Transepithelially Transported Pro-phenoloxidase in the Cuticle of the Silkworm, *Bombyx mori*

IDENTIFICATION OF ITS METHIONYL RESIDUES OXIDIZED TO METHIONINE SULFOXIDES

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Pro-phenoloxidase (proPO) in insects is activated through the action of a protease cascade triggered by minute amounts of microbial cell wall components. It is an important molecule for the defense against invading microorganisms and for the repair of wounds. In the accompanying paper (Asano, T., and Ashida, M. (2001) *J. Biol. Chem.* 276, 11100–11112), a proPO isoform, proPO-HS, in the hemolymph of the silkworm, *Bombyx mori*, is reported to be transported to the cuticle. The transported proPO isoform was recovered from the cuticle and named proPO-CS. The elution profiles of proPO-CS and proPO-HS in reversed-phase high performance liquid chromatography (HPLC) were found to be different, giving a basis to the inference that proPO-CS is a modified form of proPO-HS. In the present study, we investigated the nature of the modifications occurring in proPO-CS, in which proteolytically and chemically cleaved fragments originating from the subunits of proPO-CS and proPO-HS were analyzed by reversed-phase HPLC, amino acid sequencing, and mass spectrometry. A subunit of the heterodimeric proPO-CS was found to contain five or six methionine sulfoxides, and another subunit was found to contain one methionine residue oxidized to the sulfoxide. All of the oxidized methionyl residues were identified. Other than oxidation of the methionyl residues, no additional modification of proPO-CS was found. In the model structure of each subunit of proPO-CS constructed by protein modeling with the known structures of the horseshoe crab, *Limulus polyphemus*, hemocyanin type II subunit as templates, the methionine residues identified as methionine sulfoxide had high degrees of accessibility to the solvent. The implication of the oxidation at the methionine residues is discussed in relation to the mechanism of transepithelial transport of proPO from the hemolymph to the cuticle.

The chitinous exoskeleton of the insect is a nonliving matrix of carbohydrate and protein secreted from a monolayer of epidermal cells that covers the entire surface of the insect, including respiratory tracheae, the anterior and posterior portions of the digestive tract, and reproductive ducts (1). The cuticle is a structure featuring the body plan of the insect and displays various functions (2): a physical protective barrier between the internal tissues and the external environment, a structural support of the body that enables the insect to fly and walk, and a stage for the chemical defense against microbes by relaying the signal generated by the presence of microorganisms to epidermal cells underneath and by directing the cells to secrete anti-microbial peptides into the matrix (3). The proteins and chitin fibers secreted by epidermal cells form the complexes that make up a lamellate structure. Proteins secreted by the epidermal cells play important roles in cuticular functions. Although all of the proteins in the chitinous exoskeleton of the insect are thought to be secreted by epidermal cells, their origins have been shown to be different. Some of cuticular proteins are thought to be synthesized in hemocytes and the fat body (an insect organ equivalent to the vertebrate liver) and to be transepithelially transported to the cuticle via the plasma fraction of the hemolymph. Recently, some hemolymph proteins have been shown to be transported from the hemolymph to the cuticle and vice versa in lepidopteran insects (4, 5). However, there has been little investigation on the mechanisms for the transepithelial transport of proteins in insects, in contrast to studies on transepithelial and transendothelial protein transport in mammals in which the mechanisms of transcytosis of proteins such as polymeric immunoglobulin A and transferrin have been investigated in detail (6–10).

In our laboratory, we have been studying the activation mechanism of pro-phenoloxidase (zymogen of phenoloxidase (proPO)) (3). Our study has recently demonstrated that the silkworm cuticle contains proPO and the protease cascade for its activation. Furthermore, the epidermal cells underneath the cuticle were found not to contain the transcripts of the genes encoding proPO subunits, suggesting that cuticular proPO is transepithelially transported from the hemolymph (11).

In the silkworm, *Bombyx mori*, two proPO isoforms have been purified from both the hemolymph and cuticle (12). The isoforms from the hemolymph have been named proPO-HF and proPO-HS, and the isoforms from the cuticle have been named proPO-CF and proPO-CS. The isoforms proPO-HF and proPO-CF have the same mobilities in polyacrylamide gel electrophoresis under nondenaturing conditions (native PAGE), and they are collectively referred to as F-type proPO. Similarly,
proPO-HS and proPO-CS migrated to the same position in native PAGE and are referred to as S-type proPO. F-type proPOs have slightly greater mobility than do S-type proPOs in native PAGE. ProPOs purified from the hemolymph and cuticle have been shown to be heterodimeric proteins (12). One of the two subunits of proPO-HS and proPO-HF is common, but the other subunits are different, and five amino acid substitutions have been detected between them (12).

In the accompanying paper (12), proPO-HS is reported to be transported from the hemolymph to the cuticle and to be recovered as proPO-CS from the cuticle. ProPO-HF is thought to be transported similarly, although the direct evidence of such transportation has not yet been obtained. The isoforms proPO-CS and proPO-HS did not exhibit any appreciable difference in their molecular mass, as determined by gel permeation chromatography, or in their enzymatic properties after activation by a specific endogenous protease, but their elution profiles in reversed-phase high performance liquid chromatography (RP-HPLC) on an octadecyl (ODS) column were different. These results indicate that modifications occur in cuticular proPO. We observed that little proPO-CS purified from cuticles was transported from the hemolymph to the cuticle even if it was injected into the hemocoel of the larval silkworm (12). It seems that the sites susceptible to modification play an important role in the transport of hemolymph proPO to the cuticle. We considered elucidation of the nature of the modification to be the first step in advancing our understanding of the mechanism of transepithelial protein transport in insects.

Here, we report that the modification in proPO-CS is oxidation of methionine residues. All of the oxidized methionine residues are identified. This is the first report on the structural characterization of transepithelially transported protein in insects.

**EXPERIMENTAL PROCEDURES**

**Silkworm (B. mori)—**Silkworm larvae (Kinsyu × Showa) were reared on the artificial diet Silkmate 2M (Kyodo Shiryo, Tokyo) at 25 °C under a 12-h photoperiod.

**Purification of Cuticular and Hemolymph ProPO Isoforms—**Purification of proPO isoforms from cuticle and hemolymph were carried out as described in the accompanying paper (12).

**Separation of Subunits of Each ProPO Isoform by RP-HPLC—**Subunits of each proPO isoform were separated on an ODS column (YMC-Pack ODS-AP; pore size, 300 Å; column size, 4.6 × 250 mm) as described previously (Fig. 4 in Ref. 12). Briefly, purified proPO isoforms were diluted 5-fold with 0.1% trifluoroacetic acid and applied separately to the ODS column equilibrated with 5% acetonitrile in 0.1% trifluoroacetic acid. Adsorbed polypeptides were eluted with two consecutive acetonitrile gradients (5–30% in 5 min and 30–65%/65 min) in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. ProPO-CS was eluted in three peaks. Polypeptides contained in the peaks were designated proPO-CS-pI, proPO-CS-pII*, and proPO-CS-pII in the order of the elution (12). On the other hand, proPO-HS was eluted in two peaks. The polypeptides contained in the peaks were named proPO-HS-pI and proPO-HS-pII in the order of the elution (12). Separated proPO subunits were lyophilized and stored at −20 °C until use.

**Protein Modeling—**Molecular models of the three-dimensional structures of the subunits of proPO-HS (proPO-HS-pI and proPO-HS-pII) were generated using the knowledge-based protein modeling method that is implemented in the Swiss-Model server (15, 16). Both of the molecular models for proPO-HS-pI and proPO-HS-pII were built using known structures of Limulus hemocyanin type II subunit (Protein Data Bank codes: 1LLA, 1NOL, and 1OXY) as template structures. The portions of putative amino acid sequences deduced from the cDNAs, pPO-5 (Leu66–Asp675) and pPO-23 (Pro60–Gln678) (12, 13), were submitted as target sequences via the First Approach Mode. N-terminal and C-terminal regions other than the submitted sequences were rejected by the program because of low similarity between the target sequences and template sequences. The surface accessibility of amino acid residues in the models was calculated by submitting predicted models to a GETAREA server (17), and the accessibility of the residues is expressed as the value relative to that of Xaa in the tripeptide Gly-Xaa-Gly (18).
**RESULTS**

**Amino Acid Sequencing and Molecular Mass Analyses of the Peptides in the Digests of Pyridylethylated ProPO Subunits—**As has been reported in the accompanying paper (12), both proPO-HS and proPO-CS migrated in SDS-PAGE to the position corresponding to that of 71-kDa proteins. No appreciable difference between the isoforms was observed in the electrophoresis. On the other hand, proPO-CS was eluted in three peaks in RP-HPLC on an ODS column, whereas proPO-HS was eluted in only two peaks (Figs. 2 and 4 in Ref. 12). Polypeptides eluted in those peaks were designated as described under “Experimental Procedures.”

The polypeptides obtained in the RP-HPLC of proPO-CS and proPO-HS were pyridylethylated and digested by Lys-C. The resulting digests were subjected to RP-HPLC on an ODS column. Among the digests obtained from the pyridylethylated proPO-CS-pI and proPO-CS-pII, the peptides in the peaks that appeared at the same retention times in the chromatography (Fig. 1B) were determined to have the same molecular masses (data not shown). Some of the peptide pairs with the same retention times were chosen at random, and their N-terminal sequences were analyzed. The peptides of each pair had the same N-terminal sequences without exception (data not shown). Similarly, among the digests obtained from the pyridylethylated proPO-CS-pI and proPO-HS-pII, the peptides in the peaks that appeared at the same retention times in the chromatography (Fig. 1B) were determined to have the same molecular masses and N-terminal amino acid sequences (data not shown).

The N-terminal amino acid sequences and molecular masses of peptides of the peaks indicated by arrows in Fig. 1 were also analyzed. The results are presented in Table I. The peptides CSPId, CSPIe, HSPId, and HSPIe, each of which originated from proPO-HS, had N-terminal sequences and molecular masses corresponding to Val159–Lys227, Tyr40–Lys87, Leu419–Lys456, and Lys561–Val629, respectively, of the amino acid sequence of proPO-HS-pI deduced from the base sequence of a cDNA clone, pPO-5. The N-terminal amino acid sequence and molecular mass of the peptide HSPId was determined by electrospray ionization MS. The N-terminal amino acid sequence and molecular mass of the peptide HSPId corresponded to those of the peptide Lys639–Val692, which is the C-terminal region of the CUB domains in gel permeation chromatography (12), the results so far obtained in the present study indicate the following possibility: ProPO-CS is a mixture of two molecular species. One of them is composed of proPO-CS-pI and proPO-CS-pII, and the other is composed of proPO-CS-pI* and proPO-CS-pII. After proPO-HS

| Fragment | N-terminal sequence | Theoretical mass/Da | Observed mass (difference from the calculated mass) |
|----------|---------------------|---------------------|---------------------------------------------------|
| CSPIa    | VFRQAREVVSSVFSG.... | 7962.1              | 7979.0 (+16.9)*                                  |
| CSPI*a   | VFRQAREVVSSVFSG.... | 7978.5 (+16.4)*     |                                                   |
| HSPIa    | VFRQAREVVSSVFSG.... | 7962.3 (+0.2)*      |                                                   |
| CSPIb    | YQRVSNAGRFSG....    | 5464.4              | 5496.2 (+31.8)*                                   |
| CSPI*b   | YQRVSNAGRFSG....    | 5496.3 (+31.9)*     |                                                   |
| HSPIb    | YQRVSNAGRFSG....    | 5444.4 (+0.0)*      |                                                   |
| CSPIc    | LTPYGNRDFPFNIR....  | 11702.2             | 11720.1 (+17.9)*                                 |
| CSPI*c   | LTPYGNRDFPFNIR....  | 11720.3 (+18.1)*    |                                                   |
| HSPIc    | LTPYGNRDFPFNIR....  | 11704.1 (+1.9)*     |                                                   |
| CSPId    | LDSTVASRMPFPFA....  | 16522.7             | 16556.1 (+33.4)*                                 |
| CSPI*d   | LDSTVASRMPFPFA....  | 16540.0 (+17.3)*    |                                                   |
| HSPId    | LDSTVASRMPFPFA....  | 16525.1 (+0.4)*     |                                                   |
| CSPIe    | KYPDRAAGFFDPR....   | 6090.1              | 6106.0 (+15.9)*                                  |
| HSPIIe   | KYPDRAAGFFDPR....   | 6090.3 (+0.2)*      |                                                   |

* Fragments in the peaks indicated by arrows in Fig. 1.
* N-terminal sequence determined by Edman degradation.
* Calculated from the amino acid sequences of proPO-HS-pI and proPO-HS-pII deduced from pPO-5 and pPO-23, respectively.
* The molecular mass was determined by electrospray ionization MS.
* The molecular mass was determined by MALDI-MS at a linear mode.

**Determination of Protein—**Protein was determined according to the method of Bradford (20) with a Bio-Rad Protein Assay using bovine serum albumin fraction V as a standard.
is modified to proPO-CS, the overall change in their molecular masses is not large enough to be detected by their behaviors in gel permeation chromatography, as is reported in the accompanying paper, but the reaction that brought about the modification results in an increase in molecular mass of about 16 or 32 at the site to be subjected to the modification.

In the above-described peptide mapping of the subunits derived from proPO-CS and proPO-HS, the peptides corresponding to His\textsuperscript{88}-Arg\textsuperscript{89}-Lys\textsuperscript{90} (439.6 Da) and Tyr\textsuperscript{417}-Lys\textsuperscript{418} (309.4 Da) of the sequence deduced from pPO-5 and Ile\textsuperscript{59}-Pro\textsuperscript{60}-Leu\textsuperscript{61}-Lys\textsuperscript{62} (469.7 Da), Val\textsuperscript{144}-Arg\textsuperscript{145}-Val\textsuperscript{146}-Lys\textsuperscript{147} (500.7 Da), and Asp\textsuperscript{637}-Lys\textsuperscript{638} (261.3 Da) of the sequence deduced from pPO-23 could not be recovered in RP-HPLC as shown in Fig. 1. However, over 98% of the sequences deduced from pPO-5 and pPO-23 could be assigned to the sequences of the peptides recovered in the chromatography of the Lys-C digests of pyridylethylated subunits of proPO-HS and proPO-CS.

Only N-acetylation of the N-terminal amino acids of the nascent polypeptides of the silkworm proPO has so far been reported as a post-translational modification (12, 13). The vertical arrow indicates the cleavage site in the activation of the silkworm proPO by PPAE. Asterisks show histidine residues that have been shown to form copper binding sites in Limulus hemocyanin type II subunit. Of the Lys-C-digested fragments of proPO-HS-pI, proPO-HS-pII, proPO-CS-pI, proPO-CS-pI*, and proPO-CS-pII, the sequences of the fragments listed in Table I are indicated with highlighted letters, and the names of the fragments are indicated above the aligned sequences. Among the tryptic fragments listed in Table II, those with the sequences containing the methionine residue(s) to be oxidized to methionine sulfoxide(s) in cuticular proPO-CS are also indicated by horizontal bars with an arrowhead at each end above the aligned sequences. Open triangles with Roman numerals indicate methionine residues and residue numbers that were determined to be oxidized methionine residues in proPO-CS-pI. In proPO-CS-pI*, the same methionine residues as those in proPO-CS-pI were detected as oxidized methionine residues except for Met\textsuperscript{327}. The closed triangle with a Roman numeral indicates Met\textsuperscript{690} that was detected as a residue oxidized to methionine sulfoxide in the sequence of proPO-CS-pII.
The tryptic digests were subjected to MALDI-MS in a linear mode. The spectra of the digests derived from CSPIa, CSPI*a, and HSPIa are shown to the spectrum of the digest of HSPIa were observed, the only proPO-CS-pI and proPO-CS-pI*, respectively, spectra similar digests of peptides, CSPIa and CSPI*a, originating from analysis of a peptide mixture (22). In the case of the tryptic by the suppression of ionization, which often occurs in the Table II). The absence of this fragment may have been caused masses in which acetylation and modification of the subunits (12), were in good agreement with the theoretical molecular masses in which acetylation and modification of the subunits identified in the present study (as will be described below) were taken into account. Therefore, the above results seem to imply that all of the modifications that have occurred in proPO-CS are in peptides with names CSPIa, CSPIb, CSPIc, CSPId, CSPI*a, CSPI*b, CSPI*c, CSPI*d, and CSPIe indicated in Fig. 1 and listed in Table I. In the sequences of the Lys-C peptides that were not recovered in RP-HPLC, there is no consensus sequence for glycosylation, sulfation, and phosphorylation. Silkworm proPO in hemolymph has not been shown to be a glycoprotein by chemical analysis (21).

To make it easier to understand the experimental design and results described in the sections below, the deduced amino acid sequences of proPO-HS-pI and proPO-HS-pII and sequence of horse shoe crab (Limulus) hemocyanin type II subunit are aligned (Fig. 2). In Fig. 2, Lys-C fragments containing the modified amino acid residues in the subunits of proPO-CS and the amino acid residues that will be identified as modified amino acids are also indicated.

Identification of the Modification Occurring in Cuticular proPO—The Lys-C fragments listed in Table I were digested with trypsin, and the resulting digests were subjected directly to MALDI-MS. In the spectrum of the digest of HSPIa, all of the fragments that were expected to be produced were detected except for Ala223–Lys227 (m/z 544.68) (Fig. 3A, panel c, and Table I). The absence of this fragment may have been caused by the suppression of ionization, which often occurs in the analysis of a peptide mixture (22). In the case of the tryptic digests of peptides, CSPIa and CSPI*a, originating from proPO-CS-pI and proPO-CS-pII*, respectively, spectra similar to the spectrum of the digest of HSPIa were observed, the only major difference being that the ion at m/z 2149.4, which corresponds to the fragment Met176–Arg194, was not detected. Also instead of the peak at m/z 2149.4, another peak appeared at m/z 2165.3–2166.3 in each spectrum of the digests of CSPIa and CSPI*a (panels a and b in Fig. 3A). The PSD spectrum of the ion at m/z 2165.39 derived from the digest of CSPIa is shown in panel d of Fig. 3B. A predominant fragment 64 Da smaller than the precursor ion was detected at m/z 2102.3.16 in addition to minor peaks at m/z 2149.3, m/z 2073.5, and m/z 2016.5. This fragmentation is characteristic of a peptide with a methionine sulfoxide (Met(O)) at the N terminus as is depicted in panel e of Fig. 3B (23, 24). The 64-Da smaller size is the result of the release of methanesulfenic acid from the side chain of the oxidized methionine. Between Met176 and Arg194 of the polypeptide sequence deduced from pPO-5 (Table II), there was no methionine residue other than the terminal methionine. Therefore, Met176 of CSPIa was concluded to be an oxidized methionine residue, Met(O).

The same experiments as those performed on CSPIa were performed on CSPI*a. The results indicated that Met176 of CSPI*a was a residue oxidized to Met(O) (data not shown). Tryptic fragments corresponding to Met176–Arg194 were isolated from all of the three digests of CSPIa, CSPI*a, and HSPIa in RP-HPLC. The isolated fragments derived from CSPIa and CSPI*a were eluted in chromatography with shorter retention times than that for the fragment from HSPIa (data not shown). The isolated fragments, however, were confirmed to have the same amino acid sequence (MIPIVSNYTASDERPEQR) in Edman degradation. Met(O) was not detected in the sequencing, inferring that the observed difference (16 Da) between CSPIa
and HSPIa and between CSPI*a and HSPIa was caused by the presence of one Met(O) in CSPIa and CSPI*a and that the residue is Met176 in the amino acid sequence deduced from pPO-5.

The Lys-C peptides other than CSPIa, CSPI*a, and HSPIa listed in Table I were similarly examined as above by tryptic digestion and MALDI-MS analyses. The data are summarized in Table II. In the examination of the peptides HSPIb, HSPIc, HSPId, and HSPIle, which originated from proPO-HS, all the observed molecular masses of the fragments obtained in their tryptic digestion were almost identical to one of the molecular masses predicted from the sequences deduced from pPO-5 and pPO-20 (Table II). On the other hand, several peaks in the MALDI-MS of the tryptic digests of CSPIb, CSPI*c, CSPI*d, and CSPIe were observed at positions 16 or 32 Da larger than the expected molecular mass. Gains in the observed molecular mass were detected only with the tryptic peptides that were thought to contain one or two methionine residues (Table II). The tryptic fragments with molecular masses of 16 or 32 Da greater than the predicted molecular mass gave PSD spectra characteristic of a peptide containing Met(O) (data not shown). From these observations and the fact that Met58–Arg62 and Asn63–Lys87 of proPO-CS-pI and proPO-CS-pII and Asn687–Val692 of proPO-CS-pII contain one methionine residue in their sequences (Table II and Fig. 2), it was concluded that Met58 and Met73 of both proPO-CS-pI and proPO-CS-pII and Met690 of proPO-CS-pII are residues oxidized to Met(O)s.

In tryptic digests of CSPIc and CSPI*c, peptides with sequence corresponding to Phe475–Arg500 of proPO-CS-pI and proPO-CS-pII were expected to be produced. Two methionine residues, Met498 and Met500, are present in Phe475–Arg502. The molecular mass of Phe475–Arg502 calculated from the deduced amino acid sequence is 3246.61 Da (m/z 3247.62), but the two major peaks observed (difference from the calculated mass) at m/z 3263.92 and m/z 3247.67 were thought to depend on the position of the Met(O) residue in the peptides, as depicted in panels a and b of Fig. 4B, because CNBr does not cleave the C-terminal side of Met(O) (22). In the spectrum of the CNBr fragments of CSPIc, three major peaks were observed (panel a in Fig. 4A). A peak at m/z 4831.4 corresponded to the calculated molecular mass (4831.4 Da) of Leu419–Met463.

| Assignment | Number of Met (position of Met) | [M + H]+ calculated | [M + H]+ observed (difference from the calculated mass) |
|------------|--------------------------------|---------------------|------------------------------------------------------|
| Val159–Arg161 | 421.52 | 421.1 | 421.5 |
| Gln162–Arg164 | 374.42 | 374.8 | 374.5 |
| Glu165–Arg175 | 1088.22 | 1088.64 | 1088.04 |
| Met176–Arg184 | 2119.4 | 2116.26 ( +16.9) | 2119.89 |
| Val185–Arg193 | 655.78 | 655.55 | 655.42 |
| Glu200–Arg209 | 2843.16 | 2843.66 | 2842.46 |
| Ala223–Lys227 | 544.68 | ND | ND |
| Tyr50–Arg62 | 466.52 | 466.32 | 466.25 |
| Val73–Arg80 | 830.93 | 830.8 | 831.1 |
| Phe51–Arg57 | 709.74 | 710.1 | 709.5 |
| Met58–Arg62 | 729.85 | 646.1 ( +16.3) | 646.2 ( +16.4) |
| Asn63–Lys87 | 2904.43 | 2920.2 ( +16.8) | 2904.4 |
| Leu419–Arg426 | 936.02 | 935.1 | 936 |
| Leu427–Arg433 | 875.02 | 874.7 | 875 |
| Val434–Arg461 | 2919.2 | 2919.4 | 2919.2 |
| Gly436–Arg456 | 823.85 | 823 | 824 |
| Gly469–Arg474 | 602.72 | ND | ND |
| Phe475–Arg502 | 3263.92 ( +16.3) | 3263.4 ( +15.8) | 3264.76 |
| He503–Arg522 | 2284.55 | 2285.39 | 2285.01 |
| Lys523 | 141.17 | ND | ND |
| Leu275–Arg302 | 848.94 | 848.9 | 848.5 |
| Ala283–Arg317 | 626.74 | 627.1 | 627.2 |
| Phe288–Arg319 | 765.89 | 765.5 | 765.9 |
| Asp295–Arg304 | 1227.37 | 1227.2 | 1227.1 |
| Lys323–Arg332 | 1853.6 ( +32.4) | 1853.7 ( +16.1) | 1859.21 |
| Gln333–Arg356 | 2631.0 | 2630.8 | 2631 |
| Asn357–Arg381 | 3029.31 | 3029.3 | 3029.5 |
| His382–Arg413 | 2528.89 | 2528.2 | 2528.7 |
| Trp404–Lys416 | 1722.1 | 1722.2 | 1721.5 |
| Lys529–Arg554 | 834.96 | 834.96 | 834.96 |
| Ala454–Arg466 | 4649.3 | 4649.3 | 4649.3 |
| Asn697–Val692 | 644.77 | 661.2 ( +16.4) | 644.7 |

a Fragment subjected to trypsin digestion.

b ND, not detected.
of which the C terminus was homoserine lactone, and another peak at m/z 2716.07 corresponded to the calculated molecular mass (2715.1 Da) of Phe<sup>501</sup>–Lys<sup>523</sup>. The postulated CNBr fragment spanning from Asp<sup>464</sup> to Met<sup>500</sup> with unoxidized Met<sup>498</sup> should have had the molecular mass of 4096.6 Da, but instead one peak at m/z 4112.05 was observed in the spectrum corresponding to the theoretical molecular mass (m/z 4112.6) of the CNBr fragment Asp<sup>464</sup>–Met<sup>500</sup> with Met(O) at the 498th residue. Essentially the same results as above were obtained in the case of the peptide CSPId (data not shown). Met<sup>498</sup> of proPO-CS-pI and proPO-CS-pI* was concluded to be the oxidized residue Met(O).

The Lys-C peptides CSPId, CSPI*d, and HSPId were digested with trypsin. The digests were subjected to MALDI-MS as above. The peptides corresponding to Phe<sup>317</sup>–Arg<sup>332</sup> of CSPId should have appeared in the spectrum at m/z 1821.19, which was not observed. The equilibrium between homoserine and homoserine lactone shifts depending on the acidity of the medium. Therefore, the methionine residue that had been located at the C terminus of the peptide bond cleaved by CNBr should be detected as homoserine or homoserine lactone under the present experimental conditions. The molecular masses of the postulated fragments whose C termini are homoserine, homoserine lactone, and other amino acids are indicated in parentheses, brackets, and angle brackets, respectively, in the schemes.

**Fig. 4.** MALDI mass spectra of CNBr fragments of the Lys-C fragments, CSPIc (Leu<sup>419</sup>–Lys<sup>523</sup>) and CSPI*d (Leu<sup>275</sup>–Lys<sup>416</sup>), from proPO-CS-pI and proPO-CS-pI*. A, Lys-C fragments, CSPIc and CSPI*d, obtained as described in the legend of Fig. 1 A were treated with CNBr and subjected to MALDI-MS in a linear mode. Panel a, spectrum of the digest of CSPIc. Panel b, spectrum of the digest of CSPI*d. B, schematic representation of postulated CNBr fragments to be produced from CSPIc or CSPI*d. The cleavage with CNBr is expected to be blocked at Met(O)s. Thus, the fragmentation pattern by CNBr depends on the site of the oxidized Met in the fragments. Panel c, fragmentation of CSPIc with Met(O)<sup>498</sup>. Panel d, fragmentation of CSPIc with Met(O)<sup>500</sup>. Panel e, fragmentation of CSPI*d with Met(O)<sup>324</sup>. Panel f, fragmentation of CSPI*d with Met(O)<sup>327</sup>. The equilibrium between homoserine and homoserine lactone shifts depending on the acidity of the medium. Therefore, the methionine residue that had been located at the C terminus of the peptide bond cleaved by CNBr should be detected as homoserine or homoserine lactone under the present experimental conditions. The molecular masses of the postulated fragments whose C termini are homoserine, homoserine lactone, and other amino acids are indicated in parentheses, brackets, and angle brackets, respectively, in the schemes.
but they were observed at \( m/z \) 1853.58, 32 Da larger than the theoretical molecular mass of the peptide with the sequence of Phe\(^{317}\)–Arg\(^{322}\) of proPO-HS-pI. The PSD spectrum of the precursor ion at \( m/z \) 1853.58 showed the characteristic features of a peptide containing two Met(O)s (Fig. 5). Because two methionine residues are present in Phe\(^{317}\)–Arg\(^{322}\) of the sequence of proPO-HS-pI deduced from pPO-5, CSPId was concluded to have two oxidized methionines at Met\(^{324}\) and Met\(^{327}\).

The peptide CSPId appeared to have the same amino acid sequence as that of CSPId, but its molecular mass was found to be larger by 16 Da than the theoretical value (Table I), suggesting that one of the methionine residues was oxidized to Met(O). The results of analyses of the tryptic digest indicated that one of the two methionines, Met\(^{324}\) and Met\(^{327}\), in the postulated tryptic fragment of Phe\(^{317}\)–Arg\(^{322}\) was the oxidized residue (Table II). To identify the oxidized methionine residue, CSPId was treated with CNBr, and the resulting fragments were directly subjected to MALDI-MS (Fig. 4). The fragmentation pattern was thought to differ depending on the position of the oxidized methionine, and two possible patterns are depicted in panels e and f of Fig. 4B. The spectrum of CNBr-treated CSPId indicated the presence of a fragment with \( m/z \) 5803.83, which corresponded to the singly protonated Leu\(^{275}\)–Met\(^{324}\) because the C terminus was expected to have been converted to homoserine, and the theoretical mass of such a peptide was calculated to be 5802.1. This result indicates that CSPId was cleaved at Met\(^{324}\), suggesting that Met\(^{327}\) was not the residue oxidized to Met(O). Although a fragment with a molecular mass corresponding to Ser\(^{325}\)–Met\(^{326}\), which might contain Met(O) at the position of Met\(^{327}\), was not detected in the spectrum shown in Fig. 5, Met\(^{327}\) was concluded to be Met(O). Observations supporting this conclusion were as follows: Phe\(^{317}\)–Arg\(^{322}\), which was derived from CSPId by tryptic digestion, had two methionine residues, Met\(^{324}\) and Met\(^{327}\); the peptide Phe\(^{317}\)–Arg\(^{322}\) was larger by 16.1 Da than the theoretical molecular mass (Table II); and Met\(^{324}\) was not shown to be the residue oxidized to Met(O).

Taking into account all the data obtained in the present study on the modification of the subunits of the cuticular proPO isoform (proPO-CS), proPO-CS-pI can be said to have the same amino acid sequence as that of proPO-HS-pI except that it has six Met(O)s at Met\(^{56}\), Met\(^{73}\), Met\(^{176}\), Met\(^{324}\), Met\(^{327}\), and Met\(^{498}\). Similarly, proPO-CS-pI* has the same amino acid sequence as that of proPO-CS-pI except that its Met\(^{324}\) is not Met(O), and proPO-CS-pII has the same amino acid sequence as that of proPO-HS-pII except that it has a Met(O) at Met\(^{650}\). The theoretical molecular masses of proPO-CS-pI, proPO-CS-pI*, and proPO-CS-pII, in which acetylation of their N termini and modification at the methionyl residues were taken into account, were calculated to be 78,791, 78,775, and 80,107 Da, respectively. The observed molecular masses in MALDI-MS of the same polypeptides, proPO-CS-pI, proPO-CS-pI*, and proPO-CS-pII were 78,887, 78,861, and 80,190 Da, respectively. The observed molecular masses and theoretical masses are in good agreement within the experimental errors in the MALDI-MS (22).

**Protein Modeling of the ProPO Subunits with the Sequences Deduced from pPO-5 and pPO-23—**ProPO is a protein homologous to the arthropod hemocyanin. Because the three-dimensional structure of proPO has not been determined yet, model structures of the silkworm proPO subunits were constructed by protein modeling in which the known structures of the horseshoe crab hemocyanin (Limulus) type II subunit were employed as templates. When the entire sequences of proPO-HS-pI and proPO-HS-pII were submitted for protein modeling, they were rejected by the Swiss-Model Server (14, 15) because the sequences of the N-terminal and C-terminal regions of the proPO subunits have low identity to those of the horseshoe crab hemocyanin type II subunit. Therefore, the Lue\(^{66}\)–Asp\(^{675}\) deduced from pPO-5 and the Pro\(^{60}\)–Gln\(^{678}\) deduced from pPO-23 were submitted to the Server. The former and latter were calculated to have sequence identities of 44 and 42%, respectively, to the sequence of horseshoe crab hemocyanin type II subunit. The foldings of the models shown in Fig. 6A were very similar to those of the template structures (26–28). Two disulfide bridges, which had been observed in the template structures, were formed in each of the models (not shown). The models were used for calculation of the surface accessibility of methionine side chains. The results are summarized in Table III and visually presented in Fig. 6B. Methionine residues exposed (surface accessibility > 50%) or partially exposed (surface accessibility > 20%) to the surface are indicated in green in the figure. All of the methionine residues detected as Met(O)s except for Met\(^{58}\) of proPO-CS-pI and proPO-CS-pI* and Met\(^{650}\) of proPO-CS-pII are located at the surface in the model with surface accessibility of more than 20%. The surface accessibility of...
The mechanisms of transepithelial or transendothelial transportation of mammalian proteins such as polymeric immunoglobulin A and transferrin have become clearer in recent years (6, 7, 8, 9, 10). Because of the presence of the tight junction sealing extracellular space between the epidermal cells, macromolecules such as proteins cannot pass freely between mammalian epithelial cells. It is well documented that proteins taken up into the epithelial cells by receptor-mediated endocytosis at the basolateral surface are transported via cytoplasm to the apical surface and secreted (6, 7). However, in insects, the mechanism by which proteins are transepithelially transported has not been studied. There is not even any concrete evidence that a protein is transported by transcytosis through insect epidermal cells. We considered the silkworm proPO to be a molecule that would offer a rare opportunity to study the mechanism of transepithelial protein transport in the insect.

Purified cuticular proPO had the same enzymatic properties as those of hemolymph proPO when they were activated by a specific activating protease, pro-phenoloxidase-activating enzyme (PPOAE) (12). However, subunits of cuticular proPO were not eluted at the same retention times as those of hemolymph proPO subunits in RP-HPLC, indicating that cuticular proPO is a modified form of hemolymph proPO. There are some reports suggesting that hemolymph proteins are transepithelially transported from hemolymph to the cuticle (4, 5). However, there have been no detailed structural analyses of the transported proteins. Elucidation of the nature of the modification of transepithelially transported proteins would be the first step to understanding the mechanisms of transepithelial transport of macromolecules from hemolymph to the cuticle in insects.

In the hemolymph of the silkworm, B. mori, two proPO isoforms (referred to as proPO-HS and proPO-HF) are present. Only proPO-HS has been proved to be transported to the cuticle and become proPO-CS, but it is almost certain that proPO-HF is also transported as proPO-HS (12). In the present study, modification occurring in proPO-CS was analyzed. In RP-HPLC, proPO-CS was eluted in three peaks. The polypeptides in the peaks were named proPO-CS-pI, proPO-CS-pII, and proPO-CS-pIII. The polypeptides proPO-CS-pI and proPO-CS-pII were revealed to be modified forms of proPO-HS-pI (a subunit of proPO-HS), and the former was revealed to have six methionine sulfoxides, Met(O)s, at Met58, Met73, Met176, Met324, Met327, and Met498, and the latter was revealed to have five Met(O)s at Met58, Met73, Met176, Met327, and Met498, and the latter was revealed to have five Met(O)s at Met58, Met73, Met176, Met327, and Met498, and the latter was revealed to have five Met(O)s at Met58, Met73, Met176, Met327, and Met498. The polypeptide proPO-CS-pIII was shown to be a modified form of proPO-HS-pIII (another subunit of proPO-HS) and to have a Met(O) at Met690. Molecular mass data for the subunits of proPO-CS, results of mass mapping of the peptides in protease digests of the subunits, N-terminal amino acid sequences of the isolated peptides in the digests, and the results of analyses of peptides containing methionine sulfoxide(s) by PSD mass spectrometry were all consistent with the contention that modification of the subunits of proPO-CS involves only oxidation of the methionine residues detected in the present study. In the case of F-type proPOs (proPO-HF and proPO-CF), our preliminary analyses indicated that proPO-HF and proPO-CF have the same amino acid sequences and that essentially the same modifications as those observed in proPO-CS occur on proPO-CF.3

Oxidation of methionyl residues in many proteins caused by atmospheric oxygen or other reactive oxygen species has been reported (31, 32). It was therefore speculated that the oxidized methionyl residues detected in the cuticular proPO were artificially introduced during the purification process employed in the present study. However, the purified or crude hemolymph proPO preparations could be stored for several months

2 M. Sass, T. Asano, and M. Ashida, unpublished observation.

3 T. Asano and M. Ashida, unpublished observation.
without any appreciable change of their elution profiles in RP-HPLC, indicating that the oxidation of methionyl residues detected in cuticular proPO did not occur during storage. In another experiment, an extraction buffer supplemented with hemolymph proPO was used to extract cuticular proteins, and immune precipitate of proPOs in the resulting extract was prepared. From the precipitate, a subunit (proPO-HS-pI) of hemolymph proPO was recovered in a high yield, indicating that the oxidation of methionine residues of proPO does not take place during the extraction of cuticular proteins. Considering these observations, cuticular proPO isoforms are likely to exist in the oxidized form in situ before extraction.

The molecular mass of purified native proPO-CS was estimated to be 142 kDa by permeation gel chromatography, and the same value was also obtained for the molecular mass of proPO-HS. Because proPO-HS has been shown to be composed of two different subunits, the molecular mass data seem to indicate that proPO-CS is also a heterodimeric protein. Thus, the purified proPO-CS was concluded to be a mixture of proPO-CS variants, one of which is composed of proPO-CS-pI and proPO-CS-pII and the other of which is composed of proPO-CS-pI* and proPO-CS-pII. We have not succeeded in separating the variants by any purification procedure under nondenaturing conditions.

The silkworm proPO is a protein homologous to arthropod hemocyanins. Among the two types of arthropod hemocyanins of which the crystallographic three-dimensional structures have been determined (26, 27, 28), cheliceratan (Limulus) hemocyanin is closer to the silkworm proPO in molecular phylogeny than is crustacean (Panulirus) hemocyanin (34). Three structures of Limulus hemocyanin type II subunits have been reported. Two of them were obtained from protein crystallized in buffer containing 0.5 M NaCl, and the other one was obtained from protein crystallized in the presence of nitrate (26, 27, 28). They have been shown to have essentially the same structures (27, 28). Protein modeling of proPO subunits was carried out with these three crystallographic structures as templates. The resulting model structures were very similar to those of the templates (Fig. 6). From the model structures, surface accessibilities of methionine residues were calculated (Table III). Methionine residues identified to be Met(O)s in cuticular proPO subunits were shown to have high surface accessibilities in the model, with Met73 bearing the lowest value, 32.5%, and Met176 bearing the highest value, 87.2%. Both Met237 and Met324 are the residues of proPO-HS-pI and Met690 of proPO-HS-pII, which were both identified as methionine sulfoxides in proPO-CS, could not be assessed in the modeling because the regions containing these methionines were not included in the sequences submitted as targets in the present protein modeling. Although Met456 and Met498 of proPO-HS-pII were shown to have relatively high surface accessibilities (40.1 and 48.4%, respectively) in the model, they were not detected as oxidized methionine residues in proPO-CS-pII. This observation seems to be explainable if it is assumed that the methionyl residues are located at the surfaces where subunits of proPO interact and are therefore not exposed to the solvent surrounding the proPO molecule. Methionine residues in proteins are classified into three groups: exposed, partially exposed, and buried (31). Oxidation of methionine is thought to be restricted to the exposed or partially exposed residues (31). Considering the previous reports, the calculated high surface accessibilities of the methionine residues detected as methionine sulfoxides in proPO-CS suggest that the model structures obtained in the present protein modeling reflect rather faithfully the real structures of proPO subunits, especially that of proPO-HS-pI.

The oxidation of methionine residues of proteins sometimes induces conformational change and causes loss of their activity (31, 32). In the case of the silkworm proPO, such oxidative inactivation did not seem to occur. We have observed (12) that PPAE purified from the silkworm cuticle converted hemolymph proPO and cuticular proPO to phenoloxidases (mono-
phenol, L-3,4-dihydroxyphenylalanine:oxygen oxidoreductase, EC1.14.18.1) of which the enzymatic properties were very similar and that the conversion catalyzed by PPAE did not appear to be influenced by the oxidation of methionyl residues. The only conspicuous effect of oxidation detected was on the transport of proPO from hemolymph to the cuticle; cuticular proPO-CS injected into the hemocoel was not transported to the cuticle, whereas the injected purified proPO-HS was transported to the cuticle (12). The most plausible model for proPO transport may be receptor-mediated transcellular transport as has been observed in mammals (35). It is possible that the putative receptor for proPO does not have appreciable affinity to the oxidized proPO isolated from cuticles. Methionyl residues of some proteins have been shown to participate in molecular interaction (36–44). Oxidation of methionyl residues has been observed to have a crucial effect on interaction between molecules without causing large conformational changes of the proteins (42–44). The reduced hydrophobicity at the oxidized methionyl residues of cuticular proPO may lower the binding ability of molecules to the putative proPO receptors on epidermal cells. Such lowered affinity of the cuticular proPO for the putative receptor may imply that epidermal cells em-

Figure 7. A phylogenetic tree of proPO subunits of insects (A) and alignment of the sequences of fragments with oxidized methionine residue(s) in the silkworm cuticular proPO with the corresponding sequences of other insect proPOs (B). A, all the sequences of proPO subunits presently available in data bases were analyzed by the Nearest-Joining method (48) to construct the phylogenetic tree. ProPO-HS-pl and ProPO-HS-pII are indicated by underlining. B, sequences around methionines of proPO-HS-pI to be oxidized to Met(O) in proPO-CS-pI were aligned with the corresponding regions of other insect proPOs that belong to the cluster a in the phylogenetic tree. The methionine residue (Met690) of proPO-HS-pII that is oxidized to Met(O) in proPO-CS-pII was found not to be conserved at all in other insect proPOs. Therefore, the alignment of the sequence around Met690 is not presented here. The methionines identified as Met(O) in proPO-CS-pI are indicated with the residue numbers. The conserved methionine residues among the proPO subunits are presented with white letters on a black background. The sequences extracted from data bases and their accession numbers are as follows (in cases in which a data base other than DNA Data Bank of Japan is used, the name of the data base is given in parentheses): *Drosophila melanogaster* proPO 1a, CG2952 (fly base); *Sarcophaga bullata* proPO, AF031625; *Anopheles gambiae* proPO 1, AF004915; *Armigeres subalbatus*, AF260567; *Anopheles stephensi* proPO 1, AJ010193; *D. melanogaster* proPO 1b, CG516 (fly base); *Tenebrio molitor* proPO, AB020738; *B. mori* proPO-HS-pl, AF004916; *M. sexta* proPO 2, L42556; and *H. cunea* proPO 2, AF020391.
ploy oxidation of the methionine residues to facilitate the secretion of proPO, which is destined to be transepithelially transported from the hemolymph to the cuticle. It is also possible that the lowered affinity plays a role in preventing the cuticular proPO from being taken up from the cuticle into epidermal cells. To the best of our knowledge, no structural studies have been carried out on insect proteins that have been shown to be transported from the hemolymph to the cuticle. Accordingly, it is not known whether methionyl residues of other transepithelially transported proteins are also oxidized.

Since the first cDNA cloning of insect proPOs in 1995 (11, 45, 46), the number of reports on cloned proPO cDNAs in literature has been increasing. A phylogenetic tree of proPOs of insects was constructed from the reported sequences (Fig. 7A). Insect proPOs were grouped in two clusters. The subunits proPO-HS-I and proPO-HS-II separately belong to each cluster. ProPO subunits belonging to the cluster in which proPO-HS-I is located have often been referred to as type I proPO subunits, and those belonging to the cluster in which proPO-HS-II is located have often been referred to as type II proPO subunits. In the alignments of sequences around oxidized methionines of Bombbyx proPO subunits, the methionine residue corresponding to Met73 of proPO-HS-pl was found in all type I subunits except for a coleopteran proPO subunit. Next to Met73, Met176 is well conserved among the type I proPO subunits with the exceptions of the subunits of coleopteran proPO and Drosophila proPO-1b (Fig. 7). With regard to Met690 of proPO-HS-pl, none of the type II proPO subunits of other insects were found to have a methionine residue at the position corresponding to Met690. These alignment data seem to raise the possibility that the methionine residue corresponding to Met73 or Met176 of proPO-HS-pl plays a role in the transport of proPOs from hemolymph to the cuticle in insects. The coleopteran proPO subunit, which does not have a methionine residue corresponding to Met73 or Met176 of proPO-HS-pl, cannot rule out this possibility because all of the proPOs so far characterized at protein level have been shown to be composed of more than two subunits. There has been no report of a partner of the coleopteran proPO subunit to form a complete proPO molecule, and it is not known whether coleopteran proPO containing the subunit is transported to the cuticle. In a dipteran species, Anopheles gambiae, as many as six genes coding for proPO subunits are known to be present, but there has been no examination of whether the gene products are transported to the cuticle. Further investigation is needed to determine whether particular proPOs in other insects are transported from hemolymph to the cuticle and whether the transported proPO subunits have conserved methionine at the position corresponding to Met73 or Met176 of proPO-HS-pl. More direct evidence of the roles of these two methionines of proPO-HS-pl in the transport of proPO subunits could be obtained if mutagenized proPO molecules at the methionine residues were made. However, we have not so far succeeded in the synthesis of such mutagenized molecules.

As stated above, the oxidized methionyl residues in cuticular proPO do not seem to have been artifically introduced during the process of extraction and purification of the protein. Elucidation of the site where the oxidation takes place should contribute greatly to an understanding of the mechanisms of the transepithelial transport of hemolymph proPO. There are two possible sites for oxidation: cuticle and epithelial cells. We are currently trying to raise an antibody cross-reactive to hemolymph proPO but not to cuticular proPO by using peptides with sequences containing methionines identified as Met(O) in cuticular proPO. The use of such an antibody and an immunocytochemical technique would enable identification of the site where methionine residues are oxidized. All of the methionyl residues exposed to the surface of proPO appear to be oxidized. Therefore, there does not seem to be a mechanism by which specific methionyl residues are oxidized. It is certain that hemolymph proPO is subjected to an oxidative environment during the translocation from hemolymph to the cuticle.

To the best of our knowledge, this is the first report of a high content of methionine sulfoxide in cuticular proteins. This may be partly because methionyl residues oxidized to sulfoxide are detected as methionines in Edman degradation (25). Although the general physiological significance of the methionine residues oxidized to Met(O) in cuticular proteins remains to be determined, the implication of oxidation in the transepithelial transport of hemolymph proPO to the cuticle is worthy of further study.

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Transepithi ally Transported Pro-phenoloxidase in the Cuticle of the Silkworm, 
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