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Benjamin Alper

*Sacred Heart University*, [alperb@sacredheart.edu](mailto:alperb@sacredheart.edu)

Jarrad W. Rowse

*University of Georgia*

Walter K. Schmidt

*University of Georgia*

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## YEAST STE23P SHARES FUNCTIONAL SIMILARITIES WITH MAMMALIAN INSULIN-DEGRADING ENZYMES

**Benjamin J. Alper, Jarrad W. Rowse, Walter K. Schmidt**

Department of Biochemistry and Molecular Biology,

University of Georgia, Athens, Georgia 30602

Running title: Properties of Ste23p, the yeast IDE ortholog

Address correspondence to: Walter K. Schmidt, Ph.D., A416 Life Sciences Building, Athens, GA 30602.

Fax: 706-542-1738; E-mail: [wkschmidt@bmb.uga.edu](mailto:wkschmidt@bmb.uga.edu)

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List of abbreviations used within the text:  $\beta$ -ME:  $\beta$ -mercaptoethanol, 1,10-P: 1,10-phenanthroline, 4,7-P:

4,7-phenanthroline, A $\beta$ : amyloid beta peptide, Abz: aminobenzoic acid, AD: Alzheimer's disease,

APP: amyloid precursor protein, ATP: adenosine-5'-triphosphate, CE: capillary electrophoresis, DM2:

Type II diabetes mellitus, DMSO: dimethylsulfoxide, DTT: dithiothreitol, EDTA:

ethylenediaminetetraacetic acid, FPLC: Fast protein liquid chromatography, GST: glutathione S-

transferase, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HFIP: hexafluoroisopropanol,

IDE: insulin-degrading enzyme, NEM: N-ethylmaleimide, PMSF: phenylmethylsulfonyl fluoride, Ptr:

pitriylsin, SC: synthetic complete, SD, synthetic defined, TPEN: N,N,N'-tetrakis(2-

pyridylmethyl)ethylenediamine, YEPD: yeast extract, peptone, and dextrose

### ABSTRACT

**The *S. cerevisiae* genome encodes two M16A enzymes: Axl1p and Ste23p. Of the two, Ste23p shares significantly higher sequence identity with M16A enzymes from other species, including mammalian insulin-degrading enzymes (IDEs). In this study, recombinant Ste23p and *R. norvegicus* IDE (*RnIDE*) were isolated from *E. coli*, and their enzymatic properties compared. Ste23p was found to cleave established *RnIDE* substrates including the amyloid- $\beta$  peptide (A $\beta$  1-40) and insulin B chain. A novel internally quenched fluorogenic substrate (Abz-SEKKDNYIHKGV-nitroY-OH) based on the polypeptide sequence of the yeast P2 a-factor mating pheromone was determined to be a suitable substrate for both Ste23p and *RnIDE*, and was used to conduct comparative enzymological studies. Both enzymes were most active at 37 °C, in alkaline buffers, and in high salt environments. In addition, the proteolytic activities of both enzymes towards the fluorogenic substrate were inhibited by metal chelators, thiol modifiers, inhibitors of cysteine protease activity, and insulin. Characteristics of *STE23* expression were also evaluated. Our analysis indicates that the 5' terminus of the *STE23* gene has been mischaracterized, with the physiologically relevant initiator corresponding to residue M53 of the publicly annotated protein sequence. Finally, we demonstrate that unlike haploid-specific Axl1p, Ste23p is expressed in both haploid and diploid cell types. Our study presents the first comprehensive biochemical analysis of a yeast M16A enzyme, and provides evidence that *S. cerevisiae* Ste23p has enzymatic properties that are highly consistent with mammalian IDEs and other M16A enzymes.**

### INTRODUCTION

The M16A subfamily comprises a group of large (~100 kDa), zinc-dependent metallo-endopeptidases, which are found within all prokaryotic and eukaryotic organisms that have been thoroughly examined (Rawlings, 2008). M16A enzymes are characterized by the presence of an essential HxxEH motif generally located within 200 residues of the amino terminus. These residues are responsible for zinc coordination and

catalysis (Becker and Roth, 1992).

Structural characterization of M16A subfamily enzymes has been reported (Im, et al., 2007; Maskos, 2005; Shen, et al., 2006). M16A enzymes have quaternary structure that is similar to a clamshell in appearance, with N- and C-terminal bowl-shaped domains of approximately 50 kDa connected by a short linker region of 20-30 residues. Both the N- and C-terminal domains, as well as individual residues from highly

conserved sequences within each domain, are required for the catalytic function of M16A enzymes (Alper, et al., 2006; Becker and Roth, 1993; Ding, et al., 1992; Kim, et al., 2005; Li, et al., 2006; Perlman, et al., 1993). Recent studies of the human insulin-degrading enzyme (*HsIDE*) and bacterial pitrilysin (*EcPtr*) have demonstrated that M16A enzymes can adopt either an 'open' conformation, which is hypothesized to permit substrate access to a central catalytic compartment, or a 'closed' conformation, which is presumed to be necessary for proteolysis, yet likely prohibits entrance and exit of substrates from the catalytic site (Maskos, 2005; Shen, et al., 2006). Transitions between the 'open' and 'closed' conformations of M16A enzymes are hypothesized to represent a rate-limiting step within these enzymes' catalytic cycles. Consistent with this hypothesis, mutations that disrupt interactions between the N- and C-terminal domains of *HsIDE* increase catalytic activity by as much as 40-fold (presumably by facilitating the transition between the open and closed conformations of the enzyme) (Shen, et al., 2006). Allosteric regulation of *HsIDE* and *R. norvegicus* IDE (*RnIDE*) by ATP has been reported, although the underlying reason for such regulation is not understood at present (Camberos, 2001; Song, et al., 2004). Eukaryotic M16A enzymes also demonstrate sensitivity to N-ethylmaleimide (NEM) and other thiol modifiers, though the mechanistic importance of cysteine residues within these enzymes remains poorly elucidated (Becker and Roth, 1995; Kim, et al., 2005; Neant-Fery, et al., 2008).

M16A enzymes have broad yet selective substrate specificity. Established substrates of M16A enzymes include insulin, amyloid beta (A $\beta$ ) peptides, glucagon, amylin, plant systemin, and yeast P2 pro-*a*-factor, among others (Adames, et al., 1995; Alper, et al., 2006; Bennett, et al., 2000; Huet, et al., 2008; Kim, et al., 2005; Kirschner and Goldberg, 1983; Qiu, et al., 1998). Structural studies of *HsIDE* in complex with multiple peptide substrates have revealed several features that play a role in substrate recognition by this and presumably other M16A enzymes (Maskos, 2005; Shen, et al., 2006). To be recognized by *HsIDE*, potential substrates must be capable of anchoring within the enzyme's catalytic binding pocket and forming favorable interactions with enzymatic  $\beta$ -

strands proximal to the site of peptide hydrolysis. Potential substrates must also be small enough (~50 amino acids in length) to permit encapsulation within the central chamber of the closed enzyme. Peptides with significant positive charge at their C-terminus are generally poor substrates due to unfavorable electrostatic interactions with the positively charged face of the central chamber contributed by the C-terminal domain of the enzyme (Shen, et al., 2006). No absolute primary sequence determinant has been reported among substrates of the M16A subfamily. However, studies of *RnIDE* using synthetic fluorogenic peptides have shown that the enzyme preferentially cleaves on the amino side of hydrophobic and basic residues (Song, et al., 2005), and studies of *S. lycopersicum* IDE (*SlIDE*) using various alternative peptide substrates have further suggested a limited preference for proline one or two residues N-terminal to the site of proteolytic cleavage (Huet, et al., 2008), which may reflect an overall preference for substrates that adopt a  $\beta$ -turn structure (Kurochkin, 1998).

Medical interest in the M16A subfamily has been motivated in part by the proposal that *HsIDE* serves a protective function in forestalling the onset of Alzheimer's disease (AD) (Hardy and Selkoe, 2002; Selkoe, 2001). Human population studies have indicated that variations in and around the chromosomal locus encoding *HsIDE* are risk factors for AD and Type II diabetes mellitus (DM2) (Ertekin-Taner, et al., 2000; Groves, et al., 2003; Kim, et al., 2007; Myers, et al., 2000). Consistent with these observations, IDE deficiency or loss of function in rodent models has been correlated with increased levels of A $\beta$  and insulin, as well as increased incidence of AD and DM2 (Farris, et al., 2003; Leissring, et al., 2003; Miller, et al., 2003). Levels of soluble and insoluble A $\beta$  and premature death rates are also significantly reduced in transgenic rodent models in which both IDE and the amyloid precursor protein, APP, have been overexpressed (Leissring, et al., 2003).

Ste23p and Axl1p are the M16A enzymes native to *S. cerevisiae*. Genetic studies support involvement of Ste23p and Axl1p in exacting an N-terminal endoproteolytic cleavage of the P2 precursor of the *a*-factor mating pheromone that is produced by *MATa* haploid cells. *MATa* yeast that lack functional copies of both Ste23p and Axl1p

cannot signal mating competency to cells of the complementary mating type and thus exhibit a 'sterile' mating phenotype (Adames, et al., 1995). Axl1p expression is restricted to haploid yeast (Fujita, et al., 1994), where, in addition to its role in **a**-factor processing, the enzyme has several other known functions. These include maintenance of a haploid-specific axial budding pattern (Adames, et al., 1995; Fujita, et al., 1994), repression of haploid-specific invasive growth (Cullen and Sprague, 2002; Palecek, et al., 2000), and promotion of efficient haploid cell fusion during mating (Elia and Marsh, 1996). As documented in this study, and by contrast to the haploid-specific expression of Axl1p, Ste23p is expressed in both haploid and diploid yeast. Despite its broad expression profile, the only known function of Ste23p is in **a**-factor processing, a *MATa*-specific event. Its role in *MATa* and diploid cells, which may also be conserved in *MATa* cells, remains enigmatic. Ste23p and Axl1p are not functionally equivalent in terms of their ability to promote mature **a**-factor production. Ste23p is responsible for at most 5% of all **a**-factor production, yet it is expressed at steady state levels that are at least ten times higher than that observed for Axl1p (Adames, et al., 1995; Alper, et al., 2006). These observations suggest an alternative biological function of Ste23p that has yet to be identified.

We now report a comparative biochemical analysis of yeast Ste23p and *RnIDE*. We demonstrate that Ste23p exhibits conserved specificity towards established *RnIDE* substrates A $\beta$  1-40 and insulin B chain, and has enzymatic characteristics that are similar to those of *RnIDE* and other eukaryotic M16A enzymes. In addition, we provide genetic and biochemical evidence that the 5' terminus of the *STE23* ORF has been mischaracterized. This study presents the first detailed biochemical analysis of a fungal M16A enzyme, and supports the suitability of Ste23p as a model for continuing studies of the M16A subfamily within *S. cerevisiae*.

## MATERIALS AND METHODS

*Sequence comparisons*- Polypeptide sequences of M16A enzymes from several commonly studied organisms were evaluated using the multiple global sequence alignment function of Clustal W 2.0.10 with default parameters (Larkin, et al.,

2007; Wilbur and Lipman, 1983). Polypeptide sequences for *HsIDE*, *RnIDE*, *Drosophila melanogaster IDE* (*DmIDE*), *SIIDE* and *EcPtr* are equivalent to the UniProtKB/Swiss-Prot protein sequence entries P14735, P35559, P22817, Q93YG9, and P05458, respectively (Leinonen, et al., 2004). The polypeptide sequence for *Caenorhabditis elegans IDE* (*CeIDE*) corresponds to WormBase protein entry WP:CE37861 (Bieri, et al., 2007; Stein, et al., 2001). Polypeptide sequences for Ste23p and Axl1p correspond to the Ste23p and Axl1p protein sequences annotated within the *Saccharomyces* Genome Database (Cherry, et al., 1998). The polypeptide sequence for *Ustilago maydis* Ste23p (*UmSte23p*) was inferred from *U. maydis* genomic locus UM03257.1 (Harvard, 2008), and the polypeptide sequence for *Schizosaccharomyces pombe* (*SpSte23p*) was inferred from *S. pombe* genomic locus NP\_593966 (Wood, et al., 2002). Graphical representation of the Ste23p and *RnIDE* alignment was created using BOXSHADE 3.21.

*Yeast strains and plasmids*- The yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Yeast strains were grown at 30 °C in solid or liquid rich media (YEPD) or synthetic complete (SC) dropout medium. Plasmids p80, pRS316, pWS371, pWS375, pWS482, pWS512 and pWS514 have been previously described (Adames, et al., 1995; Kim, et al., 2005; Sikorski and Hieter, 1989). Plasmids having 5' sequence deletions (pWS759-763) were created by direct subcloning of PCR products derived from pWS375, which were designed to lack the appropriate DNA region and contain KpnI and HindIII restriction sites that were subsequently used to insert the PCR product into corresponding sites of pWS482; the KpnI site is within the pWS482 polylinker, and HindIII is internal to the *STE23* ORF. A plasmid lacking 5' sequence and the first predicted 156 nucleotides of *STE23* (pWS765) was created in a similar manner. pWS908 and pWS909 were obtained commercially (Norclone Biotech Laboratories, London, Ontario). Yeast transformations were carried out according to established methods (Elble, 1992).

*Bacterial strains and plasmids*- Bacterial strains and plasmids used within this study are included in Tables 1 and 2. DH5 $\alpha$  *E. coli* (Invitrogen Inc., San Diego, CA) was used for

amplification of plasmid DNA, and BL21(DE3) *E. coli* (Novagen Inc., Darmstadt, Germany) was used for protein expression. pET30-b(+) (Novagen Inc.) was used as the vector for recombinant expression of Ste23p and *RnIDE*. Bacterial cells were prepared for chemical transformation according to established methods (Hanahan, 1983).

Vectors for recombinant expression of *STE23* and *RnIDE* were created within pET30-b(+) such that each encoded an N-terminal polyhistidine tag immediately behind a start codon. pWS769 (6xHis-Ste23p<sub>M53-E1027</sub>) was created by subcloning a PCR fragment encoding the region of the *STE23* gene corresponding with residues M53-E1027 of the annotated Ste23p polypeptide sequence. This sequence was amplified from pWS375 such that an NdeI site, an ATG codon, and sequence encoding a 6xHis tag were created at the 5' end of the fragment and a NotI site was created at the 3' end. This strategy permitted direct subcloning into corresponding sites within the pET-30b(+) vector. Construction of pWS804 (6xHis-*RnIDE*<sub>M42-L1019</sub>) was achieved in an analogous fashion and has been described (Alper and Schmidt, 2009). DNA sequencing analysis revealed two mutations encoding single-site amino acid substitutions Y248C and E768A within the plasmid encoding *RnIDE*. The corresponding native residues are not conserved between *RnIDE* and Ste23p, and are positioned distal to the catalytic site as inferred from analogous residues within *HsIDE*, the most closely related *RnIDE* homolog for which structural data is available.

*Enzyme purification*- 6xHis-Ste23p<sub>M53-E1027</sub> and 6xHis-*IDE*<sub>M42-L1019</sub> were expressed within BL21 (DE3) *E. coli* and purified by immobilized nickel affinity chromatography according to our established methods (Alper and Schmidt, 2009). In brief, a clarified cell lysate containing the protein of interest was applied onto a HisTrap™ Fast Flow 5 mL column that was attached to an AKTApurifier™ FPLC system (GE Healthcare Inc., Piscataway, NJ). Block elution with 0.5 M imidazole was used to recover the bound protein after washes to remove unbound material. The imidazole concentration in the eluted sample was reduced by first concentrating the sample to approximately 1 mL using an Amicon Ultra™-15 mL 100 kDa MW-cutoff centrifugal filter device (Millipore Inc., Bedford MA), and then diluting

the sample approximately 10-fold with Lysis Buffer (50 mM HEPES, 140 mM NaCl, pH 7.4). The dilution and concentration steps were repeated once more prior to diluting the final sample to 1 mg/mL in Storage Buffer (Lysis Buffer containing 20% glycerol (v/v)). Molarities of the purified enzymes were determined prior to dilution from UV absorbance measurements and A<sub>280</sub> extinction coefficients according to established methods (Gill and von Hippel, 1989). These values were consistent with those determined by Bradford assay. Sample aliquots were stored at -80 °C and thawed as needed.

*Peptides and reagents*- Recombinant human Aβ 1-40 (rPeptide Inc., Athens, GA), human insulin, and insulin B chain (Sigma-Aldrich Inc., St. Louis, MO) and the quenched fluorogenic Abz-SEKKDNYIIKGV-nitroY-OH peptide (AnaSpec Inc., San Jose, CA) were all reconstituted from dry powders. Detailed preparation of Aβ 1-40 and insulin solutions has been reported elsewhere (Alper and Schmidt, 2009). In brief, Aβ 1-40 was treated with hexafluoroisopropanol (HFIP) to ensure the initial monomeric state of the peptide (Dahlgren, et al., 2002), and dissolved within dimethylsulfoxide (DMSO) to a final concentration of 4 mg/mL. Insulin was resuspended in DMSO to 4 mg/mL and sonicated using a bath sonicator (Branson Inc., Danbury, CT) for approximately 5 min to fully dissolve the peptide. Insulin B chain was resuspended in DMSO to a final concentration of 4 mg/mL, but did not require sonication for complete solubility. The Abz-SEKKDNYIIKGV-nitroY-OH peptide was resuspended directly within DMSO to a final concentration of 10 mM. After reconstitution, all peptide-containing solutions were stored at -80 °C. Ethylenediaminetetraacetic acid (EDTA), N-ethylmaleimide (NEM), free cysteine, β-mercaptoethanol (β-ME), dithiothreitol (DTT), adenosine-5'-triphosphate (ATP), and guanosine triphosphate (GTP) were prepared as 10 mM stocks in deionized water. N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), 1,10-phenanthroline (1,10-P.), 4,7-phenanthroline (4,7-P.), and phenylmethylsulphonyl fluoride (PMSF) were prepared as 50 mM stocks in DMSO.

*Capillary electrophoresis*- Aβ 1-40 and insulin B chain proteolysis were analyzed by capillary electrophoresis (CE) as previously described

(Alper and Schmidt, 2009). In brief, proteolytic reactions (40  $\mu$ L) were conducted in CE Proteolysis Buffer (10 mM potassium phosphate, pH 7.6) at 30 °C (Ste23p) or at 37 °C (*RnIDE*) using 20 nM enzyme and an initial substrate concentration of 0.1 mg/mL (A $\beta$  1-40 and insulin B chain). Enzyme and substrate mixes were independently prepared as 2x reaction pre-mixtures by dilution within CE Proteolysis Buffer that was warmed at 30 °C or 37 °C for 10 min, prior to mixing the pre-mixtures within a pre-warmed PCR tube to initiate the proteolytic reaction. Proteolysis was terminated after appropriate intervals by heating the reaction mixture to 80 °C for 3 min. Samples were then stored at -80 °C prior to analysis.

**Fluorescence assay-** An internally quenched fluorogenic dodecapeptide substrate centered on the M16A proteolytic cleavage site within the yeast P2 **a**-factor mating pheromone (Abz-SEKKDNYIIKGV-nitroY-OH) was used to monitor the activity of 6xHis-Ste23p<sub>M53-E1027</sub> and 6xHis-IDE<sub>M42-L1019</sub> under steady-state reaction conditions. This substrate contains a fluorescent aminobenzoic acid moiety (Abz) that is linked through the peptide backbone to a nitrotyrosine (nitroY) fluorescence quencher. Proteolytic cleavage results in enhanced sample fluorescence, which can be quantified using a fluorimeter.

Effects of various environmental conditions upon the enzymatic activities of Ste23p and *RnIDE* were evaluated by manipulating a component condition of the fluorescence assay reaction mixture while holding all others constant. The standard conditions for assays containing Ste23p (20 nM) were 100 mM potassium phosphate, pH 8.1 and 30 °C; the standard conditions for *RnIDE* (50 nM) were 100 mM potassium phosphate, pH 9.2, and 37 °C. Potassium phosphate buffers were prepared according to published methods (Gomori, 1956). In general, the use of phosphate buffers was found to permit maximal enzymatic activity relative to alternative buffering systems (Alper, Rowse, and Schmidt, unpublished observations). The initial Abz-SEKKDNYIIKGV-nitroY-OH peptide concentration was 17  $\mu$ M. Kinetic parameters were determined using the standard conditions described above and initial substrate concentrations ranging from 3 – 172  $\mu$ M.

Typical assembly of the reaction mixture involved 1:1 mixing of appropriately diluted substrate and enzyme solutions within an individual well of a pre-warmed, black, flat-bottomed fluorescence microtiter plate. The fluorogenic substrate was prepared as a 2x reaction pre-mixture by dilution with appropriate buffer in a PCR tube, and pre-equilibrated for 10 min at the desired temperature. Ste23p and *RnIDE* were similarly prepared. Where noted, pre-mixtures contained competing substrate (0.16 mg/mL insulin) or added compounds (2 mM), the latter preincubated with the enzyme for 10 min. Proteolysis was initiated by combining the 2x pre-mixtures. The total reaction volume of a typical assay was 100  $\mu$ L, except in the instance of experiments involving  $K_m$  determination, where the total volume was 30  $\mu$ L in an effort to permit conservation of substrate. Larger volume samples were combined in a 96-well microtiter plate; smaller volumes were combined in a 384-well plate. Sample fluorescence was analyzed at 420 nm over multiple time points over a 60 min time course using a Bio-Tek® Synergy™ HT fluorimeter equipped with a 320/420 nm excitation/emission filter set (Bio-Tek® Instruments, Inc., Winooski, VT).

As controls for the fluorescence assay, background and maximum fluorescence signals were determined as reference points for all conditions evaluated. This analysis was performed to identify reaction conditions that modulated fluorescence of the reporter. The background fluorescence signal was determined in the absence of added enzyme, while retaining all other components of the experimental reaction mixture described above. The maximum fluorescence signal was determined in the presence of trypsin (5  $\mu$ g/mL), which cleaves the Abz-SEKKDNYIIKGV-nitroY-OH substrate, after the proteolytic reaction was allowed to proceed to completion. In instances where fluorescence was affected (e.g. altered pH, certain added compounds), the absolute difference between the negative and positive controls was used as a normalization factor to calculate the relative fluorescence output of the sample. Rate values observed in the presence of added compounds are reported relative to those for the untreated enzymes.

*Rate Calculations-* Data obtained by fluorescence assay was analyzed using Microsoft Excel™ and GraphPad Prism™ 4.0 Software. Reaction velocity was determined from 10 to 30 min after the initiation of each experiment, or up to such time as a maximum of 10% substrate proteolysis was achieved, allowing for an initial lag period upon mixing of enzyme and substrate pre-mixtures. Reported values represent the average of least 3 experimental replicates each derived from 10 experimental data points satisfying the conditions described above. Maximal reaction velocity and initial reaction velocity were taken to be equivalent for purposes of this analysis. Kinetic parameters for Ste23p and RnIDE were determined using nonlinear regression curve-fitting software within Prism™ and a 4-parameter logistic equation without constraints.

Because significant intermolecular quenching effects were observed with the fluorescence-based substrate at concentrations  $>23 \mu\text{M}$ , activities observed at these concentrations were adjusted by correction factors for the purposes of kinetic analysis. These factors were concentration-dependent and derived by comparing best-fit equations for both free Abz and a 1:1 mixture of Abz and nitroY over a range of concentrations. Free Abz data points were best fit by a 2<sup>nd</sup> order polynomial function ( $R^2 = 0.9998$ ), whereas data for the Abz and nitroY mixture was best fit by a 4-parameter logistic equation ( $R^2=1.000$ ). Correction factors were determined from ratios of fluorescence (i.e.  $\text{RFU}_{\text{Abz}}/\text{RFU}_{\text{Abz+nitroY}}$ ) at specific concentrations, which were multiplied against the activities observed at those concentrations. The correction factors were minor (0.98 to 1.05) between 0-23  $\mu\text{M}$  and progressively increased (1.09 to 1.51) over the range of 34-172  $\mu\text{M}$ . Similar mathematical corrections have been reported for other datasets where intermolecular quenching has been observed (Lazure, et al., 1998).

*Yeast mating assay-* Mating tests were performed essentially as previously described (Alper, et al., 2006; Kim, et al., 2005). In brief, *MATa* yeast of the indicated genotypes were cultured to saturation in selective (SC-ura) liquid media while the *MAT $\alpha$*  mating tester (IH1783; *MAT $\alpha$  lys1*) was cultured in YEPD. The saturated cultures were then diluted with fresh media to

achieve a cell culture density of 0.95-1.05  $\text{OD}_{600}$ . The diluted *MAT $\alpha$*  cell suspension (98  $\mu\text{L}$ ) and an appropriate *MATa* suspension (2  $\mu\text{L}$ ) were mixed, and the mixed suspension spotted (5  $\mu\text{L}$ ) onto synthetic defined (SD) solid media. Mating was recorded after incubation at 30 °C for 48-72 hours. An equivalent portion of the mixed suspension was also spotted on SC-lys solid media to serve as a loading control for *MATa* cell input.

*Preparation of cell extracts and immunoblotting-* Yeast total cellular protein extracts were prepared as previously described (Fujimura-Kamada, et al., 1997). In brief, mid-log cells (2 mL of 1.0  $\text{OD}_{600}$  culture) were harvested by centrifugation, rinsed with deionized water, resuspended in 1 mL of cold deionized water, and treated with a mixture of NaOH (0.24 N final) and  $\beta$ -mercaptoethanol (0.14 M final) for 15 min while on ice. Proteins were precipitated by addition of TCA (11.5% final), recovered by centrifugation (16,000 g x 15 min), resuspended in 50  $\mu\text{L}$  of prewarmed ( $\sim 100$  °C) urea sample buffer (250 mM Tris, pH 8.0, 6 M urea, 4% SDS, and 0.01% bromophenol blue), and boiled for 3 min. Samples were subjected to SDS-PAGE using a 10% polyacrylamide gel. Immunoblot analysis was performed using anti-HA (Roche, Ltd., Basel, Switzerland) or anti-Act1p primary antibodies (gift of R. Meager, University of Georgia, Athens, GA), and appropriate HRP-conjugated secondary antibodies (GE Health, Inc.). Visualization was achieved by chemiluminescent detection methods (BM Chemiluminescence Blotting Substrate, Roche, Ltd.) according to the manufacturer's instructions.

## RESULTS

*Ste23p is the most highly conserved M16A enzyme within S. cerevisiae.* Two M16A enzymes are encoded within the *S. cerevisiae* genome: Ste23p and Ax11p. Primary sequence alignments reveal that Ste23p shares a higher degree of amino acid sequence identity with mammalian IDEs than does Ax11p (35% vs. 18% identity to *HsIDE*, respectively; Table 3). Moreover, Ste23p shares roughly equal sequence conservation to mammalian IDEs as is shared by other fungal M16A enzymes, and has higher identity than Ax11p when compared to IDE orthologs from other species. Observations from prior studies further indicate that Ste23p is the most

representative IDE ortholog in *S. cerevisiae*, with Axl1p likely having evolved to serve multiple functions that are specific to this organism (see Discussion). Hence, Ste23p was chosen as the model yeast M16A enzyme for purposes of the current analysis. A primary sequence alignment of Ste23p and *RnIDE* reveals notable features of the two enzymes, including conserved residues of the characteristic M16A motif (Fig. 1).

*Purification of Ste23p and RnIDE.* 6xHis-Ste23p<sub>M53-E1027</sub> and 6xHis-*RnIDE*<sub>M42-L1019</sub> were isolated primarily as single protein species of the expected molecular mass (Fig. 2). The purified proteins retain N-terminal polyhistidine tags, as evidenced by immunoblot analysis using an anti-6xHis antibody (Alper, Rowse, and Schmidt, unpublished observations).

Ste23p and *RnIDE* are annotated in public sequence databases as polypeptides having length of 1027 and 1019 amino acids, respectively (Cherry, et al., 1998; Leinonen, et al., 2004). Relative to the annotated Ste23p and *RnIDE* sequences, N-terminally truncated forms of these enzymes were isolated for purposes of our analysis. Our purification of ‘truncated’ enzymes – beginning, in each case, at the first in-frame methionine encoded downstream from the annotated initiator – over ‘full-length’ polypeptides was motivated by several factors. First, *HsIDE* was recently purified and crystallized as a similarly truncated protein (Shen, et al., 2006), and to our knowledge, there has not been definitive assignment of the initiator methionine for any mammalian IDE (Baumeister, et al., 1993). Second, attempts to purify the full annotated sequence of Ste23p as a C-terminal polyhistidine fusion protein led to a purification product having T54 at its N-terminus, as determined by Edmund degradation analysis (Alper, Rowse, and Schmidt, unpublished observations); M53 was presumably absent due to the action of methionine aminopeptidase. Third, the ‘truncated’ form of Ste23p most likely represents the physiologically relevant form of the enzyme, as supported by data presented elsewhere within this study (see Fig. 6).

*Ste23p proteolyzes mammalian IDE substrates Aβ 1-40 and insulin B chain.* Ste23p has no recognized physiological substrates other than the yeast P2 a-factor; however, the fungal enzyme is capable of proteolyzing the established mammalian IDE substrates Aβ 1-40 and insulin B

chain (Fig. 3). Aβ 1-40 and insulin B chain are common substrates of the M16A subfamily. These peptides have been used to study the proteolytic specificity of M16A enzymes even in instances where they are unlikely biological targets (Becker and Roth, 1992; Ding, et al., 1992; Huet, et al., 2008). Analysis of samples from time-course proteolysis experiments shows the accumulation of multiple peaks that presumably correspond with Aβ 1-40 and insulin B chain degradation products, indicating that Ste23p proteolyzes each of these substrates at multiple sites (Figs. 3B-C). These products are not observed in the presence of histidine-tagged glutathione *S*-transferase (GST-6xHis) under otherwise similar reaction conditions (Alper, Rowse, and Schmidt, unpublished observations), indicating that proteolysis is not attributable to a contaminating copurification product from the bacterial lysate. Detectable accumulation of Aβ 1-40 and insulin B chain cleavage products is apparent at the 1 hr and 4 hr timepoints, respectively, suggesting that degradation of Aβ 1-40 occurs relatively more rapidly. For both substrates, Ste23p-mediated product formation appears slower than that mediated by *RnIDE*, which cleaves Aβ 1-40 and insulin B chain to near completion within 4 hrs (Figs. 3D-E). The identity of Aβ 1-40 and insulin B chain degradation products was not determined during this study. However, *RnIDE* is known to proteolyze both peptides at multiple cleavage sites, which have been previously evaluated (Duckworth, et al., 1998; McDermott and Gibson, 1997; Mukherjee, et al., 2000).

*Comparative activity studies.* The enzymatic properties of Ste23p and *RnIDE* were compared using a continuous readout fluorescence assay under conditions of varying pH, salt concentration, temperature, and substrate concentration (Fig. 4). Both enzymes were most active within alkaline environments (Figs. 4A-B), with Ste23p having a maximal activity of  $0.49 \pm 0.031 \mu\text{mols} \times \text{mg}^{-1} \times \text{min}^{-1}$  at pH 8.1, and *RnIDE* having maximal activity of  $0.22 \pm 0.004 \mu\text{mols} \times \text{mg}^{-1} \times \text{min}^{-1}$  at pH 9.2, the most alkaline condition evaluated. These values may be compared with the reported values for *RnIDE*-mediated proteolysis of a distinct synthetic fluorogenic Abz-GGFLRKGVQ-EDDnp peptide, which exhibits a  $V_{\text{max}}$  of  $\sim 0.2 \mu\text{mols} \times \text{min}^{-1} \times \text{mg}^{-1}$ , and *RnIDE*-mediated



cleavage of  $\beta$ -endorphin, which exhibits a  $V_{\max}$  of  $2.6 \mu\text{mol} \times \text{min}^{-1} \times \text{mg}^{-1}$ , albeit under different assay conditions (Song, et al., 2001). Both enzymes displayed enhanced enzymatic activity at high salt concentrations, with Ste23p and *RnIDE* activities being stimulated 2- and 6-fold, respectively, at the highest concentration of potassium chloride evaluated (Figs. 4C-D). Both enzymes displayed optimal activity at or about 37 °C (Figs. 4E-F). Kinetic parameters for Ste23p and *RnIDE* were determined, revealing that both had roughly equivalent affinity for the Abz-SEKKDNYIIKGV-nitroY-OH peptide under the conditions evaluated (Figs. 4G-H). Observed  $K_m$  values were  $215 \pm 69 \mu\text{M}$  and  $219 \pm 22 \mu\text{M}$  for Ste23p and *RnIDE*, respectively. Negligible activity was observed by fluorescence assay using a GST-6xHis control (Alper, Rowse, and Schmidt, unpublished observations).

Given the otherwise similar enzymatic properties of Ste23p and *RnIDE* identified within our studies, we sought to determine whether the two enzymes exhibited comparable sensitivity to known modulators of M16A peptidase activity, including metal chelators, thiol modifiers, and certain inhibitors of cysteine protease activity. This analysis, which was conducted using the fluorescence assay, demonstrated that the sensitivities of Ste23p generally paralleled that observed for *RnIDE* (Fig. 5). Addition of EDTA, TPEN, and 1,10-phenanthroline, as well as NEM, free cysteine, and DTT resulted in substantial inhibition of both Ste23p and *RnIDE* proteolysis (<40% activity). By comparison, the non-chelating agent 4,7-phenanthroline had limited impact (>60% activity), and addition of  $\beta$ -mercaptoethanol had little or no effect on both enzymes (>80% activity). The serine protease inhibitor PMSF and physiological cofactors ATP and GTP also had limited impact on the activity of both enzymes (>60% activity). Addition of the competing substrate insulin to the proteolytic reaction mixture substantially inhibited both enzymes (<40% activity), but had a more pronounced affect on *RnIDE*.

*The predicted initiator methionine of Ste23p is dispensable for activity in vivo.* Our biochemical analysis revealed that the extreme N-terminus (residues 1-52) of the publicly annotated Ste23p polypeptide sequence is not essential for catalytic

function *in vitro*. To better understand the significance of this region *in vivo*, genetic and biochemical methods were used to assess its importance for expression of functional Ste23p (Fig. 6). Our standard vector for Ste23p expression contains approximately 1.5 kb of genomic sequence 5' of the *STE23* ORF, inclusive of a distinct ORF of opposite orientation that is separated from *STE23* by a 405 basepair intergenic region (Fig. 6A). A modified version of this expression vector that eliminated the entire upstream sequence relative to the 5' margin of the *STE23* ORF ( $\Delta$ -1467  $\rightarrow$  -1) supported functional Ste23p expression as well as the unmodified plasmid (Figs. 6B-C); the reduced mating observed relative to WT is due to the absence of *AXL1* in the test strain. Loss of additional nucleotide sequence ( $\Delta$ -1467  $\rightarrow$  +156), specifically that encoding the annotated initiator codon (M1) and genomic sequence up to but not including the next available methionine codon (M53), abolished Ste23p expression and function, suggesting that the genetic region +1  $\rightarrow$  +156 of *STE23* is important for gene expression.

We next evaluated the dependence of Ste23p expression on M1 and M53 codons by site directed mutagenesis (Fig. 6D). Mating and protein expression were observed with the M1A mutant, but not with the M53A mutant, implying that M53, but not M1, is necessary for expression of functional Ste23p.

*Ste23p is expressed in both haploid and diploid cell types.* Whereas expression of the yeast M16A homolog Ax11p is specific to haploid cells (Fig. 7A) (Fujita, et al., 1994), the expression pattern of Ste23p has not been previously reported. In view of the widespread occurrence of IDE across species and the broad tissue expression of M16A enzymes within multi-cellular organisms (Baumeister, et al., 1993), we hypothesized that Ste23p would be expressed in an unrestricted manner among the three cell types of *S. cerevisiae* – diploid, *MATa* and *MAT $\alpha$*  haploid. Indeed, our analysis indicates that Ste23p is expressed in both diploid and haploid cell types (Fig. 7B). Moreover, Ste23p expression levels appear relatively constant within each of the cell types evaluated. Ste23p is produced at levels that are approximately 10-fold higher than Ax11p, as judged by immunoblots of serially diluted samples

(Alper, et al., 2006). We interpret the unrestricted expression of Ste23p and its high level of production relative to Axl1p as potential indicators of an as yet undetermined function for Ste23p that is distinct from the enzyme's role in P2 **a**-factor processing. This function must be nonessential, as yeast deficient for *STE23* and/or *AXL1* are viable.

## DISCUSSION

The M16A subfamily is emerging as a remarkable class of enzymes, most notably due to the proposed role of human IDE in protecting against Alzheimer's disease and the unusual mechanism by which M16A enzymes capture and cleave their substrates (Selkoe, 2001; Shen, et al., 2006). Despite their widespread species distribution, biological functions of M16A enzymes remain poorly elucidated. Studies of M16A enzymes within well-characterized models may thus serve to distinguish functions of the M16A subfamily that have yet to be identified. In this study, we provide evidence that such studies in yeast should focus on Ste23p, rather than Axl1p. This conclusion is based on the higher degree of amino acid sequence conservation that is shared between Ste23p and other M16A enzymes and the remarkably similar biochemical properties of Ste23p and *RnIDE*.

To our knowledge, this study presents the first biochemical characterization of a fungal M16A enzyme. We have demonstrated that Ste23p is capable of proteolyzing A $\beta$  1-40 and insulin B chain (Fig. 3). While neither substrate represents a likely physiological target of the fungal enzyme, these observations are significant in that they provide the first evidence that Ste23p exhibits conserved specificity towards these established substrates of mammalian M16A enzymes. Reciprocal substrate specificity thus emerges as a general property of fungal, mammalian, and prokaryotic M16A enzymes, as we have previously demonstrated that *RnIDE* and *EcPtr* can proteolyze a Ste23p substrate – namely, the P2 precursor of the **a**-factor mating pheromone (Alper, et al., 2006; Kim, et al., 2005). Our observation that Ste23p can proteolyze both A $\beta$  1-40 and insulin B chain also indicates that substrate recognition by Ste23p does not require the presence of a farnesyl moiety, which is associated with P2 **a**-factor, the only previously recognized substrate of the yeast enzyme. Collectively, these

observations led us to develop a fluorogenic substrate (Abz-SEKKDNYIIKGV-nitroY-OH) based on the amino acid sequence of P2 **a**-factor, but lacking the farnesyl moiety of the native mating pheromone. This substrate provides a useful tool for cross-species enzymological studies of the M16A subfamily.

Using a continuous fluorescence assay to measure Ste23p and *RnIDE*-mediated proteolysis of the Abz-SEKKDNYIIKGV-nitroY-OH peptide, we have demonstrated that in addition to exhibiting reciprocal substrate specificity, Ste23p and *RnIDE* have similar activity profiles in response to alterations in pH, salt concentration, and temperature (Fig. 4), and various agents that are known modulators of M16A activity (Fig. 5). M16A enzymes are recognized as Zn<sup>2+</sup> metalloproteinases (Becker and Roth, 1992; Ding, et al., 1992; Ebrahim, et al., 1991; Huet, et al., 2008). Consistent with expectations, Ste23p and *RnIDE* were both inhibited by certain metal chelating agents. Also, like *RnIDE* and other eukaryotic M16A enzymes, but unlike bacterial *EcPtr*, Ste23p was inhibited by certain thiol modifiers (Affholter, et al., 1990; Becker and Roth, 1992; Ding, et al., 1992; Huet, et al., 2008; Kuo, et al., 1990). Neither Ste23p nor *RnIDE* was sensitive to ATP or GTP addition under conditions of our analysis. This observation may be interpreted in the context of reports that alternatively claim activation or inhibition of mammalian IDE by these cofactors (Camberos, 2001; Huet, et al., 2008; Song, et al., 2004). Ste23p also exhibits a broad pH profile, having optimal activity over the range of pH 7.6 – 9.2. Like *RnIDE*, the yeast enzyme was found to be most active under alkaline conditions. We speculate that basic environments may enhance the propensity of M16A substrates to adopt a  $\beta$ -turn structure, which could enhance their interaction with catalytic residues in the N-terminal substrate binding pockets of Ste23p or *RnIDE*, or alternatively, that rate-limiting conformational dynamics within the M16A enzymes themselves are somehow altered under alkaline conditions. The broad pH profile exhibited by Ste23p is not without precedent. Members of the M4 metalloproteinase subfamily (e.g. thermolysin) also exhibit a broad pH profile and are optimally active at alkaline pH (pH 7.0 – 8.5; Feder and Schuck, 2004). We speculate that the broad range pH

optima exhibited by these enzymes may reflect their having evolved to retain maximal activity under a wide range of environmental conditions for reasons that have yet to be elucidated. Despite the similar biochemical and enzymatic properties of *RnIDE* and Ste23p, whether Ste23p represents a true insulinase remains open to interpretation. While the yeast enzyme proteolyzes insulin B chain (Fig. 3), we were unable able to document cleavage of intact insulin by Ste23p, despite measurable inhibition of Ste23p-mediated proteolysis upon addition of insulin to the fluorogenic assay mixture (Alper, Rowse, and Schmidt, unpublished observations; Fig. 5).

Available evidence collectively indicates that Ste23p is the most representative M16A enzyme within *S. cerevisiae*. First, Ste23p shares higher primary sequence homology to mammalian IDEs and other M16A enzymes than does Ax11p (36% vs. 18% identity to *HsIDE*, respectively; Table 3). Second, like yeast-expressed *RnIDE* and *EcPtr*, Ste23p is incapable of supporting certain Ax11p-dependent functions within *S. cerevisiae* (*i.e.*, maintenance of the haploid-specific axial budding pattern and repression of haploid invasive growth (Alper, et al., 2006; Cullen and Sprague, 2002; Fujita, et al., 1994; Kim, et al., 2005). Third, Ste23p has a relatively minor role in **a**-factor processing, despite being over-expressed approximately 10-fold relative to Ax11p in *MAT $\alpha$*  haploids (Adames, et al., 1995; Alper, et al., 2006). We now demonstrate that Ste23p is expressed in diploid and *MAT $\alpha$*  haploid yeast that are incapable of producing **a**-factor (Fig. 7). This observation is consistent with the broad tissue expression of *RnIDE* mRNA transcripts (Baumeister, et al., 1993) and may ultimately imply a role for Ste23p in a conserved housekeeping function that has yet to be established.

The assignment of Ste23p as the most representative yeast M16A ortholog supports a continued focus on the biological properties of this enzyme. A number of bioinformatic, genetic, and biochemical studies have identified potential

interactors of Ste23p that may inform its physiological relevance. Yet, to our knowledge, none of these interactions have been thoroughly investigated. Any future investigation of Ste23p interactors must take into account two observations. First, we have previously reported that the *STE23* stop codon was misannotated in the original release of the yeast genome sequence (Cherry, et al., 1998, Kim, 2005 #2765); a similar conclusion was reached through comparative analysis of related fungal genomes (Brachat, et al., 2003). The net effect of this misannotation is a predicted translation product with a shortened and altered C-terminus. Second, we now report that the initiator codon of Ste23p is also misannotated (Fig. 6). Previously identified genetic interactors of Ste23p must therefore be viewed with caution, as the genetic constructs used to identify these interactors have likely encoded forms of Ste23p with non-physiological N- and C-termini.

In sum, this study provides evidence of extensive enzymatic similarities among Ste23p, *RnIDE*, and other M16A enzymes. These findings support the conclusion that Ste23p represents a suitable model for studies of the M16A subfamily within *S. cerevisiae*. We thus anticipate that continuing studies of Ste23p will serve to provide significant insight into the biological properties of this and other M16A enzymes.

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**Table 1.** *Yeast and bacterial strains used in this study*

Strain	Genotype <sup>a</sup>	Reference
IH1783	<i>MATa trp1 leu2 ura3 his4 can1</i>	(Michaelis and Herskowitz, 1988)
IH1784	<i>MATα trp1 leu2 ura3 his4 can1</i>	(Adames, et al., 1995)
IH1788	<i>MATa/α trp1 leu2 ura3 his4 can1</i>	(Michaelis and Herskowitz, 1988)
IH1793	<i>MATα lys1</i>	(Michaelis and Herskowitz, 1988)
Y272	<i>MATa trp1 leu2 ura3 his4 can1 axl1::LEU2 ste23::LEU2</i>	(Adames, et al., 1995)
DH5α	<i>F-β80lacZD(lacZYA-argF) U169deoR recA1 endA1 hsdR17(rk-mk+) phoA supE44 thi-1gyr A96 relA1 ton A</i>	Invitrogen Inc.
BL21(DE3)	<i>F- ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm (DE3)</i>	Novagen Inc.

<sup>a</sup> IH1783, IH1784, IH1788, and Y272 are isogenic.



**Table 2.** *Plasmids used in this study*

Plasmid	Genotype	Reference
p80	<i>CEN URA3 STE23</i>	(Adames, et al., 1995)
pET-30b(+)	<i>pBR322 fl KAN lacI pT7</i>	Novagen Inc.
pSM1569	<i>AMP SR<math>\alpha</math>-RnIDE</i>	(Bondy, et al., 1994; Seta and Roth, 1997)
pRS316	<i>CEN URA3</i>	(Sikorski and Hieter, 1989)
pWS371	<i>CEN URA3 AXL1-2HAc</i>	(Kim, et al., 2005)
pWS375	<i>CEN URA3 STE23</i>	(Kim, et al., 2005)
pWS482	<i>CEN URA3 STE23-2HAc</i>	(Kim, et al., 2005)
pWS759	<i>CEN URA3 STE23-2HAc (<math>\Delta</math>-1467<math>\rightarrow</math>-1)</i>	This study
pWS760	<i>CEN URA3 STE23-2HAc (<math>\Delta</math>-1467<math>\rightarrow</math>-100)</i>	This study
pWS761	<i>CEN URA3 STE23-2HAc (<math>\Delta</math>-1467<math>\rightarrow</math>-200)</i>	This study
pWS762	<i>CEN URA3 STE23-2HAc (<math>\Delta</math>-1467<math>\rightarrow</math>-300)</i>	This study
pWS763	<i>CEN URA3 STE23-2HAc (<math>\Delta</math>-1467<math>\rightarrow</math>-400)</i>	This study
pWS765	<i>CEN URA3 STE23-2HAc (<math>\Delta</math>-1467<math>\rightarrow</math>+156)</i>	This study
pWS769	<i>pBR322 fl KAN lacI pT7 6XHIS-STE23<sub>M53-E1027</sub></i>	This study
pWS804	<i>pBR322 fl KAN lacI pT7 6XHIS-RnIDE<sub>M42-L1019</sub></i>	(Alper and Schmidt, 2009)
pWS908	<i>CEN URA3 STE23<sub>M1A</sub>-2HAc</i>	This study
pWS909	<i>CEN URA3 STE23<sub>M53A</sub>-2HAc</i>	This study

**Table 3.** Conservation of sequence identity among M16A enzymes <sup>a,b</sup>

	<i>Hs</i> IDE	<i>Rn</i> IDE	<i>Dm</i> IDE	<i>Ce</i> IDE	<i>Sl</i> IDE	<i>Um</i> Ste2	<i>Sp</i> Ste23	Ste23p	<i>Ec</i> Ptr	<i>Axl</i> 1p
<i>Hs</i> IDE	100	94	45	40	38	37	36	35	25	18
<i>Rn</i> IDE		100	45	39	38	38	36	35	24	18
<i>Dm</i> IDE			100	39	35	37	32	34	24	18
<i>Ce</i> IDE				100	31	32	31	32	22	16
<i>Sl</i> IDE					100	36	35	32	24	19
<i>Um</i> Ste2						100	39	36	26	14
<i>Sp</i> Ste23							100	36	25	18
Ste23p								100	24	16
<i>Ec</i> Ptr									100	12
<i>Axl</i> 1p										100

<sup>a</sup> Percent sequence identity calculated using multiple global sequence alignment function of Clustal W 2.0.10.

<sup>b</sup> *Hs*: *Homo sapiens*, *Rn*: *Rattus norvegicus*, *Dm*: *Drosophila melanogaster*, *Ce*: *Caenorhabditis elegans*, *Sl*: *Solanum lycopersicum*, *Um*: *Ustilago maydis*, *Sp*: *Schizosaccharomyces pombe*, *Ec*: *Escherichia coli*.

**FIGURE LEGENDS****Figure 1. Sequence alignment of Ste23p and RnIDE**

Ste23p and RnIDE protein sequences as annotated within the *Saccharomyces* Genome Database and Swiss-Prot Database, respectively, were aligned using Clustal W 2.0.10. Conserved amino acids are shaded black, and those considered similar are shaded grey. Closed and open arrowheads indicate the N-terminus of recombinant Ste23p and RnIDE isolated in this study and the position of the inserted 6xHis affinity tag. The characteristic HxxEH metalloprotease motif, which is conserved among all M16A enzymes, is indicated by a black bar. The two amino acids within 6xHis-RnIDE<sub>M42-L1019</sub> that are different from the annotated sequence are denoted in parentheses below the appropriate residue that they replace.

**Figure 2. Purification recombinant Ste23p and RnIDE**

Polyhistidine tagged Ste23p and RnIDE were expressed in *E. coli* from pET expressions vectors (pWS769 and pWS804, respectively) and isolated to near homogeneity. Two-fold serial dilutions of purified 6xHis-Ste23p<sub>M53-E1027</sub> and 6xHis-RnIDE<sub>M42-L1019</sub> were analyzed by SDS-PAGE and stained using Coomassie™ Brilliant Blue R-250. The leftmost lane of each dilution series contains 7.5 µg of total protein (lanes 1 and 5). The predicted molecular mass of 6xHis-Ste23p<sub>M53-E1027</sub> is 113 kDa, and the predicted mass of 6xHis-RnIDE<sub>M42-L1019</sub> is 114 kDa.

**Figure 3. Ste23p proteolyzes Aβ 1-40 and insulin B chain**

The ability of Ste23p and RnIDE to proteolyze established IDE substrates Aβ 1-40 and insulin B chain was evaluated by capillary electrophoresis (CE). Shown are chromatogram traces depicting UV absorbance at 200 nm. **A)** Analysis of the individual components of the proteolytic reaction mixture. Solutions contain 24 µM Aβ 1-40, 30 µM insulin B chain, 20 nM Ste23p, or 20 nM RnIDE in Proteolysis Buffer, reflective of their initial concentrations within the proteolytic reaction mixture. **B, C)** Profile of Aβ 1-40 (B) and insulin B chain (C) products produced by Ste23p at indicated timepoints. Peaks contributed by substrates are denoted with arrowheads, and peaks contributed by the enzymatic experimental component are denoted with asterisks. **D, E)** Profile of Aβ 1-40 (D) and insulin B chain (E) produced by RnIDE after 4 hr incubation.

**Figure 4. Comparative enzymatic properties of Ste23p and RnIDE**

Proteolysis of the Abz-SEKKDNYIIKGV-nitroY-OH peptide by Ste23p (**A, C, E**) and RnIDE (**B, D, F**) was evaluated over the indicated range of pH (**A, B**), salt concentration (**C, D**), and temperature (**E, F**) using a continuous fluorescence assay. Proteolytic activity rates were determined from initial rates and are presented as a percentage of the maximal enzyme activity observed within an experiment for each variable evaluated. Reported values are the average of ≥ 3 experimental replicates. Error bars depict standard deviation from the mean. Velocity vs. substrate concentration curves for Ste23p (**G**) and RnIDE (**H**) were determined over a substrate concentration range of 3 – 172 µM.  $K_m$  values were derived using the nonlinear regression function of GraphPad Prism™ 4.0.

**Figure 5. Sensitivity of Ste23p and RnIDE to various agents**

Activities of Ste23p (black bars) and RnIDE (grey bars) were determined as described in Fig. 4 in the presence of the indicated agents relative to untreated and DMSO (dimethyl sulfoxide)-treated controls. Reported values are the average of three experimental replicates, and error bars indicate standard deviation from the mean. EDTA (ethylenediaminetetraacetic acid), TPEN (N,N,N'-tetrakis-[2-pyridylmethyl]ethylenediamine), 1,10-P (1,10-phenanthroline), 4,7-P (4,7-phenanthroline), NEM (N-ethylmaleimide), β-ME (β-mercaptoethanol), DTT (dithiothreitol), PMSF (phenylmethylsulfonyl fluoride), ATP (adenosine-5'-triphosphate), and GTP (guanosine-5'-triphosphate) were added to a concentration of 1 mM within the proteolytic reaction mixture. Insulin was added to an initial concentration of 0.8 mg/mL.

**Figure 6. Characterization of the translation start site of Ste23p**

**A)** Schematic of the genomic DNA sequence that is 5' to the predicted start codon of the *STE23* open reading frame within pWS482. Schematic is not drawn to scale. **B)** The predicted 5' UTR of *STE23* is not required for expression of functional Ste23p. A set of plasmids bearing deletions of the 5' UTR contained within pWS482 (pWS759-763) and a plasmid lacking part of the *STE23* ORF itself (pWS765) were evaluated for their ability to promote yeast mating (top panel) and Ste23p expression (middle panel). Yeast mating was evaluated by mixing *MAT $\alpha$*  cells (IH1793) and *MATa*-deficient *MATa* cells (Y272) carrying the indicated plasmid, followed by selection on diploid-selective media. Ste23p expression was evaluated by immunoblotting. Equivalent percentage amounts (2%) of total cellular protein extracts were separated by SDS-PAGE and transferred onto blots that were probed with anti-HA and anti-Act1p antibodies as a loading control (middle and bottom panels, respectively). **C)** Expression of Ste23p partially rescues the mating defect of an *M16A*-deficient yeast strain (Y272). Yeast mating assays and analysis of Ste23p expression were performed as described in panel B; the panel order is preserved. WT (IH1783) was transformed with an empty vector (pRS316), and Y272 was transformed with either pRS316 or a plasmid encoding Ste23p-2HA (pWS482). **D)** The annotated start codon of *STE23* is not required for expression of functional Ste23p. Yeast mating assays and analysis of Ste23p expression were performed as described in panel B using plasmid transformed Y272 cells, except that 4% of each extract was evaluated; the panel order is preserved. Plasmids used were pWS908-909.

**Figure 7. Ste23p is expressed in both haploid and diploid yeast**

Plasmid-based expression of Ste23p (A) and Axl1p (B) was examined in *MATa* (IH1783), *MAT $\alpha$*  (IH1784), and diploid (IH1788) cell types using pWS482 and pWS371, respectively. The steady-state levels of the indicated HA-tagged protein (top panel) and yeast actin (bottom panel) were detected by immunoblotting as described in Fig. 6, except that 60% of the total cellular extract preparation was evaluated in the instance of Axl1p samples due to its low abundance.