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Carboxypeptidase Y: Structural Basis for Protein Sorting and Catalytic Triad

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A yeast vacuolar protease, carboxypeptidase Y (CPY), is known to be involved in the C-terminal processing of peptides and proteins; however, its real function remains unclear. The CPY biosynthetic pathway has been used as a model system for protein sorting in eukaryotes. CPY is synthesized as a prepro-form that travels through the ER and Golgi to its final destination in vacuoles. In the course of studies on the transport mechanism of CPY, various post-translational events have been identified, *e.g.* carbohydrate modification and cleavage of the pre-segments. In addition, sorting signals and various sorting vehicles, similar to those found in higher eukaryotic cells, have been found. The catalytic triad in the active site of CPY makes this enzyme a serine protease. A unique feature distinguishing CPY from other serine proteases is its wide pH optimum, in particular its high activity at acidic pH. Several structural properties which might contribute to this unique feature exist such as a conserved free cysteine residue in the S_1 substrate binding pocket, a recognition site for a C-terminal carboxyl group, and a disulfide zipper motif. The structural bases in CPY functions are discussed in this article.

Key words: carboxypeptidase, catalytic triad, protein sorting, serine protease, vacuolar protease.

Carboxypeptidase Y (CPY) is synthesized in the nucleus as a preproenzyme and transported to vacuoles *via* the endoplasmic reticulum (ER) and Golgi apparatus where it receives carbohydrate modifications as well as N-terminal processing. In the vacuole, proCPY undergoes the final maturation step and becomes catalytically active (*1*). CPY is a popular model protein for studying protein sorting in yeast. CPY is also an attractive enzyme because it contains a catalytic triad in its active center. Initial studies have focused on enzymological aspects such as substrate specificity, and on applications such as C-terminal sequence analysis (*2*). Recently, the crystal structure of CPY has been solved, adding interest to the field and expanding the scope of CPY studies (*3*). The aim of this review is to summarize recent progress in studies on CPY in three major areas: intracellular transportation and carbohydrates (*4, 5*), three dimensional structure, and catalysis (*6, 7*).

Intracellular transport and secretory pathway

CPY is synthesized on ribosomes and sorted into vacuoles through a characteristic secretory pathway. The pathway includes translocation into the endoplasmic reticulum (ER) membrane, to the Golgi apparatus, and finally to vacuoles. During the transport process, the molecular weight of CPY changes as the protein matures (Fig. 1). The overall process resembles typical eukaryotic protein transport.

Translocation of the newly synthesized protein into the ER membrane and transport to the Golgi apparatus. The gene for CPY, PRC1, encodes an inactive pre-proenzyme with a 20 residue signal peptide, a 91 residue propeptide, and a 421 residue mature region (Fig. 1). When the newly synthesized CPY is translocated to the ER membrane, the signal peptide is removed by a signal peptidase. In the lumen of the ER, the processed protein undergoes folding and the core glycosylation occurs to give a 67-kDa form called p1-CPY (*8-10*). Folding and glycosylation seem to be completed in the ER; however, glycosylation, which is not needed for proceeding folding, vacuole sorting, or enzyme activation, is required for efficient intracellular transport (*5, 9, 10*).

In the Golgi apparatus, oligosaccharide modification is completed to give p2-CPY (69 kDa) in which 2 *N*-acetylglucosamines and 8-14 mannoses, including a 1 mannosyl phosphate group, are attached (*11*). The molecular weight of the oligosaccharides comes to 10,000; the final structure of the oligosaccharide is shown in Fig. 2.

Amino acid deletions within the pro-region of pro-CPY result in the accumulation of pro-CPY in the ER even in the presence of the targeting signal to the vacuole (*12*). The accumulated mutant pro-CPY is correctly folded in ER, indicating that the propeptide segment of CPY is essential for the correct protein folding to be completed.

Sorting to the vacuole from the Golgi apparatus by a sorting signal. Upon completion of glycosylation in the

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Abbreviations: CPY, carboxypeptidase Y; CD, circular dichroism; CNBr, cyanogen bromide; DFP, diisopropylphosphorofluoridate; -OEt, ethylester; PCMB, *p*-chloromercuribenzoic acid; PhHgCl, phenylmercuric chloride; PMSF, phenylmethylsulfonylfluoride; Z, benzyloxycarbonyl; ZPCK, chloromethylketone derivative of Z-Phe.

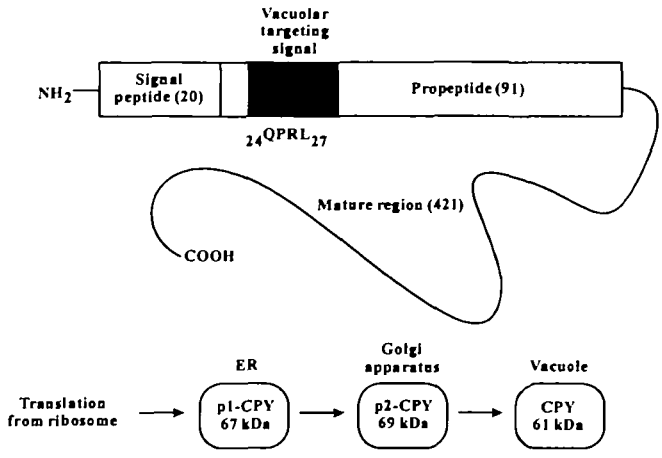

Fig. 1. **Schematic view of the *in vivo* processing of CPY.**

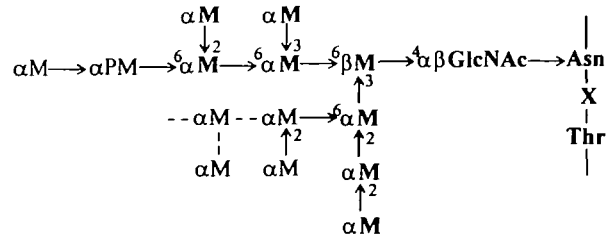

Fig. 2. **Structure of the carbohydrate moiety attached to mature CPY.** GlcNAc stands for *N*-acetyl glucosamine, M for mannose, and P for phosphate.

Golgi apparatus, pro-CPY is targeted to vacuoles. Transport from the Golgi network to the target organelle requires a specific sorting signal, otherwise the protein will be secreted for proteasome degradation (*13, 14*). The vacuolar localization signal, Gln^{24}-Arg-Pro-Leu^{27}, locates near the NH_2-terminus of the propeptide (*4, 5, 15, 16*). Many mutants defective in the gene for vacuolar protein sorting (*vps*) have been identified by genetic selection in *Saccharomyces cerevisiae*, where the *vps10* gene product, a membrane associated protein, has been identified as the sorting receptor for CPY (*17, 18*).

The analysis of *vps* gene product function predicts the existence of prevacuolar compartments serving as transport intermediates along the biogenesis pathway. Such prevacuolar compartments are analogous to the pre-lysosomal compartment. Therefore, the intracellular transport of CPY also serves as a model for the maturation machinery of lysosomal hydrolases.

Vacuolar hydrolases and activation of CPY. Maturation proceeds in the vacuoles where the pro segment of pro-CPY is cleaved by several vacuolar hydrolases, including proteinase A (PrA), proteinase B (PrB), and aminopeptidase (*19*). Vacuolar pH is maintained at 6.2 by a *vma* gene product, a multi-subunit H^+-ATPase (*20*), so that the vacuolar hydrolases retain their activities. The activation of PrA is considered to be the first step in the activation cascade of proCPY in which the protein is cleaved leaving the C-terminal 35 amino acid residue portion of the pro-segment attached to the mature CPY. Subsequently, PrB is involved in further processing to leave 5 residues attached to the mature CPY. The remaining 5 residues are finally removed by aminopeptidase (*21*). The action of PrA is thought to precede that of PrB because the deletion of the structural gene (*pep4*) of PrA leads to the accumulation of the inactive precursors of pro-CPY and pro-PrB in vacuoles (*22*).

Primary sequence of the propeptide. A characteristic of the primary sequence of the CPY propeptide is its high content of charged amino acids; polar amino acids comprise 22% of the mature region and 32% of the propeptide. This is a common feature shared with other propeptides required for folding, *i.e.* yeast vacuolar hydrolase, subtilisin and α-lytic protease (*23, 24*).

Serine carboxypeptidases show high levels of amino acid sequence homology to one another. However, this close similarity is limited to the mature regions, for example, 74% identity between CPY from *S. cerevisiae* and CPY from *Candida albicans*. Overall, there is less than 20% identity between the two propeptides (*25*). The sequence identity in the propeptide region is mainly localized on the COOH-side. This indicates that the COOH-proximal region is likely to be involved in the folding and the NH_2-proximal region in adapting to the specific host environment (*12, 26*).

Role of the propeptide as an intramolecular chaperon. Both *in vivo* and *in vitro* studies have shown that the CPY propeptide is essential for folding. Experiments with deletion mutants indicate that the C-terminal region of the propeptide is essential for *in vivo* folding (*12*). Refolding experiments on pro-CPY were carried out in 6 M guanidinium chloride. In the absence of a reducing agent, 50% of pro-CPY was successfully refolded within 5 min, whereas no refolding was observed for mature CPY. This experiment suggests that the propeptide acts as an intramolecular chaperon. When the 5 disulfide bonds were reduced, 25% of pro-CPY was refolded within 12 h. This suggests that disulfide bond formation is probably the rate limiting step in the renaturation process (*27*). The chaperon-like role of the propeptide is highly dependent on the salt concentration. In the absence of salt, the denatured mature CPY does not recover any activity; but, it shows 5–10% activity in the presence of high salt concentration, such as 0.9 M ammonium sulfate (*27*). The initial rate of folding, based on activity recovery, is not significantly influenced by the addition of salt (*28*). Properties of propeptide that affect the yield more than the overall rate suggest a mechanism for the propeptide similar to those of molecular chaperones.

The propeptide plays a role in maintaining the enzyme in the inactive form. The activation of pro-CPY is done by PrA, but acidic conditions are crucial for PrA activation. The inactive intermediate with 33 amino acid residues in the pro-region accumulates when the PrA treatment is carried out at neutral pH or when a mutant strain containing a deficient H^+-ATPase gene that no longer is able to maintain the vacuolar pH is used (*19*). Further studies of the *in vitro* processing of highly purified pro-CPY by PrA and other processing enzymes as well as the structural characterization of pro-CPY, are underway (Shirai, K., Haruta, N., Ueno, H., and Hayashi, R., manuscript in preparation).

Active site construction in ProCPY. Chemical modification of the catalytic serine residue (Ser146) in pro-CPY with diisopropylfluorophosphate fails to suggest that catalytic triad in pro-CPY is not functional. On the other hand,

Cys341 and Met398 in the pro-CPY, located at the S_1 and S'_2 substrate binding sites, respectively, are accessible to chemical modification agents: Cys341 reacts with a fairly bulky reagent, PhHgCl, and Met398 is modified even faster than in CPY (*29*). These results indicate that a certain part of the active site of pro-CPY is more exposed to the solvent than that of mature CPY.

Role of carbohydrate moieties. Four *N*-linked glycosylation sites, Asn13, Asn87, Asn168, and Asn368, are found in CPY, all with the consensus sequence, Asn-Xaa-Thr. The roles of carbohydrate moieties in CPY are not totally clear. It has been suggested that carbohydrates, especially the one at Asn87, participate in the intracellular transport mechanism (*5*). The Asn87 mutant enzyme (N87I) exhibits a reduced transport rate; however, it also shows reduced enzymatic activity (30%). Thus, the participation of the carbohydrate moiety in the folding and catalysis is suspected (*5*). Recently, extensive characterization of a mutant enzyme lacking carbohydrate modification has been carried out (*30, 31*). A comparative expression yield of the mutant to the wild type suggests that the *in vivo* role of the carbohydrate moieties of CPY might be insignificant or at least their effect can be overcome by the housekeeping system of the host cell. The *in vitro* properties of the mutant enzyme show that high pressure treatment can alter significantly more on the mutant than the wild type enzyme. It has been suggested that carbohydrate plays a role in protecting CPY functions under stress conditions (*30, 31*).

Three-dimensional structure

The secondary and tertiary structures of CPY became clear when the crystal structure of CPY was determined (*3*). Contents of α-helical and β-sheet structures are 36 and 15%, respectively. The folding pattern of CPY suggests that it belongs to a group of α/β hydrolases, a diverse family of structurally related hydrolases that includes acetylcholinesterase from *Torpedo californica*, dienelactone hydrolase from *Pseudomonas* sp. B13, haloalkane dehalogenase from *Xanthobacter autotrophicus*, lipase from *Geotrichum candidum*, protective protein from human and carboxypeptidase II from wheat (*32*). Unique properties of the CPY structure are as follows:

Disulfide zipper. There are five pairs of disulfide bonds in the CPY structure, all of which surround the active site pocket. Two pairs of disulfide bonds, Cys217-Cys240 and Cys224-Cys233, are positioned between two α-helices, one from 204 to 227 and the other from 230 to 251. These appear to connect the two α-helices in an antiparallel way and are called a "disulfide zipper" (*3*). Disulfide bridges between α-helices are generally considered to be unusual; thus, a special role for disulfide zippers has been sought (*3*). A similar disulfide zipper has been found in CPMIII but not in CPWII, a dimeric serine carboxypeptidase.

Catalysis and the active site

CPY hydrolyzes peptides, esters, amides, and anilides (*33-36*). The best substrate is an *N*-blocked-Phe-Leu and the enzyme generally prefers hydrophobic amino acids, but exhibits broad substrate specificity. Typical serine protease inhibitors such as DFP, PMSF, and ZPCK inhibit CPY activity (*2*). CPY is also sensitive to metal ions such as Hg^{2+}, Ag^{+}, and Cu^{2+} (*2, 37*). EDTA and *o*-phenanthroline have no effect on the enzyme activity (*2*). All of these chemically determined properties of CPY are consistent with the three dimensional structure.

Charge-relay system. The charge-relay system in CPY functions similarly or identically to that of the serine endopeptidases (*38, 39*). The three essential catalytic residues are Ser146, His397, and Asp338. The assignment of these catalytic residues was made by sequence comparison with other serine carboxypeptidases, by studies of the three-dimensional structure (*3*), and by site-directed mutagenesis (*40*). Direct chemical proof was obtained for Ser146 and His398 (*6, 7, 41*).

The catalytic mechanism of CPY resembles that of typical serine proteases, which hydrolyze peptide and ester substrates in a two-step reaction. In the first reaction, a tetrahedral intermediate is formed as a result of the nucleophilic attack of the essential serine hydroxyl on the carbonyl carbon atom of the substrate. The histidine residue assists in this step by accepting the proton from the serine hydroxyl and stabilizing the tetrahedral intermediate. This proton is then transferred to the newly generated amino half of the peptide bond (X in Fig. 3), which is now free to dissociate, in an acylation step (Fig. 3). Hydrolysis of the acyl-enzyme intermediate then produces a product in a deacylation step.

A key feature of this mechanism is the ability of the catalytic histidine to abstract a proton from the catalytic serine prior to or simultaneously with the nucleophilic attack on the scissile bond. Exactly the same mechanism is proposed for both CPY and serine endopeptidases, the difference is that CPY is highly active at low pH where serine endopeptidases are inactive (*42, 43*). This high activity of CPY at low pH can be explained by assuming that His397 in the enzyme substrate complex is highly perturbed. Two models for the mechanism of perturbation have recently been proposed.

One model describes the direct perturbation of the catalytic histidine by an apolar environment as described for His95 in triose phosphate isomerase and a derivative of papain (*44*). In CPY, an acidic residue, Glu145, at the substrate C-terminal recognition site, may contribute to the creation of this apolar environment (*43*). Glu145 has a pK_a at 4.3 and may take the place of the histidine residue when CPY hydrolyzes a peptide substrate at a low pH range where His397 remains protonated (*45*). A similar model has been suggested in the case of CPWII (*46*), where a proton sink for the catalytic histidine occurs when it forms a hydrogen bond network with the substrate carboxylate group. Transfer of a proton from the protonated histidine to the substrate carboxylate neutralizes both charged groups, allowing the hydrolytic process can proceed.

The second model describes a direct proton transfer from the catalytic histidine to the scissile nitrogen atom during the acylation at low pH (*39*). Serine endopeptidases do not possess a mechanism for polarizing the scissile peptide bond. Such polarization may activate the positively charged histidine by accepting a proton, hence perturbing the pK_a of the catalytic histidine. But CPY may be able to accomplish this through interaction with the α-carboxylate group of the substrate *via* two hydrogen bond donors, Asp51 and Glu145. This interaction facilitates the formation of the transition state, possibly by distorting the peptide bond leading to the required polarization; hence, the pK_a value of

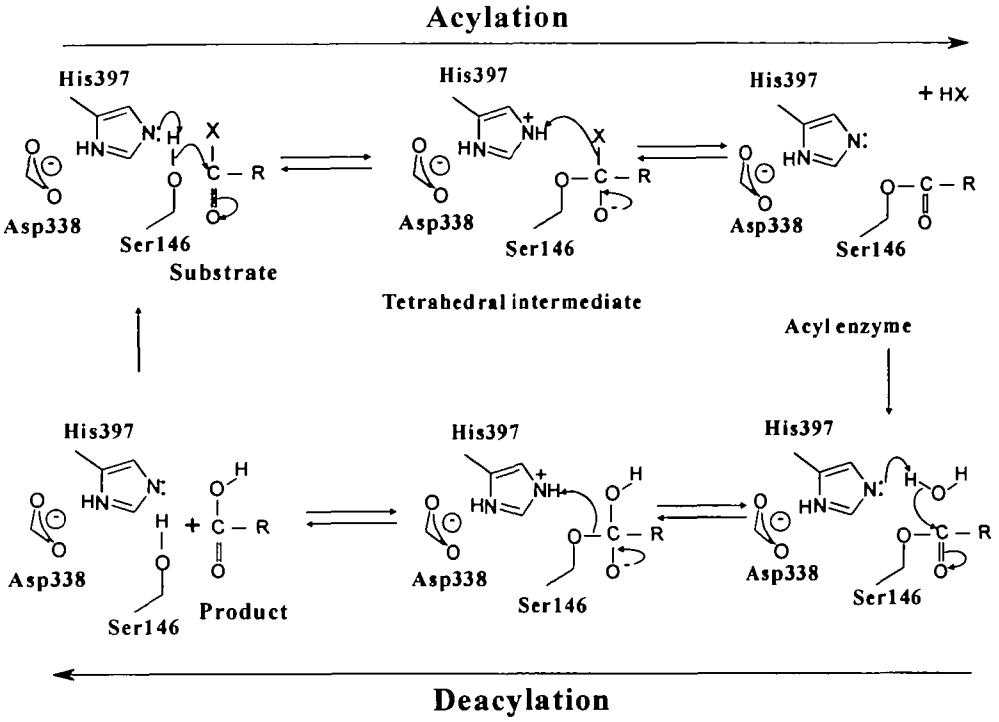

Fig. 3. **Steps in peptide bond cleavage by CPY.**

the amide nitrogen is raised. The required distortion of the peptide bond may result from stabilization of the specific tautomeric form of the substrate.

The chief difference between the charge-relay systems of CPY and serine endopeptidases lies in the fact that the geometrical positionings of Ser146, His397, and Asp338 are not identical. In CPY, the carboxylate of Asp338 and the imidazole of His397 are not coplanar, and this geometric arrangement is far from ideal for proton transfer (*47*). In addition, the spatial relationships between the aspartic acid and serine of endopeptidases and CPY are different, so that the overall orientation of the three catalytic residues cannot be superimposed.

Substrate binding properties. Two substrate binding sites, the S_1 and S_1' subsites, accommodate amino acid side chains of the substrates, P_1 and P_1'. There is also a binding site for the C-terminal carboxylate group of peptide substrates. CPY has an oxyanion hole to accommodate a tetrahedral intermediate where the oxyanion in the transition state is stabilized. The presence of additional substrate binding subsites, S_2-S_5, has been shown (*48*). By filling these additional substrate binding pockets, extensive enhancements in the catalytic efficiency are observed: a heptapeptide is hydrolyzed 52,000-fold faster than a dipeptide (*49*).

Oxyanion hole. The three-dimensional structure shows that the oxyanion hole is located just above Ser146 and consists of the backbone amides of Gly53 and Tyr147.

S_1 substrate binding site. The S_1 subsite is a deep pocket mainly constructed of hydrophobic residues, Tyr147, Leu178, Tyr185, Tyr188, Trp312, Ile340, and Cys341. This pocket actually determines the P_1 substrate preference (*50*). When Leu178, located at the bottom of the S_1 binding pocket, was replaced by charged residues, the hydrolytic preference was altered to the oppositely charged P_1 side chains (*51, 52*). When Trp312, located in the upper wall of the S_1 binding pocket, was changed to asparagine, the hydrolytic activity for basic P_1 side chains increased dramatically (*53*).

Cys341 is the only residue considered to be a polar amino acid in the S_1 subsite. Because this single cysteinyl residue is conserved in the vicinity of the active site in serine carboxypeptidases, the role of this residue has been a focus of research since the discovery of the enzyme. Chemical modification and mutagenic approaches have shown that, although Cys341 is not essential for the enzyme activity, it exerts a large effect on the catalytic efficiency (*37, 50, 54, 55*). The involvement of Cys341 in the correct positioning of His397, and also in the shielding of Asp338 from the solvent, has been suggested (*32*). Recently, the role of Cys341 was re-investigated by combining site-directed mutagenesis and a high-pressure technique. Six enzymes with mutations at Cys341, C341G, C341S, C341V, C341F, C341D, and C341H, were prepared and their activities toward Fua-Phe-Phe and Fua-Ala-Phe were measured under moderately high pressure. It was found that Cys341 plays a role in the catalytic reaction, especially of large hydrophobic substrates, by controlling the cavity size of the S_1 substrate binding pocket during the hydrolytic transition state (*56*).

S_1' substrate binding site. The S_1' subsite of CPY is constructed mainly of hydrophobic residues, Thr60, Phe64, Tyr256, Tyr269, Leu272, and Met398. Unlike the S_1 subsite, the S_1' subsite exhibits wide substrate preference by recognizing both hydrophobic and hydrophilic amino acids, alcohols, amides, and anilides. This unique property is probably explained by the fact that the S_1' binding pocket is sufficiently large to allow a multiple binding mode. Another explanation has recently been proposed that the flexible side chain of Met398 may contribute to the wide substrate preference by adjusting the distance to the hydrophobic portion of the P_1' residue or the hydrophilic

portion of the P_1' residue (*57*). Another noteworthy report shows that the P_1'-S_1' interaction depends upon the length and hydrophobicity of the P_1' residue, where a larger hydrophobic side chain gives higher activity (*58*).

C-terminal recognition by the hydrogen bond network. A prominent feature of carboxypeptidases is their ability to recognize and bind to the carboxylate terminus of peptide substrates (*59*). This C-terminal recognition mechanism differs between metallo and serine carboxypeptidases. In carboxypeptidase A, a typical metallo enzyme, the formation of a salt bridge between the α-carboxylate of the substrate and the side chain of the arginine residue is important (*60*). In CPY, C-terminal recognition is accomplished strictly by hydrogen bonds: the α-carboxylate of the substrate forms hydrogen bonds with the backbone amide of Gly52 and the side chains of Asn51 and Glu145 (*43, 61*). The latter mode of C-terminal recognition of the substrate helps CPY to release wide varieties of leaving groups, including amino acids, hydroxyacids, alcohols, ammonia, and amino acid amides (*2, 62*). The hydrogen bond network is located at the bottom of the S_1' binding pocket and contributes to the stabilization of the transition state *via* interaction with the C-terminal carboxylate group of the substrate (*43, 62*). Glu145 has been shown to be the most important residue for both substrate recognition and stabilization of the transition state. The state of ionization of Glu145 determines the hydrolysis of the peptide or peptide amide substrate.

Future prospects

Within the past decade or so, many aspects of CPY have been explained; however, new questions have also arisen. Although the activation mechanism of pro-CPY has become clear at the cellular level, more structural information is clearly needed to understand the process at the molecular level. The activation process of pro-CPY, for instance, is not totally the same *in vivo* and *in vitro*. Why and how the activity of pro-CPY is inhibited remain unanswered. The molecular recognition between pro-CPY and sorting proteins is of interest since this question relates not only to what is involved in the sorting machinery, but also to the fundamental question of protein-protein interaction. It is also of interest to clarify the real role of serine carboxypeptidases, including cathepsin A or protective protein, in eukaryotes. There is no doubt that attempts to answer these questions relating to the molecular structure of CPY would open vast avenues of future knowledge.

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