Novel Glycosidic Linkage in Aedes aegypti Chorion Peroxidase

N-MANNOSYL TRYPOTOPHAN*

Junsuo S. Li†, Liwang Cui‡, Daniel L. Rock§, and Jianyong Li*†

From the ‡Department of Pathobiology, University of Illinois, Urbana, Illinois 61802 and the *Department of Entomology, Pennsylvania State University, University Park, Pennsylvania 16802

Aedes aegypti chorion peroxidase (CPO) plays a crucial role in chorion hardening by catalyzing chorion protein cross-linking through dityrosine formation. The enzyme is extremely resistant to denaturing conditions, which seem intimately related to its post-translational modifications, including disulfide bond formation and glycosylation. In this report, we have provided data that describe a new type of glycosylation in CPO, where a mannose is linked to the N-1 atom of the indole ring of Trp residue. Through liquid chromatography/electrospray ionization/tandem mass spectrometry and de novo sequencing of CPO tryptic peptides, we determined that the mannosyl moiety is not linked via the anomeric carbon of a mannose residue and the N-1 atom of the indole ring of Trp. This is the first report that provides definitive evidence for N-mannosylation of Trp residues in proteins has been reported (2–4). It is termed C-glycosylation because this linkage is formed via a C–C bond. C-mannosylation has been proved to occur in a number of mammalian proteins, such as RNase2, interleukin-12, complements, proderpin, thrombospondin, erythropoietin receptor, mucins, and a bovine lens protein (2–11, 13–16). A Trp-X-Trp (WXXW) motif seems to serve as the specificity determinant for C-mannosylation, in which the first Trp residue is mannosylated. In addition, it has been demonstrated that the C-mannosylation is an enzyme-catalyzed event (17). The potential function of the C-mannosylation has also been discussed in a recent report (15).

In our study dealing with mosquito chorion hardening, we identified a specific chorion peroxidase (CPO)† that displayed extremely high physical stability under a number of denaturing conditions. For example, the enzyme remains active for months in 1–5% SDS (18).

CPO undergoes extensive post-translational modifications, including proteolytic processing and glycosylation (19). Recently, we have determined the N-glycosylation site and N-glycan structures in CPO (20). In our current study dealing with the characterization of CPO post-translational modifications, we determined that some Trp residues in CPO were mannosylated. Further analysis of their MS/MS spectra determined that the mannosyl moiety is not linked via the anomeric carbon to the C2 atom of the Trp indole ring as described for a number of mammalian proteins (2–4) but is covalently connected via the N-1 atom of the side chain of Trp. Although a Trp N-linked glycoconjugate has been detected in fruit (21, 22), N-mannosylation of the peptide-associated Trp has not been clearly identified as a protein post-translational event or as a new type of protein glycosylation. The detection of Trp N-mannosylation in CPO raises some interesting questions, such as questions concerning the enzymes involved in catalyzing the Trp N-mannosylation or hydrolyzing the Trp N-linked mannose in proteins and, more importantly, the physiological function of the Trp N-mannosylation in proteins, which should stimulate further research in these directions.

**Experimental Procedures**

**Materials**—PNGase A, PNGase F, and Asp-N were purchased from Sigma. Modified trypsin was from Promega (Madison). ZipTip C18 was from Millipore (Bedford, MA). Fresh Milli-Q water was used to prepare all buffers. Other laboratory chemicals were purchased from Sigma or Fisher (Fairlawn, NJ).

**CPO Purification**—Chorion isolation and CPO purification were based on a previously described method (20). Purity of the isolated CPO was assessed by SDS-PAGE. Protein concentration was determined at 280 nm with a U2001 spectrophotometer (Hitachi, Tokyo, Japan).

**In-gel Digestion**—CPO was electrophoresed on a SDS-PAGE gel and stained with Coomassie Blue. The CPO band was cut from the gel and...
FIGURE 1. LC/ESI/MS spectra of tryptic peptides of CPO. The peaks (with masses of 1,343, 1,398, and 1,763 Da) labeled with one asterisk are those not matched to the deduced CPO sequence and subsequently identified as hexosyl peptides. The peaks labeled with double asterisks, i.e., 1,236 and 1,601 Da, are subsequently verified to correspond to 1,398- and 1,763-Da hexosyl peptides, respectively, but are not modified by a hexose residue (162 Da). The peaks of 3356 and 3502 Da are typical N-linked glycopeptides.

FIGURE 2. LC/ESI/MS spectra of tryptic peptides of CPO following PNGase A deglycosylation. The peaks labeled with double asterisks are new peak or peaks with their relative intensity increased after PNGase deglycosylation. Note that all peaks of hexosyl peptides (1,343, 1,398, and 1,763 Da) shown in Fig. 1 are absent here. This result strongly implies that these glycopeptides have a novel N-linked hexose structure. The peaks around mass of 2466 Da are N-deglycosylated peptides.
FIGURE 3. LC/ESI/MS spectra of tryptic peptides of CPO following incubation in 50 mM citrate-phosphate buffer (pH 5.0) (buffer control). The peptide map, including the putative glycosylated fragments, is very similar to the untreated sample (Fig. 1), indicating that these N-glycosidic linkages are stable with regard to the incubation condition of deglycosylation. The peaks labeled with a single asterisk are those not matched to the deduced CPO sequence and subsequently identified as hexosyl peptides. Peaks labeled with double asterisks are the corresponding peptides that are not substituted by hexose.

FIGURE 4. MS/MS spectra of precursor m/z 1,344 (a mannosylated peptide) (A) and m/z 1,182 (the deglycosylated peptide) (B) after PNGase A treatment. Note that the mannosylated Trp residue (348 Da) changed to Trp residue (186 Da) following deglycosylation.
transferred into a 0.6-ml siliconized microcentrifuge tube (Fisher). After dithiothreitol reduction and iodoacetamide alkylation, CPO was in-gel digested with 0.01 μg/μl trypsin in 50 mM Tris-HCl (pH 8.0) at 37 °C for 16 h. Tryptic peptides were extracted from the gel using 50% acetonitrile in water plus sonication. After evaporation with a Speed-Vac, peptides were redissolved in 0.1% formic acid and desalted with ZipTip C18 for subsequent analysis using LC/ESI/MS/MS. 

Enzymatic Deglycosylation of CPO—In enzymatic deglycosylation, a denaturing protocol was used (23). CPO (4 μg) was first digested with trypsin. The tryptic peptides were incubated with 25 microunits of PNGase A in 5 μl of 50 mM citrate-phosphate buffer (pH 5.0) (or 0.5 unit of PNGase F in 5 μl of 20 mM NaHCO₃, pH 7.0) at 37 °C for 24 h. The deglycosylated peptides were desalted using ZipTip C18 and subsequently analyzed by LC/ESI/MS/MS. To determine the stability of the glycosidic linkage under the applied deglycosylation conditions, CPO tryptic peptides were incubated in 50 mM citrate-phosphate buffer (pH 5.0) without PNGase at 37 °C for 24 h and then applied to LC/ESI/MS/MS following desalting.

NanoLC/ESI/MS/MS for Glycopeptide Sequencing—The nanoLC/ESI/MS/MS system consists of a CapLC XE fitted with a NanoEase 75-μm C18 column, an OPTI-PACK C18 Trap column, and a Q-TOF micro™ mass spectrometer with a nanospray source (Waters Micromass, Manchester, UK). Peptide separation was achieved by gradient elution with mobile phase A (5% acetonitrile in 0.1% formic acid) and mobile phase B (90% acetonitrile in 0.1% formic acid). The following gradient profile was applied: 5% B from 0 to 5 min, 5–40% B from 6 to 40 min, and 40–90% B from 40 to 65 min. In MS analysis of peptides, precursor ions that were not matched to the deduced CPO tryptic peptide map, due presumably to post-translational modification, were selected and extracted into the collision cells for dissociation. In MS/MS analysis, identification of potential glycopeptides was based on the presence of marker ions of m/z 163, m/z 204, or m/z 366. The structures of the glycopeptides were elucidated based on their MS/MS spectra.

RESULTS

Determination of Modified CPO Tryptic Peptides—Figs. 1, 2, and 3 show the peptide maps of unincubated buffer and PNGase A incubated and buffer-alone incubated CPO tryptic peptides, respectively. When trypsin-digested CPO peptides were analyzed by LC/ESI/MS/MS and its resulting peptide ions were searched against the peptide map generated using the deduced sequence of CPO from its cDNA, a number of peptide ions did not match the deduced CPO tryptic map (see Fig. 1). Among the unmatched ions, a number of low intensity peaks with masses of 3,502–4,116 Da have been previously identified as N-glycosylated peptides (20). However, several high intensity ions, including 1,343, 1,398, and 1,763 Da, also did not match the deduced CPO sequence (Fig. 1). Among the unmatched ions, a number of low intensity peaks with masses of 3,502–4,116 Da have been previously identified as N-glycosylated peptides (20). However, several high intensity ions, including 1,343, 1,398, and 1,763 Da, also did not match the deduced CPO tryptic map (see Fig. 1). After the CPO tryptic peptides were treated with either PNGase A or F, the low intensity ions of 3,502–4,116 Da (seen in Fig. 1) essentially disappeared, which is in agreement with our previous report (20), but the peaks of 1,343, 1,398, and 1,763 Da also disappeared from the MS spectrum of the PNGase-deglycosylated peptides. At the same time, several new peaks, i.e. 1,181, 1,662, and 2,466 Da, were observed in the
MS spectrum of deglycosylated CPO peptides (see Fig. 2). In addition, the relative intensity of 1,236 and 1,601 Da was conspicuously increased in the MS spectrum of the deglycosylated peptides (see Figs. 1 and 2). When the CPO tryptic peptides were incubated in the same citrate-phosphate buffer (pH 5.0) in the absence of PNGase, disappearance of 1,343, 1,398, and 1,763 Da was not observed in the MS spectrum of the CPO tryptic peptides (Fig. 3), suggesting that the potential glycosidic linkage of the three peptide ions was stable to the applied incubation conditions. Because the disappearance of all three ions (1,343, 1,398, and 1,763 Da) from the peptide MS spectrum was directly related to PNGase-mediated reactions, their primary structures were thoroughly analyzed, which eventually led to the identification of a unusual N-linked hexose (a 162-Da substituent) structure in these peptides.

**MS/MS Spectra of the Hexosyl Peptides and Localization of Hexosyl Residues**—Based on the MS/MS spectrum of the $m/z$ 1,343, some partial sequences, NPH and DDER, were derived based on the presumed y and b series ions (Fig. 4A). Comparison of these partial sequences with the deduced CPO sequence matched them to a CPO tryptic peptide, 476INPHWDDER484. However, its calculated peptide ion is 1,181, which is 162 Da less than the $m/z$ 1,343. Search of the deglycosylated MS spectra of CPO tryptic peptides indeed revealed a new peak with $m/z$ 1,181 (see Fig. 2B), and the fragmentation pattern (MS/MS spectrum) of its peptide ion perfectly matched the INPHWDDER sequence (Fig. 4B). Comparison of the MS/MS spectra between $m/z$ 1,343 and 1,181 revealed that both corresponded to the same peptide, except that the Trp residue in the native peptide was modified, because its residue became 348 Da, which was 162 Da above its expected mass (Fig. 4A and B). Because the substituent linked to the Trp residue was released by deglycosylation and a low mass hexose marker ion of $m/z$ 163 was also present in the MS/MS spectrum of its native (undeglycosylated) peptides (Fig. 4A), it was apparent that a hexosyl moiety was linked to the Trp residue in the peptide. In our previous study (20), we showed that mannos is the only hexose in CPO monosaccharide composition. Therefore, the 162-Da substituent is considered to be a mannos.

Similarly, the $m/z$ 1,398 and $m/z$ 1,763 that also disappeared after deglycosylation were first subtracted by 162 Da and the possible presence of their $[M + H – 162]$, i.e. $m/z$ 1,236 and $m/z$ 1,601, were searched in the MS spectra of both native and deglycosylated CPO peptide samples. Interestingly, both $m/z$ 1,236 and $m/z$ 1,601 were present in the native and deglycosylated peptides, but the relative intensities of these peptide ions were much greater in the deglycosylated peptides than in the native peptides (see Figs. 1 and 2). De novo sequencing of the $m/z$ 1,398 and $m/z$ 1,236 pair, based on their MS/MS spectra, revealed that both peptide ions corresponded to the same CPO peptide of 778YDTVNLGLWR787 except that the Trp residue in $m/z$ 1,398 was conjugated with a 162-Da constituent, presumably a mannosyl residue (Fig. 5, A and B). Using the same approach, it was determined that the $m/z$ 1,763 and $m/z$ 1,601 pair corresponded to the same 250VLE-
PAYEDGVWAPR\textsuperscript{263} CPO peptide, and again the Trp residue in m/z
1,763 seemed to be conjugated with a mannosyl residue (Fig. 6, A and B).
Because both [M + H - 162] and [M + H] for the \textsuperscript{250}\textsuperscript{263}PAYEDGVWAPR and \textsuperscript{778}\textsuperscript{787}YDTVNLGLWR\textsuperscript{787} peptide ions were present in the MS spectrum of the native CPO peptides, it was clear that not all Trp residues in these two peptides were modified by a mannosyl residue. Based on the relative intensity between their [M + H - 162] and [M + H] in the MS spectrum of the native CPO peptides, we determined that \textasciitilde40–50\% of the Trp residues in the \textsuperscript{250}\textsuperscript{263}PAYEDGVWAPR and \textsuperscript{778}\textsuperscript{787}YDTVNLGLWR\textsuperscript{787} peptides were modified by a mannose. A summary of identified tryptic peptides and their modifications is provided in TABLE ONE.

### TABLE ONE

| Observed mass\textsuperscript{a} | Deduced mass | Start | End | Sequences, potential PTMs\textsuperscript{b} |
|---------------------------------|-------------|-------|-----|----------------------------------------------|
| 1448.23                         | 1447.67     | 211   | 222 | CLPPVPCSPHRS, acetyl C\textsuperscript{2+} |
| 1471.38                         | 1471.67     | 225   | 237 | TIDGSCNNPLPDR                                  |
| 1444.33                         | 1444.61     | 238   | 249 | TSWGMEMGPFDHR                                  |
| 1600.49                         | 1600.79     | 250   | 263 | VLEPAYEDGVWAPR                                |
| 1762.54                         | 1762.84     | 277   | 292 | VLEPAYEDGVWAPR, W\textsuperscript{260}-hexose |
| 1873.34                         | 1872.97     | 293   | 312 | LNILEFMQMGQFISHDFTLSR                         |
| 2428.50                         | 2429.19     | 313   | 317 | GEFTK                                         |
| 3356.34                         | 3356.39     | 318   | 338 | HGQAIECCTPNCTAPLFQGPR                         |
| 3502.33                         | 3502.45     |       |     |                                               |
| 4004.39                         | 4004.88     |       |     |                                               |
| 4166.30                         | 4166.93     |       |     |                                               |
| 2205.57                         | 2206.03     | 339   | 356 |                                               |
| 2222.31                         | 2222.03     |       |     |                                               |
| 1447.43                         | 1447.71     | 369   | 381 | LAQGPEQOLGYAK                                  |
| 2791.86                         | 2792.36     | 382   | 407 | QADLVTHFALDSHTYVGSTNDVAAELR                   |
| 1816.41                         | 1815.99     | 414   | 429 | LKDSFPGIELLPFAR                                |
| 1574.50                         | 1574.81     | 416   | 429 | DSFPNGIELLPFAR                                 |
| 1243.67                         | 1243.62     | 430   | 439 | NRTACVLPWAR                                    |
| 973.25                          | 973.46      | 432   | 439 | TACVLPWAR                                     |
| 1081.28                         | 1081.49     | 440   | 448 | VCEYGGDIR                                     |
| 1873.62                         | 1873.99     | 449   | 464 | TNQLGLTMVTHTLEMHR, H for Y\textsuperscript{782} |
| 554.159                         | 554.256     | 465   | 468 | EHNHR                                         |
| 1342.37                         | 1342.58     | 476   | 484 | INPHWDDER, W\textsuperscript{480}-hexose       |
| 934.470                         | 934.498     | 485   | 491 | LYQEEARR                                      |
| 3922.53                         | 3922.89     | 515   | 549 | VQQQLGLADPFDTYTNYYDPNLMPMTLAEVGAAMHR          |
| 1310.37                         | 1310.64     | 550   | 560 | YGHSLVGFFR                                    |
| 1159.36                         | 1159.58     | 565   | 574 | ESPPEDVFIK                                     |
| 2075.60                         | 2076.01     | 565   | 582 | ESPPEDVFIKDFNDPSK                              |
| 969.336                         | 969.528     | 607   | 614 | FLYGGLTR                                      |
| 1544.54                         | 1544.83     | 621   | 634 | KPGSDLSNLOOKR                                  |
| 1415.21                         | 1414.66     | 637   | 647 | DFAVRPYNDYR                                   |
| 5873.63                         | 5874.94     | 655   | 709 | ITDFNQLGEQAVLQAITYSPDDVDWLP                    |
| 2921.21                         | 2921.47     | 713   | 738 | ADRYFTNGPEVPGALTLQGLGEIR                      |
| 1497.50                         | 1497.76     | 740   | 753 | TTLAGRCANAHDK                                  |
| 2848.45                         | 2848.38     | 740   | 764 | TTLAGRCANADKHKEDFYQAQAEALR               |
| 1368.39                         | 1368.63     | 754   | 764 | EDFYQAQAEALR                                  |
| 1443.42                         | 1443.68     | 765   | 777 | QSSADNVPVCTR                                   |
| 1235.36                         | 1235.63     | 778   | 787 | YDTVNLGLWR                                    |
| 1397.47                         | 1397.68     |       |     |                                               |

\textsuperscript{a} Monoisotopic mass.
\textsuperscript{b} PTMs, post-translational modifications. The chemical modifications during sample preparation, such as iodoacetamide alkylation or acrylamide adduct, were not listed.
\textsuperscript{c} The modification was determined basing on observed peptide mass.
Moreover, when trypsin or Asp due is linked at the N-1 position of the Trp indole ring in these peptides. Incubation provided additional data suggesting that the mannosyl residue. In each case, there were abundant peaks at m/z 163 and [M + H - 162], whereas essentially no peak of [M + H - 120] was observed in the MS/MS spectra of native peptides (Figs. 4–6). A loss of 162 Da is characteristic of an N- or O-linked sugar complex, and a loss of 120 Da is typical for an aromatic C-linked sugar complex or peptides (2–4, 21, 24–26). In comparison with the previously reported collision-induced dissociation fragmentation patterns of C-mannosylated peptides or glycosides, such as those of C-mannosylated peptides in RNase (2), flavone C-glycopyranoside (24–26), and tryptophan-N-glucoside in fruits (21), the current results strongly suggest that the mannosyl residue at C-6 of CPO Trp residue is linked at the C-1 position of CPO, though the linkage conformation remains to be solved. A proposed structure of a C-mannosylated peptide in a CPO peptide is shown in Fig. 7. 

The release of mannosyl residues from the peptides following PNGase incubation provided additional data suggesting that the mannosyl residue is linked at the N-1 position of the Trp indole ring in these peptides. Moreover, when trypsin or Asp-N-produced peptides of complements C7 and C8 (two C-mannosylated proteins) were treated with PNGase A or F, no change was observed from their C-mannosylated peptides (data not shown), which provided indirect evidence supporting the Trp N-mannosylation in CPO.

**DISCUSSION**

This study has demonstrated that Trp residues in CPO undergo a novel type of glycosylation, N-mannosylation. In addition, both PNGase A and F are capable of hydrolyzing Trp N-linked mannosyl residues in proteins, suggesting that the amidase activity of enzymes may be involved in the degradation of the Trp N-mannose complex in proteins.

**N-Mannosylation**—An additional 162 Da higher than the calculated mass of the three CPO tryptic peptides (256VLEPAYEDGVWAPR263, 476INPHWDDER484, 779YDTVNLGLWR787) and the presence of the abundant hexose marker ion of m/z 163 in their MS/MS spectra provided the basis for suggesting that a hexose residue is associated with these tryptic peptides. The presence of the fragment with a loss of 348 Da (W + Hex), corresponding to each Trp residue position, indicated that the mannosyl is linked to the Trp residues in these peptides. To fully establish the linkage between the mannosyl and the Trp residue in CPO, it would be ideal to have the mannosyl-trypsin complex or their peptides analyzed by NMR. Because of difficulties in obtaining enough CPO (<50 µg of CPO was purified from several thousand pairs of oocytes), it is impossible to perform such a structural characterization on mannosylated CPO peptides. However, the presence of the hexose marker ion of m/z 163 in the MS/MS spectra of the three CPO peptides and the ability to hydrolyze the covalently linked mannosyl from them by PNGase suggest that the mannosyl residue is linked at the N-1 position of Trp residue in the three peptides.

Covalent linkage of a mannosyl to the Trp residues in proteins through the α-carbon of a mannosyl and the C-2 atom of the indole ring of Trp has been determined in a number of proteins (2–11, 13–16). Because of a C-C bond formation, this type of protein glycosylation has been termed protein C-mannosylation (2–4). Because of difficulty in breaking the C-C bond at low energy collision-induced dissociation, the hexose marker ion of m/z 163 is absent in the MS/MS spectra of the Trp C-mannosylated peptides, but a dominant ion derived by a loss of 120 Da is typical for aromatic C-glycosides. Our initial suspicion was that the Trp mannosylation in CPO might be C-glycosidic linkage. However, careful examination of the MS/MS spectra of the mannosyl-containing CPO peptides, in comparison with the reported MS/MS spectra of the Trp C-mannosylated peptides in the literature (2–4), clearly showed that the mannosyl was not linked at the C-2 position of Trp, because a loss of 120 Da was not observed in the MS/MS spectra of the three CPO peptides. The absence of [M + H - 120] ions in the MS/MS spectra of the Trp mannosylated peptides provides additional support for Trp N-mannosylation in CPO.

**PNGase-mediated Hydrolysis of N-Mannosyl Trp**—The ability to hydrolyze the N-linked mannosyl by PNGase supports that the mannosyl is linked to the N-1 position of the Trp residues in CPO, but it also raises some critical questions regarding the biochemical mechanism leading to the hydrolysis of the N-linked mannosyl. Both PNGase A and F hydrolyze the β-amide bond of the asparagine side chain (see Fig. 8), and the sugar moiety seems to serve principally as substrate recognition, but no typical amide bond is present in the N-mannosyl Trp structure of the CPO peptides. Study of substrate specificity of PNGase A and F revealed that both enzymes can hydrolyze glycopeptides containing only a single GlcNAc or a glucose, the 2-acetamide group on the GlcNAc may not be an absolute requirement for substrate recognition, and the 2-acetamide group on the GlcNAc may not be an absolute requirement for substrate recognition by PNGase. Our data provide clear evidence that PNGase is able to hydrolyze the N-linked mannosyl in CPO peptides, but its catalytic mechanism remains unknown and requires further experiments for elucidation.

**Possibility of Trp N-Mannosylation as a Common Post-translational Modification of Proteins**—No reports have shown unambiguously Trp N-mannosylation in other proteins, so the detection of Trp N-mannosylation in CPO raises several critical questions, e.g., whether this new type of protein glycosylation is common in living organisms and what is the biochemical mechanism leading to the Trp N-mannosylation in proteins. Formation of N-mannosyl Trp proceeds under high acidic conditions at elevated temperature (22); consequently, one may ask if the N-linked mannosyl might be produced during CPO isolation. All buffers used for CPO purification were near neutral pH, and most of the purification procedures were conducted at 4 °C. Based on our experiment conditions and the specificity and extent of Trp N-mannosylation in the three CPO tryptic peptides, it is evident that Trp N-mannosyla-

---

**FIGURE 7. Structure of N-1-mannosyl tryptan moiety in a CPO peptide.** The conformation of C-1′-N′ bond remains to be confirmed.
tion is an apparent event of in vivo post-translational modifications. Therefore, it is reasonable to consider that Trp N-mannosylation in CPO is a consequence of a specific biological process instead of an artifact of sample preparation or other in vitro experimental factors. Accordingly, mosquitoes must have specific enzyme(s) catalyzing protein N-mannosylation reaction.

If Trp N-mannosylation is an enzyme-mediated event, it is also reasonable to speculate that there should be other proteins undergoing Trp N-mannosylation in insects. An earlier report has indeed discussed the presence of a hexosyl Trp residue in an insect neuropeptide, but no definitive evidence was provided to identify its linkage nature (28).

Although techniques for glycoprotein analysis have been available, the requirement for a relatively large amount of starting material for elucidating the detailed structures of protein-associated sugars has often been the limiting factor. The technical progress in protein/peptide glycosylation site and glycan structures as well as its monosaccharide composition. Moreover, the study revealed that there is more mannose than that calculated based on the N-glycan structures. Our initial consideration was the presence of O-linked mannose in CPO. However, our present study led to the identification of Trp N-linked mannose at the Trp residues of the enzyme. Although the N-mannosylation appears to be an apparent post-translational modification in CPO, we certainly overlooked it in our previous study (20). Whether protein Trp N-mannosylation is restricted to insects or is a widespread phenomenon in higher eukaryotes remains to be determined.

Potential Physiological Functions of the CPO N-Mannosylation—CPO is present in the chorion of Aedes aegypti eggs and plays a critical role during chorion hardening by catalyzing chorion protein cross-linking through dityrosine formation (12, 18). Compared with other peroxidases, CPO is extremely resistant to some denaturing conditions. For example, CPO remains active for months in 1–5% SDS, but horseradish peroxidase completely lost its activity in 1% SDS after 2 h (18). The physical stability of the enzyme undoubtedly is intimately related to its intrinsic structure. After synthesis in follicle cells, CPO is secreted and assembled into a densely packed chorion layer. Although the results of this study did not provide evidence for the potential function of the N-linked mannose in CPO, it is reasonable to speculate that the Trp N-mannosylation might contribute to its physical stability or might be critical for its transportation and assembly into an intact chorion layer.

In summary, we have identified a new type of linkage between carbohydrate moieties and protein involving the C–N bond that is formed between the anomeric carbon of a mannose residue and the N-1 atom at the indole ring of Trp residues in CPO. The most widely distributed N-glycosidic linkages not only occur at Asn residues but also at Trp residues of proteins. This is the first report that provides definitive evidence for N-mannosylation of the Trp residue in a protein, which should stimulate further research toward a comprehensive understanding of the physiology and biochemistry of Trp N-mannosylation in protein modifications. In addition, the PNGase-mediated cleavage of the N-mannosyl Trp residue in peptides is intriguing and deserves further investigation.

REFERENCES

1. Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., and Marth, J. (1999) Essentials of Glycobiology, pp. 85–113 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Hofsteenge, J., Muller, D. R., de Beer, T., Löffler, A., Richter, W. J., and Vliegenthart, J. F. G. (1994) Biochemistry 33, 13524–13530
3. de Beer, T., Vliegenthart, J. F. G., Löffler, A., and Hofsteenge J. (1995) Biochemistry 34, 12005–12014
4. Löffler, A., Doucey, M. A., Jansson, A. M., Müller, D. R., de Beer, T., Hess, D., Meldal, M., Richter, W. J., Vliegenthart, J. F. G., and Hofsteenge J. (1996) Biochemistry 35, 12005–12014
5. Krieg, J., Glasner, W., Vicentini, A., Doucey, M. A., Löffler, A., Hess, D., and Hofsteenge J. (1997) J. Biol. Chem. 272, 26687–26692
6. Krieg, J., Hartmann, S., Vicentini, A., Glasner, W., Hess, D., and Hofsteenge J. (1998) Mol. Biol. Cell 9, 301–309
7. Doucey, M. A., Hess, D., Blommers, M. J., and Hofsteenge J. (1999) Glycobiology 9, 435–441
8. Hofsteenge, J., Blommers, M., Hess, D., Furmanek, A., and Miroshnichenko, O. (1999) J. Biol. Chem. 274, 32786–32794
9. Hartmann, S., and Hofsteenge, J. (2000) J. Biol. Chem. 275, 28569–28574
10. Gonzalez de Peredo, A., Klein, D., Macek, B., Hess, D., Peter-Katalinic, J., and Hofsteenge J. (2002) Mol. Cell. Proteomics 1, 11–18
11. Hofsteenge, J., Huwiler, K. G., Macek, B., Hess, D., Lawler, J., Mosher, D. F., and Peter-Katalinic, J. (2001) J. Biol. Chem. 276, 6485–6498
12. Li, J., Hodgerman, B. A., and Christensen, B. M. (1996) Insect Biochem. Mol. Biol. 26, 309–317
13. Perez-Vilar, J., Randell, S. H., and Boucher, R. C. (2004) Glycobiology 14, 325–337
14. Hilton, D. J., Watowich, S. S., Katz, L., and Lodish, H. F. (1996) J. Biol. Chem. 271, 4699–4708
15. Ito, Y., Manabe, S., Kanda, M., Kawano, H., Nakayama, T., Sekine, I., Kondo, T., and Ito, Y. (2005) Glycobiology 15, 383–392
16. Ervin, L. A., Ball, L. E., Crouch, R. K., and Schey, K. L. (2005) Invest. Ophthalmol. Vis. Sci. 46, 627–635
17. Doucey, M. A., Hess, D., Cacan, R., and Hofsteenge, J. (1998) Mol. Biol. Cell 9, 291–300
18. Han, Q., Li, G., and Li, J. (2000) Arch. Biochem. Biophys. 378, 107–115
19. Li, J. S., Kim, S. R., and Li, J. (2004) Insect Biochem. Mol. Biol. 34, 1195–1203
20. Li, J. S., and Li, J. (2005) Protein Sci. 14, 2370–2386
21. Diem, S., Bergmann, J., and Herderich, M. (2000) J. Agric. Food Chem. 48, 4913–4917
22. Gutsche, B., Grun, C., Scheutzow, D., and Herderich, M. (1999) Biochem. J. 343, 11–19
23. Kuster, B., Wheeler, S. F., Hunter, A. P., Dwek, R. A., and Harvey, D. J. (1997) Anal. Biochem. 250, 82–101
24. Becchi, M., and Fraisse, D. (1989) Biomed. Environ. Mass Spectrom. 18, 122–130
25. Li, Q. M., van den Heuvel, H., Dillen, L., and Claeys, M. (1992) Biol. Mass Spectrom. 21, 213–221
26. Prox, A. (1968) Tetrahedron 24, 3697–3700
27. Fan, J. Q., and Lee, Y. C. (1997) J. Biol. Chem. 272, 27058–27064
28. Gäde, G., Kellner, R., Rinehart, K. L., and Proefke, M. L. (1992) Biochem. Biophysical Res. Commun. 189, 1303–1309