

Identification of a Cytoplasm to Vacuole Targeting Determinant in Aminopeptidase I

Michael N. Oda, Sidney V. Scott, Ann Hefner-Gravink, Anthony D. Caffarelli, and Daniel J. Klionsky

Section of Microbiology, University of California, Davis, California 95616

Abstract. Aminopeptidase I (API) is a soluble leucine aminopeptidase resident in the yeast vacuole (Frey, J., and K.H. Rohm. 1978. *Biochim. Biophys. Acta.* 527:31–41). The precursor form of API contains an amino-terminal 45–amino acid propeptide, which is removed by proteinase B (PrB) upon entry into the vacuole. The propeptide of API lacks a consensus signal sequence and it has been demonstrated that vacuolar localization of API is independent of the secretory pathway (Klionsky, D.J., R. Cueva, and D.S. Yaver. 1992. *J. Cell Biol.* 119:287–299). The predicted secondary structure for the API propeptide is composed of an amphipathic α -helix followed by a β -turn and another α -helix, forming a helix-turn-helix structure. With the use of mutational analysis, we determined that the API propeptide is essential for proper transport into the vacuole. Dele-

tion of the entire propeptide from the API molecule resulted in accumulation of a mature-sized protein in the cytosol. A more detailed examination using random mutagenesis and a series of smaller deletions throughout the propeptide revealed that API localization is severely affected by alterations within the predicted first α -helix. In vitro studies indicate that mutations in this predicted helix prevent productive binding interactions from taking place. In contrast, vacuolar import is relatively insensitive to alterations in the second predicted helix of the propeptide. Examination of API folding revealed that mutations that affect entry into the vacuole did not affect the structure of API. These data indicate that the API propeptide serves as a vacuolar targeting determinant at a critical step along the cytoplasm to vacuole targeting pathway.

IN the yeast *Saccharomyces cerevisiae* soluble proteins enter the vacuole through one of four mechanisms: autophagy (Hirsch et al., 1992; Tsukada and Ohsumi, 1993; Thumm et al., 1994; reviewed in Seglen and Bohley, 1992), endocytosis (reviewed in Nothwehr and Stevens, 1994; Raths et al., 1993; Riezman, 1993), the secretory pathway (reviewed in Pryer et al., 1992), and the cytoplasm to vacuole targeting pathway (Cvt; Klionsky et al., 1992; Harding et al., 1995). In both autophagy and endocytosis there is bulk flow of soluble proteins into the vacuole but to date no soluble resident vacuolar protein has been shown to enter the yeast vacuole by these means.

The majority of resident yeast vacuolar proteins enter this organelle through the secretory pathway (reviewed in Stack and Emr, 1993). Contrary to autophagy and endocytosis, proteins that transit through the secretory pathway are not engulfed by membranes but are translocated across the endoplasmic reticulum (ER) membrane. In a series of vesicle-mediated transport steps, the proteins are carried from the ER through the subcompartments of the Golgi before being directed to the vacuole (reviewed in Pryer et al., 1992). Transit through this portion of the

secretory pathway has been carefully documented for a number of vacuolar hydrolases (reviewed in Klionsky et al., 1990). These proteins translocate across the ER membrane via an NH_2 -terminal cleavable signal sequence or noncleaved internal hydrophobic domain. The signal sequence serves no further purpose once the protein has entered the ER. Delivery to the vacuole is dependent upon the presence of a second targeting signal. For all of the characterized soluble hydrolases, this targeting determinant has been shown to reside in a propeptide domain that is proteolytically removed upon delivery to the vacuole.

Two resident vacuolar enzymes, aminopeptidase I (API)¹ and α -mannosidase, have been found to enter the vacuole in a Sec-independent manner (Yoshihisa and Anraku, 1990; Klionsky et al., 1992). Very little is known about the mechanism by which α -mannosidase enters the vacuole except that the protein contains a vacuolar targeting determinant within the last 157 amino acids (of 1,083 amino acids total) of its carboxyl terminus (Yoshihisa and Anraku,

Address correspondence to Daniel J. Klionsky, Section of Microbiology, University of California, Davis, CA 95616. Tel.: (916) 752-0277. Fax: (916) 752-9014.

1. *Abbreviations used in this paper:* API, aminopeptidase I; CPY, carboxypeptidase Y; cvt, cytoplasm to vacuole targeting; DPAP B, dipeptidyl aminopeptidase B; IB, import buffer; PGK, phosphoglycerate kinase; PrA, proteinase A; PrB, proteinase B; TX-100, Triton X-100; TTBS, Tween-20 tris buffered saline; YNB, yeast nitrogen base; YPD, 1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose.

1990). Substantially more information is available regarding the vacuolar localization of API. Genetic studies in our laboratory have isolated a number of mutants that are defective in transporting API to the vacuole (Harding et al., 1995). These cytoplasm to vacuole targeting (*cvt*) mutants are unaffected in their ability to transport proteins through the secretory pathway. In vitro studies have demonstrated that API targeting requires ATP, a functional vacuolar ATPase and a GTP-binding protein (Scott and Klionsky, 1996).

An additional element essential to understanding cytoplasm to vacuole targeting is identification of the vacuole sorting determinant that directs proteins to this pathway. Knowing the targeting information has been useful in many instances for identifying or confirming components of the cellular sorting machinery for almost every eukaryotic organelle. For example, identification of the targeting determinants in carboxypeptidase Y (CPY; Johnson et al., 1987; Valls et al., 1987) permitted a biochemical confirmation that Vps10p is the CPY receptor (Marcusson et al., 1994). Similarly, analysis of protein import in peroxisomal assembly mutants revealed that the *pas8* mutant was specifically defective in the recognition of proteins using the previously identified peroxisomal targeting signal PTS1 (SKL) (McCullum et al., 1993). In vitro translated *PAS8* protein was shown to bind the PTS1 signal suggesting its potential role as a receptor. Additional experiments including cross-linking to an SKL-containing peptide confirmed this functional role for Pas8p (Terlecky et al., 1995). Finally, nuclear localization sequences have been used in ligand binding techniques to identify binding proteins that play a role in nuclear protein import (Lee and Melese, 1989; Silver, 1989). The isolation of p67, a nuclear localization sequence binding protein, was facilitated by conjugating the histone H2B nuclear localization sequence to an affinity column resin. This in turn led to its eventual cloning (Lee et al., 1991).

Targeting signals also have been used as the basis for genetic selections or screens designed to identify proteins that comprise the targeting machinery. Hybrid proteins containing the HDEL or KKXX endoplasmic reticulum retention motifs were utilized to allow the isolation of the HDEL receptor (Pelham et al., 1988; Lewis et al., 1990) and Ret1p, respectively (Letourneur et al., 1994), involved in the recapture or retention of ER resident proteins. Identification of the *trans*-Golgi network (TGN) localization signals in the Kex2 protease (K. Redding, J. Brickner, and R.S. Fuller, personal communication) and dipeptidyl aminopeptidase A (S. Nothwehr and T. Stevens, personal communication) has enabled the design of genetic screens that have led to the identification of genes involved in localization of these late Golgi membrane proteins. Recently, a tyrosine-based sorting signal from a mammalian TGN protein was used to identify interacting clathrin-associated protein complexes through the yeast two-hybrid system (Ohno et al., 1995). Finally, the sequestration of the *HIS4* or *URA3* gene products fused behind an ER signal sequence or a matrix targeting signal, respectively, were used in genetic selections to identify components of the ER translocation machinery (Deshaies and Schekman, 1987) or mitochondrial protein import pathway (Maarse et al., 1992).

Turning to the API molecule, the Chou-Fasman index

(Chou and Fasman, 1974) for secondary structure indicates that the API propeptide has a predicted structure composed of an α -helix followed by a β -turn and a second α -helix. A helical wheel projection analysis of the helices revealed that the first α -helix is most likely amphipathic. The presence of an amphipathic α -helix in the propeptide is reminiscent of the propeptides of nuclear-encoded mitochondrial proteins. Unlike mitochondrial presequences, however, the amphipathic α -helix in the API propeptide contains both basic and acidic residues. Since targeting signals are present in the propeptides of many vacuolar proteins, we focused our examination on the propeptide of API.

We found that deleting the entire propeptide from API abrogated vacuolar localization. Smaller deletions within the propeptide combined with a series of random and site directed mutants indicated that the propeptide's amphipathic helix is responsible for the vacuolar targeting of API. The data reported here indicate that the API propeptide is not necessary for folding of the mature portion of the API polypeptide but instead plays a direct role in the entry of API into the yeast vacuole.

Materials and Methods

Strains and Media

Saccharomyces cerevisiae yeast strains used in this study were DKY6224 MAT α *ura3-52 his3- Δ 200 trp1- Δ 901 ade2-101 pep4::LEU2 suc2- Δ 9; DYY101 MAT α *leu2-3, 112 ura3-52 his3- Δ 200 trp1- Δ 901 ade2-101 suc2- Δ 9 GAL Δ ape1::LEU2* (Klionsky et al., 1992); SEY6210 MAT α *leu2-3, 112 ura3-52 his3- Δ 200 trp1- Δ 901 lys2-801 suc2- Δ 9 GAL* (Robinson et al., 1988); TVY1 MAT α *leu2-3, 112 ura3-52 his3- Δ 200 trp1- Δ 901 lys2-801 suc2- Δ 9 GAL Δ pep4::LEU2* (Cereghino et al., 1995).*

Yeast strain DYY101 was backcrossed with strain SEY6211. The resulting progeny were backcrossed with strain SEY6210 to generate strain THY101 (MAT α *leu2-3, 112 ura3-52 his3- Δ 200 trp1- Δ 901 ade2-101 suc2- Δ 9 GAL Δ ape1::LEU2*). Strain MOY101 (MAT α *leu2-3, 112 ura3-52 his3- Δ 200 trp1- Δ 901 ade2-101 suc2- Δ 9 GAL Δ ape1::LEU2 Δ pep4::URA3*) was constructed as follows: The *PEP4* gene was cloned into the plasmid pUC4. The HindIII fragment was deleted and replaced with the *URA3* gene. This *pep4* knockout plasmid was obtained from Gustav Ammerer, (Biocentrum Vienna, Austria). The plasmid was linearized with BamHI and used to transform strain THY101. Ura⁺ Leu⁺ colonies were selected and the absence of the *APE1* and *PEP4* gene products was confirmed by Western blot.

Escherichia coli strains used in this study were BW313 *HfrKLI6 POI45 [lysA(61-62)] dut-1 ung-1 thi-1 relA1 spoT-1*; MC1061 *F-araD139 Δ (araABOIC-leu) 7679 Δ (lac)x74 galU galK rpsL hsr- hsm+*; DH5 α *ϕ 80dlacZ Δ M15 recA1 endA1 gyrA96 thi-1 hsdR17 (r_K^- , m_K^+) supE44 relA1 doeR Δ (lacZYA-augF)U169*.

SLM medium (1 M sorbitol, 1% glucose, 1% proline, Wickerham's salts, pH 7.5; Guthrie and Fink, 1991), SMD medium (0.067% yeast nitrogen base [YNB], 2% glucose, and vitamins and auxotrophic amino acids as required), and YPD medium (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose; Sherman et al., 1979) were used for growing yeast. LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NaCl) was used for growing *E. coli*.

Reagents

Autofluor was from National Diagnostics, Inc. (Manville, NJ); Immobilon-P was from Millipore Corp. (Bedford, MA); DNA restriction and modifying enzymes were from New England Biolabs, Inc. (Beverly, MA); GTG and LE agarose were from FMC BioProducts (Rockland, ME); luminol was from Sigma Chem. Co. (St. Louis, MO); lyticase was obtained from EnzoGenetics (Corvallis, OR); papain and proteinase K were from Boehringer Mannheim Biochemicals (Indianapolis, IN); Sequenase was from United States Biochemicals (Cleveland, OH); ³⁵S-methionine and Expre³⁵S-label were from DuPont NEN Research Products (Boston,

MA); unstained and prestained protein molecular mass markers were from Bio-Rad Laboratories (Cambridge, MA); Wizard PCR purification system was from Promega (Madison, WI); and bacto-yeast extract, bacto peptone, and yeast nitrogen base without amino acids were from Difco Labs. (Detroit, MI). Antiserum to API was prepared as described (Klionsky et al., 1992). Antiserum to CPY and proteinase A (PrA) were prepared as described (Klionsky et al., 1988). Antiserum to phosphoglycerate kinase (PGK) was generously provided by J. Thorne (Baum et al., 1978). Goat anti-rabbit IgG-HRP antiserum was from Cappel (Durham, NC).

Plasmid Construction

A single copy of the *APE1* gene, which encodes the API enzyme (Cueva et al., 1989; Chang and Smith, 1989), was subcloned into the single copy number (centromeric) plasmid pRS414 (Sikorski and Hieter, 1989) to generate the plasmid pFBAPicΔX as follows: a 2.1-kb BamHI fragment from pRN1, encoding the promoter and the NH₂-terminal first 400 amino acids of the API ORF, was cloned into the BamHI site within the polylinker of pRS414 resulting in the plasmid pAPIBamc. The resulting pAPIBamc plasmid was then restriction digested with KpnI, cleaving within the polylinker and 382 bp from the 3' end of the *APE1* BamHI fragment. A 1.5-kb KpnI fragment from pRN1, encoding the COOH-terminal 227 amino acids of the API ORF, was cloned into the pAPIBamc plasmid, restoring the *APE1* gene to full-length and resulting in the pFBAPic plasmid. The XhoI site at the 3' end of the *APE1* gene was destroyed as follows: a NotI and a partial XhoI digest of the plasmid pFBAPic was performed to isolate the *APE1* gene. The plasmid pRS414 was digested with XhoI and the restriction site overhang was filled in with the Klenow fragment of DNA polymerase I. The newly synthesized blunt ends were ligated to produce pRS414ΔX, where the XhoI site was destroyed. The NotI/XhoI fragment of the *APE1* gene was then ligated into a NotI/Sall digested pRS414ΔX plasmid, destroying the XhoI site at the 3' end of the *APE1* gene by ligating it to the compatible Sall cohesive end, yielding the pFBAPicΔX plasmid. Finally, the filamentous phage origin of replication in pFBAPicΔX was used to synthesize a single stranded DNA template in the *E. coli dut ung* strain BW313 (el-Hajj et al., 1992). An oligonucleotide was synthesized starting at nucleotide -28 of the *APE1* gene and ending at nucleotide 11 (Cueva et al., 1989; Chang and Smith, 1989) and encoding a BglII restriction site at nucleotide -10 (APIBGL5, 5'CCA ACA AAA TTA AAA CAA GAT CTA AAA GAA TGG). This oligonucleotide and DNA template were used in an *in vitro* extension reaction to introduce a BglII restriction site into the *APE1* gene, 8 bp upstream of the ATG start codon (Kunkel, 1985; Kunkel et al., 1987) and yielded the final product pFBAPicΔX. The pFBAPicΔX plasmid was used for mutagenesis of the DNA encoding the API propeptide and the subsequent expression of the altered API. Routine molecular biology methods were carried out according to Sambrook (Sambrook et al., 1989).

Propeptide Deletion Construction

The Δ2-45, Δ4-13, and Δ31-40 deletions in the propeptide were constructed as follows: a 1.8-kb BamHI/XbaI fragment from the plasmid pRN1 (Klionsky et al., 1992) was ligated into the BamHI/XbaI sites of the M13 mp19 vector. Single strand DNA was prepared and used for a template in *dut ung* mutagenesis by the Kunkel method (Kunkel, 1985; Kunkel et al., 1987). The mutagenic oligonucleotide primers were as follows: APIΔ4-13, 5' GAA TGG AGG AAA CTC TGC AGA TGC; APIΔ31-40, 5'CAA ATC GCC AAC TCG TGG TGC ATC C; APIΔ2-45, 5'CAA GAA AAA AAA GAA TGG AGC ACA ATT ATG AGG. The BamHI/XbaI fragment containing the mutated DNA was excised from mp19 and ligated into the BamHI/XbaI sites of pSEYC306 (Johnson et al., 1987). A 1.5-kb XbaI/Sall fragment from pRN1 was cloned at the 3' end of this insert by ligating into the XbaI/Sall sites to generate the full-length *APE1* gene containing the propeptide deletions.

Random Mutagenesis

Random mutants in the API propeptide-encoding regions were generated by PCR mutagenesis as described by Muhlrad et al. (1992). In our mutagenesis the oligonucleotide primers used were: RSFWD starting at nucleotide 2055 and ending at nucleotide 2032 of the plasmid pRS414, 5'-GAC CAT GAT TAC GCC AAG CGC GCA; and APIPCRMI starting at nucleotide 211 and ending at nucleotide 188 of the *APE1* gene, 5'-ACT ACA TGG TAA GTG GTA GGG TTC. For mutagenesis, the PCR reac-

tions were conducted with a disproportionate level of either adenosine or guanosine (one-tenth the concentration of the three other nucleotides). The PCR reactions were titrated with 0 to 8 mM MnCl₂ to determine a MnCl₂ concentration where the yield of amplified product dropped to ~50% of that in the 0 mM MnCl₂ reaction (Muhlrad et al., 1992). The resulting PCR product was separated from excess primer and nucleotide by passage through a Wizard PCR purification matrix. The purified PCR product was added to a yeast transformation reaction in the presence of pFBAPicΔX, which had been restriction gapped with BglII and XhoI. The resulting transformation/recombination product incorporated mutated DNA in place of the absent propeptide-encoding DNA of the pFBAPicΔX plasmid. To identify mutants, protein extracts were prepared from transformants and analyzed by Western blot using antiserum to API (Klionsky et al., 1992). Mutations were confirmed by dideoxy sequencing. The percentage of transformants containing a mutation was ~5%.

Directed Mutagenesis

Site directed mutagenesis was carried out via PCR, using the "mega-primer" method of incorporating mutations (Landt et al., 1990; Perrin and Gilliland, 1990; Sarkar and Sommer, 1990; Marini et al., 1993). The mutagenesis was carried out in two PCR reactions. The first reaction required the use of the RSFWD primer and the mutagenic primer to amplify the promoter region and a portion of the API propeptide-encoding DNA, using the pFBAPicΔX plasmid as a template. The second reaction used the APIPCRMI primer and the product of the first reaction to extend the first product through the propeptide-encoding region to nucleotide 211 of the *APE1* gene. The product was digested with BglII and XhoI and subcloned into pFBAPicΔX. Deletion mutations were screened by molecular mass shift of a BglII/XhoI restriction fragment on a 3%/1% GTG/LE agarose gel and verified by dideoxy sequencing. Substitution mutations were screened by dideoxy sequencing.

Pulse Chase Analysis and Immunoprecipitation of API

Whole cell radiolabeling and API immunoprecipitation were carried out essentially as described previously (Klionsky et al., 1992). Yeast cells were grown to midlog phase (approximate OD₆₀₀ 0.8), isolated by centrifugation and incubated at 30°C. Cells were labeled with [³⁵S]methionine for 20 min followed by a nonradioactive chase for times indicated in the experiment. The chase reaction was terminated by the addition of trichloroacetic acid (TCA) to 10% and a protein extract was prepared by glass bead lysis. Immunoprecipitation was carried out at 4°C using antiserum to API (Klionsky et al., 1992).

Spheroplast Preparation and Subcellular Fractionation

The vacuolar localization of API was assayed as described previously (Harding et al., 1995; Scott and Klionsky, 1995). Briefly, spheroplasts were subjected to differential osmotic lysis to gently lyse the plasma membrane while maintaining vacuolar integrity. One fraction was precipitated with 10% TCA (Fig. 5, T). Two other fractions were treated with 50 μg/ml proteinase K (30 min at 4°C), in the absence or presence of 0.1% Triton X-100 (Fig. 5, T+Proteinase K, T+TX100) before precipitation with 10% TCA. Membrane pellet and supernatant fractions were separated by centrifuging at 5,000 g for 5 min. The supernatant (Fig. 5 S) containing cytosol was removed and both the supernatant and the pellet fractions (containing vacuoles; Fig. 5 P) were precipitated with 10% TCA and prepared for SDS PAGE/Western blot analysis.

Protease Studies

Yeast cells were grown to midlog phase (approximate OD₆₀₀ 0.8), isolated by centrifugation and spheroplasted as described (Klionsky et al., 1992). Cells were radiolabeled with [³⁵S]methionine for 15 min. The radiolabeled spheroplasts were washed with ice cold SLM, isolated by centrifugation, and resuspended to 10 OD₆₀₀/ml in 50 mM Tris, pH 7.5, 10 mM EDTA, 0.2% Triton X-100 for proteinase K or 50 mM Tris, pH 6.8, 0.2% Triton X-100 for papain. Proteolytic digestion was carried out by the addition of proteinase K or papain to a final concentration of 50 μg/ml. The papain and proteinase K digests were incubated 30 min at 37 and 4°C, respectively. The digests were terminated by the addition of TCA to a concentration of 10%. Immunoprecipitation from the radiolabeled digest extracts was carried out at 4°C using antiserum to API (Klionsky et al.,

1992). The immunoprecipitated API was analyzed by SDS PAGE and autoradiographed.

API Binding and In Vitro Import Studies

20 OD₆₀₀ equivalents of spheroplasts were pulse labeled for 5 min, pelleted and resuspended in IB (100 mM sorbitol, 100 mM KCl, 50 mM KOAc, 20 mM K-Pipes, pH 6.8, 5 mM MgCl₂). The resulting permeabilized cells were centrifuged at 5,000 g for 5 min. 2 OD₆₀₀ equivalents of the resulting supernatant and pellet fractions were subjected to immunoprecipitation, SDS PAGE and quantitation with a Fuji FUJIX BAS1000 Bioimaging Analyzer (Fuji Medical Systems, USA, Stamford, CT). The remaining 18 OD₆₀₀ equivalents of the pellet fraction were subjected to in vitro import reactions (Scott and Klionsky, 1995). Briefly, the pellet fraction was resuspended in 1 ml IB containing 1 mM ATP, 40 mM phosphocreatine, and 0.2 mg/ml creatine kinase. The reactions were incubated at 30°C for 2 h and API was immunoprecipitated from a vacuole-enriched fraction collected by flotation through a Ficoll gradient as described (Scott and Klionsky, 1995).

Protein Electrophoresis and Immunoblotting

All SDS PAGE was carried out as described (Laemmli, 1970). Protein samples were resolved by SDS PAGE, transferred onto Immobilon-P and blocked in 5% nonfat milk in TTBS. The blots were probed with primary rabbit antiserum to API diluted 1:20,000 in TTBS, washed, probed with secondary goat anti-rabbit IgG conjugated to horseradish peroxidase, and then developed by luminol chemiluminescence.

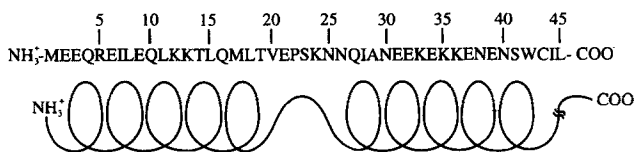
Results

The API Propeptide Is Required for Vacuolar Localization

API is synthesized as a precursor containing an amino-terminal propeptide. Analysis of the Chou-Fasman index (Chou and Fasman, 1974) for predicted secondary structure of the propeptide indicated the presence of two α -helices separated by a β -turn (Fig. 1 A). A helical wheel projection analysis of the helices indicated that the first helix may form an amphipathic structure (Fig. 1 B). The participation of propeptides in the transport of a majority of posttranslationally targeted proteins and the unusual predicted secondary structure for the API propeptide led us to examine its role in vacuolar localization.

We investigated the participation of the propeptide in vacuolar delivery through mutational analysis. Yeast cells disrupted at the chromosomal *APE1* locus (DYY101) were transformed with plasmids in which the entire propeptide-encoding segment (Δ 2-45) or smaller domains were deleted. Cells were converted to spheroplasts, labeled with [³⁵S]methionine and subjected to a differential osmotic lysis procedure which ruptures the plasma membrane while maintaining the integrity of the vacuole (Scott and Klionsky, 1995). The lysed spheroplasts were separated into membrane pellet and supernatant fractions, as described in Materials and Methods. Each fraction was immunoprecipitated with antiserum to API, carboxypeptidase Y (CPY; vacuolar marker) and phosphoglycerate kinase (PGK; cytosolic marker). Deletion of the entire propeptide (Δ 2-45) resulted in accumulation of API outside the vacuole in the supernatant fractions (Fig. 2). Similar results were obtained when 10 amino acid fragments were deleted from either of the predicted α -helices (Δ 4-13, Δ 31-40). This suggests that targeting and/or translocation of API is dependent on information within the propeptide.

A.



B.

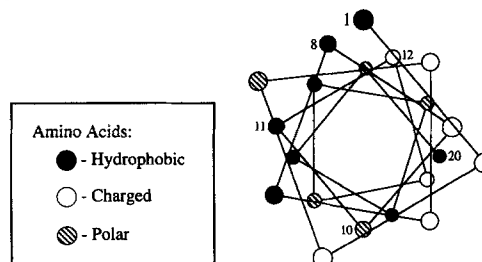


Figure 1. Amino acid sequence and predicted secondary structure of the API propeptide. Chou and Fasman analysis predicts that the first 18 amino acids exist in an α -helix, amino acids 20 to 24 form a β -turn, and the last 20 amino acids of the propeptide form a second α -helix. (A) These structures are lined up with their respective amino acid constituents. (B) The helical wheel projection shows the first 20 amino acids of the API propeptide to indicate that the hydrophobic amino acids are arrayed primarily on one face of the helix and charged or polar amino acids primarily on the other, forming an amphipathic structure. The amino acids and their respective characteristics are represented as follows: hydrophobic ●, charged ○, and polar ⊗.

The Amphipathic α -Helix Contains Targeting Information

The deletion of large stretches of amino acids from the propeptide could cause significant alterations in the secondary or tertiary structure of other targeting domains of the API protein and induce missorting events. To further characterize the targeting information and minimize structural alterations, random mutagenesis was employed to generate point mutants. Random mutations were generated in the API propeptide through PCR-mediated mutagenesis of the *APE1* gene on a plasmid as described in Materials and Methods. The mutated DNA was transformed into the yeast strain DYY101. We identified ~30 mutants (from 600 recombinant/transformants) by Western blot analysis that were defective for API processing.

Sequencing of the plasmid DNA from yeast cells expressing defectively processed API revealed that all of the mutations that affected API processing were in the predicted amphipathic α -helix (Table I). The only exception was a substitution of proline at position 22 with leucine (denoted P22L). The remaining mutations were localized to a region spanning amino acids 6-15 and affected processing to varying degrees as shown in Fig. 3. In some cases, processing was completely blocked (Fig. 3, lane 2), whereas in others it was only partially affected (Fig. 3, lane 3) or resulted in a species of intermediate molecular mass

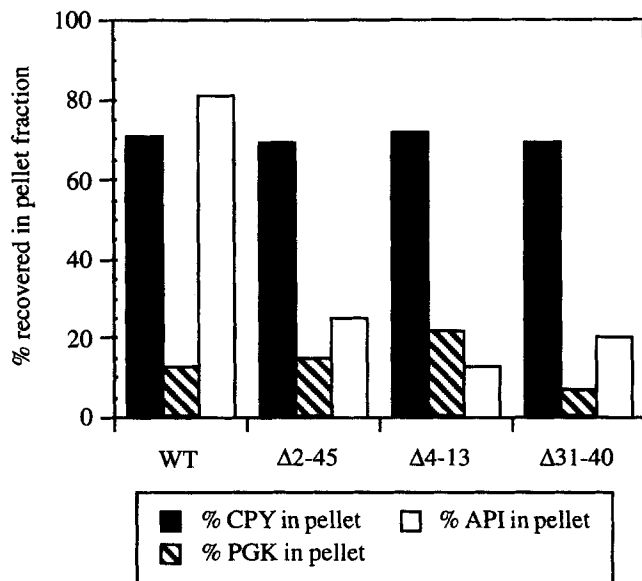


Figure 2. The API propeptide is required for vacuolar localization. DYY101 (*Δape1*) cells harboring a single copy plasmid encoding either the wild type, Δ2-45, Δ4-13, or Δ31-40 forms of API were grown, converted to spheroplasts, labeled, and separated into the vacuolar membrane pellet and cytosolic supernatant fractions as described in Materials and Methods. CPY, PGK, and API were immunoprecipitated from both fractions and analyzed by SDS PAGE and autoradiography. The relative levels of the different proteins in each fraction were then quantitated with phosphorimage analysis. The percent recovered in the pellet fraction was calculated by dividing the value for the pellet by the combined values for pellet plus supernatant fractions.

between that of the precursor and mature forms (Fig 3, lane 4). Changes that would be predicted to alter the hydrophobic nature of the amphipathic first helix such as L8R and L11S (Fig. 1 B) resulted in complete blocks in processing. A similar dramatic block in processing was seen when the helix was disrupted due to the substitution of leucine at position 15 with proline (L15P). The M1V

Table I. Random Mutations in the API Propeptide

Mutation	API Processing*
M1V	Intermediate
E2A	WT
E3G	WT
E6G	Complete Block
L8R	Complete Block
Q10R	Major Block
L11S	Complete Block
K12R	ts: Major Block
K12E	Major Block
T14A	WT
L15P	Intermediate
Q16R	WT
M17V	WT
P22L	Major Block

*Phenotype is represented as follows: WT, wild type >90% mature; Complete Block, >90% precursor; Major Block, ~50% precursor; Intermediate, processing to or synthesis of a species intermediate in relative molecular mass between precursor and mature API.

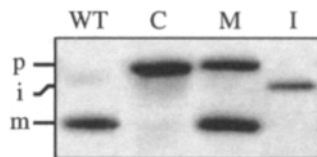


Figure 3. Mutations within the first helix of the API propeptide give rise to a variety of phenotypes. Shown is a 9% SDS PAGE immunoblot of whole cell extracts, probed with antiserum to API, depicting: WT, wild type; C, complete block to API maturation (Δ9-11); M, major block to API maturation (K12E); and I, an intermediate processing or synthesis of API (M1V). The positions of precursor, intermediate, and mature API are indicated.

mutation resulted in the synthesis of an intermediate-sized species that does not undergo further processing. The elimination of the methionine at position 1 may result in diverting the translation start site to the methionine at position 17. This start point would remove the putative first helix and, in a manner similar to the Δ4-13 deletion, would prevent processing of the remaining propeptide.

The absence of a mutation in the predicted second α-helix that blocked API import was surprising given our initial results that indicated that both helices were essential for proper transport. Since the initial survey was carried out with deletions of ten amino acids, it is possible that deletions of this size in one helix may affect the function or presentation of the other helix. Alternatively, the random mutagenesis may not have been sufficiently comprehensive to reveal critical residues in the second helix. To address these possibilities, a series of three-amino acid deletions that encompass the entire propeptide was created. Similar to the random mutagenesis results, the deletions in the predicted amphipathic α-helix severely impaired API processing (Table II; Fig. 4 A, lanes 2 and 3). In contrast, deletions in the second predicted α-helix had no effect on the processing of API (Table II; Fig. 4 A, lanes 4 and 5).

Table II. Effects of Site Directed Mutations on API Processing/Targeting

Mutation*	API Processing [†]	Localization [‡]	Processing Kinetics [§]
Δ3-5	Complete Block	N/D	None
Δ6-8	Complete Block	N/D	None
Δ9-11	Complete Block	Cytosolic	None
Δ12-14	Complete Block	Cytosolic	None
Δ15-17	Complete Block	Cytosolic	None
Δ18-20	Complete Block	Cytosolic	None
Δ25-27	WT	N/D	N/D
Δ28-30	WT	Vacuolar	N/D
Δ31-33	WT	Vacuolar	WT
Δ34-36	WT	Vacuolar	WT
Δ37-39	WT	N/D	N/D
Δ40-42	WT	N/D	N/D
Ala 11	Complete Block	Cytosolic	None
Ala 34	WT	Vacuolar	WT
P22L	Major Block	Membrane	None

*The Δ designation denotes the deletion of the indicated amino acids. Ala11 and Ala34 are insertion mutations. P22L is a substitution mutation.

[†]API processing was determined by immunoblot detection from a 9% acrylamide SDS gel as described in Fig. 4.

[‡]Localization of API mutants was based on the percent API present in the supernatant fraction versus total of the supernatant and the vacuole containing pellet fraction as described in Fig. 5.

[§]Processing kinetics were examined by pulse chase analysis of DYY101 cells expressing mutated forms of API as described in Fig. 6. In the case of processing-defective mutations, rates of propeptide cleavage were indeterminable (None). N/D, not determined.

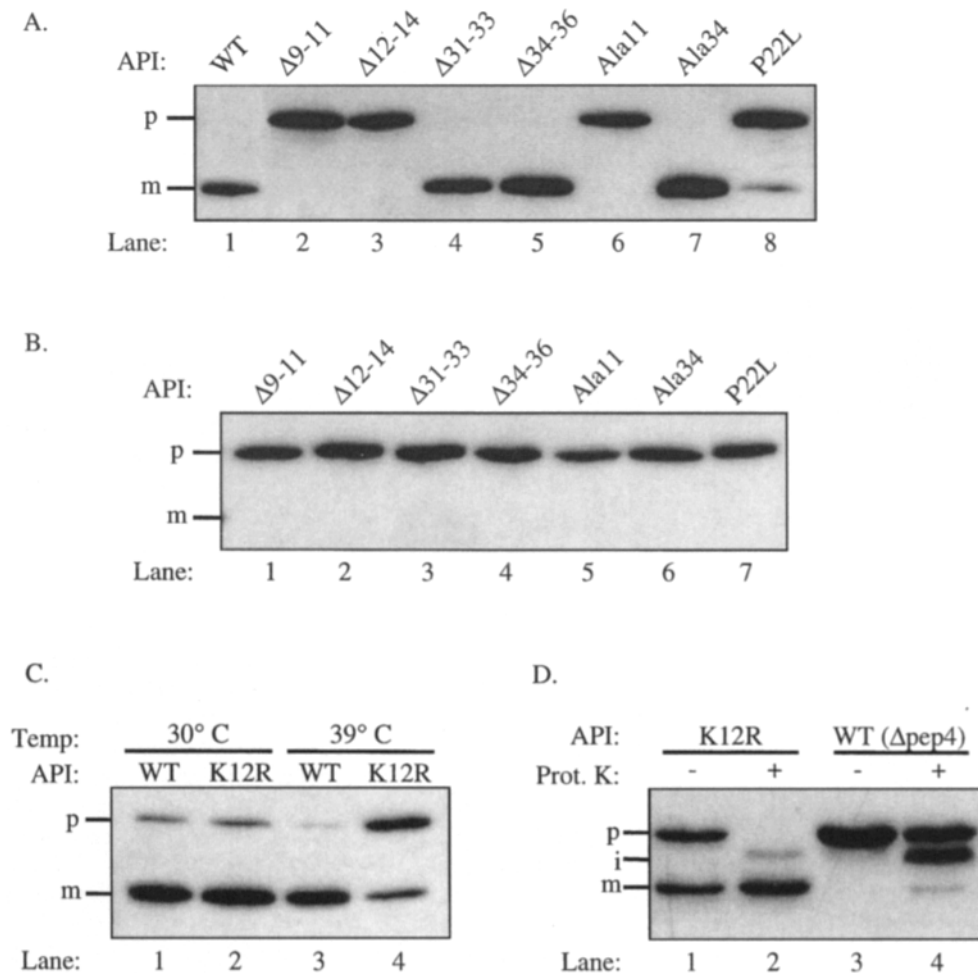


Figure 4. (A) Processing of API is affected by mutations that alter the predicted first α -helix and β -turn but not by mutations within the predicted second α -helix. Samples were prepared from whole cell extracts from DYY101 ($\Delta ape1$) cells harboring a single copy plasmid encoding the different forms of API as indicated. Protein extracts were prepared and examined by immunoblot using antiserum against API as described in Materials and Methods. (B) The processing of the second helix mutants is a proteinase A-dependent event. Protein extracts were prepared from MOY101 cells ($\Delta ape1 \Delta pep4$) harboring single copy plasmids encoding the different forms of API. Proteins were resolved by 9% SDS PAGE and API was detected by immunoblot. (C) The point mutation K12R imparts a temperature sensitive defect for API processing. DYY101 yeast cells containing a single copy plasmid expressing either wild type or the K12R mutant API were grown overnight at 30 or 39°C in SMD. Protein extracts were obtained by glass bead lysis and the re-

sultant samples were examined by 9% SDS PAGE and immunoblot. (D) The K12R mutant precursor API accumulated at 39°C is accessible to the cytosol. Spheroplasts were prepared from DYY101 cells expressing the K12R mutant API and TVY1 ($\Delta pep4$) cells containing the wild-type API-encoding plasmid pBAPIC Δ X grown at 39°C. The spheroplasts were subjected to differential osmotic lysis to preserve vacuolar integrity. The total lysate was divided into two fractions, untreated and proteinase K treated (50 μ g/ml final concentration). Both fractions were resolved by 9% SDS PAGE and API was detected by immunoblot. The positions of precursor, mature, and intermediate API are indicated.

API is processed in the vacuole from its precursor to mature form by the enzyme proteinase B (PrB) (Klionsky et al., 1992). PrB maturation is dependent on the *PEP4* gene product proteinase A (PrA) so that $\Delta pep4$ mutants accumulate precursor API. To determine whether the API second helix mutations (Ala34, $\Delta 31-33$, and $\Delta 34-36$) were being processed in the vacuole we examined maturation in a $\Delta ape1 \Delta pep4$ strain (MOY101). Whole cell extracts from the $\Delta ape1 \Delta pep4$ cells harboring plasmids encoding mutant API were examined by SDS PAGE and immunoblot analysis (Fig. 4 B). While the mutant API are processed to the mature form in a *PEP4* strain they are not processed in the $\Delta pep4$ strain (Fig. 4 A, lanes 4, 5, and 7 and Fig. 4 B, lanes 3, 4, and 6), indicating that processing of the API second helix mutants is *PEP4* dependent.

Disruption of the putative β -turn by substituting the β -turn initiating amino acid proline with the β -turn terminating amino acid leucine (P22L) resulted in a mutant exhibiting a severe block in processing (Fig. 4 A, lane 8).

Similarly, disrupting the periodicity of the first amphipathic α -helix by insertion of an alanine at position 11 (Ala11) caused a loss of processing (Fig. 4 A, lane 6). In contrast, when the periodicity of the second helix was similarly disrupted there was no apparent change in API processing as was seen with the Ala34 mutation (insertion of an alanine at position 34; Fig. 4 A, lane 7). These results indicate that API targeting is sensitive to changes in periodicity and content of the predicted amphipathic α -helix but insensitive to similar changes in the second helix.

A Conditional Mutant Identifies a Highly Sensitive Amino Acid within the Amphipathic α -Helix

Among the random mutations in the API propeptide, K12E caused a major block in API processing (Table I). This result suggests that the specific charge and/or size of this residue are important for correct vacuolar localization. During the course of examination of the random mu-

tants, the cell extracts from a series of mutants were examined by SDS PAGE and Western blot analysis after growth at 39 and at 30°C. From this survey we found one mutant which caused a temperature sensitive defect for API processing (Fig. 4 C). This mutant was the result of a substitution of a lysine residue at position 12 for arginine (K12R).

To determine if the accumulated precursor form of the K12R point mutant is cytosolic or vacuolar, DYY101 ($\Delta pep1$) cells expressing the K12R point mutant and TVY1 ($\Delta pep4$) cells containing a wild type API encoding plasmid were grown at 39°C, converted to spheroplasts, and subjected to differential osmotic lysis to preserve the integrity of the vacuole. The lysed spheroplasts were treated with proteinase K and analyzed by Western blot (Fig. 4 D); under these conditions cytosolic precursor API is cleaved to the mature size, while mature API is relatively resistant to degradation (Harding et al., 1995). The precursor form of the K12R point mutant API was sensitive to proteinase K digestion (Fig. 4 D, lanes 1 and 2), suggesting that it is not enclosed in a membrane-bound compartment and faces the cytosol. The integrity of the vacuoles in these preparations was confirmed by examining the proteinase K-sensitivity of API in the $\Delta pep4$ cells (TVY1) expressing wild-type API. API expressed in a $\Delta pep4$ strain enters the vacuole but is not processed. The precursor form of API in the $\Delta pep4$ cells is protected from degradation (Fig. 4 D, lanes 3 and 4) indicating its localization within the vacuole. The presence of an intermediate species may be due to vacuolar lysis during sample preparation. The import and processing defect seen with the K12R mutant suggests that the presence of a basic residue at this position is not sufficient for proper function; there may be a strict size limitation for this part of the helix.

Localization of API Mutants

To examine the localization of the API propeptide mutants, subcellular fractionation experiments were performed. DYY101 cells containing plasmids encoding wild type or altered forms of API were converted to spheroplasts and were subjected to differential osmotic lysis to preserve the integrity of the vacuole. The lysed spheroplasts were then separated into membrane pellet and supernatant fractions. Each fraction was examined for the presence of API and both the vacuolar and cytosolic marker proteins, PrA and PGK, respectively. Efficient lysis of spheroplasts was reflected in the recovery of only 20% of the PGK in the pellet fraction. Conversely, the recovery of greater than 80% of the soluble vacuolar hydrolyase PrA in the pellet fraction is indicative of a high yield of intact vacuoles (Fig. 5 A). Mutations in the first helix resulted in accumulation of precursor API in the supernatant fraction, while those in the β -turn or second helix resulted in mature API localizing to the pellet (containing vacuoles) fraction (Fig. 5).

To further examine the location of API, we carried out a proteinase K sensitivity assay. After differential osmotic lysis (Fig. 5 A), the samples were incubated with protease in the presence or absence of the detergent TX-100 (Fig. 5 B). Mature API is relatively resistant to degradation under these conditions, while precursor API is cleaved to the ma-

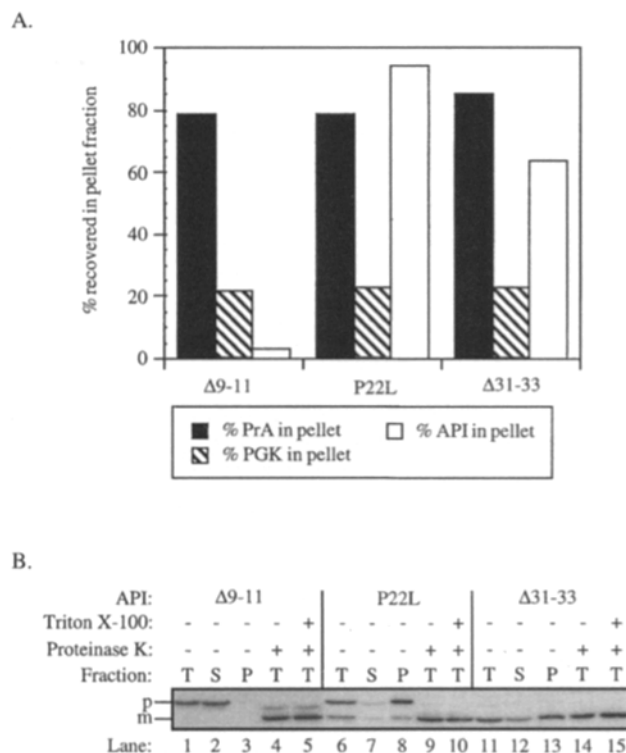


Figure 5. The processing defect in API propeptide mutants is due to the inability of the API molecule to enter the vacuole. (A) Fractionation of API propeptide mutants. DYY101 ($\Delta pep1$) cells containing plasmids encoding versions of API with altered propeptides were separated into membrane pellet and supernatant fractions as described in Materials and Methods. The presence of the vacuolar marker enzyme PrA, the cytosolic marker enzyme PGK, and API were detected in the pellet and supernatant fractions by immunoblotting. (B) Protease sensitivity assay of API propeptide mutants. DYY101 cells containing a single copy plasmid encoding the mutated forms of API were fractionated into T, total; S, cytosolic supernatant; and P, vacuolar pellet fractions, as described in Materials and Methods. The total fraction was further divided into thirds: untreated, proteinase K treated (50 μ g/ml final concentration), and proteinase K + TX-100 (0.2%) treated. Samples were resolved by SDS PAGE and detected by Western blot using antiserum to API. The $\Delta 12-14$, $\Delta 15-17$, Ala11, and L15P mutations gave results essentially identical to $\Delta 9-11$; $\Delta 28-30$, $\Delta 34-36$, and Ala34 were like $\Delta 31-33$.

ture size (Harding et al., 1995). Mutations in the amphipathic α -helix generated precursor API that was sensitive to protease in the absence of detergent (Fig. 5 B, lane 4). This indicated that these altered proteins were not accumulating within a membrane-enclosed compartment and were present in the cytosol. Analysis of the P22L mutant showed that although it fractionated with the pellet fraction (Fig. 5 A and Fig. 5 B, lanes 7 and 8), it was accessible to proteolytic digestion in the absence of detergent (Fig. 5 B, lane 9). This indicated that the P22L mutant also was not enclosed in a membrane-bound compartment and may have been associating with the cytosolic face of the vacuolar membrane. Mutations in the second helix, as exemplified by $\Delta 31-33$, did not affect localization of API. Just as the wild type API, the mature form of the protein gener-

ated from the $\Delta 31-33$ mutation was not degraded by proteinase K in the absence or presence of detergent (Harding et al., 1995; Fig. 5 B, lanes 14 and 15).

Second Helix Mutants Are Processed with Wild-type Kinetics

Mutations in the second helix of the propeptide did not affect API processing under steady state conditions. It is possible, however, that these mutations significantly slowed the rate of vacuolar import. To address this possibility we carried out a kinetic analysis of processing. Yeast cells harboring a plasmid containing either a wild-type copy of the *APE1* gene or a copy with a mutation in the sequence encoding the second helix were labeled with [35 S]methionine for 20 min and subjected to a nonradioactive chase for 0–90 min. The second helix mutants showed a rate of processing similar to that of wild-type API, implying a normal rate of vacuolar entry (Fig. 6).

Mutations in the Propeptide Do Not Alter Folding of API

Many proteins, including subtilisin (Zhu et al., 1989), PrA (Van den Hazel et al., 1993; Shinde and Inouye, 1995), CPY (Winther et al., 1994), and α -lytic protease (Silen and Agard, 1989) contain internal chaperones; domains within the protein which function to initiate proper folding. Because API is imported to the vacuole posttranslationally, one possible role for the propeptide could be to maintain the protein in an import-compatible conformation. If this

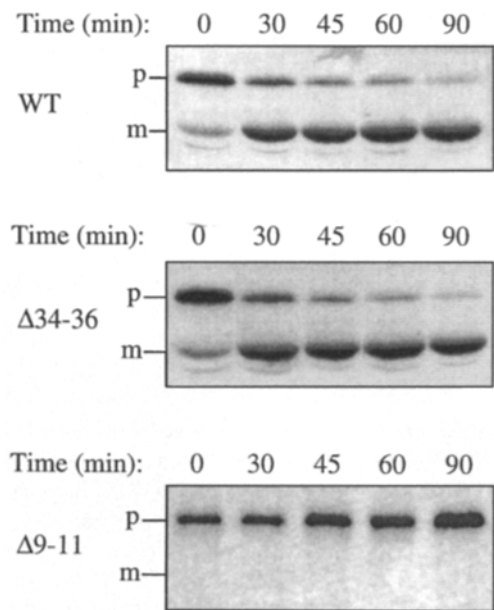


Figure 6. API processing kinetics are unaffected by mutations in the second helix of the API propeptide. DYY101 (*Δape1*) yeast cells containing a single copy plasmid expressing wild-type API, the $\Delta 9-11$ deletion, and the $\Delta 31-33$ deletion were radiolabeled for 20 min and subjected to a nonradioactive chase. At the indicated time points an aliquot was removed and precipitated with TCA. Protein extracts were prepared and immunoprecipitated with antiserum to API as described in Materials and Methods.

is the case, alterations in the propeptide could affect folding of the mature domain. To determine if the amphipathic α -helix had a chaperone function, we studied the folding state of the API mutants through limited protease digestion experiments.

API proteins with wild-type or altered propeptide sequences were subjected to limited digestion with proteinase K and papain under native conditions as described in Materials and Methods. In all propeptide mutants studied, the proteinase K digestion produced a product with a relative molecular mass equivalent to mature API (Fig. 7 A), implying proper folding of the mature portion of API and retention of its protease-resistant character. In contrast, the API 5-4 mutant, which has no mutations within the propeptide encoding sequence but contains four amino acid substitutions within the mature portion of the protein (N48D, Y49H, F73L, and W91R), was completely degraded under these conditions (Fig. 7 A, lane 10). In the papain digest (Fig. 7 B) the wild type and propeptide mutant API were digested from the precursor relative molecular mass of 61 kD down to a size of 58 kD. The 58-kD species does not have a relative molecular mass equivalent to mature (50 kD) but is of an intermediate size. The 5-4 mutant was again completely degraded (Fig. 7 B, lane 10). The papain digest results confirm the proteinase K digest results which indicate that even with mutations in the propeptide, the mature portion of API assumes a wild-type stability and is thus folded properly.

Mutations in the First α -Helix Are Defective in Membrane Association

Previous studies have demonstrated that although API has a half-time for maturation of 30–45 min, a significant amount of newly synthesized API is membrane-associated

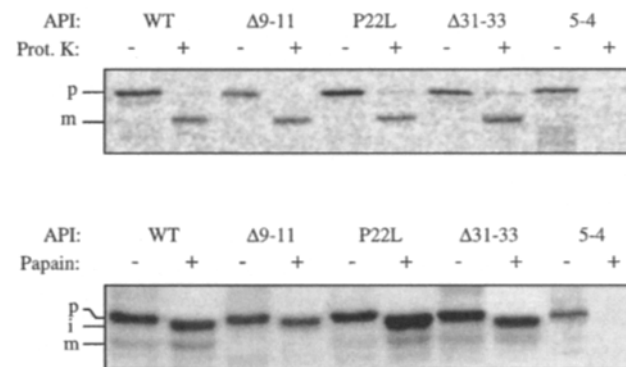


Figure 7. Mutations in the propeptide do not affect folding of API. DYY101 (*Δape1*) cells expressing the wild type, $\Delta 9-11$, P22L, $\Delta 31-33$, and 5-4 forms of API from a single copy plasmid were converted to spheroplasts and radiolabeled for 15 min. The spheroplasts were pelleted and lysed by resuspending in 50 mM Tris, pH 7.5, 10 mM EDTA, 0.2% TX-100 for proteinase K or 50 mM Tris, pH 6.8, 0.2% TX-100 for papain. Native proteins were subjected to digestion with either proteinase K (50 μ g/ml final concentration) at 4°C or papain (50 μ g/ml final concentration) at 37°C, A and B, respectively. API was subsequently immunoprecipitated as described in Materials and Methods. Both panels are composites of two gels.

(Scott and Klionsky, 1995). Since this membrane-associated API is competent for translocation into the vacuole in a subsequent *in vitro* chase reaction (Scott and Klionsky, 1995), the observed pool of membrane-associated precursor must be on the protein import pathway. To determine if the predicted first helix of the API propeptide is involved in membrane association, we performed binding studies with the collection of API propeptide mutants. Spheroplasts were pulse labeled for 5 min and then subjected to differential osmotic lysis in a buffer containing a physiological concentration of potassium salts. This method lyses the plasma membrane while maintaining the integrity of the vacuole, like the differential lysis procedure of Fig. 5 (Scott and Klionsky, 1995). The presence of physiological salt, however, preserves the interaction of API with the membrane fraction, and the population of API examined here was newly synthesized. In cells containing either a wild-type copy of API or the second helix deletion $\Delta 31-33$, over 50% of the labeled API was recovered in the membrane pellet fraction (Fig. 8 A). When the same experiment was performed in cells containing the first helix mutants $\Delta 9-11$, $\Delta 12-14$, and Ala11, the amount of API recovered in the membrane pellet fraction was greatly reduced, suggesting that these mutants are defective in membrane

recognition. Interestingly, the P22L mutation shows a wild-type level of membrane interaction.

In vitro chase reactions were performed to examine whether the bound precursor in these cells was competent to chase into the vacuole. Membrane pellets containing bound precursor were resuspended in import buffer containing ATP and an ATP regenerating system and allowed to import at 30°C for 2 h (Scott and Klionsky, 1995). The only labeled API in these reactions is contributed by the membrane pellet. In both wild-type API and $\Delta 31-33$ containing cells, ~50% of the bound API was matured (Fig. 8 B). In contrast, the membrane bound P22L was defective in translocation into the vacuole. As expected, the first helix mutants, $\Delta 9-11$, $\Delta 12-14$, and Ala11, which are defective for maturation *in vivo* were also defective *in vitro*, and matured only minimal levels of the bound precursor API. The *in vitro* chase reaction indicates that even the lower levels of precursor API that bound in the pellet fraction with the first helix mutations (Fig. 8 A), were not competent for translocation into the vacuole. This could be because the low level of binding observed in the mutants is nonproductive or because the pro-region is also important for a subsequent step on the import pathway, as suggested by the results with the P22L mutation.

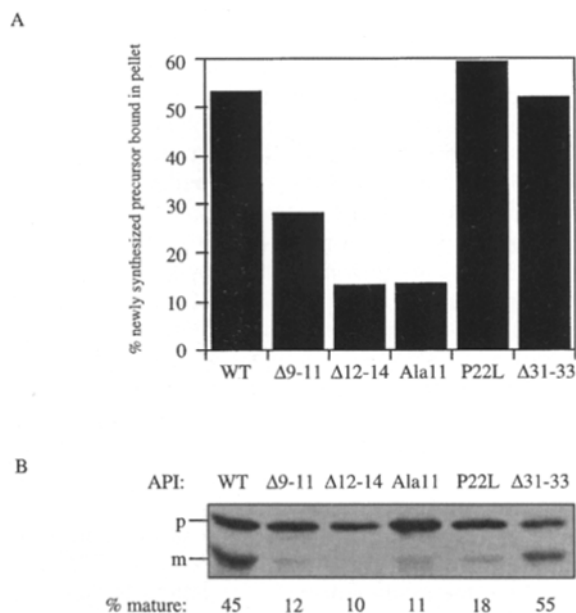


Figure 8. Mutations in the first helix of the API pro-region block the membrane recognition step of the import process. (A) Spheroplasts containing the indicated API constructs were pulse labeled for 5 min, subjected to differential osmotic lysis in IB, separated into pellet and supernatant fractions, and immunoprecipitated with anti-API antibodies as described in Materials and Methods. The percent of API precursor in the pellet fraction was calculated by comparing the amount of API in the pellet fraction to the sum of the API recovered in the supernatant and pellet fractions. (B) Pellet fractions from the experiment in A were subjected to *in vitro* import procedures as described in Materials and Methods. The percent mature API was calculated by dividing the amount of mature recovered after import by the sum of the precursor and mature forms recovered after import.

Discussion

We sought to identify the vacuolar targeting determinant within API for use as a tool to examine the mechanisms underlying the Cvt pathway. Since the vacuole targeting determinants of the known soluble vacuolar hydrolases are within their respective propeptides, we turned our attention to the API propeptide. Deletion of either the entire propeptide or 10-amino acid segments from within either of the two predicted α -helices abrogated delivery of API to the vacuolar lumen (Fig. 2). These results implicate the API propeptide in directing API to the vacuole. A series of 3-amino acid deletions across the API propeptide, site-directed insertions of alanine, and a random mutagenesis survey of the API propeptide revealed that the putative amphipathic α -helix and β -turn are sensitive to change. Both random and site-directed mutagenesis showed that all substitutions that interfered with API processing were within the predicted amphipathic α -helix (Table I and II), with only one exception (P22L). Many of the amino acid substitutions within the propeptide that disrupted API processing were changes that substituted a charged amino acid for a nonpolar amino acid (Table I), suggesting that API processing is sensitive to changes which either obliterate or mask the amphipathic nature of the first α -helix.

The essential role of the first helix for vacuolar localization of API is supported by the alanine insertion mutants. Insertion of an alanine at position 11 severely interfered with both API processing and vacuolar entry but insertion of an alanine at position 34 had no effect on API sorting or processing (Fig. 4 A, lanes 6 and 7). From these results we conclude that the periodicity of the amphipathic α -helix is essential for both API processing and vacuolar import, whereas the periodicity of the second helix does not significantly contribute to these events. Rather, the role of the second helix may be to present the amphipathic α -helix in

the proper context for recognition by the targeting machinery.

The screening method we employed after random mutagenesis was biased toward detecting mutants that affected API processing. For a comprehensive analysis we also employed site directed mutagenesis as a means of methodically examining the propeptide for regions both sensitive and insensitive to change. The findings of this study generally mirrored the findings of the random mutagenesis survey; all mutations affecting targeting were located in the first predicted helix or β -turn (Tables I and II). In addition we found that deletion of amino acids 15–17 or 18–20 blocked import (Table II). This region was not identified in the random mutagenesis survey and these deletions may be affecting API processing by altering the facing of the hydrophobic side of the amphipathic α -helix relative to the predicted β -turn. Alternatively, it is conceivable that amino acids 1–15 may need to be a minimum number of residues away from the β -turn to allow recognition. Finally, it is possible that the original random mutagenesis survey may not have been exhaustive enough to reveal amino acids 15–20 as sensitive to substitution.

To ascertain the cause of the API processing defect resulting from alterations in the first predicted helix we examined both the localization of API (Fig. 5) and the nature of any membrane interactions (Fig. 8). Lysis in the absence of physiological salts disrupts any binding interactions between extra-vacuolar precursor API and the vacuole membrane. Lysis under these conditions allows an assessment of vacuolar localization. By this criterion, alterations in the first helix prevent API from entering the vacuole. Protease sensitivity studies confirmed that these API proteins were accessible in the cytosol (Fig. 5). In the presence of physiological salts precursor API that is bound to the membrane remains bound (Scott and Klionsky, 1995). This material can be examined in subsequent *in vitro* import reactions to determine if it is competent for import. Alterations in the first helix of the propeptide resulted in reduced and nonproductive binding interactions because the low level of bound precursor was not imported *in vitro*.

The discovery that the API propeptide contains a vacuolar sorting determinant prompted us to construct a number of fusion proteins to demonstrate that the propeptide is sufficient for vacuolar localization. All attempts at fusing the API propeptide to other molecules, such as invertase, failed to carry the passenger molecule into the vacuole (data not shown). Failure to target a passenger protein implies that the import pathway or signal of API may be complex, requiring more than one targeting determinant to be present. Preliminary data indicate that a second targeting determinant may reside in the carboxyl-terminal third of API, because COOH-terminal truncations of API block entry into the vacuole (data not shown).

Given the breadth of knowledge of propeptide function in other proteins, we can propose a number of possible roles for the propeptide in API sorting. The most obvious is the possibility that the amphipathic α -helix acts as a sorting signal, which interacts directly with either a cytosolic factor, the vacuolar membrane, or a membrane-bound receptor. The fact that mutations in the first helix prevented productive binding interactions from taking place (Fig. 8) suggests that the amphipathic α -helix of the API

propeptide is necessary for the proper interaction of API with the vacuolar membrane.

An alternative possibility is that the propeptide serves as an intramolecular chaperone and maintains API in a translocation-compatible state. The appearance of charged amino acids and α -helical structures is common to domains that have chaperone activity (Winther and Sorenson, 1991). The observation that alterations in the propeptide did not affect folding or stability of API (as assessed by protease sensitivity; Fig. 7), however, argue against a role for the propeptide in nucleating protein folding. In addition, the fact that the vacuole is a protease-rich environment makes it unlikely that proteins will be imported into this organelle in an unfolded conformation.

Alternatively, API has been reported to exist in the vacuole as a dodecamer and as a less abundant hexamer (Loffler and Rohm, 1979); the amphipathic α -helix may facilitate the formation of these higher order species as demonstrated for the propeptide of Mas70p (Millar and Shore, 1993, 1994). In turn these higher order species may be a prerequisite for vacuolar membrane interaction as in the import of some peroxisomal proteins (McNew and Goodman, 1994). The possibility that the putative amphipathic α -helix is acting as a domain of interaction with a membrane or cytosolic receptor or is driving the formation of higher order API complexes remains to be addressed. The API propeptide amphipathic α -helix contains two overlapping leucine zipper motifs; one at positions 8 and 15 and the other at positions 11 and 18. Since three of these four leucines were identified in the random mutagenesis survey, this motif may play a role in the recognition of an essential component of the transport machinery. Examination of the temperature-sensitive K12R mutation also may shed light on the function of the amphipathic α -helix. The extreme sensitivity of the lysine at position 12 implies a direct functional relevance for this amino acid or a specific size limitation in this region of the propeptide. This size limitation may reflect an interaction between this part of the amphipathic α -helix and a component of the import machinery.

In addition to the functional relevance of the predicted amphipathic α -helix, our results indicate some role for the predicted β -turn. The P22L mutation caused precursor API to associate with the vacuolar membrane even under conditions where the binding interaction of the wild-type precursor was disrupted, i.e., absence of physiological salt (Fig. 5; Harding et al., 1995). This suggests that P22L does not have the same binding properties as the wild-type precursor. The P22L fractionation data imply that the β -turn does not participate in the membrane association step of vacuolar entry but with a succeeding step. This phenotype suggests that the propeptide may have a dual function in vacuolar entry: vacuolar membrane association and a yet unidentified subsequent step. Conversely, the putative second α -helix was shown to not participate in API processing or sorting and may only serve as a means of extending the amphipathic α -helix away from the mature protein and preventing it from misfolding into the hydrophobic core of the mature portion of API.

We have identified a number of mutations in API that interfere with its correct targeting to the vacuole. These mutations fall into one of three classes; those that fail to

bind to the vacuolar membrane, those that bind to the vacuolar membrane but do not enter the vacuole, and those that are temperature sensitive for vacuolar delivery. These mutants will be useful for future studies aimed at elucidating both the mechanism and the molecular components involved in API targeting.

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References

Baum, P., J. Thorner, and L. Honig. 1978. Identification of tubulin from the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*. 75:4962-4966.

Cereghino, J.L., E.G. Marcusson, and S.D. Emr. 1995. The cytoplasmic tail domain of the vacuolar protein sorting receptor vps10p and a subset of vps gene products regulate receptor stability, function, and localization. *Mol. Biol. Cell*. 6:1089-1102.

Chang, Y.H., and J.A. Smith. 1989. Molecular cloning and sequencing of genomic DNA encoding aminopeptidase I from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 264:6979-6983.

Chou, P.Y., and G.D. Fasman. 1974. Prediction of protein conformation. *Biochemistry*. 13:222-245.

Cueva, R., N. Garcia-Alvarez, and P. Suarez-Rendueles. 1989. Yeast vacuolar aminopeptidase yscI. Isolation and regulation of the *APE1 (LAP4)* structural gene. *FEBS Lett.* 259:125-129.

Deshaies, R.J., and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* 105:633-645.

el-Hajj, H.H., L. Wang, and B. Weiss. 1992. Multiple mutant of *Escherichia coli* synthesizing virtually thymineless DNA during limited growth. *J. Bacteriol.* 174:4450-4456.

Frey, J., and K.H. Rohm. 1978. Subcellular localization and levels of aminopeptidases and dipeptidase in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta.* 527:31-41.

Guthrie, C., and G.R. Fink. 1991. Guide to yeast genetics and molecular biology. In *Methods in Enzymology*. Vol. 194. J.N. Abelson and M.I. Simon, editors. Academic Press, Inc., NY. 863 pp.

Harding, T.M., K.A. Morano, S.V. Scott, and D.J. Klionsky. 1995. Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. *J. Cell Biol.* 131:591-602.

Hirsch, H.H., H.H. Schiffer, and D.H. Wolf. 1992. Biogenesis of the yeast vacuole (lysosome). Proteinase yscB contributes molecularly and kinetically to vacuolar hydrolase-precursor maturation. *Eur. J. Biochem.* 207:867-876.

Johnson, L.M., V.A. Bankaitis, and S.D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. *Cell*. 48:875-885.

Klionsky, D.J., P.K. Herman, and S.D. Emr. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* 54:266-292.

Klionsky, D.J., R. Cueva, and D.S. Yaver. 1992. Aminopeptidase I of *Saccharomyces cerevisiae* is localized to the vacuole independent of the secretory pathway. *J. Cell Biol.* 119:287-299.

Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA*. 82:488-492.

Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367-382.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.

Landt, O., H.P. Grunert, and U. Hahn. 1990. A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene*. 96:125-128.

Lee, W.C., and T. Melese. 1989. Identification and characterization of a nuclear localization sequence-binding protein in yeast. *Proc. Natl. Acad. Sci. USA*. 86:8808-8812.

Lee, W.C., Z.X. Xue, and T. Melese. 1991. The *NSR1* gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. *J. Cell Biol.* 113:1-12.

Letourneur, F., E.C. Gaynor, S. Hennecke, C. Demolliere, R. Duden, S.D. Emr, H. Riezman, and P. Cosson. 1994. Coatamer is essential for retrieval of

dilysine-tagged proteins to the endoplasmic reticulum. *Cell*. 79:1199-1207.

Lewis, M.J., D.J. Sweet, and H.R. Pelham. 1990. The *ERD2* gene determines the specificity of the luminal ER protein retention system. *Cell*. 61:1359-1363.

Loffler, H.G., and K.H. Rohm. 1979. Comparative studies on the dodecameric and hexameric forms of yeast aminopeptidase I. *Z. Naturforsch.* 34C:381-386.

Maarse, A.C., J. Blom, L.A. Grivell, and M. Meijer. 1992. *MPI1*, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:3619-3628.

Marini, F., 3d., A. Naeem, and J.N. Lapeyre. 1993. An efficient 1-tube PCR method for internal site-directed mutagenesis of large amplified molecules. *Nucleic Acids Res.* 21:2277-2278.

Marcusson, E.G., B.F. Horazdovsky, J.L. Cereghino, E. Gharakhanian, and S.D. Emr. 1994. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the *VPS10* gene. *Cell*. 77:579-586.

McCollum, D., E. Monosov, and S. Subramani. 1993. The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells—the PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family. *J. Cell Biol.* 121:761-774.

McNew, J.A., and J.M. Goodman. 1994. An oligomeric protein is imported into peroxisomes in vivo. *J. Cell Biol.* 127:1245-1257.

Millar, D.G., and G.C. Shore. 1993. The signal anchor sequence of mitochondrial Mas70p contains an oligomerization domain. *J. Biol. Chem.* 268:18403-18406.

Millar, D.G., and G.C. Shore. 1994. Mitochondrial Mas70p signal anchor sequence. Mutations in the transmembrane domain that disrupt dimerization but not targeting or membrane insertion. *J. Biol. Chem.* 269:12229-12232.

Muhlrad, D., R. Hunter, and R. Parker. 1992. A rapid method for localized mutagenesis of yeast genes. *Yeast*. 8:79-82.

Nothwehr, S.F., and T.H. Stevens. 1994. Sorting of membrane proteins in the yeast secretory pathway. *J. Biol. Chem.* 269:10185-10188.

Ohno, H., J. Stewart, M.C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J.S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science (Wash. DC)*. 269:1872-1875.

Pelham, H.R., K.G. Hardwick, and M.J. Lewis. 1988. Sorting of soluble ER proteins in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1757-1762.

Perrin, S., and G. Gilliland. 1990. Site-specific mutagenesis using asymmetric polymerase chain reaction and a single mutant primer. *Nucleic Acids Res.* 18:7433-7438.

Pryer, N.K., L.J. Wuestehube, and R. Schekman. 1992. Vesicle-mediated protein sorting. *Annu. Rev. Biochem.* 61:471-516.

Raths, S., J. Rohrer, F. Crausaz, and H. Riezman. 1993. *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* 120:55-65.

Riezman, H. 1993. Yeast Endocytosis. *Trends Cell Biol.* 3:273-277.

Robinson, J.S., D.J. Klionsky, L.M. Banta, and S.D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell Biol.* 8:4936-4948.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp.1.1-18.88.

Sarkar, G., and S.S. Sommer. 1990. The "megaprimer" method of site-directed mutagenesis. *Biotechniques*. 8:404-407.

Scott, S.V., and D.J. Klionsky. 1995. In vitro reconstitution of the cytoplasm to vacuole protein targeting pathway in yeast. *J. Cell Biol.* 131:1727-1736.

Seglen, P.O., and P. Bohley. 1992. Autophagy and other vacuolar protein degradation mechanisms. *Experientia*. 48:158-172.

Sherman, F., G.R. Fink, and L.W. Lawrence. 1979. *Methods in Yeast Genetics: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 61-62.

Shinde, U., and M. Inouye. 1995. Folding mediated by an intramolecular chaperone: autoprocessing pathway of the precursor resolved via a substrate assisted catalysis mechanism. *J. Mol. Biol.* 247:390-395.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 122:19-27.

Silen, J.L., and D.A. Agard. 1989. The alpha-lytic protease pro-region does not require a physical linkage to activate the protease domain in vivo. *Nature (Lond.)*. 341:462-464.

Silver, P., I. Sadler, and M.A. Osborne. 1989. Yeast proteins that recognize nuclear localization sequences. *J. Cell Biol.* 109:983-989.

Stack, J.H., and S.D. Emr. 1993. Genetic and biochemical studies of protein sorting to the yeast vacuole. *Curr. Opin. Cell Biol.* 5:641-646.

Terlecky, S.R., W.M. Nuttley, D. McCollum, E. Sock, and S. Subramani. 1995. The *Pichia pastoris* peroxisomal protein PAS8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:3627-3634.

Thumm, M., R. Egner, B. Koch, M. Schlumpberger, M. Straub, M. Veenhuis, and D.H. Wolf. 1994. Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* 349:275-280.

Tsukada, M., and Y. Ohsumi. 1993. Isolation and characterization of auto-

- phagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* 333:169–174.
- Valls, L.A., C.P. Hunter, J.H. Rothman, and T.H. Stevens. 1987. Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. *Cell.* 48:887–897.
- van den Hazel, H.B., M.C. Kielland-Brandt, and J.R. Winther. 1993. The propeptide is required for *in vivo* formation of stable active yeast proteinase A and can function even when not covalently linked to the mature region. *J. Biol. Chem.* 268:18002–18007.
- Winther, J.R., and P. Sorensen. 1991. Propeptide of carboxypeptidase Y provides a chaperone-like function as well as inhibition of the enzymatic activity. *Proc. Natl. Acad. Sci. USA.* 88:9330–9334.
- Winther, J.R., P. Sorensen, and M.C. Kielland-Brandt. 1994. Refolding of a carboxypeptidase Y folding intermediate *in vitro* by low-affinity binding of the proregion. *J. Biol. Chem.* 269:22007–22013.
- Yoshihisa, T., and Y. Anraku. 1990. A novel pathway of import of α -mannosidase, a marker enzyme of vacuolar membrane, in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 265:22418–22425.
- Zhu, X.L., Y. Ohta, F. Jordan, and M. Inouye. 1989. Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. *Nature (Lond.)* 339:483–484.