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Mechanism of Kex2p inhibition by its proregion

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Abstract Many proteases are produced as zymogens bearing an N-terminal proregion acting both as intramolecular chaperone and as enzyme inhibitor. We studied here the inhibition mechanism of the yeast proprotein convertase Kex2p by its proregion. A recombinant secreted and soluble form of Kex2p was produced in *Pichia pastoris* and its enzymatic properties toward a fluorogenic synthetic peptide were characterized. Recombinant *Escherichia coli*-produced Kex2p proregion specifically and potently inhibited the enzyme, with an IC_{50} of 160 nM. Exploration of the inhibition mechanism revealed that the proregion behaved as a mixed inhibitor.

Key words: Kex2p; Mixed-type inhibition; Proprotein convertase; Proregion; Protease; *Saccharomyces cerevisiae*

1. Introduction

All known proteases are synthesized as inactive precursors (or zymogens) which are converted into active enzymes within a specific subcellular compartment or in the extracellular milieu [1]. By this mechanism, early action of the enzymes is prevented and proteolytic activities are confined to the compartment where their function is required. In many cases, activation occurs by removal of an inhibitory segment usually located in the N-terminus of the protein [1–3].

In the eukaryotic secretory pathway, a group of serine proteases, known as the kexin/subtilisin-like prohormone convertases (PCs), are involved in the maturation of hormones and secreted peptides at pairs of basic residues [4,5]. These proteases belong to the subtilase superfamily and share high homology with bacterial subtilisin [6]. PCs are synthesized as precursors, bearing a 83–106 amino acid long proregion which is cleaved in an autocatalytic manner soon after completion of the enzyme biosynthesis in the cell endoplasmic reticulum (ER).

By analogy with bacterial subtilisin, two functions have been proposed for PC proregions. First, work on furin [7,8], Kex2p [9], and PC2 [10] showed that their proregion is essential for enzyme activity and transport out of the ER, strongly suggesting that it may help folding of the protein, acting as intramolecular chaperones. Second, the observation that the furin proregion remains associated with the protease domain through transport in the secretory pathway, and is released only in late compartments where calcium and pH conditions are favorable, suggested that it could act as an inhibitor [11]. This hypothesis is further supported by in vitro studies with purified proregions and enzymes [10,12,13].

The first identified member of the PC family was the *Saccharomyces cerevisiae* Kex2p endoprotease. Kex2p is a type I integral membrane protein located in the yeast late Golgi compartment. The enzyme is involved in processing the α-mating pheromone and killer toxin precursors by limited proteolysis at pairs of basic amino acid residues [14,15]. Kex2p is first synthesized with an 86 amino acid long proregion at its N-terminus that is essential for enzyme biosynthesis and activity [16]. To further explore proregion function, we tested its ability to act in vitro as an inhibitor of Kex2p. A secreted and soluble form of Kex2p, ssKex2p, and Kex2p proregion were produced by expression in *Pichia pastoris* and *Escherichia coli*, respectively. Kinetic analysis revealed that the Kex2p proregion acts as a mixed inhibitor.

2. Materials and methods

2.1. DNA manipulations and plasmid constructions

DNA manipulations were performed using standard procedures [17,18]. Plasmid pPIC3-ssKex2p for expression of ssKex2p in *P. pastoris* was constructed by first cloning a 2 kb DNA fragment containing the first 673 codons of *Kex2* from Kex2HA-pVT [9] into *Bam*HI-*Avr*II digested pPIC3 (Invitrogen, Carlsbad, CA, USA). Second, self-annealed 5′-CTA GGA **ACG CGT** TC-3′ oligonucleotide was inserted into the *Avr*II site of the obtained plasmid, introducing a unique *Mlu*I site (in bold). Finally, as previously described [9], two annealed oligonucleotides containing two hemagglutinin (HA) epitope coding sequences were inserted into the *Mlu*I site. The structure of the encoded protein is shown in Fig. 1A.

To construct a vector for production of proregions in *E. coli*, we first modified plasmid pGEX4-T3 (Amersham Pharmacia Biotech, Uppsala, Sweden) by ligating annealed oligonucleotide 5′-AAT TT**G GCG CGC** CA-3′ into the *Eco*RI restriction site. This manipulation introduced a unique *Asc*I site (in bold) and destroyed the *Eco*RI site. Correct sequence of the modified plasmid, called pGL55, and conservation of the reading frame were confirmed by DNA sequencing. Next, DNA sequences encoding full-length, N- and C-terminal fragments of Kex2p proregion were excised from plasmid pAPR2 [9] and pAPR2-derived constructs as *Mlu*I-*Xho*I fragments and subcloned into pGL55 digested by *Asc*I and *Xho*I.

2.2. Production of Kex2p in P. pastoris

Electroporation of the *P. pastoris* GS115 strain and selection of positive clones were performed according to the supplier's instructions (Invitrogen). Positive cells (JGP1 strain) were cultured for 3 days at 30°C in 100 ml BMGY as recommended by the supplier, and harvested by centrifugation at 1500×*g* for 10 min at 4°C. The cell pellet

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Abbreviations: AMC, 7-amino-4-methylcoumarin; ER, endoplasmic reticulum; HA, hemagglutinin; MCA, methylcoumarinamide; PAGE, polyacrylamide gel electrophoresis; PC, proprotein convertase; SDS, sodium dodecyl sulfate

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was resuspended in 25 ml BMMY containing 0.5% methanol, and cultured for 1 day. Medium was then collected, passed through a 0.22 μm filter, and complemented with sodium azide to 10 mM and glycerol to 40% v/v. In these conditions, culture media could be stored for at least 2 months at −20°C without detectable loss of Kex2p activity. Integrity of the produced protein was confirmed by N-terminal protein sequencing (performed at the Eastern Quebec Proteomics Core Facility, Sainte-Foy, QC, Canada) and immunoblotting using an anti-HA antibody.

2.3. Production of Kex2p proregion and its N- and C-terminal fragments in E. coli

Plasmids encoding glutathione *S*-transferase fused to either the Kex2p proregion, or the N-terminal (lacking the last 29 residues) and C-terminal (lacking the first 30 residues) proregion fragments were introduced in *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA). Induction of fusion proteins by isopropyl-1-thio-β-D-galactopyranoside, binding to glutathione-Sepharose beads and cleavage with thrombin (0.05 U/μl) were performed according to the supplier's instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). The Kex2p proregion and its fragments were characterized by SDS–PAGE. Typically, 500 ml of bacterial culture yielded about 2.5–5 mg purified protein.

2.4. Enzymatic assay for Kex2p activity

Assays (100 μl final volume) were performed in 50 mM Tris–acetate pH 7.0 and 2 mM $CaCl_2$. Substrate (pERTKR-methylcoumarinamide (MCA), Peptide international, Louisville, KY, USA) stock solution in dimethyl sulfoxide was diluted to final concentrations of 10–100 μM. Reaction was initiated by adding 2 μl enzyme (culture supernatant as prepared above) diluted to 9.6 μg/ml in reaction buffer. For inhibition assays, proregion at a concentration of 100 μM in phosphate-buffered saline, or N- and C-terminal fragments at a concentration of 50 μM in phosphate-buffered saline were diluted in assay buffer to final concentrations indicated in the figure legends. Readings were performed for 10 min at room temperature in a Perkin-Elmer HTS 7000 plate reader with 360 nm and 465 nm excitation and emission filters, respectively. A standard curve from 0 to 100 μM 7-amino-4-methylcoumarin (AMC, Sigma) allowed conversion of fluorescence units in concentration of AMC released. Reaction rates were determined by linear regression and expressed in concentration of AMC released in μM per hour. Data were collected in duplicate and each value is the mean of at least three independent experiments.

3. Results and discussion

3.1. Yeast P. pastoris synthesizes a correctly processed active ssKex2p

A *P. pastoris* strain expressing ssKex2p (Fig. 1A) was constructed (see Section 2) and named JGP1. Culture medium from strain JGP1 was analyzed by SDS–PAGE and Coomassie brilliant blue staining. A single protein of approximately 80 kDa was detected after induction of the culture with 0.5% methanol for 24 h (Fig. 1B, lane 1). Typically, 100 mg of protein per liter of culture was obtained. In the same conditions, no protein was detectable in culture of the control GS115 strain (Fig. 1B, lane 2). The identity of the secreted protein was confirmed by N-terminal sequencing. The amino acid sequence obtained (underlined in Fig. 1A) corresponds to the mature Kex2p sequence expected after autocatalytic removal of the proregion and N-terminal processing by the dipeptidyl aminopeptidase Ste13p [19]. In addition, immunoblotting using anti-HA antibodies confirmed the presence of the HA epitope in the C-terminus of ssKex2p (data not shown). It thus appears that the yeast *P. pastoris* can correctly process Kex2p zymogen.

Enzymatic assays were performed using the fluorogenic peptide pERTKR-MCA. Supernatant of control GS115 culture did not release detectable AMC fluorescence, indicating that no dibasic-specific protease is secreted by this strain (Fig. 1C). In contrast, high cleavage activity was found in supernatant of the ssKex2p-expressing strain (Fig. 1C). A K_M value of 19.1 ± 3.1 μM was determined for ssKex2p in agreement with that previously reported (K_M = 25.3 μM) for Kex2p obtained by shedding from vaccinia virus-infected cells [20]. At

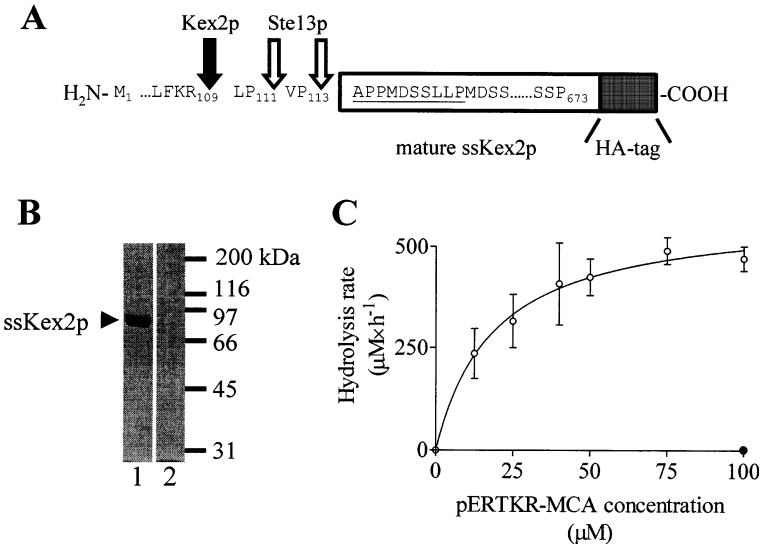

Fig. 1. Production of ssKex2p in *P. pastoris* and characterization of the enzyme. A: Schematic representation of ssKex2p. Amino acid sequence is presented in one letter code. Closed and open arrows indicate processing of precursor by Kex2p (autocatalytic) and Ste13p, respectively. Mature ssKex2p is shown boxed, residues identified by N-terminal sequencing are underlined. C-terminal copies of the HA tag are represented by the dotted box. B: SDS–PAGE analyses. Supernatants (5 μl) of methanol-induced cultures of JGP1 and control GS115 strains (lanes 1 and 2, respectively) were resolved on 8% polyacrylamide gel and stained with Coomassie brilliant blue. C: Plot of substrate hydrolysis rate against substrate concentration. JGP1 strain: open symbols; GS115 control strain: solid symbol. K_M and V_{max} values were determined by non-linear fitting to the Michaelis–Menten equation.

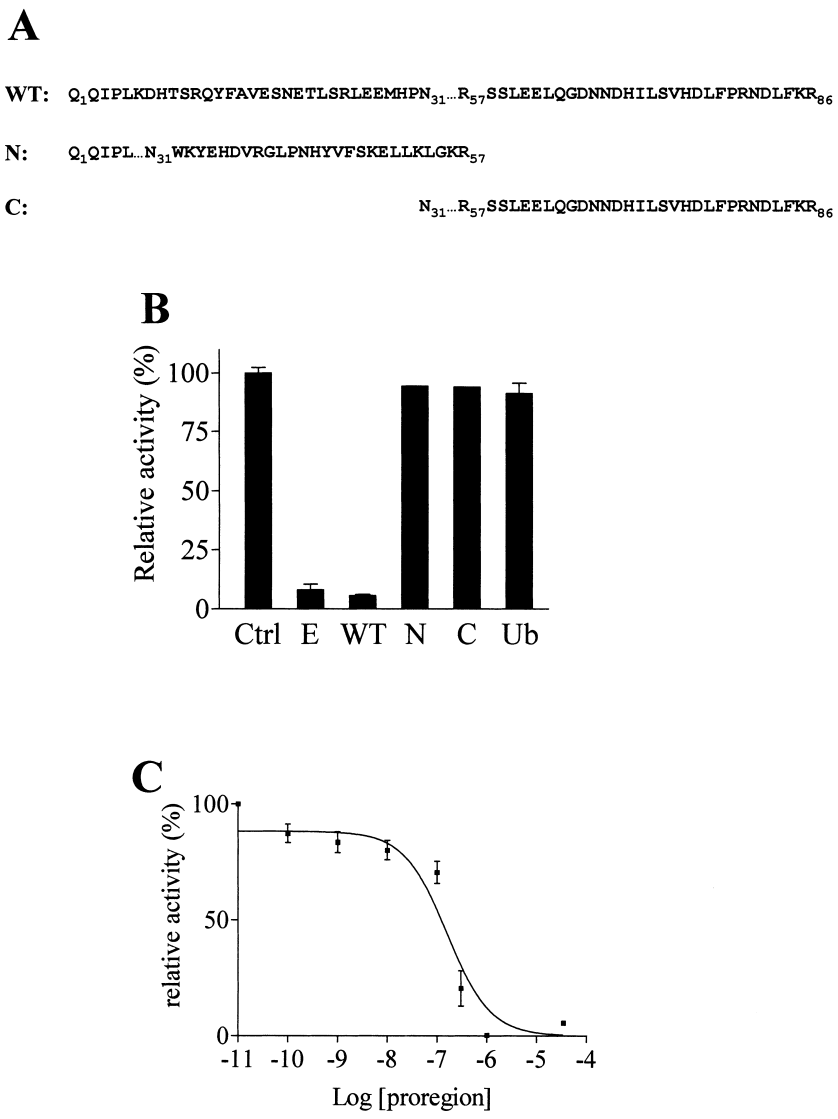

Fig. 2. Inhibition of ssKex2p by recombinant Kex2p proregion. A: Amino acid sequences of recombinant proregions used in this study. The full-length proregion (WT) is 86 residues long. The N-terminal (N) and C-terminal (C) proregion fragments encompass residues 1–57 and 31–86, respectively. B: Effect of EDTA, full-length or truncated proregions, and ubiquitin on ssKex2p activity. Substrate hydrolysis rate was determined in the presence of 10 mM EDTA (E), 1 μM full-length proregion (WT), 500 nM proregion N-terminal fragment (N), 500 nM proregion C-terminal fragment (C) or 625 nM ubiquitin (Ub) and expressed as percentage of activity of control (Ctrl). C: Determination of proregion IC_{50}. ssKex2p activity was measured in the presence of varying concentrations of proregion and expressed as percent of activity measured in the absence of proregion. Assays were performed at 100 μM of substrate.

saturation of substrate, a V_{max} value of 585.5 ± 29.2 μM/h was obtained. As expected, cleavage of the synthetic peptide was inhibited by EDTA (Fig. 2B; compare Ctrl and E) since Kex2p is a Ca^{2+}-dependent enzyme.

3.2. The proregion of Kex2p is an inhibitor of the enzyme

The Kex2p proregion and its N- and C-terminal fragments (Fig. 2A) were expressed in *E. coli*. In each case, a single protein was eluted from the glutathione-Sepharose beads upon thrombin cleavage and washes (results not shown). When added to the enzymatic assay at a concentration of 1 μM, Kex2p proregion efficiently inhibited cleavage of pERTKR-MCA by ssKex2p (WT in Fig. 2B). No inhibition was observed when 500 nM of an N-terminal (N in Fig. 2B) or a C-terminal fragment (C in Fig. 2B) of the proregion was added to the enzymatic assay. Addition of 625 nM of ubiquitin, a 77 amino acid unrelated protein lacking any dibasic site, had no effect on enzyme activity (Fig. 2B). Furthermore, addition of thrombin at 5×10^{-6} U/μl did not change the reaction velocity (data not shown) ruling out any ssKex2p degradation by residual thrombin activity in proregion preparation. We next determined the inhibitory potency of prokex2 by measuring the concentration required to inhibit 50% of total activity (IC_{50}). The proregion was found to be a good inhibitor with an IC_{50} of 160 nM (Fig. 2C).

3.3. Kex2p proregion is a mixed-type inhibitor

To explore the inhibition mechanism of Kex2p by its pro-

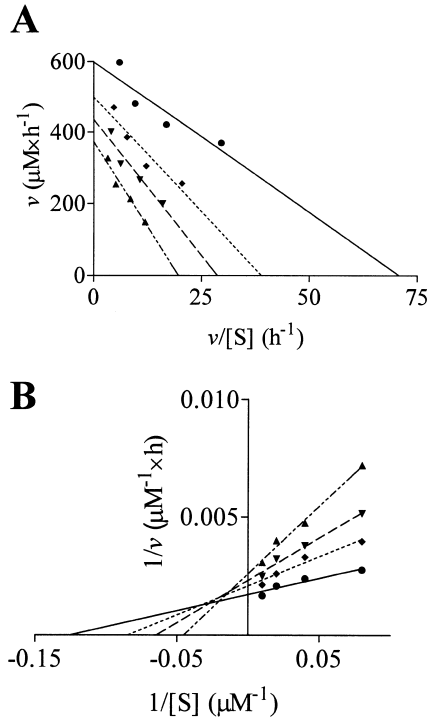

Fig. 3. Kex2p inhibition by its proregion. A: Eadie–Hofstee plot of v against v/[S]. Initial velocities were measured without (circles) or with proregion diluted to 125 nM (lozenges), 150 nM (inverted triangles), and 175 nM (triangles). Means of three independent experiments are presented. B: Lineweaver–Burk plot of $1/v$ against 1/[S]. Symbols and concentrations are the same as in A.

region, assays were performed with various substrate and proregion concentrations (10–100 μM and 50–200 nM, respectively). Fluorescence was released linearly during the experiment, indicating that the steady-state binding of the proregion to the enzyme is rapidly reached. This result suggests that the Kex2p proregion is not a slow-binding inhibitor, unlike proregions of furin, PC1 and PC7 [12,13]. Analysis of the reaction initial velocities (v) as a function of substrate concentration ([S]) for each proregion concentration showed that apparent K_M and V_{max} increased and decreased, respectively, with increasing amounts of proregion (not shown). In Eadie–Hofstee plots of v against v/[S] (Fig. 3A), both the slopes and values of ordinates at the origin decreased with increasing amounts of proregion. These data suggest a mixed-type inhibition, in which the proregion binds the free enzyme as well as the enzyme–substrate complex (ES). The Lineweaver–Burk plot of $1/v$ against 1/[S] (Fig. 3B), in which the curves intersected at a single point over the abscissa, confirmed the inhibition mechanism and indicated that the inhibition constant for binding to free enzyme (K_i) is superior to that (K_i') for binding to the ES. The enzyme–inhibitor and the ES–inhibitor complexes are likely to be inactive, since activity tends to zero with increasing proregion concentration (Fig. 2C).

To explain this mixed-type inhibition, we postulate that binding of the proregion, which is a 86 amino acid residue long polypeptide, to the protease domain occurs at more than one site. The non-competitive component of the inhibition would result from binding of the proregion to site(s) outside the catalytic pocket. Presence in the catalytic pocket of the small-size substrate used in the present study would not prevent binding of the proregion to these outside sites. Binding of the proregion to the outside site(s) would alter enzyme conformation, resulting in a catalytically inactive complex. The competitive component of the inhibition could result from substrate-like positioning of the proregion C-terminus in the catalytic site. Indeed, the autocatalytic processing of Kex2p in vivo implies that the proregion C-terminus interacts with the active site. Alternatively, covering of the catalytic pocket by part of the proregion could prevent the substrate accessing the catalytic residues. The observation that inhibition requires integrity of both N- and C-termini of the proregion suggests a cooperative binding to the enzyme. This model of inhibition is strongly supported by our recent findings that several amino acid residues located throughout the proregion structure and including the C-terminal pair of basic residues are essential for proregion function in vivo and binding to the mature protease domain in vitro (see [9] and Lesage et al., submitted).

Furin, PC1 and PC7 [12,13] proregions act as slow-binding competitive inhibitor. To explain why Kex2p proregion behaves differently, one can speculate that in furin, PC1 and PC7, binding of the proregion occurs at only one site in the catalytic pocket. Alternatively, binding to outside site(s) does not alter the conformation of those enzymes. Further work, including mutagenesis and structural studies, is required to better understand molecular mechanisms involved in the proregion binding to their cognate enzymes, and to determine what are the residues involved in the inhibition process.

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