Phenol oxidase, a copper-containing enzyme, is widely distributed not only in animals but also in plants and fungi, which is responsible for initiating the biosynthesis of melanin. Activation of prophenol oxidase in arthropods is important in host defense. However, the prophenol oxidase-activating system remains poorly understood at the molecular level. Here we show that the coagulation cascade of the horseshoe crab *Tachypleus tridentatus* is linked to prophenol oxidase activation, with the oxygen carrier hemocyanin functioning as a substitute for prophenol oxidase. *Tachypleus* clotting enzyme functionally transforms hemocyanin to phenol oxidase, and the conversion reaches a plateau at 1:1 stoichiometry without proteolytic cleavage. The active site-masked clotting enzyme also has the same effect, suggesting that complex formation of the clotting enzyme with hemocyanin is critical for the conversion. The two systems of blood coagulation and prophenol oxidase activation may have evolved from a common ancestral protease cascade.

Invertebrates lack adaptive immunity and must rely completely on innate immune systems for host defense (1). Two such systems, the blood (hemolymph) coagulation system in the horseshoe crab *Tachypleus tridentatus* and the prophenol oxidase-activating system in insects and crustaceans, are sensitive nonself-recognition cascades triggered by microbial cell wall constituents (2–4). In invertebrates, two kinds of protease cascades have been well characterized at the molecular level, the *Tachypleus* coagulation cascade (2) and the morphogenetic cascade for determining embryonic dorsal-ventral polarity in the fly *Drosophila melanogaster* (5). The structural similarity of their target proteins, a *Tachypleus* clottable protein coagulogen and a *Drosophila* Toll ligand Spätzle, as well as the sequence homology between the serine proteases of the two cascades, suggests that these two functionally different cascades may have a common origin (6–8). The microbial polysaccharide-mediated coagulation cascade in *Tachypleus* involves four-serine proteasezymogens (2). Factor C is a biosensor against lipopolysaccharides and is autocatalytically activated to factor $\tilde{C}$, which then activates factor $B$ and, in turn, factor $B$ converts the proclotting enzyme to the clotting enzyme. Another biosensor, factor $G$, is activated in the presence of $\beta$-1,3-glucans to factor $G$, which directly activates the proclotting enzyme. In insects and crustaceans, the prophenol oxidase activation system is an important part of the host defense, where it functions to detect and kill invading pathogens, as well as to synthesize melanin for wound healing and encapsulation of pathogens (3, 4). However, the molecular mechanism of the prophenol oxidase activation remains poorly understood. Prophenol oxidase-activating enzymes recently cloned from insects have been shown to be homologous to *Tachypleus* clotting enzyme, factor $B$, or *Drosophila* Easter (9–13). In the American horseshoe crab *Limulus polyphemus* the induction of prophenol oxidase activity in the blood is evident by treatment with either detergents or lipids (14), but prophenol oxidase(s) from horseshoe crabs has not been identified at the molecular level. Here we show that the *Tachypleus* coagulation cascade is linked to prophenol oxidase activation, and we propose that the two host defense systems of blood coagulation and prophenol oxidase activation have evolved from a common ancestral protease cascade.

**EXPERIMENTAL PROCEDURES**

**Protein Purification—** *Tachypleus* coagulation factors (2) and hemocyanin (15) were prepared as described previously. For the hemocyanin subunits, four fractions containing the distinct subunits were obtained (15): fraction I ($\alpha$-subunit), fraction II ($\gamma$- and $\delta$-subunits), fraction III ($\beta$- and $\epsilon$-subunits), and fraction IV ($\zeta$-subunit). Protein concentrations of hemolymph plasma, hemocyanin, and hemocyanin subunits were determined by the method of Bradford (16), using bovine serum albumin as a standard. The concentrations of *Tachypleus* coagulation factors were calculated from their extinction coefficients of $1\%$ solution at $A_{280}$ nm (2).

**Assay of Phenol Oxidase Activity—** Phenol oxidase activity was assayed spectrophotometrically according to Pye (17). Hemolymph plasma, hemocyanin, or hemocyanin subunits in the assay buffer (20 mM Tris-HCl, pH 8.0) were preincubated with the clotting enzyme or other proteases for 10 min at 20 °C. An aliquot was added to the substrate solution containing 5 mM 4-methylcatechol and 10 mM 4-hydroxyproline ethyl ester in the assay buffer. The increase at $A_{280}$ nm (or $A_{525}$ nm for determination of the specific activities) was monitored at 20 °C. An extinction coefficient of 4230 M$^{-1}$ cm$^{-1}$ at $A_{280}$ nm was used for the prolyl adduct of 4-methylcatechol (18). The activity of the aliquot was also assayed according to the method of Winder and Harris (19), with the exception that the substrate solutions contained 5 mM of substrate and 10 mM 3-methyl-2-benzothiazolinone hydrazone HCl in the assay buffer. The activity was monitored at $A_{520}$ nm.

**Active Site Masking of the Clotting Enzyme and Factor $B$—** The proteases were inactivated with 14 µM 1,5-dansyl-Glu-Gly-Arg-chloromethyl ketone (Calbiochem) in the assay buffer at 25 °C for 30 min. The complete blockage of the protease activity was confirmed by using a fluorescent peptide substrate, benzoyloxybenzoyl-Leu-Gly-Arg-4-methylcoumaryl-7-amide (20).

**RESULTS AND DISCUSSION**

*Tachypleus* coagulation factors were tested to determine whether they could produce phenol oxidase activity in plasma
Prophenol Oxidase Activation in the Horseshoe Crab

Fig. 1. Functional conversion of the horseshoe crab hemocyanin to phenol oxidase. A, phenol oxidase activity of hemolymph plasma induced by the Tachypleus coagulation factors. The plasma (1 mg/ml) was preincubated with the coagulation factors (50 μg/ml) and the phenol oxidase activity was assayed, the clotting enzyme (a) and factor B (b). The activities induced by factor C, factor G, and trypsin were equivalent to that of the background activity of the unactivated hemolymph plasma, as shown by a dotted line. B, phenol oxidase activity of the hemocyanin subunits. Phenol oxidase activity of the subunits (1 mg/ml) induced by the clotting enzyme (50 μg/ml) was assayed as follows: fraction I (α-subunit) (a), fraction IV (γ-subunit) (b), fraction II (γ- and δ-subunits) (c), and fraction III (β- and ε-subunits) (d). Each background activity of the unactivated hemocyanin subunit was subtracted from the corresponding phenol oxidase activity induced by the clotting enzyme.

free from hemocytes, using 4-methylcatechol as substrate (Fig. 1A). The clotting enzyme and factor B efficiently produced phenol oxidase activity. However, factor C and factor G, as well as trypsin, could not significantly produce phenol oxidase activity, indicating that the specific proteases are required for the production of phenol oxidase activity in Tachypleus plasma.

The phenol oxidase activity in plasma disappeared with the removal of hemocyanin by ultracentrifugation, suggesting that Tachypleus hemocyanin is originally involved in the prophenol oxidase-activating system. Prophenol oxidases and hemocyanins display significant sequence similarity, and both contain two functional copper-binding sites capable of reversibly binding an oxygen molecule (21–23). Tachypleus hemocyanin is composed of at least six subunits having the same molecular mass of 70 kDa on SDS-polyacrylamide gel electrophoresis but that are separable by an anion-exchanger column chromatography (15). All the fractions, each containing different subunit(s), expressed phenol oxidase activity (Fig. 1B). Fraction I containing the α-subunit was used for the subsequent experiments, since its primary sequence is already known (24).

Under the physiological conditions arthropod prophenol oxidases require a proteolytic cleavage for activation by a specific protease; the freshwater crayfish prophenol oxidase with a molecular mass of 76 kDa is converted into an active form with 62 kDa by the prophenol oxidase-activating enzyme (25). The tarantula Euryphomyia californicum hemocyanin was recently found to express phenol oxidase activity after limited proteolysis with trypsin or chymotrypsin (26). However, the α-subunit treated with the clotting enzyme maintained the native molecular mass of 70 kDa on SDS-polyacrylamide gel electrophoresis during incubation for 1 h (Fig. 2A), despite the fact that the aliquots showed significant phenol oxidase activity (Fig. 2B). Furthermore, amino-terminal sequence analysis of the α-subunit after the 1-h incubation showed that the first 10 residues (Thr-Ile-Lys-Glu-Lys-Gln-Ala-Ser-Ile-Leu-) were consistent with those of the native protein (24), indicating that proteolytic cleavage was not required for the functional conversion of hemocyanin to phenol oxidase.

The stoichiometry of the non-proteolytic conversion was estimated by increasing the amounts of the clotting enzyme (50 μg/ml) at 20 °C, and the aliquots at 0, 20, 40, and 60 min were taken for SDS-polyacrylamide gel electrophoresis. B, the phenol oxidase activity of the aliquots was assayed as follows: 20 (a), 40 (b), and 60 min (c). C, the reaction stoichiometry between the α-subunit and the clotting enzyme or the proclotting enzyme. The α-subunit (10 μg/ml) was incubated with different concentrations of the clotting enzyme (●) or the proclotting enzyme (▲), and the phenol oxidase activity was assayed. CE, clotting enzyme; ProCE, proclotting enzyme. D, phenol oxidase activity of the α-subunit induced by the active site-masked enzymes. The α-subunit (50 μg/ml) was incubated with the clotting enzyme (a), the active site-masked clotting enzyme (b), factor B (c), or the active site-masked factor B (d) at 1:1 molar ratio. The background activity of the unactivated α-subunit is shown by a dotted line.

The stoichiometry of the non-proteolytic conversion was estimated by increasing the amounts of the clotting enzyme under a constant concentration of the α-subunit. The clotting enzyme transformed the α-subunit in a dose-dependent manner, and the resulting phenol oxidase activity reached a plateau at 1:1 molar ratio (Fig. 2C). The specific activity was 37 ± 8 nmol/min/mg, equivalent to that of the insect Holotrichia diomphalia phenol oxidase, 47 nmol/min/mg, calculated from the published data (27). Typical inhibitors for arthropod phenol oxidases, such as phenylthiourea (12.5 mM) and EDTA (25 mM), completely inhibited the activity of the α-subunit induced by the clotting enzyme.
A conformational change of the a-subunit and concomitantly induce phenol oxidase activity. The reaction stoichiometry also suggests that the Tachypleus prophenol oxidase-activating system is equipped with a self-regulating mechanism to localize phenol oxidase activity at the site of injury and to prevent unnecessary diffusion of this activity through a proteolytic activation. Interestingly, the zymogen of the clotting enzyme (proclotting enzyme) also induced the phenol oxidase activity, and the stoichiometric interaction of the extrapolated line occurred at an [α-subunit]/[proclotting enzyme] ratio of about 1.0 (Fig. 2C).

A common structural feature of the clotting enzyme and factor B is the presence of an amino-terminal disulfide-knotted domain (termed the clip domain) consisting of three disulfide bridges (9, 10). Homologous clip domains are also present in the amino-terminal regions of insect prophenol oxidase-activating enzymes (11–13) but not in those of Tachypleus factor C (28) and factor G (29). Therefore, the clip domains of the clotting enzyme and factor B may mediate the interaction of these proteases with hemocyanin to turn on the switch for functional conversion.

Like other arthropod phenol oxidases, the functionally transformed α-subunit showed an α-diphenol oxidase activity. 4-Methylcatechol and catechol were readily oxidized, but tyrosine (monophenol) and hydroquinone (p-diphenol) were not significantly oxidized (Fig. 3A). Arthropod prophenol oxidases are known to be non-enzymatically activated by treatment with detergents, lipids, or organic solvents (30). Hemocyanins from the crab Carcinus maenas and the lobster Homarus americanus express significant phenol oxidase activity in the presence of perchlorate (31). Expectedly, SDS and phosphatidylyethanolamine also induced the phenol oxidase activity of the α-subunit, and the specific activity of the SDS-activated α-subunit for 4-methylcatechol was 35 ± 1 nmol/min/mg, approximately equal to that activated by the clotting enzyme (Fig. 3B). The substrate specificity of the SDS-activated α-subunit was also quite similar to that obtained with the α-subunit activated by the clotting enzyme (Fig. 3C).

We clearly showed here that the Tachypleus coagulation cascade is linked to prophenol oxidase activation through the non-enzymatic interaction of the specific coagulation factors with hemocyanin, which leads to the functional conversion of hemocyanin to phenol oxidase (Fig. 4). Phenol oxidases in insects and crustaceans participate in wound healing and repair of the damaged exoskeleton, as well as hardening of the exoskeleton during molting (32). Although Tachypleus hemocyanin functions as an oxygen carrier under normal conditions, it may be converted to phenol oxidase at the site of injury to prevent microbial invasion and to promote wound repair. The Tachypleus coagulation cascade appears to be a very rational and sophisticated system by which the host defense can simultaneously trigger both blood coagulation and prophenol oxidase.

**FIG. 4.** A link between the horseshoe crab clotting and prophenol oxidase-activating cascades, and a comparison between this linkage and those of the insect and crustacean prophenol oxidase-activating cascades. Open arrows indicate non-proteolytic events. proPO, prophenol oxidase; PO, phenol oxidase.
activation. In insects and crustaceans, an ancestral protease cascade corresponding to the bifunctional cascade found in horseshoe crabs may have evolved into an exclusive system of prophenol oxidase activation.

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