Differential Utilization of Enzyme-Substrate Interactions for Acylation but Not Deacylation during the Catalytic Cycle of Kex2 Protease*

Nathan C. Rockwell§§ and Robert S. Fuller¶

From the ‡Department of Biological Chemistry, University of Michigan Medical Center, Ann Arbor, Michigan 48109 and the §Department of Biochemistry, Stanford University, Stanford, California 94305

Kex2 protease from Saccharomyces cerevisiae is the prototype for a family of eukaryotic proprotein processing proteases belonging to the subtilase superfamily of serine proteases. Kex2 can be distinguished from degradative subtilisins on the basis of stringent substrate specificity and distinct pre-steady-state behavior. To better understand these mechanistic differences, we have examined the effects of substrate residues at P1 and P2 on individual steps in the Kex2 catalytic cycle with a systematic series of isosteric peptidyl amide and ester substrates. The results demonstrate that substrates based on known, physiological cleavage sites exhibit high acylation rates (≥550 s⁻¹) with Kex2. Substitution of Lys for Arg at P1 resulted in a ≥200-fold drop in acylation rate with almost no apparent effect on binding or deacylation. In contrast, substitution of the physiologically incorrect Ala for Nle at P4 resulted in a much smaller defect in acylation and a modest but significant effect on binding with Lys at P1. This substitution also had no effect on deacylation. These results demonstrate that Kex2 utilizes enzyme-substrate interactions in different ways at different steps in the catalytic cycle, with the S4-P1 contact providing a key specificity determinant at the acylation step.

Kex2 protease from the yeast Saccharomyces cerevisiae (kexin, EC 3.4.21.61) is the prototype for a family of eukaryotic processing proteases that carry out specific endoproteolytic cleavage of proprotein and prohormone precursors in the secretory pathway (1–3). These enzymes are found in both the subtilase superfamily of serine proteases and the subtilisins have distinct physiological functions. Whereas the subtilisins are digestive enzymes acting either extracellularly or in proteolytically active compartments such as the lysosome or the yeast vacuole, the processing proteases operate intracellularly in sorting and secretion. As might be expected, the processing proteases are much more specific than the degradative subtilisins (13–17). Additionally, these enzymes can be distinguished on the basis of primary sequence and domain structure (18). Somewhat surprisingly, it has also been possible to distinguish these two families of serine proteases on the basis of their pre-steady-state behavior (9, 14, 16, 19). This last finding suggests that the processing proteases may have adapted the classical serine protease mechanism (10, 20, 21) to their in vivo function during evolution in a manner not previously appreciated.

Serine proteases hydrolyze ester and amide substrates via a mechanism involving the generation and subsequent hydrolysis (or transamidation) of a covalent acylenzyme intermediate (10, 21–23). It had been thought classically that in cleavage of amide substrates, serine proteases were limited by the rate of acylation (commonly referred to as kₐ) because this step involves the hydrolysis of a resonance-stabilized amide bond rather than the more labile ester cleaved in the deacylation step (whose rate is referred to as kₑ). Early experiments with chromogenic and fluorogenic substrates analogous to single amino acids seemed to confirm this notion (9, 21). Furthermore, substrates incorporating scissile esters rather than amides exhibited an initial burst of product formation caused by rate-limiting deacylation (21, 24). For the subtilisin family, work with larger oligopeptide substrates also confirmed this expectation (9), in contrast to the evolutionarily unrelated serine proteases of the trypsin/chymotrypsin family (25).

However, the pre-steady-state behavior of Kex2 protease is at odds with this model, because the enzyme exhibits burst kinetics in cleavage of amide substrates based on known, physiological Kex2 cleavage sites (aliphatic/basic P₄, basic P₂, Arg at P₃; Refs. 14, 16, and 26).1 Substitution of Lys for Arg at the P₁ position resulted in the loss of burst kinetics, indicating a change in rate-limiting step upon this substitution (16). Burst kinetics were also observed in cleavage of model amide substrates by the homologous enzyme furin (17, 19). This work utilized tetrapeptide substrates with synthetic coumarin leaving groups (7-amino-4-methylcoumarin or 7-hydroxy-4-methylcoumarin; Refs. 14, 16, 17, 26, and 27). It was thus possible that this behavior was an artifact of the activated leaving group. However, otherwise identical substrates with either coumarin leaving groups or authentic peptide bonds are cleaved with essentially identical values of kₑ/Kₐ (15). Moreover, recent work has demonstrated that burst kinetics are also seen in

---

* This work was supported by National Institutes of Health Grant GM 39697 (to R. S. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: 401 Barker Hall, MCB Dept., University of California, Berkeley, CA 94720.

§ To whom correspondence should be addressed. E-mail: bfuller@umich.edu.

1 Throughout we use the nomenclature of Schechter and Berger (32) in designating the substrate residues surrounding the cleavage site as -P₋₄-P₋₃-P₋₂-P₋₁-P₋₀, with the scissile bond designated by the arrow. Individual subsites on the enzyme are designated S₄, S₃, etc.
Kex2 cleavage of an actual peptide bond, and this behavior requires the presence of the physiologically correct P1, Arg (28). Thus, the acylation rate for Kex2 cleavage of peptide bonds must be substantially higher than some subsequent step, such as deacylation or product release. It has also recently been demonstrated that hydrolysis of the acylenzyme is rate-limiting for Kex2 protease in cleaving a substrate that exhibits burst hydrolysis (28).

This body of work suggested that catalysis by processing proteases such as Kex2 and furin involves an adaptation of the serine protease mechanism to allow rapid acylation in cleavage of sites resembling those in physiological substrates, e.g. with Arg at P1, but not in cleavage of nonphysiological sites. The much slower rate of deacylation relative to acylation in cleavage of physiologically correct sequences suggested that the energy of enzyme-substrate interactions might be used differentially during different points in the catalytic cycle. To test this model and better understand the means by which processing enzymes such as Kex2 and furin may distinguish authentic physiological substrates from other potential cleavage sites, we have measured or estimated substrate binding, acylation rate, and deacylation rate for sequences containing correct or incorrect P1 and P1 residues. The results are consistent with a model whereby correct enzyme-substrate contacts are used to drive acylation, with less effect on binding and essentially no effect on deacylation.

MATERIALS AND METHODS

Substrates and Reagents—Kex2 protease was purified as described (15). The peptidyl-MCA2 substrates Ac-Nle-Tyr-Lys-Arg-MCA, Ac-Nle-Tyr-Lys-Arg-MCA, and Z-Ala-Tyr-Lys-Arg-MCA have been described previously (16). Z-Nle-Tyr-Lys-Arg-MCA and Z-Ala-Tyr-Lys-Arg-MCA were prepared as described (15) with the use of suprastoichiometric LiCl to solubilize the peptides (17). The synthesis of Z-Nle-Tyr-Lys-Arg-MCA and Z-Ala-Tyr-Lys-Arg-MCA were prepared as described elsewhere (27). The preparation of Z-Nle-Tyr-Lys-Arg-MCA used in these experiments was contaminated with a minor amount of Z-Nle-Tyr-Lys-Arg-MCE, apparently arising from contaminants in commercially available Z-Nle (Bachem), but this minor species did not affect our results (data not shown).

Steady-state Kinetics with Peptidyl-MCA Substrates—Kex2 protease was reacted with substrates in 0.2M sodium acetate, pH 6.0, 1 mM CaCl2, 0.1% Triton X-100 at 21 °C (standard conditions for this work). The reactions were quenched with 1 M acetic acid (600 µl added to a reaction volume of 100 µl), and product formation was measured by fluorescence as described (15). Some reactions were run in the above conditions but at 37 °C, as reported previously (15, 16). The difference in pH associated with the temperature change was negligible for these experiments (data not shown). For measurement of deacylation rates, some substrates were examined in 0.2 M sodium acetate, pH 6.0, 1 mM CaCl2, 0.1% Triton X-100 at 21 °C (standard conditions for this work). The reactions were quenched with 1 N acetic acid (600 µl added to a reaction volume of 100 µl), and product formation was measured by fluorescence as described (15). Some reactions were run in the above conditions but at 37 °C, as reported previously (15, 16). The difference in pH associated with the temperature change was negligible for these experiments (data not shown).

Rapid Quenched Flow Measurements—Z-Nle-Tyr-Lys-Arg-MCA, Ac-Nle-Tyr-Lys-Arg-MCA, and Z-Ala-Tyr-Lys-Arg-MCA were prepared in 0.1% aqueous trifluoroacetic acid and reacted with Kex2 in 0.2 M sodium acetate, pH 6.0, 1 mM CaCl2, 0.1% Triton X-100 in a Knick RQF-3 rapid quenched flow mixer exactly as described (27). This protocol minimized nonspecific ester hydrolysis. Ac-Nle-Tyr-Lys-Arg-MCA was examined using either this procedure or the method previously reported (16), and no difference between these methods was observed.

stopped Flow Florimetry—Z-Nle-Tyr-Lys-Arg-MCA, Ac-Nle-Tyr-Lys-Arg-MCA, and Z-Ala-Tyr-Lys-Arg-MCA (1–200 µM) were reacted with Kex2 (260–520 nM) in an Applied Photophysics stopped flow fluorimeter. The data were typically collected at photomultiplier tube voltages between 550 and 650 V with an offset of 4.99 V using an excitation wavelength of 380 or 385 nm with a 1-nm bandwidth. Emission was monitored with a 460-nm band pass interference filter (10 nm full width at half-maximum band pass; CVI Laser, Albuquerque, NM). Four data points/ms were collected for 100–1000 ms, and the data were transferred to Kaleidagraph for analysis. For comparison, substrate base lines were prepared using mock reactions lacking enzyme. Base lines were stable, with no significant enzyme-independent change during the time course of the reaction. At least four trials were recorded at each substrate concentration.

Stopped Flow Data Analysis—The data from the individual time courses described above showed an initial drop in fluorescence attributed to completion of mixing in the cuvette, followed by an initial burst and a linear steady state (representative trials shown in Fig. 1). Time courses were fit by using the known base line as a starting point and an initial burst phase, so Equation 1 was fit to Equation 1 using nonlinear regression. Equation 1 describes an initial exponential transient followed by a linear steady state with an arbitrary intercept (to accommodate the arbitrary fluorescence units).

\[ F(t) = F_0 + A(1 - e^{-kt}) + V_{st}t \]

(Eq. 1)

In this equation, \( F(t) \) is the fluorescence at time \( t \), \( F_0 \) is the arbitrary intercept (in V), \( A \) is the apparent amplitude of the transient (also in V), \( k \) is the observed rate of the burst (in s\(^{-1}\)), and \( V_{st} \) is the steady-state rate (in V/s). Residuals were calculated as (observed fluorescence – calculated fluorescence)/calculated fluorescence and showed no systematic deviation from the origin as a function of time (data not shown), indicating that the data were adequately described by this model. For Ac-Nle-Tyr-Lys-Arg-MCA it was occasionally necessary to constrain the fit by using the known base line as \( F_0 \).

The steady-state rate \( V_{st} \) should follow the Michaelis-Menten equation when followed as a function of substrate concentration (21), so \( V_{st} \) was plotted versus [S]. The resulting plots showed well behaved saturation behavior without apparent substrate inhibition or other complications (Fig. 2 and data not shown). Kinetic parameters derived from these plots agreed reasonably well with values determined using other procedures (all values within 50%; data not shown).

To examine the acylation phase itself, we plotted the burst rate \( k_{obs} \) as a function of substrate concentration [S]. This quantity should follow a modified Michaelis-Menten relationship (Equation 2), with the acylation rate \( k_{ac} \) as the maximal quantity (\( V_{max} \) equivalent) and the enzyme-substrate affinity constant \( K_s \) as the maximal binding constant (pre-steady-state \( K_s \); Ref. 21).

\[ k_{obs}([S]) = k_{ac} [S] / (K_s + [S]) \]

(Eq. 2)

The observed data did follow an apparent saturation relationship (Fig. 3 and data not shown), but it did not seem well behaved. Observed burst rates at higher substrate concentrations deviated from predicted behavior, and data at lower substrate concentrations were equally well described by a linear model until a rate of ~500 s\(^{-1}\) was reached. This rate corresponds to a half-time of 1.5 ms, comparable with the apparent dead time for the instrument of 1.5 ms. These deviations from ideal behavior as predicted by both the saturation relationship and simulations suggested that the apparent saturation could be caused by a machine limit and not by true saturation of the initial burst phase, so we consider the apparent \( k_{ac} \) values to be lower bounds.

RESULTS

Pre-steady-state Rate Constants for the Nle-Tyr-Lys-Arg\(^{-}\) Cleavage Site—We wished to characterize Kex2 specificity throughout the catalytic cycle to expand upon our understanding of how this enzyme recognizes physiologically correct cleavage sites. We therefore focused on the Nle-Tyr-Lys-Arg\(^{-}\) sequence, as we had previously used to examine Nle-Tyr-Lys-Arg\(^{-}\) cleavage site found in the Kex2 substate pro-α-factor (3, 28). Deacylation is known to be rate-limiting for this sequence with amide substrates (28), so the deacylation rate \( k_{ac} \) for this sequence is simply given by \( k_{ac} \). However, we also wished to examine earlier events in the catalytic cycle. We utilized Z-Nle-Tyr-Lys-Arg-MCA and Ac-Nle-Tyr-Lys-Arg-MCA for this

---

2 The abbreviations used are: MCA, methylcoumarinamide; Ac, acetyl; Bistris, bis(2-hydroxyethyl)aminotrihydroxymethyl)methane; MCE, methylcoumarinester; Nle, norleucine; Z, benzoyloxy carbonyl.
purpose. Z-Nle-Tyr-Lys-Arg \textendash \textmd{MCA} was chosen because we suspected that substitutions at P1 resulted in rate-limiting acylation, necessitating the use of ester substrates to examine the deacylation step, and we had found that tetrapeptidyl methyl-coumarin ester substrates (peptidyl MCEs; Ref. 27) and synthetic intermediates leading to them were easier to obtain as solid products with N-terminal Z groups than with N-terminal Ac groups,\(^3\) whereas Ac-Nle-Tyr-Lys-Arg \textendash \textmd{MCA} had previously been used to demonstrate rate-limiting deacylation (28). The use of peptidyl-MCE substrates also necessitated a lower pH to reduce nonspecific hydrolysis (27), but preliminary experiments using rapid quenched flow techniques at either pH 7.26 or pH 6.0 indicated that the pre-steady-state behavior of Kex2 cleavage of Ac-Nle-Tyr-Lys-Arg \textendash \textmd{MCA} was not significantly altered at the lower pH (data not shown).

To examine early events in the Kex2 catalytic cycle (binding and acylation, referred to as \(K_S\) and \(k_2\) in the tables), we attempted to examine the saturation behavior of the burst phase of Kex2 cleavage of these substrates. When deacylation is rate-limiting, the initial approach to the steady state occurs as an exponential transient whose rate is a function of the substrate concentration, binding constant, and acylation rate (21). Therefore, examination of the apparent burst rate \((k_{\text{obs}}\) in Equation 1) as a function of substrate concentration should in turn follow a Michaelis-Menten relationship with the known steady-state parameters. Initial experiments using rapid quenched flow techniques suggested that the acylation rate was extremely rapid (data not shown), so we attempted to examine these processes by stopped flow fluorimetry.

Kex2 cleavage of Z-Nle-Tyr-Lys-Arg \textendash \textmd{MCA} was examined at 21°C over a range of substrate concentrations from 1 to 100 \(\mu\text{M}\), and the resulting data were fit to Equation 1 (representative trials at 5 and 25 \(\mu\text{M}\) substrate are shown in Fig. 1).

Analysis of the fitted data showed that the steady-state rate \(V_{\text{obs}}\) did indeed follow the expected behavior (Fig. 2), and the apparent burst rates initially seemed to exhibit saturation. However, detailed examination of the pre-steady-state rates \((k_{\text{obs}})\) showed that there might be significant deviations from the predicted behavior at higher substrate concentrations, where the observed burst rate was \(-550\text{ s}^{-1}\) (Fig. 3). The observed rate constants were approximately linear with substrate concentration until this point was reached and then diverged from predicted saturation behavior to remain at this apparent limiting rate (Fig. 3B). We were therefore concerned that the apparent saturation was due to a machine limit, in keeping with the observation that a rate constant of 550 \(\text{s}^{-1}\) corresponds to a half-time that is quite close to the dead time of the instrument (1.3 ms \textit{versus} 1.5 ms; “Materials and Methods”). Therefore, we have chosen to report this value as a lower bound for the acylation rate constant \((k_2\geq 550\text{ s}^{-1});\) Table I.

The lack of a reliable value for \(k_2\) precludes reliable determination of \(K_S\) using this approach, because the binding pre-equilibrium is measured as the pre-steady-state \(K_T\) and hence as the substrate concentration giving half-maximal \(V_{\text{obs}}\). However, the linear relationship between \(k_{\text{obs}}\) and substrate concentration below the machine limit implies that this range of substrate concentrations \((\text{[S]}=25\text{ \(\mu\text{M}\)})\) is at or below \(K_S\) for this substrate. Therefore, we are able to place a lower boundary on the binding constant for this substrate (Table I). Additionally, this suggests that the true acylation rate could be at least 2-fold higher than the apparent value, because a range of \([S]\) at or below \(K_S\) would imply that the observed rate constant \(k_{\text{obs}}\) is at or below \(V_2\) of the maximal \(k_{\text{obs}}\) (from the definition of \(K_S\) and \(k_{\text{obs}}\)). However, we have chosen to report the experimental values throughout this paper.

Similar results were obtained in stopped flow characterization of Ac-Nle-Tyr-Lys-Arg \textendash \textmd{MCA} (Table I). We again found that the acylation rate was equal to or greater than 550 \(\text{s}^{-1}\). However, we were able to estimate a slightly lower boundary for \(K_S\) with Ac-Nle-Tyr-Lys-Arg \textendash \textmd{MCA} (Table I), although this

\(^3\) N. C. Rockwell and R. S. Fuller, unpublished observations.
Pre-steady-state Examination of Kex2 Protease

The apparent burst rate \( k_{\text{obs}} \) was plotted as a function of substrate concentration and fit to the Michaelis-Menten equation (solid curve). The data for substrate concentrations at or below 25 \( \mu \text{M} \) were also fit by linear regression (dashed line). The equivalent results yielded by these two fitting models and the deviation from Michaelis-Menten behavior at higher substrate concentrations suggest that the apparent saturation could be due to a machine limit. The data from A are presented on a double-reciprocal plot and fit by linear regression. Insert, data with [S] = 10 \( \mu \text{M} \) are shown to aid in examination of the linear fit.

**Table I**

| Substrate | 21 °C, pH 7.26 | 21 °C, pH 6.0 | 37 °C, pH 7.26 |
|-----------|----------------|--------------|---------------|
| \( K_{M} \) | \( k_{2} \) | \( k_{3} \) | \( k_{3} \) |
| Z-βYKR | MCA \( ^{a} \) | \( >25^{b} \) | \( >550^{b} \) | 19 \( ^{b} \) | 27 \( ^{c} \) | 87 \( ^{c} \) |
| Ac-βYKR | MCA | \( >12^{c} \) | \( >550^{c} \) | 15 \( ^{c} \) | 4.7 \( ^{d} \) | 45 \( ^{d} \) |

\( ^{a} \) \( g \) = Nle.

\( ^{b} \) Measured or estimated by stopped flow at 21 °C in 0.2 mM Bistris, pH 7.26, 1 mM CaCl\(_2\), 0.1% Triton X-100, \( k_{2} \) and \( K_{M} \) were derived from plotting \( k_{\text{cat}} \) versus [S] as shown in Fig. 3. \( k_{2} \) was determined from plots of \( V_{\text{cat}} \), versus [S] as shown in Fig. 2.

\( ^{c} \) Measured or estimated by stopped flow at 21 °C in 0.2 mM Bistris, pH 7.26, 1 mM CaCl\(_2\), 0.1% Triton X-100. \( k_{2} \) and \( K_{M} \) were derived from plotting \( k_{\text{cat}} \) versus [S] (Fig. 3).

\( ^{d} \) Measured by rapid quenched flow at multiple substrate concentrations in 0.2 mM Bistris, pH 7.26, 1 mM CaCl\(_2\), 0.1% Triton X-100 at 21 °C. The observed rate was insensitive to substrate concentration, indicating that the reaction was saturated. Analysis of stopped flow data and a previously published temperature dependence (28) also gave values within 50% of each other and of the reported value.

\( ^{e} \) Measured by saturation kinetics at 21 °C in 0.2 mM sodium acetate, pH 6.0, 1 mM CaCl\(_2\), 0.1% Triton X-100.

\( ^{f} \) Measured by rapid quenched flow at multiple substrate concentrations in 0.2 mM sodium acetate, pH 6.0, 1 mM CaCl\(_2\), 0.1% Triton X-100 at 21 °C.

\( ^{g} \) Measured by saturation kinetics at 37 °C in 0.2 mM Bistris, pH 7.26, 1 mM CaCl\(_2\), 0.1% Triton X-100.

The enzyme has evolved for fast acylation, because the attack on the amide bond is at least 30-fold faster than subsequent hydrolysis of the resulting ester. We therefore examined how substitutions in this substrate sequence altered the enzyme-substrate interactions during the catalytic cycle.

**Pre-steady-state Rate Constants for the Nle-Tyr-Lys-Lys \( 2^{c} \)**

Cleavage Site: Effects of a P1 Substitution—Previous work with Kex2 protease had shown that substitution of Lys for Arg at P1 results in significant decreases in \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_{M} \) (14, 16). This substitution also results in a change in rate-limiting step, because substrates with Lys at P1 no longer exhibit burst kinetics in formation of the acylation product (16, 28). This lack of an initial burst with such sequences suggests that acylation is severely impaired for such sequences, such that it has become rate-limiting. To test this hypothesis, we compared Kex2 cleavage of a tetrapeptidyl methylcoumarin ester, Z-Nle-Tyr-Lys-Lys \( 2^{c} \), MCE (27), to a similar methylcoumarinamide, Ac-Nle-Tyr-Lys-Lys \( 2^{c} \), MCA (16). If the amide substrate is limited by acylation, the ester substrate should show an increase in \( k_{\text{cat}} \) caused by its more rapid acylation, as is the case here (Table II and Ref. 27). This indicates that the substitution of Lys for Arg at P1 does produce a dramatic reduction in acylation rate, such that acylation itself is now rate-limiting. This also implies that simple saturation kinetics with the amide substrate will measure \( K_{S} \) and \( k_{2} \).

We therefore measured these quantities under conditions equivalent to those used in stopped flow characterization of equivalent substrates with P1 Arg (Ac-Nle-Tyr-Lys-Lys \( 2^{c} \), MCA versus Ac-Nle-Tyr-Lys-Arg \( 2^{c} \), MCA; Table II). Although we were only able to place lower boundaries on these values for the Arg-containing cleavage sites, the comparison is nonetheless informative. Comparison of \( K_{S} \) values shows that the physiologically correct (i.e. P1 Arg) substrate is only bound at most 4-fold more tightly than the incorrect substrate. However, the acylation rates are dramatically different; in fact, the P1 substitution of Lys for Arg results in a minimum defect of over 200-fold (over 3 kcal/mol in \( \Delta G^{\circ} \), calculated as in Ref. 21). Thus, the enzyme-substrate interaction at P1 is utilized almost entirely for transition state stabilization at the first irreversible step, acylation.
To measure deacetylation, we examined the ester substrate Z-Nle-Tyr-Lys-Lys \( \downarrow \) MCE. Unlike the equivalent amide, this substrate does exhibit burst kinetics (27). Therefore, the steady-state \( k_{\text{cat}} \) for this substrate provides a convenient approximation to the deacetylation rate constant \( k_2 \) (25). Comparison of the deacetylation rate for this sequence with the deacetylation rate for the physiologically correct equivalent (Z-Nle-Tyr-Lys-Arg \( \downarrow \) MCA versus Z-Nle-Tyr-Lys-Lys \( \downarrow \) MCE; Table II) shows that these rate constants are within 50% of each other, in dramatic contrast to the results obtained at the acylation step. Thus, an enzyme-substrate interaction that is key for efficient acylation is essentially uninvolved in deacetylation.

**TABLE II**

| P₁ residue | \( K_a \) | \( k_a \) | \( k_b \) |
|------------|----------|----------|----------|
| Arg        | \( >2 \) | \( 550 \) | 27 |
| Lys        | 48 \( ^{a} \) | 2.6 \( ^{b} \) | 38 \( ^{a} \) |

\( ^{a} \) Obtained by stopped flow analysis of Kex2 cleavage of Ac-Nle-Tyr-Lys-Arg \( \downarrow \) MCA in 0.2 M Bistris, pH 7.26, 1 mM CaCl₂, 0.1% Triton X-100 at 21 °C.

\( ^{b} \) Obtained by saturation kinetics with Ac-Nle-Tyr-Lys-Lys \( \downarrow \) MCA in 0.2 M sodium acetate, pH 6.0, 1 mM CaCl₂, 0.1% Triton X-100, using Z-Nle-Tyr-Lys-Arg \( \downarrow \) MCA and Z-Nle-Tyr-Lys-Lys \( \downarrow \) MCE. For these substrates, \( k_{\text{cat}} = k_2 \).

**TABLE III**

| P₄ residue | P₁ residue | \( K_a \) | \( k_a \) | \( k_b \) |
|------------|------------|----------|----------|----------|
| Nle        | Arg        | \( >25 \) | \( 550 \) | 27 |
| Ala        | Arg        | 30 \( ^{c} \) | \( 550 \) | 73 |
| Nle        | Lys        | 48 \( ^{c} \) | 2.6 \( ^{c} \) | 38 |
| Ala        | Lys        | 330 \( ^{d} \) | 0.12 \( ^{d} \) | 44 |

\( ^{a} \) Obtained by saturation kinetics with Ac-Nle-Tyr-Lys-Arg \( \downarrow \) MCA in 0.2 M Bistris, pH 7.26, 1 mM CaCl₂, 0.1% Triton X-100 at 21 °C.

\( ^{b} \) Obtained by saturation kinetics with Z-Nle-Tyr-Lys-Arg \( \downarrow \) MCA in 0.2 M Bistris, pH 7.26, 1 mM CaCl₂, 0.1% Triton X-100 at 21 °C.

\( ^{c} \) Obtained by stopped flow analysis of Kex2 cleavage of Z-Ala-Tyr-Lys-Arg \( \downarrow \) MCA in 0.2 M Bistris, pH 7.26, 1 mM CaCl₂, 0.1% Triton X-100 at 21 °C, using Equation 2.

\( ^{d} \) Obtained by stopped flow analysis of Z-Ala-Tyr-Lys-Arg \( \downarrow \) MCA in 0.2 M Bistris, pH 7.26, 1 mM CaCl₂, 0.1% Triton X-100 at 21 °C, using Equation 2.

**DISCUSSION**

We have characterized the roles different enzyme-substrate interactions play at different steps in the Kex2 catalytic cycle. We found that the P₄ residue is used for both acylation (\( -1.8 \) kcal/mol) and binding (\( -1.1 \) kcal/mol) with little effect on deacetylation (Table III). In contrast, the P₁ residue seems to primarily act at the acylation step (\( \geq 3.1 \) kcal/mol), with little effect on either binding or deacetylation (Tables II and III). Even the relatively conservative substitution of Lys for Arg at P₁ results in a large defect in acylation such that it becomes rate-determining. Thus, this enzyme seems to generate specificity primarily at the acylation step and not to utilize the same interactions for the chemically related deacetylation step.

The approach we have taken to measure binding and acylation presupposes a one-step binding process with rapid pre-equilibrium. It is important to note that we have not experimentally validated this assumption, nor have we been able to satisfactorily rule out the related possibility that Kex2 cleavage of substrates with P₁ Arg is diffusion-controlled. However, the results of experiments with viscosogens such as sucrose or glycerol are inconsistent with a diffusion-limited reaction. Additionally, although two-step binding processes are not unknown for some serine proteases (28), we know of no evidence that this enzyme behaves in such a manner.

To better understand the interplay between these two positions, we also examined the effect of a P₁ substitution on the individual steps in cleavage of sequences containing Lys at P₁ (Table III). In these substrates, acylation was rate-limiting, so \( K_a \) and \( k_a \) were measured by saturation kinetics with the amide substrates, and \( k_b \) was measured by saturation kinetics with the equivalent esters. This work demonstrates that the P₄ side chain can impact binding, with \( K_a \) for Ac-Nle-Tyr-Lys-Lys \( \downarrow \) MCA being 7-fold lower than that for Ac-Ala-Tyr-Lys-Lys \( \downarrow \) MCA (a 1.1 kcal/mol difference in ground-state binding).

Additionally, in the context of the incorrect Lys at P₁, the correct P₄ side chain did accelerate acylation (Table III), as we had previously proposed (16). This effect corresponds to \( -1.8 \) kcal/mol utilized for transition state stabilization during the acylation reaction.

Examination of deacetylation rates for Z-Nle-Tyr-Lys-Lys \( \downarrow \) MCE and Z-Ala-Tyr-Lys-Lys \( \downarrow \) MCE showed that they were essentially identical (Table III). Thus, Kex2 seems to utilize neither the enzyme-substrate contact at P₁ nor that at P₁ at the deacetylation step, in marked contrast to acylation.
synthetic tetrapeptidyl substrates with coumarin leaving groups will accurately mimic the cleavage of authentic precursors. Although these substrates are more soluble than substrates in which cleavage occurs at a peptide bond and are better behaved kinetically (15), coumarin leaving groups display very low $pK_a$ values for substituents at the 7 position. For example, the 7-amino-4-methyl coumarin $pK_a$ is below 3, whereas the $pK_a$ for the 7-hydroxy group in the ester substrates is $\sim$5.3.3 Thus, these leaving groups are significantly more susceptible to nucleophilic attack by the active site serine than the peptide leaving groups found in authentic Kex2 substrates in vivo (21, 22). However, peptidyl MCA substrates are cleaved by Kex2 with $k_{\text{cat}}/K_M$ values essentially identical to those obtained in cleavage of actual peptide bonds (15). For serine proteases, $k_{\text{cat}}/K_M$ is determined by $k_f/K_S$, the ratio of the acylation rate to the enzyme-substrate binding constant (21). Therefore, for the values to be equivalent for these two types of substrates, any increase in acylation rate seen with coumarin leaving groups must be offset by an approximately equal decrease in affinity. Thus, the peptide leaving group may not be able to interact with the substrate backbone are sufficient to position the acylenzyme ester for hydrolysis, a hypothesis that could not be tested in this study. Taken together with other work on P2 substitutions,3 these data suggest that side chain interactions are not utilized at all during the deacylation step. It is thus likely that interactions with the substrate backbone are sufficient to position the acylenzyme ester for hydrolysis, a hypothesis that could conceivably be examined by synthesis of substrates containing substitutions within the peptide backbone.

It is also intriguing to speculate as to why the deacylation rate constant is so low. Non-specific Kex2 homologs in the

ACKNOWLEDGMENTS—We thank Jack Thomas for assistance with preparation of Kex2 protease and Rick Neubig (University of Michigan) and Jeff Scholten (Pfizer Corporation) for use of stopped flow fluorimeters. We also acknowledge Damian Krysan, Dan Herschlag, and the members of the Fuller lab for helpful discussions.

REFERENCES

1. Seidah, N. G., Chretien, M., and Day, R. (1994) Biochimie (Paris) 76, 197–209
2. Rouillé, Y., Duguay S. J., Lund, K., Furuta, M., Gong, Q., Lipkind, G., Oliva, A. A., Jr., Chan S. J., and Steiner, D. F. (1996) Prost. Neuroendoocrino. 16, 322–361
3. Fuller, R. S., Sterne, R. E., and Thorner, J. (1988) Annu. Rev. Physiol. 50, 345–362
4. Jackson, R. S., Creemers, W. J., Ohagi, S., Raffin-Sanson, M. L., Sanders, L., Montague, C. T., Hutton, J. C., and O’Rahilly, S. (1997) Nat. Genet. 16, 303–306
5. Furuta, M., Yano, H., Zhou, A., Rouillé, Y., Holst, J. J., Carroll, R., Ravazzola, M., Ori, L., Furuta, H., and Steiner, D. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6646–6651
6. Takahashi, S., Nakagawa, T., Banno, T., Watanabe, T., Murakami, K., and Nakamura, K. (1995) J. Biol. Chem. 270, 28397–28401
7. Wan, L., Moyer, S. S., Thomas, L., Liu, G., Xiang, Y., Rytuba, S. L., and Thomas, G. (1998) Cell 94, 205–216
8. Redding, K., Holcomb, C., and Fuller, R. S. (1991) J. Cell Biol. 113, 527–538
9. Philipp, M., and Bender, M. L. (1983) Mol. Cell. Biochem. 51, 5–32
10. Perona, J. J., and Craik, C. S. (1995) Protein Sci. 4, 337–360
11. Siezen, R. J., de Vos, W. M., Leunissen, J. A. M., and Dijkstra, B. W. (1991) Protein Eng. 4, 719–737
12. Siezen, R. J., and Leunissen, J. A. M. (1997) Protein Sci. 6, 501–523
13. Gren, H., Meldal, M., and Breddam, K. (1992) Biochemistry 31, 6011–6018
14. Brenner, C., and Fuller, R. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 922–926
15. Rockwell, N. C., Wang, G. T., Krafft, G. A., and Fuller, R. S. (1997) Biochemistry 36, 1912–1917
16. Rockwell, N. C., and Fuller, R. S. (1998) Biochemistry 37, 3586–3591
17. Krysan, D. J., Rockwell, N. C., and Fuller, R. S. (1999) J. Biol. Chem. 274, 23229–23234
18. Gluschankof, P., and Fuller, R. S. (1994) EMBO J. 13, 2280–2288
19. Bereznay, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) J. Biol. Chem. 269, 25830–25837
20. Farstrez, J., and Fersht, A. R. (1973) Biochemistry 12, 2025–2034
21. Fersht, A. R. (1985) Enzyme Structure and Mechanism, W. H. Freeman and Company, New York
22. Jencks, W. P. (1987) Catalysis in Chemistry and Enzymology, Dover Publications, New York
23. Schellenberger, V., Turck, C. W., Hedstrom, L., and Rutter, W. J. (1993) Biochemistry 32, 4349–4353
24. Hartley, B. S., and Kilby, B. A. (1954) Biochem. J. 56, 288–297
25. Hedstrom, L., Szalay, L., and Rutter, W. J. (1992) Science 255, 1249–1253
26. Brenner, C., Bevan, A., and Fuller, R. S. (1994) Methods Enzymol. 244, 152–167
27. Rockwell, N. C., Krysan, D. J., and Fuller, R. S. (2000) Anal. Biochem. 286, 201–208
28. Rockwell, N. C., and Fuller, R. S. (2001) Biochem. 40, 3657–3665
29. Takeuchi, Y., Satow, Y., Nakamura, K. T., and Mitsui, Y. (1991) J. Mol. Biol. 214, 329–335
30. Yennawar, N. H., Yennawar, H. P., and Farber, G. K. (1994) Biochemistry 33, 7526–7536
31. Wilmut, R. C., Clifton, I. J., Robinson, C. V., Aplin, R. T., Westwood, N. J., Hajdu, J., and Schofield, C. J. (1997) Nat. Struct. 4, 456–462
32. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162