Synthesis and Cleavage-Secretion of Enzymatically Active Rabbit Angiotensin-converting Enzyme in Pichia pastoris*

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Ranmkrishna Sadhukhan‡, Ganes C. Sen§, and Indira Sen¶

From the Departments of ‡Molecular Cardiology and §Molecular Biology, Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Many biologically important ectoproteins that are anchored in the plasma membrane via a hydrophobic domain undergo a proteolytic cleavage process, which releases the ectodomain to the extracellular milieu in a regulated fashion. Angiotensin-converting enzyme (ACE) is one such protein that is secreted from human and mouse cells by its cleavage at one of two alternative sites in the ectodomain. Here, we report similar cleavage-secretion of ACE in the yeast Pichia pastoris. The cleavage site used in yeasts was identical to one of the two sites used in mouse cells. Moreover, as in mammalian cells, ACE secretion in yeast was inhibited by compound 3, a potent inhibitor of the metalloprotease family of metalloproteases. ACE proteins cleavage-secreted from yeast and from mammalian cells had identical enzymatic properties. These results demonstrate the existence of a secretabe activity in yeast whose properties closely resemble those of the mammalian ACE secretase.

Conversion of a cell-bound protein to a secreted form through the action of specific cell-surface proteases have been reported for several biologically important proteins. These include various membrane-anchored growth factors, cytokine receptors, ectoenzymes, cell adhesion molecules, and β-amyloidprecursor protein (1). This mechanism converts membrane-anchored growth factors into diffusible factors and membrane receptors into soluble binding proteins that regulate ligand access to the cell. Alterations in the balance between anchored and soluble forms of proteins can lead to a diseased state. For example, failure to cleave the β-amyloid precursor protein ectodomain properly in brain cells may lead to fragment formation in the protein in the lysosomes with generation of fragments that accumulate, forming the β-amyloid plaques characteristics of Alzheimer’s disease (2). Similarly, genetic studies of the mouse steel locus have demonstrated that the membrane-anchored forms of Kit ligand/stem cell growth factors are indispensable for normal development and the soluble forms cannot entirely substitute for them (3). Despite the biological importance of this process, little is known about the nature of the responsible proteases.

We have been studying the characteristics of cleavage-secretion of rabbit ACE in mammalian cells. ACE 89, a mouse epithelial cell line permanently transfected with a rabbit ACE expression vector, synthesizes and secretes enzymatically active ACE (4). Since the transfected cell system produces both cell-bound and secreted forms of ACE, it has been used to understand the process of secretion. Secretion of natural soluble ACE is slow in unstimulated cells, but the process can be enhanced by treatment of cells with phorbol esters (5). The secreted form of native ACE is slightly smaller than the cell-bound form, suggesting that a proteolytic process might be involved in secretion. Indeed, an antibody generated against a C-terminal peptide of ACE recognizes the cell-bound but not the secreted ACE (6). Thus, secretion involves proteolytic removal of the C-terminal domain of membrane-anchored ACE. The exact peptide bond cleaved in this process has been identified by sequencing both the N-terminal residues of the purified C-terminal tail left in the cells after ACE is secreted and C-terminal residues of secreted ACE (5). Cleavage occurs at two different sites. The major cleavage is between Arg663 and Ser664, and a minor alternative cleavage occurs between Arg673 and Val674. Cleavage-secretion of ACE has also been demonstrated in rabbit lung membranes (7) and calf vascular endothelial and rabbit renal epithelial cells (8), where the other isozyme of ACE, ACE, is cleaved. The cleavage activity, for both isozymes of ACE was resistant to inhibitors of serine, chymotrypsin, trypsin, cysteine, aspartate, and elastase type proteases. However, the activity was sensitive to a synthetic hydroxamic acid derivative, compound 3, a known inhibitor of certain metalloproteases. These results, taken together, indicate that the cleavage-secretion of both isozymes of ACE is carried out by an integral membrane metalloprotease which is inhibited by compound 3. Among the other known secretases, tumor necrosis factor-α secretase is also inhibited by hydroxamates (9–11). It, however, cleaves tumor necrosis factor-α between Ala and Val residues, which is different from the specificities of ACE secretase. Other partially characterized secretases, which cleave and secrete transforming growth factor-β, KL-1, KL-2, and β-amyloid precursor protein, exhibit inhibitor profiles (12, 13) and cleavage specificities that are different from those of ACE secretase. Thus, from the limited information available, it appears that ACE secretase is distinct from other known partially characterized secretases.

Several proteolytic processing enzymes have been identified in yeasts (14–17), some of which are homologous to their mammalian counterparts. Recently, a secretase activity has been identified in the yeast, Saccharomyces cerevisiae, that processes human amyloid precursor protein (18). As yeasts offer a versatile experimental system for cellular and molecular biological studies with well characterized strategies for manipulation of genes, we expressed mammalian ACE in the methylotrophic yeast P. pastoris. The expressed rabbit ACE was

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† To whom correspondence should be addressed: Dept. of Molecular Cardiology, FF3, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-444-9057; Fax: 216-444-5263.

The abbreviations used are: ACE, angiotensin-converting enzyme; ACE, tissucal angiotensin-converting enzyme; ACE, pulmonary angiotensin-converting enzyme; CAPS, 3-cydocamino)propanesulfonic acid; Hip-His-Leu, hippuryl-L-histidyl-L-leucine.

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subjected to a proteolytic cleavage that resulted in the release of a soluble ectodomain into the medium, thus demonstrating the presence of an ACE secretase activity in yeasts. Determination of the exact peptide bond cleaved and sensitivity toward the presence of an ACE secretase activity in yeasts. Determination of the exact peptide bond cleaved and sensitivity toward the presence of an ACE secretase activity in yeasts.

Expression of ACET was assessed by measuring enzyme activity in the supernatant media, which was dialyzed extensively against 50 mM potassium phosphate buffer, pH 7.0, containing 150 mM NaCl, prior to enzyme activity measurements. To purify secreted ACET from Pichia ACET, the dialyzed medium was loaded onto a lisinopril-affinity column and eluted as described previously (7, 21). The purified ACET exhibited a single band on SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue. To prepare the cell extract, yeast cell pellets were washed once in ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5% glycerol; 2 ml/g, wet weight), mixed with an equal volume of acid-washed glass beads (500 μm), and lysed by vortexing for 8 × 30 s with intermittent cooling. The lysed membranes were centrifuged for 10 min at 10,000 g, supernatant solution collected and centrifuged again for 2 h at 100,000 g. The resulting pellet was homogenized in 0.1% Triton X-100 in 50 mM Tris-HCl, pH 7.5, kept on ice for 1 h and centrifuged for 2 h at 100,000 g. The supernatant was used as the source of cell-associated ACET (Table II and Fig. 5).

N- and C-terminal Sequencing of Yeast ACET—Purified secreted ACET was desalted on a Hewlett Packard G1004B protein chemistry station and eluted from the reverse phase support by 80% isopropanol alcohol, 0.1% trifluoroacetic acid. The eluted ACET was dried, reconstituted with SDS-sample buffer, and run on a 12.5% mini SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to a Teflon tape using 10 mM CAPS buffer containing 10% methanol in a semidy blotting apparatus. The Teflon tape containing the ACET protein was stained (0.1% Amido Black in 40% methanol, 0.1% acetic acid), destained (30% methanol) and used for N- and C-terminal sequence analysis. N-terminal sequence analysis was performed using an Applied Biosystems model 477 protein sequencer equipped with a blot cartridge. To determine the C-terminal sequence, chemical sequencing of the C terminus was performed using 100–200 pmol ACET protein transferred on the Teflon tape and a Hewlett Packard G1000A C-terminal sequencer.

Western Analysis and Enzyme Activity Measurements—Western analysis was carried out using either anti-rabbit lung ACET (anti-ACE) or anti-C-terminal peptide (anti-peptide) antibody. ACE enzyme activity was measured using hippuryl-l-histidyl-l-leucine (Hip-His-Leu) as substrate (6).

RESULTS AND DISCUSSION

Expression of Rabbit Testicular ACE in P. pastoris—P. pastoris transformed with pHIL-S1-ACE WT (Fig. 1), secreted a low molecular weight form of ACET into the medium (Fig. 3, inset). The ACET activity could be detected in the culture medium within 24 h after addition of methanol to induce production and increased progressively up to 5–7 days (Fig. 3). 0.5% methanol concentration was found to be optimum for the induction of alcohol oxidase (AOX1) promoter (Fig. 1), as increasing or decreasing the amount of methanol decreased the production of ACET. The secreted ACET was purified to homogeneity by a single affinity chromatography step (see "Experimental Procedures"). Pichia ACET exhibited a molecular mass of 90 kDa, which is considerably smaller than 110–102 kDa ACET proteins found in the culture media of ACE 89 cells or transiently transfected HeLa cells (4, 6). After total deglycosylation, the Pichia ACET exhibited a molecular mass of 70 kDa (20), which is similar to the polypeptide molecular mass of secreted ACE in ACE 89 and HeLa systems. Hence the difference in the mass of ACET in mammalian and yeast systems is probably due to a difference in their carbohydrate structure. As

2 The N- and C-terminal sequencing was carried out at the Protein and Carbohydrate Structure Facility of the University of Michigan.
the construct pHIL-S1-ACE WT contains two signal sequences, one from PHO1 and the other from ACE T cDNA, to determine the exact structure of mature secreted Pichia ACE T, the protein was subjected to N-terminal sequencing by Edman degradation. Two sequences were observed: one starting with Arg24 and the other starting with Arg24 of the primary sequence of ACE T (Ref. 19 and “Experimental Procedures”). The rest of the 15 residues sequenced matched perfectly with ACE T sequences from Val35 to Ser49 (19). This indicated that, in the Pichia system, signal cleavage occurred after the ACE signal sequence and not the PHO1 signal sequence. In addition, the cleavage was either between Ala25 and Arg24 or between Arg13 and Arg34. These N-terminal sequences together with the C-terminal sequence (discussed below) indicated that the resulting mature Pichia ACE T is a non-fusion protein and resembled the native enzyme from mammalian cells.

Catalytic Properties of Pichia ACE T—The enzymatic properties of secreted ACE T were determined by its ability to hydrolyze Hip-His-Leu. As earlier kinetic studies have shown that chloride ions are activators of ACE activity (22, 23), the hydrolysis of Hip-His-Leu by the purified enzyme was analyzed in the presence of 0–1 M NaCl (Fig. 4A). The enzyme activity is very low in the absence of added NaCl, reaches a maximum at 250–300 mM, and decreases by 20% at 800 mM NaCl. At 300 mM NaCl, hydrolysis of Hip-His-Leu by ACE T at varying concentrations of the substrate is shown in Fig. 4B. The enzyme exhibited a K_m of 1.1 10^{-3} mM and an apparent turnover number K_cat determined are presented in Table I.

![Fig. 3. Expression of rabbit testicular ACE in P. pastoris. Induction with methanol.](image)

![Fig. 4. Catalytic properties of Pichia ACE T.](image)

![Fig. 5. Secreted Pichia ACE T is C-terminally truncated.](image)

| Source of ACE T | K_m (mM) | K_cat (s^{-1}) | No chloride addition | Lisinopril K_i (nM) |
|-----------------|----------|----------------|----------------------|-------------------|
| ACE 89 cells    | 1.7      | 152            | % activity           | 1                 |
| Transfected HeLa cells | 0.6 | 136            | 1                    | 0.09              |
| Transformed P. pastoris | 1.1 | 146            | 2                    | 0.12              |

*Activity given as a percentage of that at 300 mM chloride.

Evidence that the P. pastoris ACE Secretase Activity and Mammalian ACE Secretase Activity Could Be Related—We have shown previously that ACE secretase activity present in ACE 89 cells, as well as in the primary cultures of rabbit renal proximal tubular epithelial cells, is resistant to inhibitors of various known classes of proteases. None of the inhibitors of trypsin, chymotrypsin, elastase, cysteine, serine, and aspartate, as well as certain inhibitors of metalloproteases, had any effect on cleavage-secretion of ACE T by renal epithelial cells or ACE T by ACE 89 cells. On the contrary, a hydroxamic acid-based metalloprotease inhibitor, compound 3, significantly in-
hindered secretion of both ACET$_T$ and ACET$_P$. Hence, the effect of compound 3 on secretion of Pichia ACET$_T$ was studied. Aliquots of transformed P. pastoris cell suspensions were grown without or with (50 $\mu$M or 100 $\mu$M) compound 3 for 2 days. Methanol was then added to induce synthesis of ACET$_T$, and its secretion was monitored by measuring enzyme activity in the culture media. As shown in Fig. 6, compound 3 significantly inhibited the appearance of ACET$_P$ in the culture medium. As the amount of ACE activity present in the detergent extract of P. pastoris incubated with compound 3 was higher than those grown without any additives (data not shown), it appears that secretion of ACET$_T$, rather than its synthesis, was affected by compound 3. Since ACE is also a metalloprotease, we had to ensure that the observed inhibition of ACE enzyme activity was not due to a direct inhibition of activity by compound 3. This was accomplished by dialyzing the samples extensively and diluting appropriately before enzyme activity assay (see "Experimental Procedures"). Control experiments established that inhibition by compound 3 is completely reversible and that up to 1 $\mu$M concentration, compound 3 does not inhibit ACE enzyme activity, although higher concentrations (100 $\mu$M) does partly inhibit it.

To determine the precise site of cleavage in ACET$_T$ by the yeast secretase activity, the secreted Pichia ACET$_T$ was purified to apparent homogeneity and subjected to automated C-terminal chemical analysis. The derived sequence indicated the three C-terminal residues to be Ser-Gly-Arg. These sequence matched with that of ACET$_T$ residues 671–673, establishing clearly that the cleavage site in Pichia pastoris is between the Arg residue at position 673 and Val residue at position 674. Interestingly, this is one of the two sites used by the mammalian secretase (5). Thus, the yeast secretase not only has the same sensitivity to inhibitors as the mammalian secretases but also cleaves the same peptide bond in ACET$_T$ as the mouse and human enzymes. Although there is an increasing appreciation that cleavage-secretion of ectoproteins is an important regulatory process and that an imbalance in the process may contribute to major clinical conditions such as Alzheimer’s disease or sarcoidosis, the specific secretases responsible for cleaving the corresponding proteins, remain uncharacterized. The results reported here offer the possibility of a new approach to study the mammalian ACE secretase. It appears that P. pastoris contains a very similar enzyme. Since powerful genetic tools can complement biochemical approaches in yeast, it may be more fruitful to pursue the yeast ACE secretase first and then apply the knowledge to study mammalian ACE secretases.

### Table II

| Days after methanol induction | ACE activity | Secreted | % Secreted activity (percent of total*) |
|------------------------------|-------------|---------|---------------------------------------|
|                              | Cell-bound | Secreted|                                       |
| 3                            | 70         | 19      | 21                                    |
| 4                            | 120        | 55      | 32                                    |
| 6                            | 139        | 122     | 47                                    |

* Total activity is the sum of cell-bound and secreted activity at any particular time.

![Fig. 6. Compound 3 inhibits secretion of ACET$_P$ in P. pastoris.](image)

Yeast cells expressing ACET$_P$ (see legend of Fig. 3) were grown in the absence (○) or presence (●, 50 $\mu$M; Δ, 100 $\mu$M) of compound 3. After induction with methanol, culture media were analyzed for ACE activity as described in the legend of Fig. 3. Compound 3, where indicated, was present throughout the experiment.

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