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Nucleotide sequence of the cDNA encoding the proenzyme of phenol oxidase A_1 of *Drosophila melanogaster*

(hemocyanin)

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ABSTRACT Clones encoding pro-phenol oxidase [pro-PO; zymogen of phenol oxidase (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1)] A_1 were isolated from a λgt10 library that originated from *Drosophila melanogaster* strain Oregon-R male adults. The 2294 bp of the cDNA included a 13-bp 5′-noncoding region, a 2070-bp encoding open reading frame of 690 amino acids, and a 211-bp 3′-noncoding region. A hydrophobic NH_2-terminal sequence for a signal peptide is absent in the protein. Furthermore, there are six potential N-glycosylation sites in the sequence, but no amino sugar was detected in the purified protein by amino acid analysis, indicating the lack of an N-linked sugar chain. The potential copper-binding sites, amino acids 200–248 and 359–414, are highly homologous to the corresponding sites of hemocyanin of the tarantula *Eurypelma californicum*, the horseshoe crab *Limulus polyphemus*, and the spiny lobster *Panulirus interruptus*. On the basis of the phylogenetic tree constructed by the neighbor-joining method, vertebrate tyrosinases and molluscan hemocyanins constitute one family, whereas pro-POs and arthropod hemocyanins group with another family. It seems, therefore, likely that pro-PO originates from a common ancestor with arthropod hemocyanins, independently to the vertebrate and microbial tyrosinases.

In insects, phenol oxidase (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) plays key roles in various aspects of life such as sclerotization and pigmentation of cuticles, wound healing, and defense reactions (1). The enzyme occurs in hemolymph, as well as in cuticles, mostly in an inactive precursor, pro-phenol oxidase (pro-PO). Activation of the pro-PO was first found in the fruit fly *Drosophila melanogaster* (2, 3). The pro-PO has been isolated in a homogeneous state from the silkworm *Bombyx mori* (4), the tobacco hornworm *Manduca sexta* (5), the saturniid *Hyalophora cecropia* (6), the housefly *Musca domestica* (7), and the fruit fly *D. melanogaster* (8).

In *Drosophila*, the pro-PO occurs as multiple forms, which could be separated by ammonium sulfate fractionation and electrophoresis (9). Two molecular forms, A_1 and A_3, have been isolated from pupae and well characterized (8). The gene encoding A_1 (*Mox*) is mapped at 79.6 on the right arm of chromosome 2, and the deletion mapping indicates the cytological position at 55A–B (10). Pentz and Wright (11) have cloned and sequenced *Dox-A2* gene, which seems to encode one of the components in the activating cascade system of pro-PO.

For the present study, we have cloned and sequenced cDNA coding for pro-PO A_1 of *D. melanogaster*.§ The deduced amino acid sequence indicates a close relationship of this protein to arthropod hemocyanins but a rather remote relationship to vertebrate tyrosinases.

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MATERIALS AND METHODS

Materials. A λgt10 cDNA library for *D. melanogaster* Oregon-R strain, prepared by Poole *et al.* (12), was used for screening. Other sources of materials used in this work were as follows: a plasmid SK from Takara Shuzo (Kyoto), a Chemcosorb 5-ODS-H column from Chemco Science (Osaka), *N*-tosylphenylalanine chloromethyl ketone (TPCK)-trypsin from Worthington, and a DNA-labeling kit and restriction enzymes from Nippon Gene (Tokyo).

Peptide Preparation and Sequencing. pro-PO A_1 was purified from pupae, 24–48 hr after pupariation, as described (8). Because the NH_2-terminal amino acid of pro-PO A_1 was found to be blocked, the purified protein was fragmented to obtain partial amino acid sequences. Purified pro-PO A_1 (0.3 mg of protein) was reduced with dithiothreitol, S-alkylated with iodoacetamide, and then digested with TPCK-trypsin at 37°C for 12 hr. Peptides were separated by reversed-phase HPLC using a Chemcosorb 5-ODS-H column (2.2 × 150 mm). Peptides (designated TR for tryptic) were eluted by a linear gradient of 0–72% acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 0.2 ml/min. Amino acid sequence of the purified peptides was determined by 473A and 477A gas-phase protein sequencers (Applied Biosystems) with the chemicals and program supplied by the manufacturer.

Cloning Strategy. PCR with degenerate oligonucleotides was used to amplify a cDNA fragment encoding the protein. Sense and antisense oligonucleotide primers were synthesized with *Eco*RI site at 5′ end using a DNA synthesizer (model 380B, Applied Biosystems), based on the amino acid sequences of TR22 (-DYQPKI-), TR51 (-DFNGIT-), and TR53 (-IEMDKF-). PCRs with the cDNA library and each primer were done in a Perkin–Elmer/Cetus thermal cycler for 30 cycles of denaturation (94°C, 30 sec), annealing (50°C, 1 min), and extension (70°C, 2 min). Amplified PCR products were sequenced, and one PCR product, P1 (TR53S primer-λgt10 reverse primer), was found to contain the sequence of pro-PO A_1. The P1 probe was labeled with [α-^{32}P]dCTP by means of a DNA labeling kit and used to screen the λgt10 library. Hybridization and screening of positive plaques were done as described (13). The cDNA inserts of the plaque-purified positive clones were then ligated into plasmid SK, and their nucleotide sequences were determined. The dideoxynucleotide chain-termination method was used to sequence cDNA fragments with the dye terminator sequencing kit (Applied Biosystems), and nucleotide sequences were determined by an Applied Biosystems model 370A DNA sequencer.

Computer Analysis of Sequence Data and Homology Search. The amino acid sequence of pro-PO A_1 was compared with all entries in the Swiss-Prot data base (release 14.0, October 1994) and the Gene Works system (IntelliGenetics).

Abbreviations: TPCK, *N*-tosylphenylalanine chloromethyl kinase; pro-PO, pro-phenol oxidase.
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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. D45835).

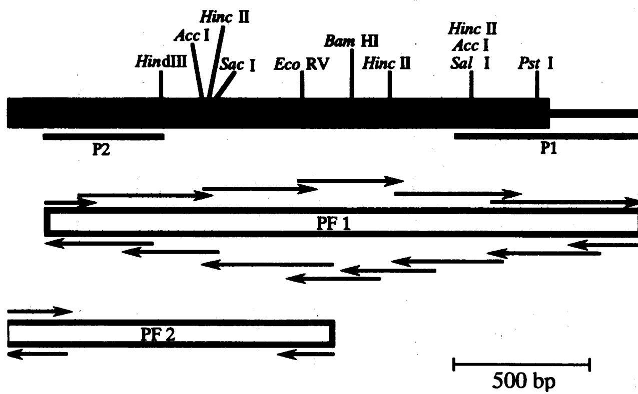

FIG. 1. Restriction map and sequencing strategy for *D. melanogaster* pro-PO A_1. Horizontal arrows indicate the direction of sequencing and the extent of information obtained from each cDNA clone. The two clones (PF1 and PF2) are shown by open bars, and the two probes (P1 and P2) are shown by solid bars.

Phylogenetic tree construction by the neighbor-joining method (14) was done with the CLUSTAL V computer software (15).

RESULTS AND DISCUSSION

Peptide Sequencing. No phenylthiohydantoin amino acid derivative was obtained by Edman reaction with the purified pro-PO A_1, suggesting the protein has a blocked NH_2 terminus. To obtain partial amino acid sequences, the protein was reduced, S-alkylated, and digested with TPCK-trypsin, and the resulting peptides were separated by reversed-phase HPLC. Among >60 peptides, 14 peptides were selected and sequenced. Sequences totaling 191 amino acids, which cover ≈30% of the protein, were identified (underlined amino acids in Fig. 2).

Cloning and Nucleotide Sequence of Pro-PO A_1. Screening of 4.5×10^5 recombinant phages (adult male cDNA library) with the P1 probe gave one positive clone, designated PF1, which has a 2.1-kb insert, as shown in Fig. 1. The insert in PF1 was sequenced, and parts of the sequence corresponded to the

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GATAGAGAAAACCATGACTAACACGGATCTGAAAGCCTTGGAACTTATGTTCCAGCGACCCCTGGAACCGGCATTCACCACTCGTGACTC   90
              M  T  N  T  D  L  K  A  L  E  L  M  F  Q  R  P  L  E  P  A  F  T  T  R  D  S   26
CGGAAAAACTGTATTGGAACTGCCCGATTCCTTCTATACGGATCGCTATCGCAATGACACCGAGGAGGTGGGCAATCGATTCTCTAAGGA  180
 G  K  T  V  L  E  L  P  D  S  F  Y  T  D  R  Y  R  N  D  T  E  E  V  G  N  R  F  S  K  D   56
CGTGGATCTGAAGATCCCAATTCAGGAGTTGAGCAACGTTCCCAGTCTGGAGTTCACCAAAAAGATTGGTCTCAAGAATCAATTTTCGCT  270
 V  D  L  K  I  P  I  Q  E  L  S  N  V  P  S  L  E  F  T  K  K  I  G  L  K  N  Q  F  S  L   86
GTTTAACAATCGCCATCGTGAGATCGCCAGCGAGCTGATCACCCTCTTCATGAGCGCACCAAATCTCAGACAATTCGTGTCGCTTTCCGT  360
 F  N  N  R  H  R  E  I  A  S  E  L  I  T  L  F  M  S  A  P  N  L  R  Q  F  V  S  L  S  V  116
TTACACCAAGGATCGTGTGAATCCTGTGTTGTTCCAATACGCCTATGCCGTCGCCGTTGCCCATCGCCCGGATACGCGTGAAGTGCCCAT  450
 Y  T  K  D  R  V  N  P  V  L  F  Q  Y  A  Y  A  V  A  V  A  H  R  P  D  T  R  E  V  P  I  146
TACCAACATTTCCCAGATCTTTCCGAGCAACTTTGTAGAACCTTCCGCCTTCCGGGATGCCCGTCAGGAAGCTTCTGTCATTGGAGAGAG  540
 T  N  I  S  Q  I  F  P  S  N  F  V  E  P  S  A  F  R  D  A  R  Q  E  A  S  V  I  G  E  S  176
CGGCGCTCGTGTCCATGTGGACATTCCGCAAAACTACACGGCCTCGGATCGCGAGGATGAGCAGCGCCTGGCGTACTTCCGTGAGGACAT  630
 G  A  R  V  H  V  D  I  P  Q  N  Y  T  A  S  D  R  E  D  E  Q  R  L  A  Y  F  R  E  D  I  206
CGGTGTTAATAGCCATCATTGGCACTGGCACTTGGTCTACCCGACCACCGGACCCACGGAGGTGGTCAACAAGGATCGTCGTGGCGAGCT  720
 G  V  N  S  H  H  W  H  W  H  L  V  Y  P  T  T  G  P  T  E  V  V  N  K  D  R  R  G  E  L  236
CTTCTACTACATGCACCACCAGATACTCGCGCGCTACAATGTGGAGCGCTTCTGCAACAATCTGAAGAAGGTTCAGCCGCTGAACAATCT  810
 F  Y  Y  M  H  H  Q  I  L  A  R  Y  N  V  E  R  F  C  N  N  L  K  K  V  Q  P  L  N  N  L  266
GCGTGTGGAGGTTCCCGAGGGCTACTTCCCCAAGATCCTGTCCAGCACGAATAACCGCACCTATCCCGCCCGAGTGACCAACCAAAAGCT  900
 R  V  E  V  P  E  G  Y  F  P  K  I  L  S  S  T  N  N  R  T  Y  P  A  R  V  T  N  Q  K  L  296
ACGGGACGTGGACCGCCATGATGGGCGTGTGGAGATCTCCGACGTGGAGCGCTGGCGTGATCGCGTGCTGGCTGCCATAGATCAAGGATA  990
 R  D  V  D  R  H  D  G  R  V  E  I  S  D  V  E  R  W  R  D  R  V  L  A  A  I  D  Q  G  Y  326
CGTTGAAGATTCTTCTGGCAATCGTATACCATTGGACGAGGTTCGTGGCATCGATATCCTGGGCAACATGATTGAGGCATCGCCAGTCCT 1080
 V  E  D  S  S  G  N  R  I  P  L  D  E  V  R  G  I  D  I  L  G  N  M  I  E  A  S  P  V  L  356
ATCCATCAACTATAATTTCTATGGCAATCTGCACAACGAGGGACACAACATAATCTCCTTTGCCCACGATCCCGATTACCGTCACTTGGA 1170
 S  I  N  Y  N  F  Y  G  N  L  H  N  E  G  H  N  I  I  S  F  A  H  D  P  D  Y  R  H  L  E  386
GGACTTCGGTGTGATGGGCGATGTGACCACGGCCATGAGGGATCCAATCTTCTACAGGTGGCACGGATTCATCGACACGGTGTTCAACAA 1260
 D  F  G  V  M  G  D  V  T  T  A  M  R  D  P  I  F  Y  R  W  H  G  F  I  D  T  V  F  N  K  416
ATTCAAGACTCGCCTGAACCCGTACAATGCCGGTGAGCTTAACTTCGATGGTATTACCGTGGACTACATTGAGGCCAAGATTGGCAAGTC 1350
 F  K  T  R  L  N  P  Y  N  A  G  E  L  N  F  D  G  I  T  V  D  Y  I  E  A  K  I  G  K  S  446
CAATACCAAGGCCAACACCCTGTTGACCTACTGGCAGAAGTCGAGTGCCGACTTGGCAGCTGGCCTGGACTTTGGACCAACGACCGATCG 1440
 N  T  K  A  N  T  L  L  T  Y  W  Q  K  S  S  A  D  L  A  A  G  L  D  F  G  P  T  T  D  R  476
CAACATATTCGCCTCGTTCACCCATCTCCAGAATGCCCCCTTCACTTACACCTTCAATGTGACCAATAATGGAGCCAGACGAACGGGTAC 1530
 N  I  F  A  S  F  T  H  L  Q  N  A  P  F  T  Y  T  F  N  V  T  N  N  G  A  R  R  T  G  T  506
TTGCCGTATCTTTATCTGCCCCAAGGTGGATGAGCGTAATCAGGCTTTGAATCTGGAGGAGCAGCGACTGTTGGCCATCGAAATGGACAA 1620
 C  R  I  F  I  C  P  K  V  D  E  R  N  Q  A  L  N  L  E  E  Q  R  L  L  A  I  E  M  D  K  536
GTTCACAGTTGATTTGGTGCCTGGTGAGAACACCATACGCCGCCAGTCGACAGAGTCCTCGGTGGCCATTCCCTTCGAGCGCTCATTCCG 1710
 F  T  V  D  L  V  P  G  E  N  T  I  R  R  Q  S  T  E  S  S  V  A  I  P  F  E  R  S  F  R  566
CCCGGTTGGAGCAGACTATCAGCCCAAGGCCGCCGATGAGTTGGCACGCTTCAAGTTTTGCGGATGTGGGTGGCCACAGCATCTCCTGCT 1800
 P  V  G  A  D  Y  Q  P  K  A  A  D  E  L  A  R  F  K  F  C  G  C  G  W  P  Q  H  L  L  L  596
GCCCAAGGGCAATGCCCAGGGAATGCTCTTCGACCTGTTCGTCATGATTTCCGATTACTCGCAGGACTCCGTTGAGCAGCCCAAAACACC 1890
 P  K  G  N  A  Q  G  M  L  F  D  L  F  V  M  I  S  D  Y  S  Q  D  S  V  E  Q  P  K  T  P  626
CAACGATGCCTGCAGCACGGCGTACTCGTTCTGTGGTCTGAAGGACAAATTGTATCCTGACAGACGCACCATGGGCTATCCCTTCGATAG 1980
 N  D  A  C  S  T  A  Y  S  F  C  G  L  K  D  K  L  Y  P  D  R  R  T  M  G  Y  P  F  D  R  656
GCGCCTGCCCAATGCCAATCTCACCGAATTGGTCGGTGCCTTTGGCAATATGGCCAAGACCGATTTAAGGATTGTCTTCAATGATCGTGT 2070
 R  L  P  N  A  N  L  T  E  L  V  G  A  F  G  N  M  A  K  T  D  L  R  I  V  F  N  D  R  V  686
GATTGACAAGGCCTAGATGATTGCTCCAACTAACAGAGATGATTTTAAGAATCAATGGAAACGGACTTGAAAACGGAAAACTCTTGGAAT 2160
 I  D  K  A                                                                                690
CATATACACCTTTTGCTATTAACTTATAACTAACCAAACTTGTGCGCGTTTATGTGGTGTTTTATCGTTTCTAATTACACTCTACAAAAA 2250
TAAATGTATTGTAATATGTGCAGGCAGTAAAAAAAAAAAAAAAA                                              2294
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FIG. 2. Nucleotide sequence and deduced amino acid sequence of *D. melanogaster* pro-PO A_1. Nucleotides and amino acid residues are numbered at right. Single underlinings represent sequences determined by amino acid sequence analysis of isolated peptides. Double-underlinings represent potential N-glycosylation sites. A poly(A) signal is boxed.

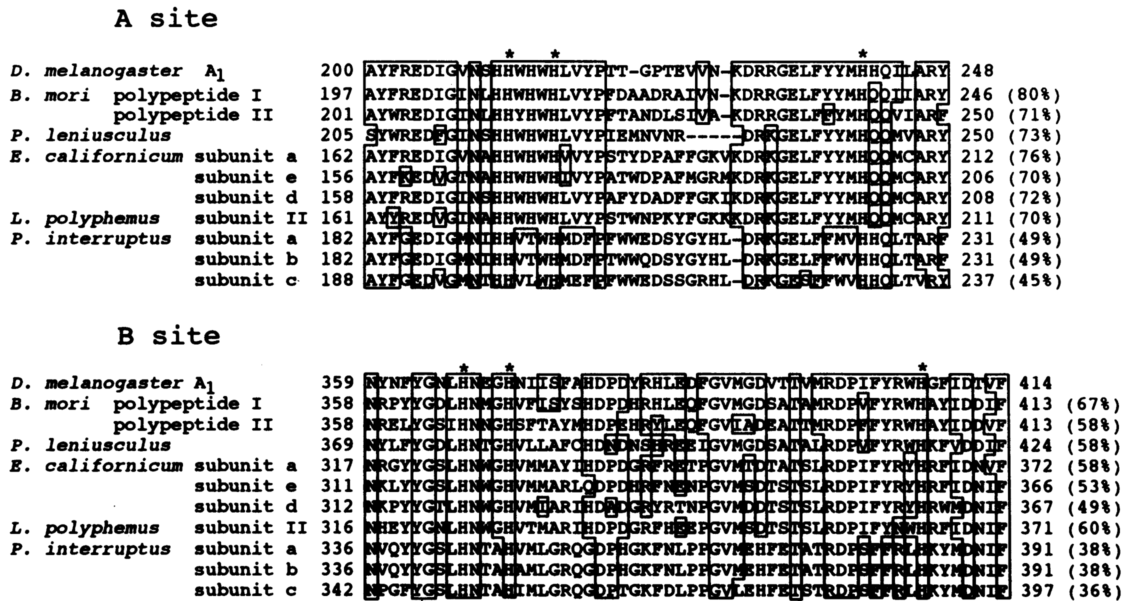

FIG. 3. Alignment of the deduced amino acid sequence at two copper-binding regions (A site and B site) of *D. melanogaster* pro-PO A_1 with those of *B. mori* pro-PO polypeptide I and II, *Pacifastacus leniusculus* pro-PO, and corresponding regions of hemocyanins from *Eurypelma californicum*, *Limulus polyphemus*, and *Panulirus interruptus*. Gaps (-) are introduced to optimize the alignment. Numbers indicate positions of amino acid residues in each amino acid sequence. Stars indicate the putative copper ligands of histidine residues.

amino acid sequences of 13 of the 14 peptides derived from a tryptic digest of pro-PO A_1. This clone was not full length, however, because the amino acid sequence of the remaining peptide was not encoded in its nucleotide sequence and it did not contain an ATG initiation codon. The same cDNA library was then rescreened with P2, the digested fragment of PF1 with *Hin*dIII (Fig. 1). A second clone, PF2, was obtained with a 1.2-kb insert that covered the 5′ region of pro-PO A_1 containing the remaining peptide sequence. Clones PF1 and PF2 were subjected to restriction mapping, and their sequences were determined on both strands by the strategy shown in Fig. 1. The composite cDNA sequence of the two clones included 2294 nt. There is one open reading frame, beginning at nt 14, corresponding to the first nucleotide of the putative initiation methionine codon (ATG), and ending at nt 2084, followed by a 3′-noncoding region including a poly(A) signal. The sequence around the methionine is ACCATGA, which is nearly identical to the optimal initiation sequence of ACCATGG for eukaryotic ribosomes (16), suggesting that this methionine codon is the initiation codon for the protein.

Deduced Amino Acid Sequence. The open reading frame codes for a protein of 690 amino acid residues (Fig. 2). The calculated M_r of 78,903 agrees well with that of the purified protein, which has been estimated by SDS/PAGE (M_r = 78,000) (8). Amino acid sequences of 14 peptides corresponded to the protein sequence deduced from the cDNA sequence, except for 6 residues: Met-12, Asn-422, Asn-425, Asn-430, Asp-432, and Asn-437. These residues had been identified as leucine, aspartate, aspartate, aspartate, asparagine, and aspartate in the peptide sequencing, respectively. The reason for this discrepancy is unclear. Possibly asparagine in certain amino acid sequences is liable to be deamidated during the sequencing procedure, as has been shown for *Panulirus* hemocyanin (17). No hydrophobic sorting signal sequence for the endoplasmic reticulum is found in the NH_2-terminal region. There are six potential glycosylation sites in

Table 1. Homology of *Drosophila* pro-PO A_1 and other proteins

Protein or subunit	Copper-binding site, % A site	Copper-binding site, % B site	Total protein, %
Bombyx mori (silkworm) pro-PO polypeptide I (25)	80	67	45
pro-PO polypeptide II (25)	71	58	43
Pacifastacus leniusculus (crayfish) pro-PO (26)	73	58	37
Mus musculus (mouse) tyrosinase (21)		10	9
Oryzias latipes (medaka fish) tyrosinase (24)		8	13
Eurypelma californicum (tarantula) hemocyanin subunit a (27)	76	58	36
hemocyanin subunit d (28)	70	53	35
hemocyanin subunit e (27)	72	49	35
Tachypleus tridentatus (Japanese horseshoe crab) hemocyanin subunit a*	62	59	35
Limulus polyphemus (American horseshoe crab) hemocyanin II (29)	70	60	36
Panulirus interruptus (lobster) hemocyanin subunit a (30)	49	38	28
hemocyanin subunit b (17)	49	38	29
hemocyanin subunit c (31)	45	36	27
Bombyx mori (silkworm) storage protein 1 (SP1) (32)			23
storage protein 2 (SP2) (32)			23
Drosophila melanogaster (fruit fly) larval serum protein 2 (LSP-2) (32)			24

The sequences appeared in references indicated by the numbers in parentheses.
*T. Takagi, personal communication.

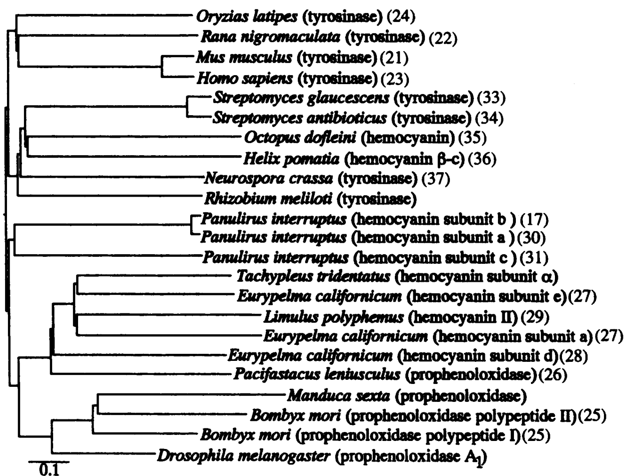

FIG. 4. A phylogenetic tree showing the evolutionary relationship among the pro-POs, tyrosinases, and hemocyanins. The scale bar indicates a branch length of 0.1, and the phylogenetic tree is constructed by the neighbor-joining method (14). Construction of this phylogenetic tree is based on the sequences that appeared in the references indicated by numbers in parentheses. The *T. tridentatus* sequence is from T. Takagi (personal communication), the *M. sexta* sequence is from J. H. Law (personal communication), and the amino acid sequence of *R. meliloti* will appear in the Swiss-Prot data base under accession no. T33180.

the protein (underlined with double lines). Because no amino sugar was detected in amino acid analysis of the purified protein, absence of N-linked sugar chain is assumed (data not shown). Although we have not yet obtained direct evidence on the cleavage site of the pro-PO A_1 at activation, we assume that the site is between Arg-52 and Phe-53 for the following reasons. (*i*) The protease responsible for the activation is trypsin-type serine protease (18). (*ii*) In *Drosophila* pro-PO, similar sequence around the cleavage sites of *Bombyx* pro-PO subunits is found (*Drosophila*, -VGNRFS-, *Bombyx* polypeptide I, -IGNRFG-, polypeptide II, -LNNRFG-). (*iii*) *Drosophila* pro-PO could be activated with the purified *Bombyx* pro-PO-activating enzyme (unpublished work).

Amino Acid Sequence Homologies. A search of Swiss-Prot revealed significant sequence similarity of pro-PO A_1 to hemocyanins of arthropod (Fig. 3). Arthropod hemocyanins form hexameric or multihexameric complexes of several homologous subunits (19, 20). The overall sequence identities between pro-PO A_1 and hemocyanins subunits are ≈30%. However, the two regions from 200 to 248 and from 359 to 414, which are very probably the copper-binding sites of pro-PO A_1, are quite homologous to the corresponding copper-binding sites (A site and B site) of arthropod hemocyanins. The first region (A site) is more conserved than the second region (B site). In addition, the putative copper-binding ligands, six histidines, are all conserved (Fig. 3). On the other hand, the sequence similarities between *Drosophila* pro-PO and molluscan hemocyanins are quite low. The surprising fact is its poor sequence similarity to those of vertebrate tyrosinases, including mouse (9% identity) (21), frog (8%) (22), human (7%) (23), and medaka fish (13%) (24). Pro-PO A_1 exhibits some homology with the insect storage proteins, although these proteins have no copper-binding capacity (Table 1).

Recently, Ashida and his colleagues (25) succeeded in cloning and sequencing the cDNA of *Bombyx* pro-PO, and Söderhäll's group (26) also sequenced cDNA of the pro-PO of the crayfish *P. leniusculus*. It is of interest that the main characteristics of *Drosophila* pro-PO A_1 are found not only in the *Bombyx* pro-PO but also in the *Pacifastacus* pro-PO. Namely, both proteins show the sequences similar to those of arthropod hemocyanins, especially at the copper-binding sites, but show low sequence similarity to vertebrate tyrosinases (Fig. 3). A phylogenetic tree has been constructed by the neighbor-joining method using the sequences of insect pro-PO, hemocyanins, and tyrosinase (Fig. 4). It is inferred from Fig. 4 that tyrosinases and molluscan hemocyanins constitute one family, whereas pro-POs and arthropod hemocyanins group with another family. Thus, it seems likely that arthropod pro-POs originate from a common ancestor with arthropod hemocyanins, independently of the vertebrate and microbial tyrosinases. It is interesting that insect pro-POs are rather closer to the chelicerate hemocyanins than to that of the spiny lobster, contrary to the phylogenetic relationship. The insect has lost hemocyanin, and pro-PO has come to play vital roles in development before diversification into various classes. The significant difference between *Drosophila* and *Bombyx* pro-POs is that the native state of *Drosophila* protein is a homodimer (8), whereas that of *Bombyx* is a heterodimer (25). Sequence identity in the protein between the two species is 45% between *Drosophila* pro-PO A_1 and *Bombyx* polypeptide I, one subunit of the dimer, and 43% with *Bombyx* polypeptide II, the other subunit of the dimer. We previously determined the chromosomal locus of the structural gene (*Mox*) of pro-PO A_1 (10).

Note. After submission of this manuscript for review, we learned that Professor John H. Law and his group at the University of Arizona had succeeded in sequencing the cDNA of the pro-PO of *M. sexta* (see ref. 38). They kindly informed us of their unpublished data, which have been included in our phylogenetic tree.

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