Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7774–7778, August 1995
Biochemistry

Molecular cloning of insect pro-phenol oxidase: A copper-containing protein homologous to arthropod hemocyanin

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Communicated by Norman H. Horowitz, California Institute of Technology, Pasadena, CA, May 3, 1995 (received for review February 6, 1995)

ABSTRACT **Pro-phenol oxidase [pro-PO; zymogen of phenol oxidase (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1)] is present in the hemolymph plasma of the silkworm *Bombyx mori*. Pro-PO is a heterodimeric protein synthesized by hemocytes. A specific serine proteinase activates both subunits through a limited proteolysis. The amino acid sequences of both subunits were deduced from their respective cDNAs; amino acid sequence homology between the subunits was 51%. The deduced amino acid sequences revealed domains highly homologous to the copper-binding site sequences (copper-binding sites A and B) of arthropod hemocyanins. The overall sequence homology between silkworm pro-PO and arthropod hemocyanins ranged from 29 to 39%. Phenol oxidases from prokaryotes, fungi, and vertebrates have sequences homologous to only the copper-binding site B of arthropod hemocyanins. Thus, silkworm pro-PO DNA described here appears distinctive and more closely related to arthropod hemocyanins. The pro-PO-activating serine proteinase was shown to hydrolyze peptide bonds at the carboxyl side of arginine in the sequence -Asn-49–Arg-50–Phe-51–Gly-52- of both subunits. Amino groups of N termini of both subunits were indicated to be N-acetylated. The cDNAs of both pro-PO subunits lacked signal peptide sequences. This result supports our contention that mature pro-PO accumulates in the cytoplasm of hemocytes and is released by cell rupture, as for arthropod hemocyanins.**

Phenol oxidase (PO; monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1; also trivially named polyphenol oxidase and tyrosinase) is a copper protein that catalyzes the oxygenation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to the corresponding *o*-quinones (1). The enzyme is present in animals and plants and functions as a key enzyme for melanin synthesis (1). In insects the enzyme is present in hemolymph (2) and cuticle (ref. 2; M.A. and P. T. Brey, unpublished work) as inactive precursor (pro-phenol oxidase, pro-PO). Recently, insect pro-PO was indicated to contain two copper atoms per subunit (3, 4). Pro-PO is activated through a limited proteolysis by a specific serine-type proteinase that is itself in a zymogen form and is regulated by a cascade (2). Insect PO is implicated in defense reactions, such as wound healing and melanization during pathogen or parasite invasion (2). POs from mammals, higher plants, fungi, and bacteria have been characterized, and their encoding genes have been cloned (5–9). In 1991, Pentz and Wright (10) reported a cDNA corresponding to *Drosophila* PO; however, they could not demonstrate sequence homology to other POs and other copper-containing proteins.

Many primitive arthropods—e.g., horseshoe crabs, spiders, and crustaceans—rely on another hemolymph copper protein, hemocyanin, for oxygen transport (11). To our knowledge, no insect has been shown to contain hemocyanin in its hemolymph. The insect has developed an intricate tracheal system to distribute atmospheric oxygen directly to tissues (12). For this reason it is thought that hemocyanin was no longer necessary and subsequently disappeared from insect hemolymph. Proteins with certain degrees of homology to arthropod hemocyanin have been reported from insects, but they are not copper-containing proteins (13).

We report here the molecular cloning of an insect pro-PO§ and its sequence homology to the copper-binding sites [Cu(A) and Cu(B)] of arthropod hemocyanin, sequences highly homologous among arthropod hemocyanins (11). These data suggest that insect pro-PO has evolved from a common ancestral arthropod copper protein and distinguish insect pro-PO from prokaryotic and other eukaryotic POs.

MATERIALS AND METHODS

Experimental Animal. Larvae (fifth-instar, day 5 or 6) of the silkworm *Bombyx mori* were used for all experiments.

Pro-PO, Pro-PO-Activating Enzyme (PPAE), and Polyclonal Anti-Pro-PO/IgG. Pro-PO was purified from silkworm hemolymph (3) with minor modifications (4), and pro-PO activating enzyme (a serine-type proteinase) was purified from larval cuticles (14). Polyclonal anti-silkworm pro-PO IgG was prepared as described (4, 15).

Construction of cDNA Library. Hemocytes were obtained from silkworms (16). Total RNA from the hemocytes was extracted as described (17), and purification of poly(A)$^+$ RNA on oligo(dT)-cellulose was done according to standard methods (18). The cDNAs of the hemocyte poly(A)$^+$ RNAs were synthesized by using a cDNA synthesis kit (Pharmacia). cDNAs were introduced into a λ ZAPII phage vector (Stratagene) according to the manufacturer's instructions. The recombinant phage DNAs were packaged into bacteriophage λ particles by Gigapak II packaging extracts (Stratagene). The cDNA library contained 4.2×10^5 plaque-forming units.

Identification of Pro-PO Clones. The λ ZAPII phage vectors containing pro-PO cDNA were screened by using an anti-pro-PO/IgG and picoBlue immunoscreening kit. After restriction endonuclease analyses, putative pro-PO cDNA inserts of the positive λ phage clones were subcloned into phagemid vector pBluescript SK(−) (Stratagene).

DNA Sequencing Analysis. After preparation of deletion mutants of the putative pro-PO clones by use of a deletion kit (Wako Pure Chemical, Osaka), the DNA fragments of each mutant were sequenced by Sanger's dideoxynucleotide chain-termination method (18). All sequencing data were analyzed

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Abbreviations: PO, phenol oxidase; pro-PO, pro-phenol oxidase; PPAE, pro-PO activating enzyme; Cu(A) and Cu(B), copper-binding sites present in the second domain of the arthropod hemocyanin subunit; MALDI, matrix-assisted laser desorption ionization; ORF, open reading frame.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D49370 and D49371 for pro-PO polypeptides I and II, respectively).

by GENETYX software (Software Development, Tokyo). One pro-PO cDNA clone (pPO5) did not contain an ATG initiation codon in the 5′-upstream region. To isolate a pro-PO cDNA clone that included the 5′ upstream region of pPO5, the λ ZAPII cDNA library of the silkworm hemocyte was rescreened by plaque hybridization according to the standard method (18), using the 0.6-kb *Sac* I–*Eco*RI fragment of pPO5. The positive clones thus obtained were subjected to restriction endonuclease analysis and also subcloned into pBluescript SK(−) plasmid vector.

Isolation of Propeptides Released from Pro-PO by PPAE. Pro-PO (267 μg) was activated as described (4) in the presence of thiourea to inhibit the PO activity. The reaction mixture for the activation was treated at 100°C for 5 min to denature PO and then centrifuged at 105,000 × *g* for 20 min. The supernatant was treated with 4-vinylpyridine (4) for S-pyridylethylation of cysteine or cystine of propeptides. The resultant propeptides were purified by reversed-phase chromatography on an octadecyl column (YMC-pack ODS column; pore size, 300 Å; column size, 4 mm × 300 mm). The peptides were eluted by linear gradients of acetonitrile in 0.1% trifluoroacetic acid (0% acetonitrile–5% acetonitrile in 5 min, 5% acetonitrile–65% acetonitrile in 60 min; flow rate, 1.0 ml/min). The propeptides were eluted in two peaks at acetonitrile concentrations of 42% and 53%, respectively. Propeptides in the first peak and second peak were named propeptides I and II, respectively.

Isolation of PO Polypeptides I and II. Pro-PO was activated as described in the preceding section. The column chromatography of the reaction mixture on an octadecyl column was done under the same elution scheme as described (4) for the separation of pro-PO polypeptides I and II. A single but rather broad peak appeared at acetonitrile concentrations of 63–64%. The effluent containing the peak was collected in three equal-volume fractions (fractions A, B, and C from the peak beginning). Fractions A and C were rechromatographed on the octadecyl column under the same conditions. Polypeptides obtained from fractions A and C were designated as PO polypeptides II and I, respectively.

Amino Acid Sequence Analysis. Samples were sequenced according to Edman and Begg (19) on a Shimadzu protein sequencer PSQ-10.

Determination of the Mass of Propeptides. Propeptides purified on octadecyl column chromatography were analyzed for their mass numbers by matrix-assisted laser desorption ionization (MALDI) mass spectrometry as described (4), following Seraglia *et al.* (20).

RESULTS

Molecular Cloning and Sequencing Analysis of Pro-PO cDNA. Eight clones were isolated by immunoscreening with rabbit anti-pro-PO polyclonal IgG from ≈3.0 × 10^5 plaque-forming units of the cDNA library of silkworm hemocytes. The restriction maps of these clones were constructed. Two of these clones, pPO5 and pPO17, which belonged to the different classes by restriction endonuclease analysis, were selected for base sequencing. Clone pPO5 contained a 3520-bp insert including the 3′ untranslated region and the poly(A) tail but not the ATG initiation codon in the 5′ upstream region. To isolate a clone containing the initiation codon, the cDNA library of the silkworm hemocytes was rescreened by using a 0.6-kb *Sac* I–*Eco*RI fragment from pPO5 as a probe. Seventeen clones were isolated from the amplified library of 9.0 × 10^5 plaque-forming units of recombinant phages, and one of them, pPO5N1, was picked up according to restriction endonuclease analysis. Its nucleotide sequence was also determined. Clone pPO5N1 contained a 3615-bp insert of the pro-PO cDNA including the 5′ untranslated region, the initiation codon, and a part of the poly(A) tail. The insert was 102 bp longer at the 5′ upstream region and 7 bp shorter at the 3′ downstream region than pPO5. The complete nucleotide sequence of 3622 bp from the inserts of pPO5 and pPO5N1 has been determined, and the cDNA with the complete sequence was designated as SPT-I. The cDNA insert of pPO17 was named SPT-II. The restriction maps of SPT-I and SPT-II are presented in Fig. 1.

The open reading frame (ORF) of SPT-I, nt 21–2075, encodes 685 amino acids and terminates at the TAG termination codon. The amino acid sequence deduced from SPT-I ORF matched the amino acid sequences so far determined for 12 peptides, including 213 amino acid residues from pro-PO polypeptide I, except that the 171st residue, deduced to be valine, appeared to be alanine in direct sequencing by the Edman method. Another cDNA, SPT-II, contained 2785 bp, including the intact ORF. This ORF, nt 45–2123, encodes 693 amino acids and terminates at the TAA termination codon presented at position 2124. In the 3′ untranslated region, no poly(A) signal was detected. The deduced amino acid sequence matched those of nine peptides, including 178 amino acid residues from pro-PO polypeptide II, except for the 433rd residue, which was deduced to be proline but appeared to be glutamine in Edman degradation. The reasons for the inconsistencies between the deduced and directly determined amino sequences are not clear and were not further studied.

The molecular weights of pro-PO polypeptides I and II were calculated to be 78,653 and 80,003, respectively, from the deduced amino acid sequences presented in Fig. 2. In the calculation N-terminal methionines of the deduced sequences were excluded. The molecular weights are very close to those previously determined by MALDI mass spectometry (78,880 and 81,105 for pro-PO polypeptides I and II, respectively), supporting our observation that pro-PO lacks a detectable amount of carbohydrate (4).

Comparison of Amino Acid Sequences Deduced from ORF Base Sequences of SPT-I and SPT-II. Fig. 2 shows the comparison between amino acid sequences of pro-PO polypeptides deduced from SPT-I and SPT-II. In each deduced sequence five possible N-linked glycosylation sites were found, and they are indicated in Fig. 2 by horizontal lines. The homology between the deduced amino acid sequences is 51%. Significant conservation was noted in the regions of I–IV shown in Fig. 2. Two polypeptides encoded by SPT-I and SPT-II appear to have similar secondary structures because 7 of 10 cysteine residues were conserved at the same positions in the sequences. Neither of the deduced sequences of pro-PO polypeptides I and II has a leader sequence for secretion of the molecule.

Amino Acid Sequence Homology. Homologous sequences to the deduced amino acid sequences were searched out through the National Biomedical Research Foundation data base. It was found that both amino acid sequences of pro-PO polypeptides I and II deduced from SPT-I and SPT-II had significant

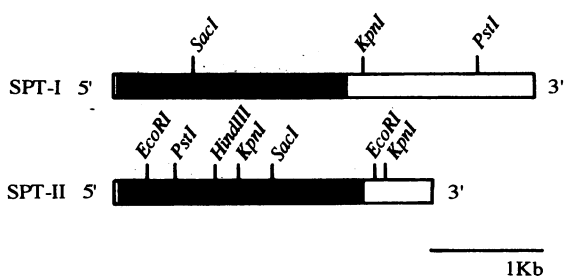

FIG. 1. Restriction site maps of SPT-I (cDNA of pro-PO polypeptide I) and SPT-II (cDNA of pro-PO polypeptide II). Direction of transcription is from left to right. The dark end and open areas represent open reading frames and 5′ and 3′ untranslated regions, respectively.

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proPO-P1   1 MSDAKNNLLLFFDRPSEPCFMQKGEENAVFEIPDNYYPEKYQRVSNAIGN
proPO-P2   1 MADVFESLELLFDRPNEPLITPKGENNSVFQLTEQFLTEDYANNGIELNN

proPO-P1  51 RFGSDAGRMIPIRNIA-LPNLDLPMELPYNEQFSLFVPKHRKLAGRLIDI
proPO-P2  51 RFGDDASEKIPLKNLSKLPEFKIATQLPKDAEFSLFLPKHQEMANELLGV

proPO-P1 100 FMGM--RDVEDLQSVCSYCQLRINPYMFNYCLSVAILHRPDTKGLSIPTF
proPO-P2 101 LMDVPENELQDLLSTCAFARVNLNPQLFNYCYSVALMHRRDTRKVRVKNF

proPO-P1 148 AESFPDKFMDPKVFRQAREVSSVVPSG-ARMPIVIPSNYTASDTEPEQRV
proPO-P2 151 AEVFPSKFLDSQVFTQARETAAVIPPDVPRIPIIIPRDYTATDLEEEHRL

                              I
proPO-P1 197 AYFREDIGINLHHWHWHLVYPFDAADRAIVNKDRRGELFYYMHQQIIARY
proPO-P2 201 AYWREDIGINLHHYHWHLVYPFTANDLSIVAKDRRGELFFYMHQQVIARF

proPO-P1 247 NVERMCNNLSRVRRYNNFRAAIEEGYFPKLDSTVASRAWPPRFAGTTIRD
proPO-P2 251 NCERLCNSLKRVKKFSNWREPIPEAYFPKLDSLTSSRGWPPRQSGMQWQD

proPO-P1 297 LDRPVDQIRSDVSELETWRDRFLQAIENMSVMLPNGRQLPLDEETGIDVL
proPO-P2 301 LNRAAEGLFVTIDEMERWRRNVEEAIATGTVRLPNGQTRPLD----IDTL

                                        II
proPO-P1 347 GNLMESSIISRNRPYYGDLHNMGHVFISYSHDPDHRHLEQFGVMGDSATA
proPO-P2 347 GNMLESSALSPNRELYGSIHNNGHSFTAYMHDPEHRYLEQFGVIADEATT

proPO-P1 397 MRDPVFYRWHAYIDDIFHLYK---YKLTPYGNDRLDFPNIRVSSVSIEG-
proPO-P2 397 MRDPFFYRWHAYIDDVFQKHKESAY-VRPYTRSELENPGVQVRSVSVETP

proPO-P1 443 GGTPNTLNTLWEQSTVDLGRGMDFTPRGSVLARFTHLQHDEYNYVIEVNN
proPO-P2 446 GGQPNTLNTFWMLSDVNLSRGLDFSDNGPVYARFTHLNYRHFSYRINVNN

proPO-P1 493 TGGSSVMGMFRIFIAPTVDESGKPFSFDEQRKLMIELDKFSQGVKPGNNT
proPO-P2 496 TGSSRRTTV-RIFITPKFDERNVPWIFSDQRKMCIEMDRFVTVLNAGENN

                                                    III
proPO-P1 543 IRRKSIDSSVTIPYERTFRNQADRPADPGTAGAAEFDFCGCGWPHHMLVP
proPO-P2 545 IVRQSTESSITIPFEQTFRDLSAQGNDPRRDELATFNYCGCGWPQHMLVP

proPO-P1 593 KGTTQGYPMVLFVMVSNWNDDRVEQDLV--GSCNDAASYCGIRDRKYPDR
proPO-P2 595 KGTEAGMPFQLFVMLSNYDLDRIDQDDGKQLTCVEASSFCGLKDKKYPDR

             IV
proPO-P1 641 RAMGFPFDRPAPAATTLSDFLRPNMAVRDCIVRFTDRTRQRGQQG
proPO-P2 645 RAMGFPFDRPSSSATSLQDFILPNMGLQDITIQLQNVTEPNPRNPPMSV
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FIG. 2. Comparison between predicted amino acid sequences of pro-PO polypeptides I and II. Residues of the predicted amino acid sequences are numbered at left. Stars refer to the identical amino acid residues between pro-PO-P1 and pro-PO-P2. An arrow indicates peptide bonds to be hydrolyzed by PPAE. Open triangles represent the conserved cysteine residues. Possible N-linked glycosylation sites are underlined. Regions of I, II, III, and IV represent highly homologous sequences among pro-PO polypeptides I, II, and the hemocyanins of several species. proPO-P1 and proPO-P2 represent pro-PO polypeptides I and II, respectively.

homologies with amino acid sequences of arthropod hemocyanins. The amino acid sequence deduced from SPT-I showed 34–39% homology with chains A, D, and E from *Eurypelma californicum* hemocyanin (11), 29% with the chain A from *Panulirus interruptus* hemocyanin (11), and 37% with subunit II from *Limulus polyphemus* hemocyanin (11). The amino acid sequence deduced from SPT-II also showed 34–38% homology with chains A, D, and E from *E. californicum* hemocyanin, 29% with chain A of *P. interruptus* hemocyanin, and 36% with subunit II from *L. polyphemus* hemocyanin. Conspicuous results emerged from the homology search in that amino acid sequences of regions I and II (Fig. 2) of the deduced pro-PO polypeptide sequences were in good agreement with those of Cu(A) and Cu(B) of arthropod hemocyanins, respectively. In the case of spiny lobster (*P. interruptus*) hemocyanin, each copper ion was shown by x-ray crystallography to be chelated by three histidine residues in Cu(A) and Cu(B), and binuclear copper atoms play a role in its oxygen-carrying capacity (21). Fig. 3 shows the comparison of amino acid sequences between region I and II of silkworm pro-PO polypeptides and pro-POs from other arthropods (22, 24, 26) and the sequences of Cu(A) and Cu(B) of some arthropod hemocyanins (11). Six histidine residues, which were found to be copper-binding ligands in spiny lobster hemocyanin, perfectly aligned to histidine residues found in regions I and II of pro-PO sequences.

N-Terminal Amino Acid Sequences of PO Polypeptides I and II. N-terminal amino acid sequences were analyzed by automated Edman degradation up to 10 residues from the N termini of the polypeptides. The results were FGSDAGRMIP- and FGDDASEKIP- for PO polypeptides I and II, respectively. The sequences were identical with those found between residues 52 and 61 of pro-PO polypeptide sequences deduced from base sequences of cDNAs of pro-PO polypeptides I and II (Fig. 2), indicating that PO polypeptides I and II originated from pro-PO polypeptides I and II, respectively. This result indicated that the PPAE hydrolyzes peptide bond between residues 51 (arginine) and 52 (phenylalanine) of the deduced sequences of pro-PO polypeptides I and II. The sequence motif around the peptide bond to be hydrolyzed was Asn-Arg-Phe-Gly.

N-Terminal Sequence Analyses of Propeptides I and II. Propeptides I and II were subjected to automated Edman degradation. Neither of the pro-peptides gave an N-terminal amino acid, indicating that α-amino groups of the N-terminal amino acids of the peptides are blocked.

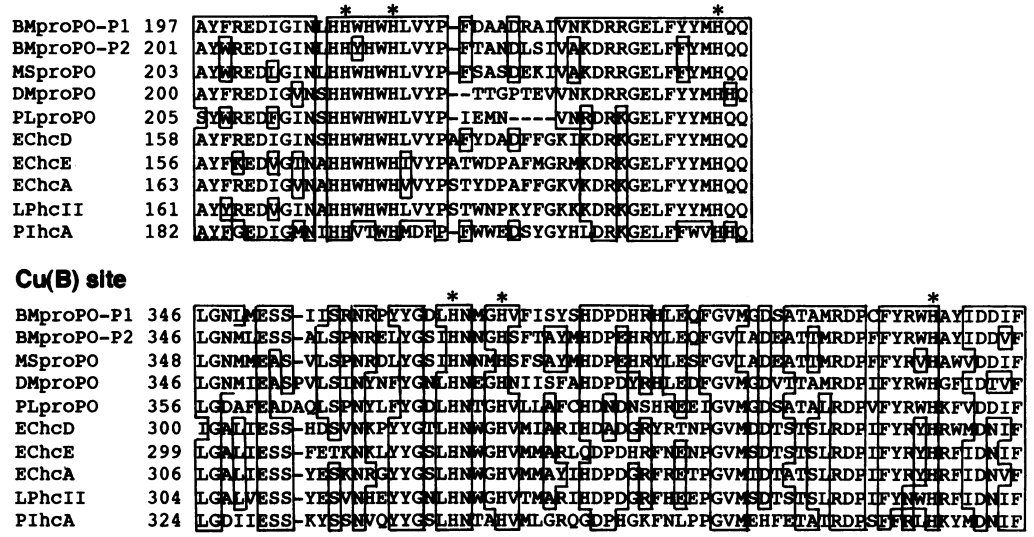

FIG. 3. Comparison of putative copper-binding sequences in silkworm pro-PO with those of other arthropod pro-POs and with sequences at Cu(A) and Cu(B) of arthropod hemocyanins. Numbers at left of each lane represent the amino acid residues of proteins. Gaps (−) are introduced to optimize alignment. The highly conservative amino acid residues are enclosed by solid lines. Asterisks show the position of histidine residues that are identified as ligands for copper atoms in *P. interruptus* hemocyanin. BMproPO-P1 and BMproPO-P2, *B. mori* (silkworm) pro-PO polypeptides I and II, respectively; MSproPO, *Manduca sexta* (tobacco hornworm) pro-PO (22); DMproPO, *Drosophila melanogaster* (fruit fly) pro-PO (23); PLproPO, *Pacifastacus leniusculus* (crayfish) pro-PO (24); EChcD, EChcE, and EChcA, *E. californicum* (tarantula) hemocyanin chains D, E, and A, respectively (11); LPhcII, *L. polyphemus* (horseshoe crab) hemocyanin subunit II (11); PIhcA, *P. interruptus* (spiny lobster) hemocyanin subunit A (25).

Molecular Masses of the Propeptides Being Cleaved from Pro-PO by PPAE. The molecular masses of propeptides I and II were studied by MALDI mass spectrometry. As the cysteine sulfhydryl group of one of the propeptides was S-pyridylethylated, theoretical molecular masses of propeptides I and II calculated from the sequence data presented in Fig. 2 should be 5961.4 Da and 5756.2 Da, respectively, where the N-terminal methionine residues in the predicted sequences are excluded. The observed mass numbers of propeptides I and II were determined as 6000 and 5796, respectively. Taking into account the accuracy of MALDI mass spectrometry, N-acetylation of the N-terminal amino acids of the propeptides practically accounts for the differences between the calculated and the observed mass numbers. Together with our previous observation that PO polypeptides I and II are not further degraded by PPAE (4), the results of MALDI mass spectrometry of the propeptides indicated that PPAE hydrolyzes one peptide bond in each of the pro-PO polypeptides and that propeptides I and II are cleaved from pro-PO polypeptides I and II, respectively.

DISCUSSION

The oxygen carrier protein hemocyanin is found in the plasma fraction of hemolymph of molluscs and primitive arthropods such as horseshoe crab, crustaceans, and spiders. The primary structures of the hemocyanins have been worked out (11, 23). All arthropod hemocyanin subunits so far sequenced have molecular masses of ≈80 kDa and contain two copper atoms. The current oxygen-binding center [Cu(A) and Cu(B)] of arthropod hemocyanin is speculated to have evolved from an ancestral mono-copper protein by gene duplication and subsequent gene fusion (23, 27). It is clear from Fig. 3 that each of the silkworm pro-PO polypeptides contains polypeptide segments showing high homology to the sequences of Cu(A) and Cu(B). Recently, pro-POs from fruit fly, tobacco hornworm, and crayfish were also shown to have domains homologous to Cu(A) and Cu(B) sequences (22, 24, 26). The histidine residues in regions I and II in Fig. 2 of the silkworm pro-PO polypeptides could be perfectly aligned to histidine residues that have been demonstrated to be copper ligands of arthropod hemocyanins (21) (Fig. 3). As the silkworm pro-PO has been shown to contain two copper atoms per 80,000 Da (3, 4), it is highly probable that the histidine residues are also the ligands for copper atoms of silkworm pro-PO. This finding is surprising because all POs so far sequenced have been shown to have a polypeptide segment homologous to Cu(B) sequence, but the sequence of the second copper-binding site is very different from the sequences of Cu(A)s of arthropod hemocyanins (23, 28). It has been speculated that POs have evolved from two separate mono-copper proteins by gene fusion (23). The same situation is inferred on the evolution of molluscan hemocyanin, which has a homologous copper-binding site to Cu(B), but another copper-binding site that is not homologous to the copper-binding-site sequences of arthropod hemocyanins (23). A phylogenetic relationship among arthropod hemocyanin, molluscan hemocyanin, and POs has been proposed (23) in which molluscan hemocyanins are claimed to be more closely related to POs than to arthropod hemocyanins. A phylogenetic tree was constructed by the neighbor-joining method (29) based on the sequences of arthropod pro-POs, hemocyanins, and tyrosinases. As is seen in Fig. 4, arthropod hemocyanins and arthropod pro-POs grouped in a family, whereas tyrosinases and molluscan hemocyanins are in another family. We suspect silkworm pro-PO has evolved from the same ancestral binuclear copper protein as arthropod hemocyanins.

In this study PPAE was shown to hydrolyze the peptide bond at the carboxyl side of arginine in the -Asn-Arg-Phe-Gly- sequence in pro-PO polypeptides I and II (Fig. 2), thus activating pro-PO to PO. Structural changes incurred by this hydrolysis are likely to endow the proenzyme with catalytic activity as PO. However, the physical process involved in this transformation of silkworm pro-PO to PO remains to be studied. Pro-POs from *M. sexta* and *D. melanogaster* were shown to have Asn-Arg-Phe sequences at positions 50–52 and 51–53, respectively (22, 26), suggesting that the Arg-Phe bond is also hydrolyzed when these pro-POs are activated *in vivo*.

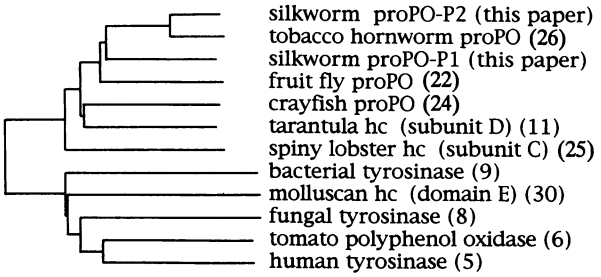

FIG. 4. Phylogenetic tree of hemocyanins, tyrosinases, and arthropod pro-POs. The phylogenetic tree is constructed by the neighbor-joining method (29). Amino acid sequences of proteins are from references indicated by numbers in parentheses. proPO-P1 and proPO-P2, pro-PO polypeptides I and II, respectively; hc, hemocyanin.

Another feature of amino acid sequences of pro-PO is the lack of a signal peptide to facilitate its secretion. Our previous studies indicated oenocytoid hemocytes as the major site of pro-PO synthesis (15, 16). Ultrastructurally, oenocytoids are atypical in that they are composed almost exclusively of ribosomes and mitochondria, with no visible rough endoplasmic reticulum (15). Accordingly, our result may suggest that pro-PO is released from oenocytoids by cell rupture. This contention again implies the close relation of silkworm pro-PO to arthropod hemocyanin because arthropod hemocyanin cDNA lacked signal peptide sequences and, hence, arthropod hemocyanin is claimed to be released from hemocytes by cell rupture (31).

The high homology between silkworm pro-PO and arthropod hemocyanins raises the intriguing question whether pro-PO is capable of, and involved in, O_2 transport in insect hemolymph and to tissue. We have no experimental data supporting this question. However, recent observations (M.A. and P. T. Brey, unpublished work) show the presence of pro-PO in the trachial cuticle, through which atmospheric O_2 is supplied to tissues (12). These observations warrant further investigation as to the role of pro-PO in the respiratory physiology of insects.

We are grateful to Drs. Eiji Ohnishi and John H. Law for sharing their data with us before publication and to Miss Yoshiko Koizumi at The Institute of Low Temperature Science, Hokkaido University, for her technical assistance. This work was supported, in part, by research grants from the Japan Ministry of Education, Science, and Culture (Grants 06554037 and 06454023) and the Japan Ministry of Agriculture, Forestry and Fisheries (BMP 95-III-2-1-2) to M.A.

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