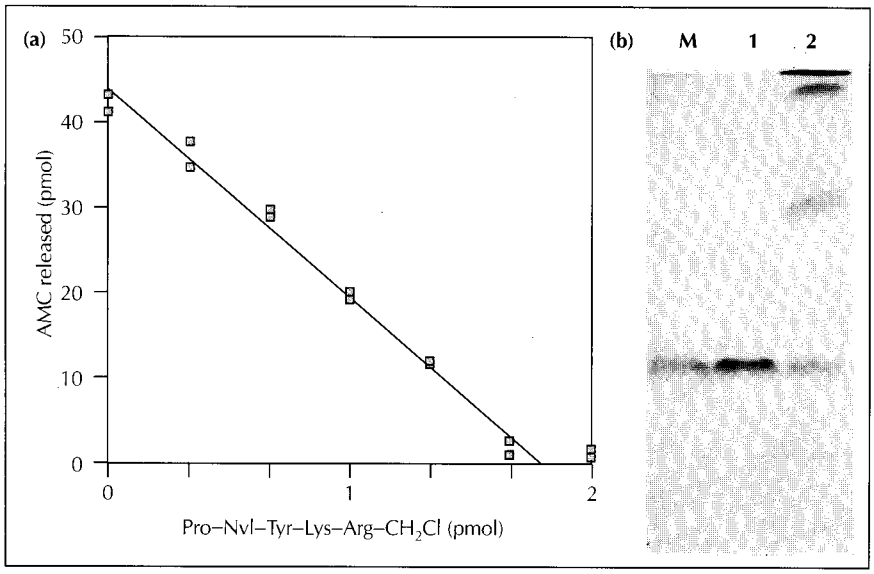

Fig. 5. Concentration and active-site titration of Asp 314 ss-Kex2. **(a)** Amount of aminomethylcoumarin (AMC) released in a 4 minutes incubation was plotted against amount of inhibitor that had been added in a 1 hour pre-incubation. Assays contained 0.1 µl of Asp 314 ss-Kex2. Linear regression of the data (the highest concentration of inhibitor not included) indicated that 1.8 pmol of active sites were present in the assays. **(b)** Coomassie-stained SDS–10 % polyacrylamide gel. Lanes are as follows: M, marker proteins; 1, 3.3 µg (60 pmol of active sites) purified wild-type ss-Kex2 protease [11]; 2, 3.3 µg (50 pmol of active sites) of Asp 314 ss-Kex2 preparation.

Steady-state rate constants were obtained for cleavage of four Lys–Arg and Arg–Arg-containing methylcoumarinamide (MCA) substrates (Table 3) that, previously, had exhibited the highest k_{cat}/K_M values with wild-type ss-Kex2 protease [11]. The wild-type enzyme discriminated between these substrates primarily on the basis of K_M differences, having k_{cat} values that were virtually identical ($\sim$25 s^{-1}; [11]). The Asp 314 ss-Kex2 enzyme cleaved substrates 2, 3 and 4 with a $\sim$4500-fold decrease in k_{cat}, offset slightly by a $\sim$4-fold decrease in K_M (Table 4). The effect on cleavage of these three substrates was similar to the effect on cleavage of each substrate examined with the corresponding mutation in subtilisin [5], which resulted in a 2500-fold decrease in k_{cat} and a 5-fold decrease in K_M. In contrast, the best substrate for wild-type ss-Kex2, Ac–Pro–Met–Tyr–Lys–Arg–MCA (see Table 4, substrate 1), the sequence of which is derived from the sequence of cleavage sites in the physiological substrate pro-α-factor, was cleaved by Asp 314 ss-Kex2 with a 3-fold increase in K_M and a 125-fold decrease in k_{cat}.

Table 4. Kinetic consequences of substitution of Asp for Asn314.

Substrate	k_{cat}wt/ k_{cat}N314D	$K_M$wt/ K_MN314D	(k_{cat}/K_M)wt/ (k_{cat}/K_M)N314D
(1)	125	0.36	330
(2)	4200	3.25	1300
(3)	5300	4.25	1200
(4)	4200	4.80	890

Introduction of an ionizable group in the vicinity of the active site could change the pH-dependence of catalysis, either by affecting protonation of the active site His or by introducing another group on whose ionization the reaction directly depends. However, the pH-dependence of k_{cat}/K_M in hydrolysis of Ac–Pro–Met–Tyr–Lys–Arg–MCA was virtually unchanged by mutation of Asn 314 to Asp. When the values of k_{cat}/K_M of the mutant enzyme were plotted on a scale 1000 times as sensitive as that of the wild-type enzyme, the titration curves of the wild-type and mutant activities were nearly superimposable (Fig. 6).

Discussion

A yeast genetic system for protein engineering

Efficient homologous recombination in mitotic cells has been crucial to the rapid progress in yeast molecular genetics. Studies can proceed not only from mutation to gene to protein, but also in the reverse order with methods such as 'one-step gene disruption' [21]. Homologous recombination can also facilitate fine-structure mapping of mutations [22] and plasmid construction [23].

Homologous recombination strategies continue to play important roles when, as in the analysis of macromolecular function, mutations more subtle than nulls are needed. Transformation of yeast with synthetic oligonucleotides has been used to mutagenize the chromosomal iso-1-cytochrome C gene [24], and libraries of randomly mutated plasmid DNA have been created by recombination of PCR products with a linearized plasmid [25]. In the latter approach, non-mutagenic primers and low fidelity PCR conditions were used to generate random mutations. The present work uses a mutagenic primer and high-fidelity PCR conditions to generate a site-directed change and shows that physiological, molecular, and biochemical methods can be used to identify mutated plasmid transformants. Although cleaved plasmids with a non-ligatable gap create a stronger selection for recombinational transformants [23,25], this work shows that yeast transformants generated by recombination between two linear DNA molecules can be obtained readily when one is a singly cleaved plasmid and the other a short PCR product. This method significantly reduces the number of DNA manipulations required to generate and to evaluate novel site-directed mutations.

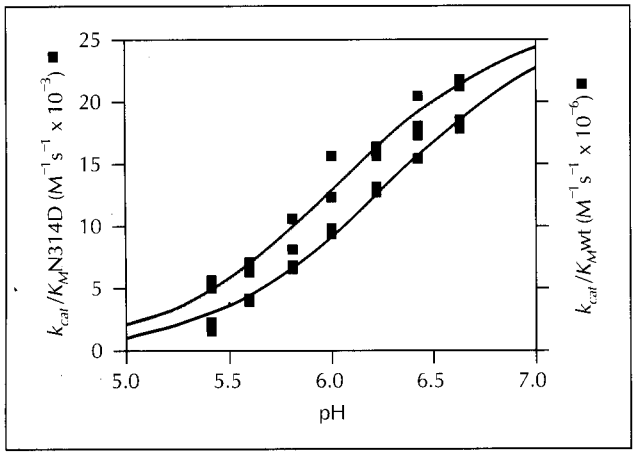

Fig. 6. pH-Titration of wild-type and Asp 314 ss-Kex2 proteases. blue □, Asp ss-Kex2; red ■, wild-type enzyme. The scale for mutant enzyme activity is 1000-fold more sensitive than is the scale for wild-type enzyme activity. The previous pH titration that measured initial rates at saturating concentrations of substrate 4 arrived at a pK_a of 5.7 for the wild-type enzyme [11]. The current method used first order curves at low concentrations of substrate 1 to measure k_{cat}/K_M directly and is considered a more reliable titration. With this assay, plots of k_{cat}/K_M against pH gave pK_a values for wild-type and Asp 314 ss-Kex2 enzymes of 6.30 and 6.05, respectively.

Both site-directed mutagenesis and random mutagenesis, coupled with thoughtful screens and selections for altered function, are valuable tools for the dissection of macromolecular function. One-step site-directed mutagenesis is readily adaptable to create both directed and chance mutations simultaneously by use of a mutagenic primer in combination with low-fidelity PCR conditions. The combined incorporation of a specific mutation along with random alterations could be valuable, because modeling often suggests which substitutions to make but it cannot provide assurance that a mutant protein will fold properly. Additional, random mutations may allow the expression of an intended substitution or may modify its environment in informative ways.

Pro-domain processing in Asp 314 Kex2

Cleavage of the Kex2 pro-domain is intramolecular, requiring the catalytic His 213 and P domain even when wild-type Kex2 protease is present *in trans* (P Gluschankof and RS Fuller, unpublished data). However, the Asn 314–Asp substitution did not reduce the rate or the extent of production of the mature protease, despite the low activity of secreted enzyme (Figs 5 and 6; Table 2). Why does intramolecular pro-domain cleavage appear to be independent of the oxyanion hole Asn? One possibility is that intramolecular cleavage differs mechanistically from substrate cleavage by the mature enzyme. For example, structural features that are unique to the pro-enzyme might position moieties other than Asn 314 to provide oxyanion stabilization during pro-domain cleavage.

Alternatively, a step before cleavage, such as folding of the pro-enzyme, might be rate-limiting and mask the Asn-dependence of the cleavage step. According to this view, cleavage can be assumed to occur at a rate equal to k_{cat} for a corresponding substrate. Calculated from k_{cat}, the half-time for cleavage of a good peptide substrate by wild-type ss-Kex2 is 0.028 seconds at 37 °C (from [11]) and 0.04 seconds at 30 °C (our unpublished observations). If the intramolecular site behaved like substrate 1, then the half-time for cleavage of the site in the Asp 314 mutant pro-enzyme would be 5 seconds at 30 °C, negligible in comparison to the overall half-time for pro-enzyme maturation (Table 2). Note, however, that a 4500-fold reduction in k_{cat}, such as that exhibited by Asp 314 ss-Kex2 in cleavage of substrates 2, 3 and 4, would delay the appearance of I_2 by 3 minutes. The intramolecular cleavage site, –Leu–Phe–Lys–Arg 109, shares with substrate 1 a hydrophobic residue at P_4 and an aromatic residue at P_3 that may be components of Kex2 specificity that confer a K_M advantage to the wild-type enzyme and reduce the k_{cat} defect of the Asp 314 form of the enzyme (see below). Mutagenesis of the P_4 and P_3 positions of the pro-domain cleavage site in Asp 314 ss-Kex2 by one-step site-directed mutagenesis should provide a direct test of these models.

Kinetics of Asp 314 ss-Kex2 activity

Mutations in the P_2 binding site of α-lytic protease that reduced k_{cat} without increasing K_M were interpreted as meaning that a conformational change was required in mutant enzyme–substrate complexes to convert them from ground to transition state [26]. As expected for the alteration of a catalytic residue, substitution of Asp for Asn 314 in Kex2 destabilized transition-state interactions with little effect on the ground state. However, the observed effects on k_{cat} depended on the substrate. The Asn 314–Asp substitution reduced k_{cat} ~4500-fold for substrates 2, 3 and 4, but substrate 1 and, apparently, the intramolecular site were cleaved with k_{cat} nearly 1 % that of the wild-type enzyme. The wild-type enzyme prefers substrate 1 to substrate 2 by only a factor of two on the basis of K_M. The mutant enzyme presumably uses the additional binding interactions with substrate 1 to increase k_{cat} 40-fold relative to substrates 2, 3 and 4, possibly using binding energy to repel or reorganize Asp 314. The additional specificity subsites of substrate 1 may allow it to be bound correctly, with its carbonyl oxygen pointing into what is normally the oxyanion hole. Formation of such a complex may depend on rotating side-chain torsion angles of Asp and/or reorganizing water or cations to minimize unfavorable electrostatic interactions. Substrates with weaker enzyme contacts might be repelled by Asp 314 and bind non-productively. On mutation of Asn to Asp (Table 4), the increased K_M for substrate 1 and reduced K_M for substrates 2, 3, and 4 are consistent with substrate 1 complexes paying a price in K_M to become catalytically competent and the other complexes going to a deeper ground state, more remote from the transition state.

Implications for pro-hormone processing by PC2

Recently, the mammalian Kex2 homologue PC2 has been identified as the type 2 endopeptidase in islets of Langerhans that cleaves the C peptide from the pro-insulin A chain at a Lys–Arg site [17,27,28]. When co-expressed with pro-insulin, PC2 cleaved the C–A junction preferentially, but Kex2 cleaved both the C–A and the B–C processing sites [18]. Given the profound effects of the Asn 314–Asp substitution on Kex2, the appearance of Asp in place of the conserved Asn in PC2 [16] is remarkable. One model suggested that protonated Asp might function as an oxyanion hole, restricting PC2 activity to low pH [29]. Because His acts as a general base in serine proteases [30], the active-site environment would have to allow the Asp to be protonated and the His to be unprotonated. A bell-shaped pH curve, with maximal activity between the pK_as of the Asp and His, would be expected if Asp 314 ss-Kex2 required a protonated Asp. However, the Asp substitution did not alter the sigmoidal shape of the pH-k_{cat}/K_M profile. The curves for both the wild-type and the mutant enzymes were consistent with inactivation by binding a single proton. If Asp 314 must be protonated for ss-Kex2 to function, then it must be protonated throughout the range of pH examined (5–7, limited by low activity below pH 5 and instability above pH 7) and have a pK_a well above 7. It is more likely that the Asp is unprotonated, and catalysis proceeds without a function analogous to that of Asn.

PC2 may have accumulated additional substitutions that create a new oxyanion binding site or that allow it to function efficiently without one. Specific substrates of PC2 might bind tightly in a way that deflects the Asp side chain from the transition state. For instance, Asp might project into the oxyanion hole in the free enzyme or when substrates are bound weakly but move away from the carbonyl oxygen of a substrate that binds well enough to displace it.

Pro-insulin is a highly concentrated substrate in nascent secretory granules [31]. It is potentially significant, therefore, that under conditions of substrate saturation, Asp314 ss-Kex2 retains substantial activity against its best substrate. Reduced enzyme activity is the cost for such discrimination between substrates. However, processing in dense core granules may be limited not by enzyme activity but by diffusion of inabundant proteases through aggregated pro-hormone substrates. This limitation and a need to minimize inappropriate proteolysis may have driven the selection of PC2, not to perfect its chemistry to the diffusion controlled limit but to repel most potential oxyanions with an oxyanion of its own.

Materials and methods

Polymerase chain reaction and DNA sequencing

Preparative reactions (100 µl) contained 25 mM Na MES pH 6.5, 50 mM KCl, 3 mM $MgCl_2$, 250 µM each dNTP, 1 µM primer NA or ND, 1 µM primer Nrev, 100 µg/ml BSA, and 1 ng pKX9 (a pBR322 subclone of *KEX2*). Pre-mixes were heated 7 min at 94°C, 5 u Taq polymerase (Perkin Elmer) were added, and then reactions were performed in 30 cycles of 30 seconds at 55°C, 60 seconds at 70°C, and 30 seconds at 94°C. Diagnostic reactions (25 µl with 1.25 u of Taq polymerase) used NX as the 5′ primer and 1 µl of yeast culture or 1 ng of purified plasmid DNA as the target. Plasmids were recovered into *Escherichia coli* by electroporation of total DNA extracted from yeast [32]. Sequences were obtained with the cycle sequencing kit (BRL) from double stranded plasmid DNA using [^{32}P]end-labeled primers Nrev and Nseq (GTAAAAGGTGTTACTGAGGG, the 3′ end of which is 11 nucleotides 5′ to primer NX).

Yeast strains and methods

Yeast strain genotypes are as follows: ABY01, *MAT**a** ura3 trp1 ade2 his3*; CB017, *MATα kex2Δ leu2 trp1 ade2 his3 ura3*; and DC14, *MAT**a** his1*. For mating assays, CB017 transformants were grown on SDC uracil-lacking (− ura) plates, replica-plated onto a lawn of ~5 x 10^5 DC14 cells on a YPD plate, incubated for 12 h, and then replica-plated onto a SD plate. Yeast media were prepared as described [33].

Pulse–chase immunoprecipitation

CB017 carrying pG5-*KEX2Δ613* and CB017 carrying pG5-*KEX2Δ613-Asp314* were grown in low sulfate medium and labeled with 300 µCi/ml [^{35}S]amino acids (Dupont) as described [8]. Chase was initiated after 2 min by addition of cold Cys and Met to 1 mM. 15 sec after the addition of chase, and 3 and 6 min thereafter, 1 ml samples were added to 10 µl 1 M NaN_3 and frozen in liquid nitrogen. Cellular and secreted proteins were subjected to two cycles of immunoprecipitation with 2 µl anti-ss-Kex2 antibody and 10 µl Pansorbin (Calbiochem) and separated on SDS–10 % PAGE. Gels were exposed to storage phosphor screens for three days before quantitation and imaging with a Molecular Dynamics PhosphorImager (Fig. 4; Table 2).

Concentration and active-site titration of Asp 314 ss-Kex2

314 ml of cell-free culture medium from ABY01 [pG5-*KEX2Δ613-Asp314*] was concentrated 150-fold and exchanged into storage buffer (100 mM NaCl, 40 mM bis-Tris Cl pH 7.0, 10 mM $CaCl_2$) by pressure and spin dialysis with 30 000 molecular weight cut-off membranes (Amicon). Total protein was assayed with bicinchonicic acid (Pierce) according to the supplier's recommendations. Samples of concentrated and desalted Asp 314 ss-Kex2 protease were mixed with 0.00, 0.33, 0.67, 1.00, 1.33, 1.67, and 2.00 pmol Pro–Nvl–Tyr–Lys–Arg–CH_2Cl [20] and incubated for 60 min at 21°C. Ac–Pro–Met–Tyr–Lys–Arg–MCA was added to 40 µM and reactions were incubated for 4 min at 37°C and terminated by the addition of $ZnSO_4$. Remaining activity was plotted against the amount of inhibitor added [20].

Determination of kinetic constants

Wild-type ss-Kex2 was purified and active-site titration was performed as described [11]. Routine assays of enzyme activity and individual measurements of k_{cat} and K_M were performed as described [11]. For determination of the pK_a of k_{cat}/K_M, the values of k_{cat}/K_M over a range of pH values were determined directly under k_{cat}/K_M conditions [34]. 100 nM Ac-Pro-Met-Tyr-Lys-Arg-MCA (initial concentration 20- to 50-fold below its K_M) was cleaved to completion at 21°C and the first order rate of product formation was divided by the concentration of active sites to determine k_{cat}/K_M. Reactions contained 100 mM NaCl, 2.5 mM $CaCl_2$, 0.01 % Triton X-100, and 40 mM imidazole·Cl at pH 5.41, 5.60, 5.81, 6.00, 6.23, 6.43 and 6.63. Wild-type ss-Kex2 was used at 1 nM and Asp 314 ss-Kex2 was used at 139 nM. pK_a values and the limiting value of k_{cat}/K_M were obtained by nonlinear regression to the equation: $k_{cat}/K_M = \text{limit}\ (10^{(pH-pKa)})/(10^{(pH-pKa)}+1)$.

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Erratum

One-step site-directed mutagenesis of the Kex2 protease oxyanion hole

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We regret that the gel in Figure 5 of this research paper was printed upside down. The correct version is printed below. Also, in the legend to Figure 2 (c), ND2 and ND13 should have been ND7 and ND9.