From Protease to Decarboxylase

THE MOLECULAR METAMORPHOSIS OF PHOSPHATIDYLSERINE DECARBOXYLASE*

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Background: Phosphatidylserine decarboxylase (PSD) undergoes an autoendoproteolytic cleavage but the mechanism has not been defined.

Results: PSD proenzyme processing occurs by a canonical serine protease mechanism catalyzed by a conserved aspartate-histidine-serine triad.

Conclusion: PSD proenzyme executes a proteolytic reaction in cis that creates the active site of the decarboxylase.

Significance: The mechanism of autoendoproteolytic processing of PSDs across phyla has been elucidated.

Phosphatidylserine decarboxylase (PSDs) play a central role in the synthesis of phosphatidylethanolamine in numerous species of prokaryotes and eukaryotes. PSDs are unusual decarboxylase containing a pyruvyl prosthetic group within the active site. The covalently attached pyruvyl moiety is formed in a concerted reaction when the PSD proenzyme undergoes an endoproteolytic cleavage into a large β-subunit, and a smaller α-subunit, which harbors the prosthetic group at its N terminus. The mechanism of PSD proenzyme cleavage has long been unclear. Using a coupled in vitro transcription/translation system with the soluble Plasmodium knowlesi enzyme (PkJPSD), we demonstrate that the post-translational processing is inhibited by the serine protease inhibitor, phenylmethylsulfonyl fluoride. Comparison of PSD sequences across multiple phyla reveals a uniquely conserved aspartic acid residue within a D-H-S-R motif, two uniquely conserved histidine residues within a PXXYHXHXXP motif, and a uniquely conserved serine residue within a GS(S/T) motif, suggesting that PSDs belong to the D-H-S serine protease family. The function of the conserved D-H-S residues was probed using site-directed mutagenesis of PkJPSD. The results from these mutagenesis experiments reveal that Asp-139, His-198, and Ser-308 are all essential for PSD processing across multiple phyla. The Gly-307 residue is also essential, but the Ser/Thr-309 is non-essential. These results define the mechanism whereby PSDs begin their biochemical existence as proteases that execute one autoendoproteolytic cleavage reaction to give rise to a mature PSD harboring a pyruvyl prosthetic group.

Phosphatidylethanolamine (PE)2 is an abundant membrane phospholipid in both prokaryotes and eukaryotes. Multiple synthetic routes produce PE including: 1) decarboxylation of phosphatidylserine (PS) (1); 2) transfer of P-ethanolamine from CDP-ethanolamine to a diacylglycerol acceptor (2); 3) phospholipid headgroup exchange (3, 4); and 4) acylation of lyso-PE (5, 6). Despite the multiple routes for PE synthesis, several studies demonstrate that the PE pools produced by different pathways are not readily interchangeable. For example, yeast harboring a null allele for PSD1, which encodes the mitochondrial enzyme, cannot restore the mitochondrial membrane defect in PE through the CDP-ethanolamine pathway, or through the PE pool produced by the Golgi/vacuole/endosomial enzyme PSD2 (7) (8). In contrast, lyso-PE transported into yeast cells by P-type ATPases and acylated by ALE1-encoded acyltransferase can restore the mitochondrial deficiency in PE caused by deletion of PSD1 (5, 9). In mammalian systems, deletion of mitochondrial PSD is an embryonic lethal mutation (10). Thus, mitochondrial phosphatidylserine decarboxylase (PSD) appears to play an essential role in the autonomous generation of PE within the organelle.

PSDs are unusual enzymes that utilize a pyruvyl prosthetic group for catalysis (11). In all PSDs this pyruvyl moiety is generated in a concerted reaction that occurs within a consensus GS*(S/T) sequence present in the proenzyme (1, 12–14). The reaction scheme involves the activation of the hydroxyl group of the first serine within the motif (denoted by the asterisk), and its nucleophilic attack of the Gly-Ser* peptide bond. The peptide bond cleavage yields an acyl-enzyme intermediate with the carbonyl of the Gly esterified to the Ser* hydroxyl group. This acyl-enzyme intermediate is the same type of intermediate formed by canonical serine proteases (15). In contrast to common serine proteases that form acyl-enzyme intermediates with extrinsic substrates, PSDs are postulated to execute their own intrinsic cleavage of specific peptide bonds. Subsequent to cleavage of the peptide bond, the acyl-envelope undergoes an α,β-elimination reaction followed by loss of NH3 to produce a mature enzyme consisting of a large β-subunit derived from the amino-terminal portion of the proenzyme, and a small α-subunit derived from the C-terminal portion of the proenzyme. After processing, the mature α-subunit harbors an N-terminal

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1 The abbreviations used are: PE, phosphatidylethanolamine; Pk, Plasmodium knowlesi; PS, phosphatidylserine; PSD, phosphatidylserine decarboxylase; MBP, maltose-binding protein; IPTG, isopropylthiogalactoside.
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pyruvoyl moiety. It is noteworthy that the Ser* predicted to constitute part of the protease active site of the proenzyme, subsequently becomes the pyruvoyl prosthetic group, which constitutes an essential element of the active site of the mature decarboxylase.

Although the general features of pyruvoyl prosthetic group formation in PSDs have been known for decades (11) (12, 16, 17), the exact mechanism of the process has remained elusive. Recent studies have provided new tools for examining the maturation of PSDs, including soluble forms of the enzyme that undergo processing events in the absence of membrane integration and organelle import processes. In this report we describe the use of soluble versions of the PSD from Plasmodium knowlesi (PkPSD) to investigate the processing of the proenzyme to mature enzyme. Using phylogenetic sequence information, we identified 1 contextually conserved aspartic acid (Asp-139) and 2 contextually conserved histidines (His-195, His-198) that could potentially partner with the proteolytic active site serine of the proenzyme protease inhibition; 2) the roles of Asp-139, His-195, His-198, and Ser-308 as protease active site amino acids; 3) the roles of each of the residues in the GS*(S/T) motif in enzyme maturation; and 4) the ability of the proenzyme to execute the proteolytic reaction in trans. Our findings define the essential amino acids and mechanism for autoendoproteolytic processing of PSD proenzymes.

EXPERIMENTAL PROCEDURES

Materials—All chemicals for Escherichia coli growth were purchased from Sigma, Fisher Scientific, and Difco. Phospholipids were purchased from Avanti Polar Lipids. Reagents for protein determination were from Bio-Rad. Pre-cast SDS-polyacrylamide gels were purchased from Invitrogen. Mouse monoclonal antibodies against His$_6$ epitope tags and MBP tags of the PkPSD fusion protein were obtained from Clontech and New England Biolabs, respectively. Other reagents used for ligand blotting were purchased from Bio-Rad and Sigma.

PMSF Inhibition of Maturation of MBP-His$_6$-Δ34PKPD Expressed in E. coli—Rosetta DE3 strains harboring the pMAL-C2X-His$_6$-Δ34PKPD vector were grown to saturation overnight on LB, 0.2% glucose, ampicillin (100 μg/ml), and chloramphenicol (34 μg/ml), then diluted 100-fold, and grown to A$_{600}$ = 0.5 (for 20 min induction) or A$_{600}$ = 1.0 (for 2 h induction) at 37 °C. Expression of MBP-His$_6$-Δ34PKPD was induced by addition of 0.3 mM isopropyl β-D-thiogalactoside (IPTG). The cells were harvested by centrifugation after the indicated times of induction (4,000 × g, 20 min, 4 °C) and washed by resuspension in water and centrifugation. The cells were resuspended in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol, frozen in a dry ice-ethanol bath, stored overnight at −20 °C, and subsequently thawed on ice water. Cell extracts were obtained by sonication (15 s burst at 20% amplitude, 8 times with 30-s cooling intervals) followed by centrifugation at 20,000 × g for 20 min.

Site-directed Mutagenesis of Conserved Amino Acids in PkPSD—Amino acid residues, including Asp-139, His-195, His-198, Gly-307, Ser*-308, and Ser-309 of PkPSD were introduced as a DNA template.

Construction of Vectors to Express MBP-His$_6$-Δ34PKPD and MBP-Δ34PKPD in E. coli—Expression vectors harboring truncated PkPSD (lacking the first 34 amino acids) and either a MBP-His$_6$ double epitope tag (MBP-His$_6$-Δ34PkPSD), or a MBP single epitope, were generated using the pMAL-C2X vector (18). Briefly, specific primers for the individual constructs were generated and used to amplify DNAs from a pET45-His$_6$-Δ34PKPD template using Phusion High-Fidelity DNA polymerase (New England Biolabs Inc.). The His$_6$-Δ34PKPD and Δ34PKPD constructs containing 5′ and 3′ flanking sequences that are homologous to those of the vector were purified by agarose gel electrophoresis. The pMAL-C2X E. coli expression vector was digested with BamHI and PstI restriction enzymes, and the resultant DNA fragment was purified. Ligation reactions by In-Fusion HD Cloning Kits (Clontech Laboratories, Inc.) yielded pMAL-C2X-His$_6$-Δ34PKPD and pMAL-C2X-Δ34PKPD. The plasmids were introduced into a Rosetta strain to express the constructs.
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Biochemical Evidence for cis-Endopeptolytic Processing of PSD—A 20-μl aliquot of cell-free extracts containing wild type MBP-His34PKPDS, or catalytically inactive mutant forms of MBP-His34PKPDS (H198A, D139A, or S308A, 0.1 μg/μl final) and 20 μl of cell-free extracts containing wild type MBP-Δ34PKPDS (without His6 tag, 0.1 μg/μl final) were mixed in 60 μl of the processing reaction buffer. The reaction mixture was incubated for 2 h at 30 °C, or 20 h at 25 °C. The samples were analyzed by Western blot analysis using anti-His6 and anti-MBP antibodies.

Measurement of PSD Activity—E. coli cell-free extracts containing various MBP-His34PKPDSs were prepared as described above and used for PSD enzyme assays. Control endogenous E. coli PSD activity was measured using Rosetta E. coli harboring no plasmid. The assay for PSD activity utilized 0.2 mM Ptd-[1-14C]Ser (400 cpm/nmol) as the substrate, and the reaction product was trapped as 14CO2 on 2 μl KOH-impregnated filter paper, as described previously (13). PSD activities are reported as enzyme specific activity (nmol/mg of protein/45 min). All enzyme reactions were performed at substrate and protein concentrations that produced a linear response with the amount of enzyme added to the reaction.

Mass Spectrometry Analysis—Experiments were performed using a standard protein identification strategy by mass spectrometry (19). Briefly, candidate proteins were excised from electrophoretic gels, followed by reduction using dithiothreitol (DTT), and alkylation using iodoacetamide. Bands were digested with trypsin overnight and extracted peptides were subsequently dried under vacuum to remove organic solvent. Peptides were chromatographically resolved on-line using a C18 column and 1200 series high performance liquid chromatography (HPLC, Agilent Technologies) and analyzed using a 6550 LCMS Q-TOF mass spectrometer (Agilent Technologies, Palo Alto, CA) at the National Jewish Health Proteomics Facility. The mass spectrometry system includes a HPLC-chip interface (Agilent Technologies), and an Agilent “long” HPLC chip (G4240-62002) was used, which consisted of a 40-nl enrichment column and a 150 mm × 75-μm analytical column combined on a single chip. Raw data were extracted and searched using Mass Hunter Bioconfirm software (B.06.00, Agilent Technologies). For peptide sequence matching, extracted data were searched against an MBP-His34PKPDS sequence.

RESULTS

Phenylmethylsulfonyl Fluoride Inhibits Processing of the PkPSD Proenzyme—In previous studies we utilized an in vitro coupled transcription translation system to demonstrate post-translational processing of a form of the PkPSD proenzyme engineered to contain a deletion of hydrophobic residues at the N terminus, and the inclusion of a His6 epitope tag at the N terminus (His6-Δ34PKPDS). The processing of the nascent proenzyme required the addition of PS, which appears to induce a conformational change that activates the proteolytic processing reaction (18). The His6-Δ34PKPDS enzyme is a particularly useful model for probing PSD processing and structure because, unlike many of its counterparts in other organisms, it is soluble. We used the His6-Δ34PKPDS construct to examine the sensitivity of the processing to the protease inhibitor PMSF. In these reactions, transcription and translation proceeded for 40 min, with PMSF added after 20 min. After the 40-min period, cycloheximide was added to arrest translation. After an additional 40 min, processing of the proenzyme was determined by monitoring the appearance of the mature β-subunit using Western blotting. Fig. 1 shows this processing reaction performed in the presence of varying concentrations of the generic serine protease inhibitor PMSF. In the absence of PMSF, 31% of the newly translated PkPSD is converted to mature enzyme during the incubation period. With increasing concentrations of PMSF, post-translational processing progressively declines. At 5 mM PMSF there is no detectable processing of the proenzyme to the mature form. These data support a model for PkPSD processing that conforms to that expected of a canonical serine protease. Relatively high levels of PMSF are required for this inhibition because this was expected to be an intramolecular proteolytic reaction occurring between two adjacent amino acids.

E. coli-expressed MBP-His34PKPDS Proenzyme Is Processed in Vivo—We have previously shown that the majority of full-length PkPSD expressed in E. coli was an inactive proenzyme, and only ~5% of the protein was processed (18). Interestingly, we found that the processing efficiency of E. coli expressed PkPSD was dramatically increased by fusing MBP to the N-terminal end of PkPSD. MBP has been reported to enhance protein solubility and folding for other proteins (20–22). As shown in Fig. 2A, when MBP-His6-Δ34PKPDS was expressed for 2 h following IPTG induction in an E. coli Rosetta strain, ~50% of the enzyme was processed to its mature form. Expression of the MBP-His6-Δ34PKPDS in an E. coli strain (EH150) with a temperature-sensitive growth defect due to a mutation in bacterial PSD (23) resulted in normal growth at 42 °C. In cell extracts, the MBP-His6-Δ34PKPDS construct yields robust synthesis of active enzyme that exceeds endogenous PSD activity of E. coli (0.27 μmol/45 min/mg of protein) by a factor of 70 (18.4 μmol/45 min/mg of protein). Mass spectrometry analysis of the purified α-subunit of PkPSD shows the pyruvoyl moiety is detected at the N termini of α subunits (Fig.

**FIGURE 1. Phenylmethylsulfonyl fluoride inhibits processing of in vitro expressed His6-Δ34PKPDS proenzyme.** In vitro expression of His6-Δ34PKPDS was conducted for 40 min at 30 °C using a TNT Quick-coupled transcription/translation system. After the first 20 min of the reaction, PMSF was added to final concentrations of 0, 0.5, 2.5, or 5 mM. After 40 min of incubation time, translation was arrested by the addition of 0.2 mM cycloheximide and the post-translational processing reaction continued for 40 min in the presence of 1.0 mg/ml of dioleophosphatidylserine liposomes. Upon the completion of the reactions, electrophoresis and Western blot analysis was conducted to detect the His6 epitope present in both the nascent and mature His6-Δ34PKPDS proteins. The percentages of proenzymes (PROENZ) and processed mature β-subunit (β-SUB) are shown below the blots. The protein band intensities of the proenzymes and processed β-subunits were quantified using ImageJ software. Data are mean ± S.D. for 3 experiments.
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2B). The result confirms that PkPSD is a pyruvyl enzyme as reported previously for other PSD enzymes (24) (11).

PKS Inhibits Processing of the E. coli-expressed MBP-His$_6$-$\Delta$34PkPSD Proenzyme in Cell Extracts—The relative amounts of PkPSD precursor and mature form detected in bacteria are a result of the rate of synthesis of new enzyme and its rate of processing. When MBP-His$_6$-$\Delta$34PkPSD was induced for 20 min, more than 80% of the protein was found in the proenzyme form (Fig. 2A), and cell extracts from these preparations were used to follow precursor processing in vitro. Fig. 3A describes the experimental design for examining MBP-His$_6$-$\Delta$34PkPSD processing in vitro. After IPTG induction for 20 min, cells were harvested and lysed and cell-free extracts were prepared, and the time-dependent processing of MBP-His$_6$-$\Delta$34PkPSD was followed for 90 min. Fig. 3, B and C, show that further processing occurs during in vitro incubation of the cell-free extract. When PMSF was added to the reaction, as shown in Fig. 3C, processing was significantly inhibited. These data demonstrate that PMSF is an inhibitor of MBP-His$_6$-$\Delta$34PkPSD processing in vitro.

Asp-139 and His-198 Are Essential for PkPSD Proenzyme Proteolysis—Analysis of the aligned sequences of PSD proenzymes from E. coli, Saccharomyces cerevisiae, Arabidopsis thaliana, Plasmodium falciparum, P. knowlesi, Mus musculus, and Homo sapiens reveal only one conserved Asp residue and two conserved His residues in addition to the conserved GS*(S/T) motif present in all PSD enzymes. The Asp residues occur within a conserved FFXRX$_2$RX$_1$PXD sequence, and the His residues occur within a conserved PAXXYHXHXP sequence (see Fig. 4). Both of these motifs are also demarcated by prolines proximal to the candidate residues. In the PkPSD sequence the positions are Asp-139, His-195, His-198 and Ser*-308. To test the potential involvement of the Asp, His, and Ser* residues in the proteolytic processing of the PkPSD, we constructed D139A, D139N, H195A, H198A, and tandem (H195A/H198A) and Ser308A mutants; and expressed these variants as MBP-His$_6$-$\Delta$34PkPSD fusion proteins in E. coli. Expression of the constructs was induced with IPTG, and Western blotting was used to visualize both the 84-kDa chimeric proenzyme and the 77-kDa chimeric $\beta$-subunit of the mature enzyme, as shown in Fig. 5A. This system provided a convenient method for assessing the effects of specific mutations upon the processing reaction in vivo.

The data in Fig. 5A demonstrate the synthesis and processing of wild type and mutant forms of nascent MBP-His$_6$-$\Delta$34PkPSD after 2 h of induction. Western blotting for the His$_6$ epitope demonstrates that the wild type enzyme is detectable as both proenzyme and mature $\beta$-subunit forms. In contrast, the D139A, D139N, H198A, and H195A/H198A mutations prevented processing of the proenzyme. However, the H195A mutation produced significant levels of enzyme processing, although the level of the $\beta$-subunit was reduced compared with that found for the wild type enzyme. These data provide strong evidence that the conserved Asp-139 and His-198 amino acids play an essential role in enzyme processing, by functioning as constituents of the protease active site.

In some D-H-S catalytic triad proteases an Asn substitution for Asp enables proteolysis to occur at elevated pH (25). We performed additional studies to try to recover proteolysis of the precursor by pH shift of the D139N mutant but these experiments did not result in processing.

The PS decarboxylase activities of the mutant constructs are shown in Fig. 5B, and reveal the expected result that no decarboxylase catalytic activity can occur in the absence of proenzyme processing. In addition, the data show that although the H195A mutant undergoes detectable processing, it has only trace levels of decarboxylase activity.

The role of His-195 in proenzyme processing and PSD enzyme activity was further analyzed. After a 2-h IPTG induction, cells expressing H195A were further incubated for 1 h under conditions where new protein synthesis was blocked by addition of tetracycline and removal of IPTG. The processing of the H195A mutant continued to occur after arrest of protein synthesis. Up to 46% of the H195A proenzyme was processed during the continued incubation time as shown in Fig. 5C. However, the increase in proenzyme processing was not accompanied by increased PS decarboxylase activity measured in cell extracts (Fig. 5D). The result demonstrates that His-195 is not essential for proenzyme processing, but is important for the decarboxylase activity of the mature enzyme.

Within the Canonical GS*(S/T) Motif of PkPSD Only Gly-307 and Ser*-308 Are Essential for Proteolytic Processing—The pioneering work of Satre and Kennedy (11) and later studies by...
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Dowhan and Li (24) established the classification of PSDs as pyruvoyl enzymes, with the prosthetic group being derived from the Ser* within the GS*(S/T) motif, shared by all PSD enzymes. Li and Dowhan (12, 24) also demonstrated that enfeebled PSDs could be produced by substituting Thr or Cys amino acids for Ser* in E. coli. In this study we probed the function of all three residues in the proenzyme processing reaction using G307A, G307P, S*308A, S*308T, S309A, and S309T mutations. The Gly-307–Ser*-308 peptide bond in PkPSD is the site of nucleophilic attack by the activated Ser*-308 hydroxyl group, which our above experiments indicate is likely to occur via a proton shuttling mechanism between Asp-139 and His-198 common to serine proteases (15). Although previous studies provide strong evidence for the role of Ser* in other PSDs in the reaction, the contributions of the Gly and Ser/Thr residues within the conserved GS*(S/T) motif is less certain. As shown in Fig. 6A, introduction of the G307A mutation eliminated proenzyme processing or decarboxylase activity. As expected, the S309A and S309T mutants, which undergo processing, reveal modest reductions in catalytic activity. When accounting for the amount of mature enzyme produced for these mutants, both have —30% of the specific activity of the wild type MBP-His$_6$-$\Delta$34PkPSD. Thus, despite the high degree of conservation at the Ser/Thr position within the motif, it is not essential for either processing or decarboxylase activity.

Analysis of the decarboxylase activity of the S309A and S309T mutants, which undergo processing, reveals modest reductions in catalytic activity. When accounting for the amount of mature enzyme produced for these mutants, both have —30% of the specific activity of the wild type MBP-His$_6$-$\Delta$34PkPSD. Thus, despite the high degree of conservation at the Ser/Thr position within the motif, it is not essential for either processing or decarboxylase activity.

Proteolytic Processing of PkPSD Does Not Occur in Trans—The proposed scheme for autoproteolytic processing of PSDs does not make the distinction between reactions occurring in cis or trans. In principle, oligomers of PSD could assemble a D-H-S protease active site from different protomers. To test for a proteolytic reaction occurring in trans we expressed an MBP-$\Delta$34PkPSD (lacking the His$_6$ epitope tag) construct in E. coli as a source of wild type enzyme, and MBP-His$_6$-$\Delta$34PkPSD as a source of wild type and mutant forms of putative substrate. If proteolysis can occur in trans then the wild type proenzyme should act upon one or more of the processing defective substrates and produce a His$_6$-tagged $\beta$-subunit. As shown in Fig. 7A, the MBP-$\Delta$34PkPSD recovered from bacterial extracts and detected by blotting with anti-MBP consists of both precursor and processed forms. Only the precursor form can participate in a proteolytic reaction, because the processed form no lon-
ger contains the active site serine. The incubation of the wild type MBP-His6-\Delta34PkPSD proenzyme with the wild type and mutant variants (D139A, H195A, and S*308A) of MBP-His6-\Delta34PkPSD, failed to produce His6-labeled \beta-subunits for any of the mutant proteins during a 2-h incubation period. In Fig. 7B, the incubation period was extended to 20 h, but still no evidence for proteolysis in trans could be found. From these data we conclude that proteolytic processing of PSD, required for formation of \alpha- and \beta-subunits, and pyruvoyl prosthetic group formation, does not occur in trans, but instead proceeds as an intramolecular proteolytic reaction acting in cis.

**DISCUSSION**

PSDs play an important role in phospholipid metabolism and organelle biogenesis by generating PE pools at specific subcellular organelles using PS substrates generated in different organelles (26). The autonomous generation of PE at specific subcellular sites appears to play an important role in organelle structure and function (7, 8, 10). PSDs belong to a small family of enzymes that contain a pyruvoyl prosthetic group, which includes S-adenosylmethionine decarboxylase (27), histidine decarboxylase (28), aspartate decarboxylase (29), and arginine decarboxylase (30).

Pyruvoyl enzymes are first synthesized as single polypeptide precursors that undergo autoendoproteolytic cleavage to produce dissimilar \alpha- and \beta-subunits. The proteolytic cleavage is initiated by an activated serine residue, which attacks the peptide bond that links the activated serine (e.g. Ser*-308 in PkPSD) with its adjacent N-terminal amino acid (e.g. Gly-307 in PkPSD). The reaction proceeds through a cyclic oxyoxazolidine intermediate that upon protonation converts the previous peptide bond to an ester bond between the carbonyl of the N-terminal amino acid (e.g. Gly-307) and the OH group of the serine (e.g. Ser*-308 (1)). Typically the ester is cleaved by general base catalysis to yield a free \beta chain and a free \alpha chain harboring a dehydroalanine residue in lieu of the original serine at its N terminus. The addition of water to the imine tautomer of dehydroalanine, followed by the elimination of NH3, results in the formation of a pyruvoyl group at the N terminus of the \alpha chain (28). The processing of PkPSD proceeds at a slow rate in the coupled transcription/translation systems in vitro, and in intact...
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prokaryotic and eukaryotic cells, with the proenzyme form being long lived. The in vitro system requires PS for the processing reaction consistent with the lipid inducing a conformational change to the proenzyme (18) that promotes the proteolytic step.

Among the different pyruvoyl enzymes, the mechanism of activation of the critical serine, and the amino acids involved in the general base catalysis for ester cleavage are highly divergent (27–30), and do not provide insights into the likely constituents of the active site of PSDs. Our inspection of the conserved residues and motifs in the PSDs led us to postulate that a canonical D-H-S catalytic triad would likely be involved in the processing of this group of enzymes. Support for this hypothesis was provided by experiments demonstrating the in vitro processing of newly synthesized His6-Δ34PpkPSD fusion protein and mutagenized proteins of G307A, G307P, S308A, S308T, S309A, and S309T are shown. The proteins were expressed for 2 h following IPTG induction from Rosetta E. coli strains harboring no vector (Mock), or plasmids encoding the designated mutant proteins. The percentages of proenzymes (PROENZ) and processed enzymes (β-SUB) are shown below the blot. Data are mean ± S.E. for 3 experiments. B, PSD enzyme assays were performed with cell free extracts from Rosetta strains expressing the designated mutant proteins. Data are mean ± S.E. for 3 experiments each performed in duplicate.

FIGURE 6. Gly-307 and Ser-308 are essential for MBP-His6-Δ34PpkPSD processing, but Ser-309 is non-essential. A, the schematic shows the amino acids targeted in these experiments in black. Western blot analyses of wild type MBP-His6-Δ34PpkPSD fusion protein and mutagenized proteins of G307A, G307P, S308A, S308T, S309A, and S309T are shown. The proteins were expressed for 2 h following IPTG induction from Rosetta E. coli strains harboring no vector (Mock), or plasmids encoding the designated mutant proteins. The percentages of proenzymes (PROENZ) and processed enzymes (β-SUB) are shown below the blot. Data are mean ± S.E. for 3 experiments. B, PSD enzyme assays were performed with cell free extracts from Rosetta strains expressing the designated mutant proteins. Data are mean ± S.E. for 3 experiments each performed in duplicate.

We tested the involvement of the phylogenetically conserved aspartic acid (Asp-139) and two phylogenetically conserved histidines (His-195 and His-198) using site-directed mutagenesis with alanine substitutions. The results from these experiments demonstrate that Asp-139 and His-198 are essential for processing of MBP-His6-Δ34PpkPSD to the mature form. Mutation of His-195 did not prevent processing of the proenzyme, although the rate of processing was significantly reduced (Fig. 5, A and C). As expected, unprocessed forms of the enzyme failed to decarboxylate PS, because the required pyruvoyl prosthetic group could not be generated. However, despite significant levels of processing, the H195A form of the enzyme showed only trace levels of catalytic activity. These latter findings demonstrate that His-195 is not required for proteolytic processing, but plays some role in the catalytic activity of the mature enzyme.

The G*S(T) motif is found in all PSDs, and for PkPSD, both Gly-307 and Ser*-308 are essential for the autodendoproteolytic processing. Work with the E. coli enzyme has demonstrated there is some plasticity allowing for substitution of the Ser*-308 equivalent in the bacterial enzyme with cysteine or threonine (24), although our construct failed to show any processing using the S*308T substitution. The Ser/Thr-309 of PkPSD is phylo-
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**Figure 8. Serine protease motifs and processing of PSD enzymes.** A, the PSD proenzyme contains a D-H-S catalytic triad that is conformationally activated by PS. B, activation of Ser*-308 in PKPc1 results in attack of the peptide bond between Gly-307 and Ser*-308, and formation of an acyl-enzyme intermediate. The acyl-enzyme intermediate is cleaved by general base catalysis by His-198 to produce an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate, and formation of an acyl-enzyme intermediate. C, the enzyme molecules could interact to form an intermolecular active site capable of entering the active site of the protease. The active site Ser*-308 for the protease reaction becomes the active site of the protease for the protease reaction.

Genetically conserved, the S309A substitution yielded a mature enzyme with 30% of the specific activity. Thus, despite the high level of conservation of the Ser/thr-309 position of PKPc1 in other species, it does not play an essential role in either the processing or the decarboxylation reactions.

The autoendoproteolytic processing of PSDs has been proposed to proceed as a unimolecular reaction occurring in cis (12, 24). We designed experiments to test whether two proenzyme molecules could interact to form an intermolecular active site capable of acting in trans. We combined one structurally distinct wild type enzyme, MBP-Δ34Pc1, with several structurally distinct mutant enzymes, MBP-His$_6$-Δ34Pc1, harboring H198A, D139A, or S308A to determine whether any of the mutant forms could be processed. The results shown in Fig. 7, A and B, demonstrate that the wild type enzyme cannot combine with any of the mutants to catalyze processing of the mutant proenzymes. These findings strongly support the model for autoendoproteolytic processing as a unimolecular reaction. A schematic summary of the steps in the processing reactions is shown in Fig. 8.

In summary, our findings identify amino acids essential for the autoendoproteolytic processing of PKPc1, and identify the D-H-S catalytic triad that is conserved across phyla as the mechanism for processing. The proenzyme executes one catalytic reaction in cis at a conserved and essential Gly-Ser$_6$ sequence, which converts the active site serine of the protease to the active site pyruvyl prothetic group of the decarboxylase.

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