Expression, Purification, and Characterization of the Yeast KEX1 Gene Product, a Polypeptide Precursor Processing Carboxypeptidase*

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The Saccharomyces cerevisiae KEX1 gene encodes a protease with carboxypeptidase B-like activity involved in K1 and K2 killer toxins and α-factor (mating pheromone) precursors processing. The gene has been expressed using the baculovirus/insect cell system, and the KEX1 encoded protein (Kex1p) was purified to apparent homogeneity from detergent-solubilized membrane preparations of insect cells infected with the recombinant virus. The specific activity of the enzyme was enriched 126-fold as compared with the cell lysate, with a recovery of 29%. The NH2-terminal sequence of the purified active enzyme was identical to the predicted sequence after the removal of the signal peptide. This provides evidence that Kex1p, at least in insect cells, is not made as a precursor. The optimum pH for activity was 6.0, and the apparent pI value of the protein was below pH 3.0. The enzyme cleaves arginine or lysine from the COOH terminal of peptides over the COOH-terminal lysine. Insect-derived Kex1p processes α-factor-Lys-Arg, its known natural substrate, to mature active α-factor. In this study and its high affinity for a-factor-Lys-Arg reveals that Kexlp preferentially cleaves the COOH-terminal arginine of peptides over the COOH-terminal lysine. The specificity of the enzyme for COOH-terminal basic amino acid residues of the peptides used is similar to the yeast KEX1 gene product, a membrane-bound endoprotease that cleaves on the carboxyl side of pairs of basic residues. Further processing at the COOH terminus requires a CP B-like activity to remove the flanking basic amino acids from processing intermediates.

The yeast Saccharomyces cerevisiae cleaves the precursor proteins of α-factor (mating pheromone) and K1 and K2 killer toxins by a similar processing pathway (Achstetter and Wolf, 1985; Bussey, 1988; Fuller et al., 1988; Dignard et al., 1991; Bourbonnais et al., 1992). Maturation is initiated by the KEX2 gene product, a membrane-bound endoprotease that cleaves on the carboxyl side of pairs of basic residues. Further processing at the COOH terminus requires a CP B-like activity to remove the flanking basic amino acids from processing intermediates. Previous genetic, molecular, as well as preliminary biochemical studies provided evidence that the KEX1 gene product fulfills this function (Dmochowska et al., 1987; Wagner and Wolf, 1987; Cooper and Bussey, 1989). The isolation of the KEX2 gene by yeast genetics established a paradigm which allowed the identification of the enzymes that carry out endoproteolytic processing at dibasic sites. However, it is presently uncertain if the KEX1 gene will similarly help in the identification of mammalian homologues as there is some evidence that COOH-terminal processing of precursor peptides in mammalian cells is fulfilled by metallo-CPs that apparently bear no homology with Kex1p (Fricker, 1988; Skidgel, 1988).

The KEX1 gene was cloned by complementation of a mutant (kex1) unable to produce active extracellular K1 killer toxin (Dmochowska et al., 1987). The gene encodes a protein consisting of 729 amino acids and analysis of the sequence demonstrated the presence of two hydrophobic regions: the first, situated at the N terminus, is a good candidate for a signal sequence; the second is near the COOH terminus and probably functions as a membrane-spanning region. Subsequent studies demonstrated that this putative transmembrane domain located at residues 613 to 637 serves to tightly associate the protein to yeast membranes (Cooper and Bussey, 1989). The predicted catalytic domain of the enzyme possesses two tracts of significant homology with CP Y (Dmochowska et al., 1987), a well-characterized yeast serine CP with broad substrate specificity (Breddam, 1986). The protein is also

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homologous to plant serine CPs and to mammalian lysosomal proteolytic proteins (Galjart et al., 1988, 1990). KEX1 yeast strains expressed CP activity which was absent from kex1Δ strains (Cooper and Bussey, 1989). This activity correlated with the presence of Kex1p and was shown to be a serine CP sensitive to phenylmethylsulfonyl fluoride (Dmochowska et al., 1987; Cooper and Bussey, 1989). The co-expression of KEX1 using a vaccinia virus system, together with the yeast KEX2 and a pro-opiomelanocortin cDNA in mammalian maturation-deficient cells, resulted in the conversion of the processing intermediate γ-lipotropin-Lys-Arg to mature γ-lipotropin (Thomas et al., 1990). This study demonstrated the ability of Kex1p to recognize and process an intermediate of mammalian origin in a non-yeast cellular environment, and hence, the interest in further dissecting the biochemical and physical properties of this CP.

The substrate specificity of mammalian basic CPs, namely CP N, M, and H, plays an important role in the regulation of the activity of bioactive peptides (Skidgel, 1988). By analogy, for Kex1p to accomplish its role in the final maturation step of α-factor and K1 killer toxin it must have strict specificity for substrates ending with basic residues. To date, no definitive data are available to indicate that Kex1p exhibits such a strict specificity. Specificity of this CP was determined by the ability of its amino acids or lysine, and N-blocked peptides with a COOH-terminal basic residue to inhibit the hydrolysis of Bz-FAR when added to the incubation mixture along with yeast membrane proteins (Cooper and Bussey, 1989). Furthermore, the enzyme from yeast had never been purified and characterized.

Overexpression of the KEX1 gene in yeast appears to be detrimental to growth. The baculovirus (Autographa californica) insect cell expression system has been successfully applied for the expression of a wide variety of heterologous eukaryotic genes (Luckow and Summers, 1988). Expression of the KEX1 gene in this system gave high yields of functional Kex1p. We have therefore used this to purify the protein to apparent homogeneity and to examine some of its properties. The results presented here demonstrate that purified Kex1p is a serine CP which specifically cleaves only basic amino acids from the COOH terminus of a variety of synthetic and biological peptides including one of its known natural substrates, α-factor-KR. The enzyme demonstrates a significant preference for a COOH-terminal Arg residue over Lys, and its apparent homogeneity and COOH-terminal basic residue resulted in the hydrolysis of Bz-FAR when added to the incubation mixture along with yeast membrane proteins (Cooper and Bussey, 1989). Furthermore, the enzyme from yeast had never been purified and characterized.

EXPERIMENTAL PROCEDURES

Buffers and Solvents—The following buffers were used: A, 0.1 M bis-Tris-HCl, pH 6.0, containing 2 mM EDTA, and 5 μg/ml of each of aprotinin and pepstatin A; B, 25 mM bis-Tris-HCl, pH 6.0, containing 1 mM EDTA, and 5 μg/ml of each of the above protease inhibitors; C, 25 mM bis-Tris-HCl, pH 6.0, containing 0.1% Triton X-100 (Mallinkrodt) and 1 mM EDTA; D, same as buffer C without EDTA; E, polybuffer 74-HCl (Pharmacia Biotechnology Inc.), pH 3.0, diluted with water 1:10 (v/v) and containing 0.1% Triton X-100; F, 0.2 M bis-Tris-HCl, pH 6.0. The following solvents were used for HPLC: A, 5% acetonitrile, 0.1% trifluoroacetic acid, 0.1% Triton X-100, 0.01% EDTA, and 0.1% phenylmethylsulfonyl fluoride; B, 10% acetonitrile, 0.1% trifluoroacetic acid, and 0.1% phenylmethylsulfonyl fluoride; C, 25 mM bis-Tris-HCl, pH 6.0, containing 2 mM EDTA, and 5 μg/ml of each of the above protease inhibitors.

Construction of Recombinant Virus Ac[KEX1]—The entire coding region of KEX1 (2.2 kb) with 4 bp at the 5′-end was generated by PCR, using the 3.1-kb HindIII fragment carrying the KEX1 gene excised from pAD8 as template. The plasmid pAD8 is derived from pAD7 (Dmochowska et al., 1987) and it contains the KEX1 gene on the 3.1-kb HindIII fragment inserted into the YCp50 HindIII site. The sense primer for PCR corresponded to the sequence encoding amino acids 1 to 7 with a 5′-extension of four bases (5′-GAAAGATCT-ATCGATTTTACAAATAGGTGGCTC-3′). The reverse primer was complementary to the sequence encoding amino acids 723 to 729 and to encode stop codon (5′-GGAAGATCTTTTAAATCATCCTCT- CAAAGG-3′). A BglII restriction enzyme site was included in both primers to facilitate the cloning of the PCR fragment into the BglII site of pETL, a baculovirus expression transfer vector (Walker and Richardson, 1992), to create plasmid pETL-KEX1. The pETL carries the Lac Z gene which upon expression results in the formation of blue plaques when β-galactosidase indicator (X-gal) is present in the agarose overlay. The vector was generously provided by C. Richardson (Biotechnology Research Institute, BRI, National Research Council (NRC), Montreal, Quebec). The KEX1/Neo-Stul 1450-bp fragment from plasmid pETL-KEX1 was then replaced with the same fragment from pAD8. The rest of the KEX1 sequence generated by PCR was then confirmed by DNA sequencing (Sanger et al., 1977). The 1.24-kb pETL-KEX1 was isolated and purified from CsCl maxipreps. The DNA was subcloned into the unique NdeI site of pETL to create plasmid pETL-KEX1Δ, which was then confirmed by DNA sequencing (Sanger et al., 1977). Occlusion-negative blue plaques were identified, and the presence of the KEX1 gene was confirmed by nucleic acid dot-blot hybridization using labeled 3.1-kb HindIII KEX1 fragment, excised from pAD8. Three rounds of plaque assays were sufficient to purify a recombinant virus designated Ac[KEX1], from the wild type virus. The recombinant virus was used to infect SF9 cells to produce viral stocks with titres of 109 plaque-forming units/ml.

Properties of Purified Recombinant Kex1p—All steps were conducted at 4°C, and all chromatographic steps were performed using an fast-protein liquid chromatography system (Pharmacia). The membrane fraction was subjected to ion-exchange chromatography on Mono Q (Pharmacia) packed in an XK26 column (Pharmacia) to 50-ml bed volume. The column was pre-equilibrated with buffer C, washed with five bed volumes of the same buffer, and the bound Kex1p was eluted with a linear salt gradient (500 mM NaCl in buffer C). Fractions of 5 ml were collected and assayed for CP activity using Bz-FAR as substrate. The active fractions which eluted at 0.5 M NaCl concentration were pooled, concentrated, and desalted using Centricon-30 concentrators (Amicon). The Q-Sepharose-purified Kex1p was applied to the affinity support, arginine-Sepharose 4B (Pharmacia), packed in a C10 column (Pharmacia) which was pre-equilibrated with buffer C. The column was washed with five bed volumes of buffer C, and the bound proteins were eluted with a linear gradient (100 ml) of 0-4 M arginine in buffer C. Fractions of 3 ml were collected and assayed for CP activity by applying the radiometric assay. The active fractions which eluted at 0.25 M arginine were pooled, concentrated, and desalted before chromatofocusing on Mono P HR 5/20 (5 × 200 mm) column (Pharmacia) that had been previously equilibrated with buffer D. Elution of bound proteins was carried out using buffer E which generated a pH gradient between pH 5 and 3. At the end of the gradient 1 M NaCl in buffer D was applied. Fractions of 1 ml were collected in tubes containing 0.5 ml of EDTA and CP activity was assayed. The preparation of the purified enzyme could be stored after desalting as aliquots at −20°C for 3 months without any loss of CP activity.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Various protein samples were analyzed by gel electrophoresis under reducing conditions according to the method of Laemmli (1970) using 7.5% polyacrylamide gels. Proteins were stained using Coomassie Blue staining. Alternatively, the active fractions eluted from Mono P were electrophoresed on 8-25% gradient gels (PhastGel
gradient 8–25, Pharmacia) followed by silver staining using the PhastSystem (Pharmacia) according to the manufacturer's instructions. For Western blotting, proteins from 7.5% gels were electrophoretically transferred to nitrocellulose membranes according to the Bio-Rad Mini Trans-Blot apparatus protocol. The blots were probed with rabbit anti-α-factor-Kex1p antisemur generated against the fusion protein according to a previously described procedure (Cooper and Bussey, 1989). The antigen-antibody complexes on immunoblots were detected with goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) as secondary antibody. Alternatively, the blots were theoretically transferred to nitrocellulose membranes according to the method of Bussey, 1989). The antigen-antibody complexes on immunoblots were detected with goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad).

**Carboxypeptidase Assays**—All assays were conducted at 30 °C. The kinetic constants Vmax and Km were obtained by linear regression analysis using the Enzfitter program (Leatherbarn, 1987).

**Radiometric Assay**—CP assay was performed by the method of Rossier et al. (1989). The principle of the assay relies on a change in solubility of Bz-FAR upon COOH-terminal cleavage of the arginine residue resulting in a product, Bz-FA, which is soluble in the organic scintillant Econofluor-2 (Du Pont-New England Nuclear). Briefly, the standard assay mix (100 μl) contained the unlabeled substrate Bz-FA (0.2 mM) (Peninsula Laboratories), the radioactive [3H]Bz-FAR (Du Pont-New England Nuclear) and up to 10 μg of protein (or 5–25 ng of purified Kex1p) in buffer D. The reaction was started by adding the enzyme, incubated for 5–30 min, and then stopped and counted for radioactivity. This assay was routinely applied to screen column fractions for CP activity.

**HPLC Assay**—CP activity was also measured by a HPLC assay (Skidgel et al., 1984) that followed the hydrolysis of enkephalins with COOH-terminal mono- or dibasic amino acid residues (Arg and/or Lys) (Peninsula Laboratories). Purified Kex1p (25–100 ng) was incubated with a given peptide (0.1 mM) in a final volume of 500 μl in buffer D. The reaction mix was incubated for up to 8 h, and aliquots (50 μl) were spotted at different time points with 10 μl of 5% trifluoroacetic acid and analyzed by HPLC. Peptides and their hydrolysis products were resolved on a Vydac RP-18 column (Chromatography Sciences Co.) using a Waters HPLC system. Elution was performed by applying a shallow linear gradient of 80% solvent A/20% solvent B to 77.5% solvent A/22.5% solvent B in 15 min. Under these conditions the following retention times were observed: [Met]Enk, 6.4 min; [Met]Enk-Arg, 4.9 min; [Met]Enk-Lys, 4.7 min; [Met]Enk-Lys-Lys, 4.2 min; [Leu]Enk, 9.4 min; [Leu]Enk-Arg, 6.0 min; [Leu]Enk-Lys, 5.6 min; [Leu]Enk-Arg2, 5.1 min; and [Leu]Enk-Arg2-Lys, 4.8 min. Peaks of products were quantified by integration and comparison of the peak area to that of a known quantity of authentic standard injected during the analysis. Peptides were detected at 214 nm.

**Biological Halo Assay and HPLC Analysis** of α-Factor-KR—Twenty ng of α-factor (Sigma), 25 ng of synthetic α-factor-KR (Dmochowska et al., 1987), or the same amount of α-factor-KR incubated for 1 h at 30 °C either in buffer D or with 2 ng of purified Kex1p in buffer D, in a final volume of 5 μl were spotted onto a 0.7% agar in a YEPD plate previously seeded with tester strain A232–4A (MATa leu2 trpl can1 sst1::LEU2), and the plates were incubated overnight at 30 °C (Chan and Otte, 1982). The yeast strain was generously provided by M. Whiteway (BRI, NRC).

HPLC analysis of α-factor-KR and its hydrolysis products was conducted as described for the enkephalins (see above) except for the elution which was performed with a linear gradient of 80% solvent A, 20% solvent B to 67% solvent A, 33% solvent B in 30 min. The retention times of α-factor-KR, α-factor-K, and α-factor were 18.1, 20.1, and 25.5 min, respectively. The identities of the last two peaks were confirmed by amino acid analysis.

**Effect of pH on Activity**—The optimum pH for Kex1p activity was empirically determined with Bz-FAR in different buffers at pH values ranging from 4.0 to 9.0. The best results were obtained using 50 mM bis-Tris-HCl, pH 6.0.

**Protein Determination**—Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein reagent and bovine serum albumin as the standard protein.

**NH2-terminal Sequence Analysis**—NH2-terminal sequencing of recombinant Kex1p was achieved by direct sequencing from Problott membrane (Applied Biosystems) by the method of Matsudaira (1987).

The purified enzyme was resolved by SDS-polyacrylamide gel electrophoresis, electrophoretically transferred onto the membrane, and stained with Coomassie Blue. The Kex1p band was then excised and subjected to NH2-terminal sequencing. Sequencing was achieved by the Edman degradation method using an Applied Biosystems 470A gas-phase sequencer equipped with an on-line Applied Biosystems 120A phenylthiohydantoin analyzer.

**RESULTS**

**Expression of Kex1p in Insect Cells**—At various time points over the course of 96 h of infection cells were harvested, washed, and lysed, and the resulting total cell lysates were analyzed by Western blotting and by assaying for CP activity using Bz-FAR as substrate (Fig. 1 A and B, respectively). The antisemur used was raised against a LacZ::KEX1 gene fusion protein, and therefore it detects both β-gal and Kex1p in cells infected with the recombinant virus Ac[KEX1] which express both proteins. The identity of the β-gal band (115 kDa) was confirmed by immunodetecting blots with an anti-β-gal monoclonal antibody (data not shown). An approximately 105-kDa immunoreactive band appears in cells as early as 18 h and increases in intensity at later times (Fig. 1A). The appearance of this band correlates well with the appearance of specific CP activity which reaches a maximal level by 72 h (Fig. 1B). Kex1p was therefore purified from cells harvested at 72-h postinfection. The immunoreactive bands and the CP activity were absent in uninfected cells or cells infected with wild type baculovirus, and neither a 105-kDa immunoreactive band nor Kex1p enzymatic activity were detected in the culture medium (data not shown). Starting at 72 h post-infection, CP activity could be detected extracellularly and this was attributed to cell lysis (not shown).

**Association of Kex1p with Insect Cell Membranes**—Previous...
Properties of Purified Recombinant Kex1p

work has demonstrated that Kex1p in yeast is tightly associated with membranes (Cooper and Bussey, 1989). To determine whether the enzyme in Sf9 cells is also membrane-bound, total cell lysates were subjected to ultracentrifugation. The resulting supernatant and pellet fractions were analyzed by immunoblotting and by the radiometric CP assay. As shown in Fig. 2A, the high speed centrifugation resulted in the disappearance of the immunoreactive Kex1p band from the cytosolic fraction and as a consequence the enrichment of the membrane pellet with it. Furthermore, the membrane fraction retained only about 15% of the total protein and the majority of the CP activity expressed as nmol/min/mg of protein was tightly associated with it (Fig. 2B). This step was therefore included in the purification protocol.

Purification of Kex1p—The enzyme was purified from a solubilized membrane preparation by successive chromatography on Q-Sepharose, arginine-Sepharose, and finally on a Mono P column (Table I). The majority of the enzyme was recovered from the Mono P column as a sharp peak when 1 M NaCl was applied (Fig. 3) suggesting that the PI value of Kex1p is below pH 3.0, although the calculated PI is 4.07. On average, a 126-fold increase in specific activity could be achieved with a recovery of about 29%. The final preparation had a specific activity of 22.5 μmol/min/mg of protein and was used as the source of enzyme for further studies.

Effect of Metal Ions and Protease Inhibitors—The effect of a variety of agents on Kex1p activity is shown in Table II. The serine protease inhibitors, phenylmethylsulfonyl fluoride and 1-chloro-3-tosylamido-7-amino-2-heptanone, inhibit the CP activity of recombinant Kex1p. Guanidinoethylmercaptoacetic acid, a byproduct analog of arginine, acts as a potent and selective inhibitor of CP H in the nanomolar range (Fricker et al., 1983). This compound also inhibits Kex1p activity but only in the micromolar range presumably by binding to the active site. Hg2+ strongly inhibits Kex1p activity and incubation of the inactivated enzyme with 5 mM dithioerythritol and 1 mM EDTA regenerated the majority of the CP activity. Ca2+ and Mg2+ at low concentrations activate the enzyme, although at 5 mM concentrations both cations barely affect the enzyme activity.

Maturation of α-Factor-KR by Purified Kex1p—To determine whether purified recombinant Kex1p is able to process α-factor-KR, a processing intermediate of α-factor, to active pheromone, a biological assay was applied and a HPLC analysis of the maturation event was conducted. The formation of a halo in Fig. 5 is indicative of the presence of mature pheromone which in turn causes growth arrest of cells from a strain supersensitive to α-factor. α-Factor-KR does not exhibit a similar biological activity on the tester strain used (Fig. 5B), however, when it is incubated with purified enzyme it becomes active (Fig. 5C). As a negative control, α-factor-KR is incubated for the same length of time and at the same temperature in buffer D before assaying (Fig. 5D). Incubation of α-factor with Kex1p does not change the size of the halo (data not shown). HPLC analysis of the peptide demonstrates the kinetics of the processing (Fig. 6). Incubation of α-factor-KR (peak 1, Fig. 6) with the purified enzyme results in the sequential processing of this peptide to α-factor-K (peak 2, Fig. 6) and then to α-factor (peak 3, Fig. 6). After 1 h of incubation at 30 °C processing to mature pheromone is com-

![Fig. 2. Association of Kex1p with the membrane fraction of Sf9 cells](image)

**Table I**

Purification of Kex1p from insect cells infected with Ac[KEX1]

Carboxypeptidase activity of Kex1p was determined by the radiometric assay, and protein was determined by the Bradford assay as described under "Experimental Procedures." These data represent a typical purification table from 500 ml of Sf9 cell culture infected with the recombinant virus.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>μmol/min/mg</td>
<td>μmol/min</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>Total cell lysate</td>
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<td>32</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
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<td>26.6</td>
<td>6</td>
<td>83</td>
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<td>Q-Sepharose</td>
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<td>64</td>
</tr>
<tr>
<td>Arginine-Sepharose</td>
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<td>15.2</td>
<td>19.4</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td>Mono P</td>
<td>0.415</td>
<td>22.5</td>
<td>9.34</td>
<td>126</td>
<td>29</td>
</tr>
</tbody>
</table>

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Fig. 2. Association of Kex1p with the membrane fraction of Sf9 cells. Cells were infected with Ac[KEX1], at 72-h postinfection a total cell lysate (T) was prepared and subjected to high speed centrifugation. The resulting 100,000 × g supernatant (S) and membrane (M) fractions were analyzed either by Western blotting using anti-β-gal-Kex1p polyclonal antibodies (A); or by applying the radiometric CP assay and the Bradford protein assay (B), as described under "Experimental Procedures." Molecular masses of standards (kDa) are indicated in the left margin. The arrows indicate the positions of β-gal (upper) and Kex1p (lower).
Properties of Purified Recombinant Kex1p

**Inhibition or activation of Kex1p activity**

Ions and inhibitors were incubated with purified enzyme for 2 h on ice. Residual carboxypeptidase activity was then determined by applying the radiometric assay as described under “Experimental Procedures.” The following compounds had no effect on enzyme activity of Kex1p: 1 mM EDTA, 5 mM diithioerythritol, 100 μM E64, 15 μM aprotinin, and 14.5 μM pepstatin A. PMSF, phenylmethylsulfonyl fluoride; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; GEMSA, guanidinoethylmercaptosuccinic acid.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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<td>100</td>
</tr>
<tr>
<td>PMSF</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TLCK</td>
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<td>65</td>
</tr>
<tr>
<td>GEMSA</td>
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<tr>
<td>Leupeptin</td>
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<tr>
<td>MgCl₂</td>
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</tr>
<tr>
<td>CaCl₂</td>
<td>0.1</td>
<td>111</td>
</tr>
<tr>
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</tbody>
</table>

*After incubating Kex1p with 10 μM HgCl₂ on ice for 2 h, 1 mM EDTA, and 5 mM diithioerythritol were added, the mixture was incubated on ice for 30 min and then the activity was measured as above.

**Kinetic Properties of Kex1p Activity**

The purified Kex1p hydrolyzed synthetic peptides containing COOH-terminal Arg or Lys (Table III). The Kₘ for the enzyme was almost 2-fold higher with fa-AR than Bz-FAR. This implied that this CP has higher affinity for the tripeptide and somewhat lower affinity for the dipeptide. However, this difference may be attributed to the benzyol or furylacryloyl N-blocking groups rather than the length of the synthetic peptides. fa-AK possessed a Kₘ value which was almost 2-fold that of fa-AR which indicates that the enzyme had a preference for Arg over Lys as a carboxyl-terminal amino acid. The same enzyme preparation was used to determine the kinetic constants listed in Table III and therefore, a direct comparison of the Vₘₐₓ/Kₘ values, which reflects the efficiency of hydrolysis, is possible. The rate of hydrolysis of the arginyl residue of Bz-FAR was approximately 5-fold that of fa-AR, and the rate of hydrolysis of the lysyl residue of fa-AR was about one-fourth the rate of hydrolysis of Arg from fa-AR (Table III). Preference for arginyll residues over lysyl residues is also demonstrated when comparing the rates of hydrolysis of [Met]- and [Leu]enkephalins with COOH-terminal basic amino acids (Table IV). The enzyme cleaves [Met]- and [Leu]Enk-Arg, respectively, about 2.4- and 2.2-fold faster than the corresponding lysyl peptides. The penultimate amino acid did not seem to have an effect on the rate of hydrolysis. Kex1p has a very high affinity for one of its known biological substrates, namely α-factor-KR (15 amino acids long) and a somewhat...
Properties of Purified Recombinant Kexlp

Inhibition constants of α-factor-KR and enkephalin heptapeptides

In the present study we have expressed the entire coding region of the yeast KEX1 gene using the insect cell/baculovirus system. Sf9 cells infected with the recombinant virus carrying the gene downstream of the strong polyhedrin promoter express and accumulate the CP intracellularly. The expression of Kexlp was detected as early as 18 h postinfection, and it reached a maximum by 72 h. It is interesting to note that at 96 h postinfection, where the rate of protein synthesis is presumably minimal due to cell death, the specific CP activity as well as the immunoreactive Kexlp band on blots were constant. The inherent stability of this protease became apparent during the course of its purification and characterization. For example, the enzyme could withstand ultrafiltration, harsh column chromatographic conditions such as exposure to extreme pH values during chromatofocusing, as well as repeated freeze/thaw cycles without any significant loss of activity. Expression of the KEX1 gene results in the accumulation of a 105-kDa Kexlp immunoreactive protein as compared to the 113-kDa protein present in yeast membranes (Cooper and Bussey, 1989). This size difference might be due to larger side chains in Asn-linked glycosylation in yeast. Approximately 98% of the total CP activity was recovered from the 100,000 × g membrane pellet, and no Kexlp activity could be detected in the culture medium. This selective association of the enzyme to the high speed pellet of Sf9 cells is consistent with the membrane localization of Kexlp in yeast (Cooper and Bussey, 1989) and in BSC-40 cells (Thomas et al., 1990). The protein was therefore purified to apparent homogeneity from solubilized membrane preparations using a three-step purification protocol which achieved an overall purification of 126-fold with a recovery of 29%. This fairly high yield together with the inherent stability of this enzyme have made it possible to conduct studies defining some of its properties. The tight binding of Kexlp to the Mono P column and consequently its low pI value (below pH 3.0) are attributed to a tract of 105 residues very rich in aspartic and glutamic acids NH2-terminal to the transmembrane domain (Dmochowska et al., 1987). This property was used with advantage since it resulted in effective binding of the protein to the chromatofocusing column, and most contaminant proteins were released from the column by the pH gradient such that when the salt was applied Kexlp eluted free from other impurities.

Evidence for the authenticity of the recombinant Kexlp was provided by NH2-terminal sequence analysis which demonstrated that the sequence of the mature enzyme starts at the site predicted for signal peptide cleavage (Dmochowska et al., 1987). This result indicates that Kexlp, when expressed...
Properties of Purified Recombinant Kex1p

in insect cells, does not have a pro-region that has to be cleaved to yield active enzyme, providing the first evidence which suggests that Kex1p is not made as a proenzyme. It is unusual for a protease not to be made as a catalytically inactive precursor; we assume that the exquisite specificity of Kex1p and its cellular location obviate this requirement. It has been demonstrated that bovine CP H exists as two soluble catalytically active forms with very similar properties and which differ only in their NH2-terminal sequence (Parkinson, 1990). The sequence of the longer polypeptide starts at the site predicted for signal peptide cleavage, whereas the shorter form is the result of endoproteolysis at a string of 5 arginine residues at the NH2 terminus. The NH2-terminal region of the predicted Kex1p sequence lacks putative dibasic or multibasic processing sites, and it is not known whether processing at any other site does occur in yeast.

Properties such as Kn for Bz-FAR, and effect of some protease inhibitors and metal ions, of purified recombinant Kex1p were similar to those reported for the yeast enzyme in crude membrane pellets (Cooper and Bussey, 1989). Like CP Y, Kex1p is strongly inhibited by HgCl2 and the mercuric-inactivated enzyme is efficiently reactivated by dithioerythritol and EDTA. Inhibition of enzyme activity by Hg2+ may be attributed to the modification of Cys-408 which aligns in primary sequence with Cys-341 of CP Y (Dmochowska et al., 1987). Cys-341 is at the substrate binding site of CP Y, and its sulphydryl group is modified by mercurials (Breddam, 1986). Although the enzyme is EDTA-insensitive, its activity is slightly activated by Ca2+ and Mg2+ at 1 mM or lower concentrations. This may be due to a stabilizing effect of these divalent cations on the enzymatic activity. The basis of the loss of such an effect at higher cation concentrations is unknown.

The specificity of the purified enzyme for COOH-terminal basic residues is demonstrated by following the hydrolysis of a number of synthetic and biological peptides with COOH-terminal extensions of basic amino acid(s). The kinetic data obtained reveal that, like CP H and M, Kex1p preferentially cleaves the COOH-terminal arginine of peptides over the COOH-terminal lysine (Fricker and Snyder, 1982; Skidgel, 1986). COOH-terminal basic residues is demonstrated by following the hydrolysis of the peptides used in this study and its higher affinity for the peptides used in this study and its higher affinity of the enzyme for peptide substrates is directly related to their length, that is, the longer the peptide the greater the affinity as reflected in the Kn (K) values. The specificity of the enzyme for basic amino acids at the COOH terminus of the peptides used in this study and its higher affinity for longer peptides correlates well with the role Kex1p plays in processing α-factor and K1 killer toxin intermediates. Although Kex1p displays an overall similarity in substrate specificity to CP B, H, M, and N, it is a distinctly different enzyme. Kex1p is not a metallo-CP like the others, rather it belongs to the family of serine CPs which includes the yeast vacuolar CP Y, plant CPs (Breddam, 1986), and the recently identified mammalian protective proteins which display cathepsin A-like activity (Galjart et al., 1991). In primary structure Kex1p is homologous to members of the latter family, and, like them, it is EDTA-insensitive. To date, of the serine CPs only the structure of wheat CP II has been determined only to 3.5-Å resolution (Liao and Remington, 1990). The availability of pure active recombinant Kex1p will allow detailed structural and physical studies which will greatly facilitate protein engineering of this serine CP to investigate and alter its specificity and hence to increase the possibilities for its application to COOH-terminal protein sequencing as well as stepwise synthesis of polypeptides (Breddam, 1986). Furthermore, the production and purification of recombinant Kex1p in good yield combined with its strict specificity for COOH-terminal basic amino acid residues makes feasible the use of this enzyme, at a practical level, to fully process recombinant peptide hormone precursors (e.g. insulin) intended for human therapeutic use. Work is in progress to increase the yield of pure recombinant Kex1p by expressing a truncated form of the enzyme that lacks the transmembrane domain but retains the entire NH2-terminal CP Y homology region. The resulting soluble protein once purified will allow the study of the three-dimensional structure of this unique member of the serine CP family.

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