Direct Measurement of Acylenzyme Hydrolysis Demonstrates Rate-Limiting Deacylation in Cleavage of Physiological Sequences by the Processing Protease Kex2[†]

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ABSTRACT: *Saccharomyces cerevisiae* Kex2 protease is the prototype for the family of eukaryotic proprotein convertases that includes furin, PC1/3, and PC2. These enzymes belong to the subtilase superfamily of serine proteases and are distinguished from degradative subtilisins by structural features and by their much more stringent substrate specificity. Pre-steady-state studies have shown that both Kex2 and furin exhibit an initial burst of 7-amino-4-methylcoumarin release in cleavage of peptidyl methylcoumarinamide substrates that are based on physiological cleavage sites. Thus, in cleavage of such substrates, formation of the acylenzyme intermediate is fast relative to some later step (deacylation or N-terminal product release). This behavior is significant, because Kex2 also exhibits burst kinetics in cleavage of peptide bonds. k_{cat} for cleavage of a tetrapeptidyl methylcoumarinamide substrate based on the physiological yeast substrate pro-α-factor exhibits a weak solvent isotope effect, but neither this isotope effect nor temperature dependence studies with this substrate conclusively identify the rate-limiting step for Kex2 cleavage of this substrate. We therefore developed an assay to measure deacylation directly by pulse−chase incorporation of $H_2{}^{18}O$ in a rapid-quenched-flow mixer followed by mass spectrometric quantitation. The results given by this assay rule out rate-limiting product release for cleavage of this substrate by Kex2. These experiments demonstrate that cleavage of the acylenzyme ester bond, as opposed to either the initial attack on the amide bond or product release, is rate-limiting for the action of Kex2 at physiological sequences. This work demonstrates a fundamental difference in the catalytic strategy of proprotein processing enzymes and degradative subtilisins.

Many eukaryotic proproteins and propeptides are endoproteolytically processed in late secretory compartments such as the trans Golgi network and the secretory granule. Such processing reactions frequently occur at motifs of basic residues such as Lys-Arg↓ or Arg-Xaa-Xaa-Arg↓,[1] and a family of serine proteases homologous to the subtilisins has been shown to carry out many if not the majority of these processing events (*1*−*4*). The prototype for this family of proprotein convertases is Kex2 protease from *Saccharomyces cerevisiae* (kexin, EC 3.4.21.61). Kex2 is responsible for processing a variety of precursors at Lys-Arg↓ cleavage sites, including the mating pheromone precursor pro-α-factor (*1*, *2*, *5*−*7*). Kex2 serves as a useful model for this family of enzymes by virtue of its extensive in vitro and in vivo characterization (*8*−*13*).

Studies of Kex2 have underscored the structural and functional differences between processing proteases of the Kex2 family and the subtilisins (*8*−*14*). Unlike the degradative enzymes of the subtilisin family, Kex2 exhibits exceptionally stringent substrate specificity. The enzyme primarily recognizes substrates through interaction with three substrate side chains, P_4, P_2, and P_1 (using the nomenclature of *15*). Recognition of favorable P_4 side chains relies on either hydrophobic or electrostatic interactions (*12*), and enzyme−substrate interactions at P_2 utilize electrostatic recognition as well as steric exclusion of certain residues (*11*). Recognition of Arg at P_1 is extremely stringent; both electrostatic and steric factors in the Arg side chain are required to confer efficient catalysis on substrates (*11*, *12*). Unlike enzymes such as subtilisin BPN′, Kex2 displays burst kinetics in the formation of the C-terminal cleavage product (released after acylation) with good amide substrates (*8*, *12*, *16*), indicating that formation of the acylenzyme need not be rate-limiting for this processing protease. Such behavior is also seen with the metazoan Kex2 homologue furin (*17*, *18*). For Kex2, burst kinetics are seen with substrates having Arg at P_1, but substrates having P_1 Lys do not exhibit such

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[1] Abbreviations: Ac, acetyl; AMC, 7-amino-4-methylcoumarin; d_3-Ac, trideuterioacetyl; fwhm, full width at half-maximum; Glu(EDANS), 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid conjugated to the δ-carboxyl group of glutamate; Lys(DABCYL), 4-[4-(dimethylamino)-phenyl]azobenzoic acid conjugated to the ϵ-amino group of lysine; peptidyl↓MCA, methylcoumarinamide; Nle, norleucine; Xaa, an arbitrary amino acid. Throughout we designate the cleavage site as -P_3-P_2-P_1↓P_1'-P_2'-P_3'- in accordance with the nomenclature of Schechter and Berger (*15*). The scissile bond is designated with an arrow (↓).

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behavior, suggesting that Arg at P_1 confers rapid acylation on a Kex2 substrate (*12*).

The fact that burst kinetics are seen in cleavage of physiologically correct (i.e., P_1 Arg) sequences with both Kex2 and furin, but not with the related degradative subtilisins, suggests that rapid acylation by correct sequences is an adaptation for the function of these processing enzymes. However, this fact also raises the question of what is rate-limiting for cleavage of such substrates by Kex2 and furin. For instance, deacylation itself could be rate-limiting, as could product release or some previously unrecognized type of conformational change. Understanding the pre-steady-state behavior of the Kex2 family of processing proteases will provide a counterpoint to the extensively studied enzymes of the subtilisin family, allowing a better appreciation of the way in which these families of enzymes have adapted an ancestral structure and a common catalytic mechanism to carry out different functions with different substrate specificities.

In this study, we have examined the rate-limiting step in Kex2 cleavage of a tetrapeptide Lys-Arg substrate based on a physiological cleavage site in pro-α-factor. The temperature dependence and solvent isotope effect of the rate-limiting step seem more consistent with a chemical rather than a physical process, but these experiments cannot be considered conclusive. Therefore, we have developed a new assay that allows direct, quantitative measurement of deacylation by pulse−chase incorporation of an ^{18}O label followed by mass spectrometry. Results obtained with this assay conclusively rule out the possibility of rate-determining product release for this enzyme. Our results demonstrate the deacylation step itself is most likely to be rate-limiting in cleaving physiologically correct amide substrates for this member of the proprotein convertase family.

MATERIALS AND METHODS

Enzymes, Substrates, and Reagents. Secreted, soluble Kex2 protease was prepared as described (*11*). For mass spectrometry, the peak of Kex2 activity after HPLC was pressure-dialyzed into 100 mM ethanolamine/0.1% β-octyl glucoside (natural abundance water, adjusted to pH 7 with acetic acid) for four cycles with a 50 mL cell and a YM30 membrane (Amicon). Enzyme was then aliquotted, frozen in liquid nitrogen, and stored at −80 °C. Ac-Nle-Tyr-Lys-Arg↓MCA (methylcoumarinamide, *19*) and d_3-Ac-Nle-Tyr-Lys-Arg↓MCA were prepared using a previously published modification of Zimmerman's chloroformate coupling procedure (*11*, *19*). Substrates were purified on both C_8 and C_{18} reverse-phase columns (*11*). $H_2{}^{18}O$ was purchased from Isotec and was 97.5% enriched for ^{18}O. Ac-Pro-Met-Tyr-Lys-Arg↓MCA was the generous gift of J. Thorner. Other reagents were from Aldrich, Sigma, or Fisher.

Steady-State Kinetics. Saturation kinetics were performed essentially as previously described (*12*). For temperature dependence studies, values of k_{cat} were determined for the substrate Ac-Nle-Tyr-Lys-Arg↓MCA over a range of temperatures from 5 to 37 °C at a substrate concentration of 200 μM in 0.2 M Bistris, 1 mM $CaCl_2$, and 0.1% Triton X-100, with a minimum of three separate measurements at each temperature. The data were then plotted using the treatment of Eyring [$\ln(k_{cat}/T)$ vs $1/T$, *20*], and activation parameters were determined from the slope and intercept of the best-fit line as determined by linear regression. For comparison, thermodynamic activation parameters for subtilisin 72 were calculated from an Eyring plot of previously reported temperature dependence data (*21*). A published temperature dependence for the first-order hydrolysis of *p*-nitrophenyl acetate (*22*) was also examined. In this case, data were extracted from the published plot (scanned at 600 dpi) using DataThief 2.0b software and evaluated on an Eyring plot to extract activation parameters.

For determination of solvent isotope effects, the indicated k_{cat} ratios (Table 1) were determined at 37 °C and 45−200 μM substrate with an $H_2{}^{18}O$ enrichment of 75% and a D_2O enrichment of 77% in 0.2 M Bistris (pH 7.26), 1 mM $CaCl_2$, and 0.1% Triton X-100 (*11*, *12*). K_M was 1.0 μM (±0.3 μM) for both Ac-Nle-Tyr-Lys-Arg↓MCA and d_3-Ac−Nle-Tyr-Lys-Arg↓MCA in natural abundance H_2O. The reported ratios are the mean values of at least three independent experiments.

Product inhibition was examined by adding increasing amounts of Ac-Nle-Tyr-Lys-Arg-COOH (University of Michigan core facility) to Kex2 cleavage reactions containing 50 μM Ac-Pro-Met-Tyr-Lys-Arg↓MCA at 37 °C under the buffer conditions described above. The formation of product (free AMC) was monitored by fluorescence as described previously (*11*, *12*). Peptide was dissolved in aqueous acetonitrile with 0.1% trifluoroacetic acid and quantitated by UV absorbance prior to use. No inhibition of Kex2 cleavage was observed at peptide concentrations as high as 1 mM.

Stopped-Flow Fluorometry. Kex2 protease (100−800 nM) was reacted with 5 μM Arg-Lys(DABCYL)-Nle-Tyr-Lys-Arg↓Glu-Ala-Glu-Ala-Glu(EDANS)-Arg or 52 μM Arg-Lys-(DABCYL)-Nle-Tyr-Lys-Lys↓Glu-Ala-Glu-Ala-Glu(EDANS)-Arg (*11*, *23*) in 0.2 M Bistris (pH 7.26), 1 mM $CaCl_2$, 0.1% Triton X-100 in an Applied Photophysics stopped-flow fluorometer at 22.4 °C. The excitation monochromator was set to 340 nm, and emission was monitored with a 490 nm band-pass interference filter (10 nm bandwidth, fwhm; CVI Laser, Albuquerque, NM). Fluorescence traces were transferred to Kaleidagraph for fitting to an equation describing burst kinetics with an arbitrary intercept (eq 1), where $F(t)$ is the fluorescence at time t, F_0 is the fluorescence at time 0, A is the observed exponential amplitude, k is the burst rate constant, and v is the steady-state reaction velocity (in V/s). For both substrates, multiple runs ($n \geq 6$) confirmed that the described behavior is indeed representative.

$$F(t) = F_0 + A(1 - e^{-kt}) + vt \quad (1)$$

Measurement of Deacylation by Mass Spectrometry. For each experiment, an aliquot of Kex2 in 100 mM ethanolamine (pH 7, acetic acid), 0.1% octyl glucoside was thawed and diluted 5- or 10-fold into $H_2{}^{18}O$. The substrate d_3-Ac-Nle-Tyr-Lys-Arg↓MCA was prepared by diluting a 20 mM stock in 0.1 M acetic acid/water with acetonitrile and adjusting the pH to 7 with ethanolamine and acetic acid. This stock was kept at −80 °C. Prior to use, substrate was thawed and diluted 1:100 into $H_2{}^{18}O$. Enzyme and substrate were then reacted at 21 °C in a volume of 41.2 μL in a Kintek RQF-3 rapid-quenched-flow mixer at an ^{18}O enrichment of 86.2% prior to quenching with 69 μL of 1 M acetic acid in natural abundance water, followed about 16 ms later by an

additional 150 μL of quench. The initial quench volume was sufficient to stop the reaction. Final enzyme and substrate concentrations were 400−800 nM and 89.5 μM, respectively. Appearance of product arising during the chase could not be reliably detected above product of identical mass arising during the pulse due to incomplete ^{18}O enrichment (data not shown), indicating that the acylenzyme may be stable under these conditions. The substrate Ac-Nle-Tyr-Lys-Arg↓MCA was prepared in the same manner, diluted 10-fold with acetonitrile, and cleaved at the same or 2-fold lower enzyme concentration for 30 min. Enzyme for this control digestion was diluted into either ^{16}O water (99.99%, Aldrich) or natural abundance water. No difference between these dilution conditions for the control digestion was observed. Duplicate aliquots were assayed for fluorescence to quantify the cleavage products, and then a small amount (typically <100 pmol) was added to each timepoint for mass spectrometry. In a typical experiment such as those shown in Figure 5, duplicate samples under these conditions were taken at each timepoint; one was diluted with an additional 350 μL of acid quench and fluorescence was measured, and the other was diluted 2-fold with acetonitrile and standard was added for mass spectrometry. All mass spectra were taken within 6 h of addition of chase to the sample. Mass spectra were taken between *m*/*z* of 300 and 800 (containing the singly and doubly ionized peaks of both substrate and product) for 5 min (5 s scan time, 0.1 s delay between scans) on a VG Platform electrospray mass spectrometer. Spectra were processed using MassLynx software, and the region between *m*/*z* of 615 and 635 (corresponding to the singly ionized product peaks) was transferred to Kaleidagraph for quantitation of peak heights. The observed ratio of peak heights and the known amount of internal standard were used to calculate the amount of deuterated product which incorporated ^{18}O. This value was then corrected for the ^{18}O enrichment in the pulse (86.2%). The doubly ionized product peaks could not be analyzed due to the presence of buffer contaminants in that region of the spectrum. All manipulations were done in 1.5 mL screw cap microcentrifuge tubes (Sarstedt) or the rapid-quenched-flow mixer. Substrate stocks, reagents, and timepoints were handled with microcentrifuge tubes and pipet tips which had first been soaked in *N*,*N*-dimethylformamide overnight, then rinsed with acetone and individually vacuum-dried. This procedure was necessary to remove contaminants thought to be plasticizers which sometimes appeared in the 600−700 nm region of the spectrum and prevented quantitation when present. Multiple trials ($n = 4$, including multiple enzyme concentrations) and duplicate timepoints suggested that precision with this assay was comparable to that of the fluorescence assay used to measure Kex2 protease activity (*8*, *11*, *12*).

Data from fluorescence measurements (the acylation product) were fit to eq 2, which describes an initial burst followed by a linear steady state. Data from mass spectrometry (the deacylation product) were also fit to eq 2 to describe linear accumulation of product after an initial lag. In this equation, $P(t)$ is product at time t, π is the burst amplitude, k is the burst rate constant, and V_{max} is the steady-state reaction velocity at saturating substrate.

$$P(t) = \pi(1 - e^{-kt}) + V_{max}t \qquad (2)$$

In our hands, eq 2 described the formation of N-terminal cleavage product in simulations very well, with substoichiometric, negative exponential amplitudes. However, in all cases we found that the predicted lag phase on the basis of these simulations resulted in a very small deviation from linear behavior which was only significant at very short times ($t \leq 5$ ms, data not shown). Unfortunately, the experimental data at short times were not of sufficiently high precision to allow a good description of this lag phase. In fact, attempts to fit the data for the appearance of the deacylation product to such a model consistently gave fits which were essentially equivalent to those obtained by simple linear regression, with substoichiometric exponential amplitudes of varying sign. We have presented one example of such an analysis in Figure 5, along with an example in which the data for the deacylation product were fit by linear regression.

RESULTS

Acylation Is Not Rate-Limiting for Cleavage of Physiologically Correct Peptide Bonds. Previous work has shown that both yeast Kex2 protease and the homologous mammalian enzyme furin display burst kinetics in cleavage of peptidyl↓MCA substrates (*8*, *12*, *17*, *18*), indicating that acylation is not rate-limiting for cleavage of these substrates. For Kex2, it has also been shown that substitution of Lys for Arg at P_1 is sufficient to cause the loss of such behavior (*12*), suggesting that this rapid acylation is an adaptation seen in cleavage of physiologically correct sequences. However, the AMC used as a reporter in such substrates is a much better leaving group than amino acids, and preliminary experiments using an internally quenched peptide substrate in which cleavage occurs at an actual peptide bond were inconclusive (*24*). Therefore, the observed kinetics could have been unique to the cleavage of such activated substrates and not been seen with actual peptide bonds.

To explore this possibility, we examined Kex2 cleavage of an internally quenched peptide substrate with a Lys-Arg cleavage site (*11*, *23*, Figure 1). As can be seen, kinetics of cleavage are clearly biphasic, indicating that the first turnover is faster than the subsequent steady-state rate. This result indicates that acylation is not rate-limiting in cleavage of an actual peptide bond and validates the use of peptidyl↓MCA substrates as models to explore this unusual kinetic behavior. Moreover, such biphasic kinetics were not observed in cleavage of the substrate Arg-Lys(DABCYL)-Nle-Tyr-Lys-Lys↓Glu-Ala-Glu-Ala-Glu(EDANS)-Arg (data not shown), consistent with the absence of burst kinetics in cleavage of peptidyl↓MCA substrates having Lys at P_1 (*11*, *12*). The lack of burst kinetics with an internally quenched substrate having Lys at P_1 indicates that burst kinetics seen with the Lys-Arg substrate arise from rapid cleavage of the peptide bond followed by a slower steady state and not from formation of a bound intermediate in which fluorescence quenching is less efficient.

Mechanistic Probes of the Rate-Limiting Step Are Inconclusive. We focused on the substrate Ac-Nle-Tyr-Lys-Arg↓MCA for further characterization of the rate-limiting step for Kex2 cleavage of such sequences. Although the leaving group in this substrate is both nonphysiological and a better leaving group than physiological amino acids, we believe that it will provide an appropriate model for the cleavage of

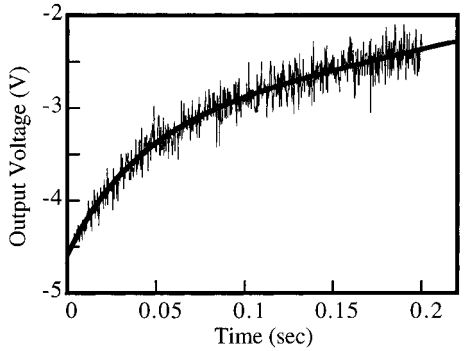

FIGURE 1: Kex2 protease exhibits burst kinetics in cleavage of a peptide bond. The internally quenched, fluorogenic peptide substrate Arg-Lys(DABCYL)-Nle-Tyr-Lys-Arg↓Glu-Ala-Glu-Ala-Glu-(EDANS)-Arg (*11*, *23*) was cleaved by Kex2 in an Applied Photophysics stopped-flow fluorometer at 22.4 °C. Substrate concentration was 5 μM, and other reaction conditions were as described under Materials and Methods. The biphasic formation of product indicates that acylation is not rate-limiting in cleavage of an actual peptide bond. Parallel reactions with varying enzyme concentration or longer time scales continued to exhibit a linear second phase, indicating that the observed curvature was not due to depletion of substrate on this time scale. Data were fit to eq 1, describing an initial burst followed by a subsequent linear steady state.

actual peptide bonds in this case. Peptidyl↓MCA substrates and peptide bonds are cleaved by Kex2 with comparable k_{cat}/K_M values (*11*, *12*). More importantly, we have now demonstrated that Kex2 cleavage of both peptide bonds and peptidyl↓MCA substrates exhibits burst kinetics in formation of the acylation product (*8*, *12*; Figure 1). This result indicates that Kex2 cleavage of true physiological substrates must also proceed with rapid acylation. While it is not yet known whether acylation rates are actually equivalent for Kex2 cleavage of peptide bonds and methylcoumarinamide linkages, it is clear that in both cases acylation is significantly faster than the rate-limiting step. Moreover, these two classes of substrate primarily differ in the nature of the leaving group, which is released prior to the deacylation step (*25*). Therefore, these types of substrates give rise to essentially identical acylenzyme intermediates, so their deacylation and product release should be quite comparable.

The two most likely candidates for the rate-determining step in Kex2 cleavage of Ac-Nle-Tyr-Lys-Arg↓MCA are product release (a physical process after deacylation) and deacylation itself (a chemical process). Assuming that product release proceeds via a single, reversible equilibrium, addition of excess deacylation product (e.g., the tetrapeptide Ac-Nle-Tyr-Lys-Arg-COOH) should lead to accumulation of the enzyme as a product-bound intermediate and inhibit turnover. However, addition of this peptide to Kex2 cleavage reactions at concentrations as high as 1 mM produced no effect (data not shown). This negative result must be interpreted with caution, but it prompted us to seek other mechanistic probes for the reaction.

Solvent isotope effects can provide useful information about the course of enzymatic reactions. Significant isotope effects ($k_H/k_D \geq 2$) are good indicators of proton transfer in the transition state, and the deacylation step does involve proton transfer (*25*). We therefore examined the effect of D_2O upon Kex2 cleavage of Ac-Nle-Tyr-Lys-Arg↓MCA. As can be seen in Table 1, the solvent isotope effect on this reaction is measurable but quite small. Indeed, this effect is too small to distinguish between deacylation and alternatives such as product release or conformational change.

Table 1: Steady-State Characterization of Substrates[a]

substrate	k_{cat} (s^{-1})	$^{18}O/^{16}O$ ratio[b]	H/D ratio[c]
Ac-Nle-Tyr-Lys-Arg↓MCA	45 ± 3	n/d[d]	n/d[d]
d_3-Ac-Nle-Tyr-Lys-Arg↓MCA	49 ± 4	0.96 ± 0.05	1.24 ± 0.05

[a] Cleavage of substrates by Kex2 protease was performed at 37 °C as described under Materials and Methods. For both substrates, K_M was 1.0 ± 0.3 μM in natural abundance water. [b] k_{cat} was determined in 75% $H_2{}^{18}O$ and in natural abundance H_2O (99.76% ^{16}O), and the indicated ratio is reported. [c] k_{cat} was determined in 77% D_2O and in natural abundance H_2O (>99% ^1H), and the indicated ratio is reported. [d] n/d, not determined.

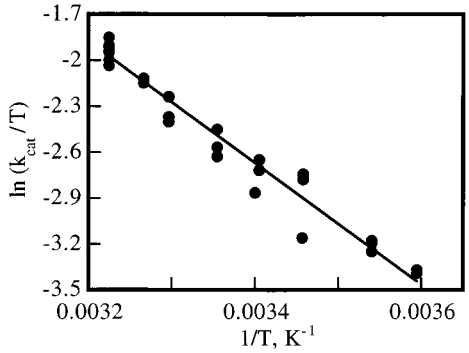

FIGURE 2: Determination of thermodynamic activation parameters for Ac-Nle-Tyr-Lys-Arg↓MCA. k_{cat} was measured at temperatures between 5 and 37 °C as described under Materials and Methods and plotted according to the formulation of Eyring (*20*) to determine $\Delta H^‡$ and $\Delta S^‡$. Activation parameters were then derived from the best fit of the data by linear regression.

The temperature dependence of an enzymatic reaction may also be used to probe the structure of the transition state to some degree. For example, HIV protease has been shown to exhibit rate-limiting chemistry with some substrates and rate-limiting product release with others, and these two classes of substrates exhibit characteristically different activation parameters ($\Delta H^‡$ and $\Delta S^‡$, *20*) and hence different temperature dependence profiles (*26*). The temperature dependence of k_{cat} in cleavage of a short amide substrate by a degradative subtilisin, subtilisin 72, has been reported (*16*, *21*). This reaction, which reflects the acylation step, has a positive enthalpy of activation ($\Delta H^‡ = 6.1-6.5$ kcal/mol) and a small, negative entropy of activation [$\Delta S^‡ = -(28.8-30.1)$ cal/(mol·K); *21*; also see Materials and Methods]. We found k_{cat} for Kex2 cleavage of Ac-Nle-Tyr-Lys-Arg↓MCA to have quite similar parameters [$\Delta H^‡ = 7.9$ kcal/mol; $\Delta S^‡ = -25.6$ cal/(mol·K); Figure 2]. These parameters are also similar to those reported for k_{cat} in HIV protease cleavage of multiple substrates limited by a chemical step [$\Delta H^‡ = 6.9-8.7$ kcal/mol; $\Delta S^‡ = -(25.7-31.7)$ cal/(mol·K); *26*]. The hydrolysis of *p*-nitrophenyl acetate in solution also proceeds with a small, negative entropy of activation [−13 cal/(mol·K); *22*; also see Materials and Methods], although this reaction has a lower enthalpy of activation (2.1 kcal/mol), as might be expected for hydrolysis of an activated ester. Thus, a number of acyl transfer transition states proceed with qualitatively similar activation parameters, even though different catalytic mechanisms and leaving groups are involved, and the activation parameters for Kex2 are quite comparable to these values.

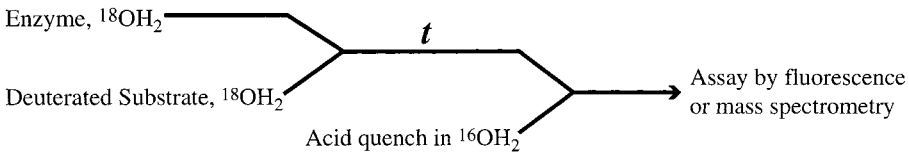

FIGURE 3: Scheme for the pulse−chase experiment to monitor deacylation directly. Enzyme is reacted with saturating deuterated substrate in $H_2{}^{18}O$ for time *t*. N-terminal product formed during this time will incorporate ^{18}O at the new C-terminus. The reaction is then quenched by addition of excess 1 M acetic acid in $H_2{}^{16}O$ (the chase). Acylenzyme present when quench is added may break down after addition of quench, but it will incorporate ^{16}O due to the excess of $H_2{}^{16}O$ in the quench. Standard is generated by cleavage of nondeuterated substrate to completion in parallel with the pulse−chase experiment. This standard is quantified by fluorescence of the released AMC, and a known amount of product is added to the quenched pulse−chase reaction to serve as an internal standard for mass spectrometry.

On this basis, it is tempting to argue that these results are most consistent with rate-limiting deacylation for Kex2 cleavage of Ac-Nle-Tyr-Lys-Arg↓MCA. However, such an interpretation would seem inconclusive at the present time. The temperature dependence of a bona fide case of product release for a member of the subtilisin superfamily is not available for comparison. Moreover, while the observed coincidence between the activation parameters for Kex2 and those for subtilisin 72 and HIV protease may indicate that enzymatic acyl-transfer transition states are generally comparable, it could also be purely coincidental and indicate that temperature dependence cannot be used in this manner.

Clearly, then, neither solvent isotope effects nor activation parameters have provided an unequivocal identification of the Kex2 rate-limiting step in this reaction. We therefore sought a more direct means of addressing this question. Classically, this problem has been addressed by the addition of exogenous nucleophiles such as hydroxylamine to the reaction, thereby competing with the solvent water molecule normally incorporated into the new C-terminus of the N-terminal proteolytic fragment and speeding the overall rate of deacylation (*27*, *28*). Unfortunately, the use of such nucleophiles with Kex2 proved difficult because of rapid inactivation of the enzyme in the presence of such compounds (data not shown). We therefore devised a new assay which allowed us to monitor the incorporation of solvent water into the N-terminal cleavage product directly and quantitatively.

A Mass Spectrometric Assay for Deacylation. In the deacylation step, water is incorporated into a carboxylate that will rapidly exchange protons with bulk solvent, precluding the use of hydrogen isotopes as labels. In addition, oxygen radioisotopes decay rapidly, so a pulse−chase measurement of hydrolysis must assay incorporation of stable isotopes. Electrospray mass spectroscopy provides a sensitive way to assay such incorporation into peptides, but the quantitative intensity of the observed signal from a sample is variable and highly dependent on that sample's ability to form ions in the vapor phase (*29*). We reasoned that chemically identical ions that differ only by stable-isotope substitution should mobilize similarly, allowing quantitative interpretation of the ratio of their observed intensities. Product could thus be quantified by comparing its intensity to that of a chemically identical standard added in a known amount.

Therefore, a pulse−chase experiment to observe deacylation was designed according to the scheme shown in Figure 3. Kex2 and saturating substrate, both in $H_2{}^{18}O$, are reacted in a rapid-quenched-flow mixer. As acylation is not rate-limiting, a pre-steady-state burst of C-terminal product is formed. Should acylenzyme hydrolysis be rate-limiting, the N-terminal product will accumulate linearly with time after an initial lag (*25*). However, should product release be rate-determining, the formation of both N- and C-terminal cleavage products will display burst kinetics, because the deacylation step will proceed more rapidly than the steady state. Acylenzyme that is present when quench is added to terminate the $H_2{}^{18}O$ pulse could break down during subsequent handling of the sample, so that an intensity ratio of product generated in $H_2{}^{16}O$ to product which incorporated $H_2{}^{18}O$ might suffer from systematic error introduced by the unknown quantity of acylenzyme present at the end of the pulse. We reasoned that such error could be significant, because the ratio of acylenzyme to product is large during the first few turnovers in the reaction.

We therefore used the peptide cleavage product from a second substrate as a standard in this experiment. One substrate (the standard) contained an ordinary acetyl group; this substrate was cleaved to completion in $H_2{}^{16}O$, and cleavage was quantified by measuring released AMC by fluorescence. This allowed a precise amount of the resulting tetrapeptide product to be added to each timepoint to serve as an internal standard. The second substrate contained a triply deuterated acetyl group within an otherwise identical molecule (d_3-Ac-Nle-Tyr-Lys-Arg↓MCA). Cleavage of this deuterated substrate in $H_2{}^{16}O$ would generate a product differing from the nondeuterated standard by three mass units, while cleavage of this substrate in $H_2{}^{18}O$ would generate a product differing from the standard by five mass units. Were a pulse−chase experiment carried out with the deuterated substrate in a rapid-quenched-flow mixer, product generated during the pulse would incorporate $H_2{}^{18}O$ and therefore weigh five mass units more than the internal standard, while product resulting from hydrolysis of acylenzyme after addition of acid quench and beginning of the $H_2{}^{16}O$ chase would only weigh three mass units more than the peptide standard. The nondeuterated standard would be cleaved to completion in $H_2{}^{16}O$ in parallel with the pulse−chase experiment, quantified by fluorescence, and then added to each sample before mass spectrometry.

Pilot experiments with deuterated and nondeuterated substrates showed that there was no difference in cleavage of these substrates and that no solvent isotope effect of $H_2{}^{18}O$ on steady-state turnover could be observed (Table 1). When the deuterated product incorporated $H_2{}^{18}O$, a product with mass/charge ratio (*m*/*z*) of 626.7−627.1 could be seen, distinct from the nondeuterated standard peptide which had *m*/*z* of 621.7−621.9 (Figure 4A). Furthermore, standard curves generated by cleaving both substrates to completion and mixing known amounts of the two products were linear (Figure 4B), indicating that this approach would allow quantitation of product. Oxygen exchange after addition of quench could not be detected over a time scale of several

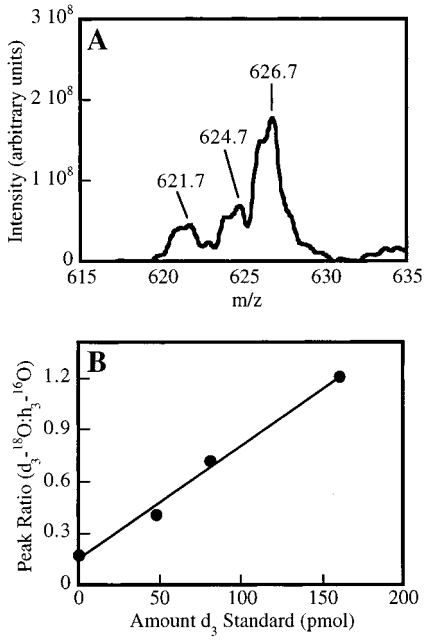

FIGURE 4: Mass spectrometry can be used as a quantitative assay for deacylation. (A) A spectrum of the region containing singly ionized cleavage products Ac-Nle-Tyr-Lys-Arg and d_3-Ac-Nle-Tyr-Lys-Arg. The reaction was carried out and examined by electrospray mass spectrometry as described under Materials and Methods. This timepoint was reacted for 400 ms. The peak at *m*/*z* 621.7 arises from internal standard, the peak at *m*/*z* 626.7 is deuterated product which incorporated ^{18}O upon deacylation, and the peak at *m*/*z* 624.7 is deuterated product which incorporated ^{16}O upon deacylation during the $H_2{}^{18}O$ pulse due to incomplete $H_2{}^{18}O$ enrichment (see Materials and Methods). The region between *m*/*z* 622 and 624 contains contaminants associated with the ethanolamine/octyl glucoside buffer. (B) The substrates Ac-Nle-Tyr-Lys-Arg↓MCA and d_3-Ac-Nle-Tyr-Lys-Arg↓MCA were cleaved to completion and quantified by fluorescence. Known amounts of each product were then mixed, and mass spectra were taken from *m*/*z* 200 to *m*/*z* 2000 for 10 min with a 10 s scan time and 0.1 s delay between scans. The peak heights corresponding to the standard and the deuterated, ^{18}O-containing product were then determined, and their ratio was calculated. These ratios were then plotted against the known amount of standard in each sample to give the linear standard curve shown. The data are not corrected for ^{18}O enrichment. Substantially greater amounts of product led to a nonlinear relationship between peak ratio and amount of product (data not shown).

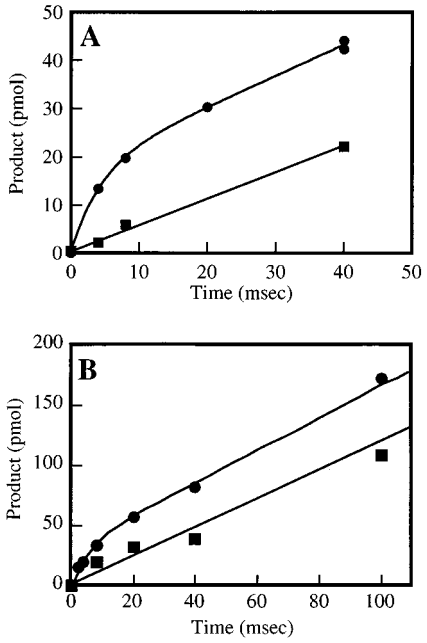

FIGURE 5: Measurement of deacylation by mass spectrometry. d_3-Ac-Nle-Tyr-Lys-Arg↓MCA was cleaved with Kex2 protease at 21 °C as described under Materials and Methods, and the indicated timepoints were assayed either by fluorescence (circles) or by mass spectrometry (squares). (A) The initial burst of AMC released during the first turnover indicates that acylation is not rate-limiting. However, the N-terminal product assayed by mass spectrometry increases essentially linearly with time at the same steady-state rate, indicating that deacylation occurs at or after the rate-determining step. In this instance, the data for both acylation and deacylation products were fit to eq 2. For the deacylation product, an exponential amplitude of −0.2 was employed, but essentially no difference was observed between this fitting procedure and linear regression (data not shown). (B) A similar experiment with longer timepoints. In this case, the data for the acylation product were fit to eq 2, while the data for the deacylation product were fit by linear regression for comparison with the model used in panel A. The equivalent results produced by these two fitting procedures indicate that the initial lag phase in formation of the deacylation product cannot be resolved under these conditions. For both experiments, the value of k_{cat} determined by mass spectrometry is within 15% of the value obtained by fluorescence. These experiments are representative of multiple trials. In all instances, the values of k_{cat} obtained with either assay were within 30% of each other (values were within 15% for all experiments but one). When duplicate points were examined by mass spectrometry, the ratios of standard to double-labeled product (d_3 and ^{18}O) were typically within 15% of each other. In our hands, this mass spectrometric assay is of comparable sensitivity and precision to the fluorescence assay used to monitor acylation, although it is much more sensitive to buffer contaminants and salt concentrations.

hours and was therefore presumed to be slow under the acidic conditions in which quenched samples were stored (data not shown).

Measurement of Deacylation by Mass Spectrometry. We examined cleavage of d_3-Ac-Nle-Tyr-Lys-Arg↓MCA by both fluorescence (monitoring AMC released at the acylation step) and mass spectrometry (monitoring deacylation). As can be seen in Figure 5, the initial burst of AMC formation was not mirrored by a burst of peptide product. Instead, labeled peptide accumulated essentially linearly with time in the presence of saturating substrate, and the rate of N-terminal product formation was identical to the steady-state rate of C-terminal product formation. We were unable to detect significant deviations from linearity at short times, indicating that the signal/noise ratio under those conditions was too small to detect the predicted lag phase. However, the absence of burst kinetics rules out the possibility that product release is rate-limiting with this enzyme, and the excellent agreement between the steady-state rates measured with these two assays convincingly demonstrates that the assay for direct hydrolysis provides reliable, sensitive mechanistic information. While the possibility of some rate-limiting conformational change after acylation but before deacylation cannot be ruled out on the basis of these data, the minimal interpretation is that deacylation itself is rate-limiting for this processing protease.

DISCUSSION

Deacylation has classically been considered to be rate-limiting for cleavage of ester substrates by serine proteases, but cleavage of amide substrates was thought to be limited by acylation rather than by cleavage of the labile ester linkage of the acylenzyme (*16*, *30*). This is in keeping with the fact

that esters are generally more susceptible to nucleophilic attack than equivalent amides, and characterization of degradative subtilisins is consistent with this view (*16*). However, Kex2 protease is limited by deacylation in cleavage of substrates based on physiological Lys-Arg cleavage sites (Figure 5). Substitution of Lys for Arg results in a loss of burst kinetics in Kex2 cleavage (*12*), suggesting that cleavage of such Lys-Lys substrates proceeds with rate-limiting acylation.

The rapid acylation and slow deacylation seen with the processing proteases (Figures 1 and 5) may have consequences for their function. First, this behavior makes these enzymes much easier to saturate, because K_M for serine proteases is determined by both enzyme−substrate affinity and the relative rates of acylation and deacylation (*25*, *30*). Second, in the presence of saturating substrate, the majority of Kex2 will be in the form of the acylenzyme intermediate, covalently linked to correct cleavage sites. This pool of enzyme is thereby prevented from binding and potentially cleaving incorrect sites.

However, this bound pool of enzyme is also unable to bind and cleave correct sites, so one might expect this behavior to affect processing efficiency adversely. If, instead, Kex2 had evolved to maximize the efficiency of processing, one would predict that the enzyme should exhibit high specificity (rigorously defined as the ratio of the k_{cat}/K_M values for competing substrates, *25*), but also high turnover. However, the subtilisin homologues of Kex2 typically exhibit higher turnover numbers than the processing proteases of the Kex2 family. Moreover, there is no obvious reason processing enzymes such as Kex2 and furin should be effectively crippled at the deacylation step, particularly as Kex2 is actually faster than the subtilisins at the acylation step with correct sequences (our unpublished experiments). Thus, while Kex2 does exhibit high specificity, it would seem to exhibit anomalously slow steady-state turnover, suggesting that it has not evolved to maximize efficiency simply in terms of high turnover with high specificity. However, this work clearly demonstrates that deacylation itself is rate-limiting for Kex2, so there is presumably some selective advantage resulting from this behavior. To better understand possible reasons for the fast acylation and slow deacylation seen with Kex2, it would then seem appropriate to consider the physiological milieu of this enzyme and the possible selective pressures that may be acting upon it.

Kex2 is localized in multiple compartments in the yeast secretory pathway, including the trans Golgi network and the endosome (*31*, *32*). These compartments are quite small, with an average diameter of 0.1−0.2 times the diameter of the mother cell (*31*). Approximating the cell and the compartments as spheres and using the known average volume of a yeast cell (70 μm^3 for haploids and 120 μm^3 for diploids, *33*), it is possible to estimate the volumes for the Kex2-containing compartments as $(0.1-1) \times 10^{-15}$ L (0.1−1 fL). These small compartment volumes and the reported K_M values for Kex2 (typically around 1 μM for correct sequences; for example, see Table 1) would indicate that a mere 3000 correct cleavage sites would result in a substrate concentration of approximately 5 μM, sufficient to saturate the enzyme. A complete list of physiological Kex2 substrates is not yet available, but is there is a steadily growing list of candidates (*1*, *7*, *34*, *35*), many of which have significant constitutive expression. Thus, the enzyme is likely to be saturated in vivo. Moreover, Kex2 resides in compartments which are thought to either contain or serve as sorting intermediates for a large number of proteins which contain incorrect cleavage sites, including the essential yeast plasma membrane ATPase encoded by the *PMA1* gene (*36*). The time scale of events in the yeast secretory pathway is on the order of minutes, so both correct and incorrect substrates are exposed to the enzyme for multiple turnovers. Kex2 is itself quite inabundant, but it is possible to overexpress either a physiological Kex2 substrate or an exogenous fusion protein containing a Kex2 cleavage site without accumulation of uncleaved precursor, suggesting enzyme is in excess in vivo. In this context, it is noteworthy that a number of possible Kex2 substrates (such as cell wall enzymes and heat shock proteins) could well be induced in response to physiological stimuli, so the presence of excess Kex2 may well constitute a reserve pool which can be mobilized when such proteins are induced, possibly by altering Kex2 localization or by presenting a theoretical allosteric effector. Thus, this enzyme is likely to be saturated with correct substrates in the presence of excess incorrect substrates, and there is likely to be some excess enzyme in vivo.

The presence of excess incorrect substrates and excess enzyme in vivo underscores the importance of high specificity (as k_{cat}/K_M ratio) for Kex2, and the enzyme is indeed very specific (*8*, *11*, *12*). However, the actual *rates* of correct and incorrect cleavages may also be important considerations, and this could explain the observed behavior. The rate of cleavage of a given substrate in the presence of other substrates can always be expressed as its k_{cat}/K_M multiplied by the concentrations of this substrate and the *free* enzyme (as opposed to the total enzyme concentration; *25*). For instance, if an incorrect substrate were cleaved with 1000-fold less efficiency than a correct one (k_{cat}/K_M ratio of 1000 for correct to incorrect) but the incorrect substrate were present at a concentration 1000-fold higher than the correct one, the actual rates of cleavage would be equal. The slow deacylation seen with Kex2 leads to accumulation of acylenzyme and thus to a lower free enzyme concentration than would be the case were the ratio of deacylation rate to acylation rate ≥ 1. This reduction in free enzyme concentration should reduce the cleavage rates for both correct and incorrect sites, and indeed simulations of Kex2 cleavage of correct substrate in the presence of excess incorrect substrate demonstrate that a low deacylation rate for the correct substrate results in lower rates of overall cleavage of both correct and incorrect substrates (data not shown). However, the relative disadvantage imposed by the slower cleavage of correct sites would be offset by a selective advantage if the in vivo rate of incorrect cleavage is reduced below a threshold of toxicity. Moreover, the presence of excess enzyme in vivo could well be viewed as a reserve pool in such a model, because such excess enzyme would of necessity raise the rate of incorrect cleavage unless the excess active sites are sequestered or occluded, as is the case here. While this model is obviously hypothetical, it does provide a possible explanation for the unusual kinetic behavior of Kex2 that is consistent with the known data about this processing enzyme and suggests that the physiological advantages resulting from this behavior may well be quite subtle.

If the selective advantage conferred by slow deacylation is simply caused by reducing the free enzyme (and hence incorrect cleavage) in vivo, one might speculate as to why the acylenzyme intermediate is accumulated rather than a different intermediate such as a Michaelis complex. Kex2 recognizes substrates at three side chains (P_4, P_2, and P_1; *11, 12*), but incorrect cleavage sites with two suitable residues out of three are common in yeast secretory proteins. Were the enzyme to bind uncleaved substrate very tightly in a stabilized Michaelis complex, such incorrect sites would also bind Kex2 more tightly, so that specificity (the actual k_{cat}/K_M ratio for the correct and incorrect substrates) could be adversely affected. An induced-fit strategy would avoid this problem, but such a strategy would adversely affect catalysis, since energy used to remodel the active site to bind the ground state cannot be simultaneously used to differentially stabilize the transition state as is required for catalysis (*37*). Moreover, it is possible that some substrates may consist of an N-terminal inhibitory segment and a C-terminal effector which is activated by cleavage (as is the case for Kex2 itself, *14*), in which case a stable acylenzyme intermediate would clearly be preferable because the effector is generated while the free enzyme concentration is still kept low. Though such considerations are currently quite speculative, they show the remarkable extent to which quite subtle features of the enzyme−substrate interaction could be optimized for in vivo advantage.

This convergence of kinetic behavior and physiological role can be contrasted with that of the subtilisins. These enzymes are nonspecific degradative or digestive enzymes active in the extracellular medium, where they function to cleave a wide variety of possible substrates at rather low substrate concentration. As a consequence, they have evolved low specificity to allow cleavage of many sites together with higher turnover to maximize free enzyme and hence overall turnover. Since there is no pressure to accumulate acylenzyme for the degradative subtilisins, these enzymes have not slowed their deacylation rates, and acylation remains rate-limiting.

The rapid acylation and rate-determining deacylation seen in cleavage of physiological sequences by Kex2 may thus reflect an adaptation for its function as a processing protease rather than as a subtilisin. The still lower k_{cat} values seen in mammalian processing proteases (*17, 18, 38*) may reflect the slower progress of potential substrates through the mammalian secretory pathway, necessitating a still lower deacylation rate to protect secretory proteins from proteolysis while they are in a processing compartment. In essence, the processing protease deacylation rate could be tuned to optimize the free enzyme concentration for its precise physiological milieu, while the acylation rate remains rapid. Thus, these results demonstrate a fundamental distinction between the degradative and processing enzymes of the subtilisin superfamily in their use of the serine protease mechanism to generate specificity and turnover.

Last, mass spectrometry has been used to examine phosphate exchange in the mitochondrial ATPase active site and to track oxygen atoms in other enzymatic reactions (*39*−*41*). It has also been used to demonstrate the existence of a covalent intermediate in the mechanism of epoxide hydrolase and to detect stable acylenzyme intermediates formed by serine proteases such as pancreatic elastase (*29, 42*−*44*). Ratio mass spectrometry has also been employed to probe enzymatic transition state structure through detection of subtle isotope effects on formation of products (*45*−*47*). We have devised a new technique that allows the use of electrospray mass spectrometry as a quantitative assay for the formation of hydrolytic products from an enzymatic reaction (Figures 4 and 5). This technique has successfully been applied to generate new insight into a classic problem in mechanistic enzymology, the serine protease deacylation step. It is hoped that this approach will prove useful not only in examining other members of the Kex2 family but also in characterizing other hydrolytic enzymes as well.

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