Phenoloxidase, widely distributed among animals, plants, and fungi, is involved in many biologically essential functions including sclerotization and host defense. In chelicerates, the oxygen carrier hemocyanin seems to function as the phenoloxidase. Here, we show that hemocyanins from two ancient chelicerates, the horseshoe crab Limulus polyphemus and the tarantula Euryphilma californicum, exhibit O-diphenoloxidase activity induced by submicellar concentrations of SDS, a reagent frequently used to identify phenoloxidase activity. The enzymatic activity seems to be restricted to only a few of the heterogeneous subunits. These active subunit types share similar topological positions in the quaternary structures as linkers of the two tightly connected 2×6-mers. Because no other phenoloxidase activity was found in the hemolymph of these animals, their hemocyanins may act as a phenoloxidase and thus be involved in the primary immune response and sclerotization of the cuticle. In contrast, hemolymph of a more recent arthropod, the crab Cancer magister, contains both hemocyanin with weak phenoloxidase activity and another hemolymph protein with relatively strong phenoloxidase activity. The chelicerate hemocyanin subunits showing phenoloxidase activity may have evolved into a separate phenoloxidase in crustaceans.

Phenoloxidases are widespread in animals, plants, fungi, and some bacteria. They are involved in several biological functions. They initiate the synthesis of melanin, are responsible for the browning of fruits, vegetables, and fungi, and are crucial for sclerotization of arthropod exoskeleton. Their central role in a common defense cascade for Deuterostoma and Prostoma has been reviewed (1). Phenoloxidases catalyze the O-hydroxylation of monophenols (cresolase) or oxidation of O-diphenols to quinones (catecholoxidase) or both (tyrosinase) (2–4). Both melanin and phenoloxidase are considered to be integral parts of the arthropod immune system (5, 6).

Among the Arthropoda, prophenoloxidases and their activated form, phenoloxidases, have been well characterized in insects and crustaceans but not in chelicerates. Hemocyanin, which is closely related to crustacean phenoloxidase based on sequence data (7–9), is the major constituent of the hemolymph of crustaceans and chelicerates but is absent in insects. Interestingly, the clotting system of the horseshoe crab, Limulus polyphemus, an ancient chelicerate, bears several functional and biochemical similarities to the prophenoloxidase system in insects and crustacea, including activation induced by lipopolysaccharides or β-1,3-glucans (10, 11). A marked difference from insects and crustaceans, however, is that L. polyphemus amoebocyte lyase supernatant lacks phenoloxidase activity (10). Recently Nellaiappan and Sugumaran (12) reported the presence of a phenoloxidase in the hemolymph of L. polyphemus. Although other phenoloxidases are often easily activated by limited proteolysis, the phenoloxidase of L. polyphemus could be only weakly activated by proteolysis, but activity could be induced by detergents such as SDS or cetylpyridinium chloride (12). Based on gel electrophoresis and substrate specificities they determined that the enzyme is an O-diphenoloxidase with a molecular mass of about 70 kDa. Our experiments suggest that this phenoloxidase is hemocyanin, which is present in high concentrations in the hemolymph of L. polyphemus.

Hemocyanins are large multisubunit copper proteins composed of different subunit types and found freely dissolved in the hemolymph of arthropods and molluscs (13–16). Their primary biological function is the transport of oxygen. Several physicochemical properties of hemocyanins are very similar to those of phenoloxidases (2, 15, 17, 18). Both molecules are copper proteins. Based on x-ray structures, dioxygen is bound in a μ-η2:η2 side-on coordination in hemocyanins (19), which should also hold for phenoloxidases because of the very similar spectroscopic properties of the two proteins (2, 20, 21). The high degree of similarity between the active sites of phenoloxidases and hemocyanins of arthropods and molluscs is also supported by the capability of hemocyanins to exhibit O-diphenoloxidase activity after exposure to chaotropic salts, SDS, or low pH values (22, 23). The hemocyanin from a chelicerate, the tarantula Euryphilma californicum, has shown phenoloxidase activity after proteolysis (24). E. californicum hemocyanin is sequentially and structurally closely related to the hemocyanin from L. polyphemus (25). In another chelicerate, Tachypleus tridentatus, it has recently been shown that the naturally occurring coagulation cascade is linked to phenoloxidase activation with hemocyanin functioning as a phenoloxidase (26).

We demonstrate here that hemocyanins from two ancient species of chelicerate, L. polyphemus and E. californicum, develop O-diphenoloxidase activity in response to submicellar concentrations of SDS, a reagent commonly used to identify phenoloxidase activity (12, 27). The functional conversion of hemocyanin may indicate an induced conformational change. This activation is restricted to only a few of the various subunit types of each hemocyanin. We compare the enzymatic activities of these chelicerate hemocyanins with that of Cancer magister, a crustacean.
Phenoloxidase Activity of Chelicerate and Crustacean Hemocyanins

Experimental Procedures

Purification Procedures—L. polyphemus was purchased through Carolina Biological Supply. The hemolymph was withdrawn from the pericardial cavity and immediately centrifuged at 1000 × g for 5 min at 4 °C using a Beckman J2-HS centrifuge. The supernatant was then centrifuged at 12,000 × g for 10 min. The supernatant was chromatographed at 4 °C on a BioGel A5m column (400-mesh) column (120 × 2.5 cm) that was equilibrated with a 0.1 M Tris/HCl buffer, 10 mM CaCl₂, 10 mM MgCl₂, and 0.1 mM NaCl, pH 7.5. The purification of hemocyanins from E. californicum and C. magister has been described elsewhere (24, 28). The purified proteins were stored at 4 °C. The concentrations of the proteins were determined spectroscopically using the molar extinction coefficient at 278 nm, ε₂₇₈ = 1.1 ml mg⁻¹ cm⁻¹ for chelicerate hemocyanin. Concentrations were expressed on a molar basis using the value of 75 kDa for an arthropod hemocyanin subunit (16).

Polyacrylamide Gel Electrophoresis Assay of Phenoloxidase Activity—Native PAGE of hemolymph and hemocyanin was carried out at pH 7.4 using a 3% gel (see Ref. 29, as modified by Ref. 28). Dissociating, non-denaturing PAGE at pH 8.9 in EDTA was done on a 7.5% gel. Prior to pH 7.4 PAGE, hemocyanins were dissociated into subunits by overnight dialysis at 4 °C in 0.05 M glycine/NaOH buffer, pH 9.5, 5 mM in EDTA. According to electrophoresis results, more than 90% of the hemocyanin was dissociated under these conditions. Phenoloxidase activity after electrophoresis on both pH 7.4 and pH 8.9 gels was demonstrated using dopa and its derivatives by the absorbance at 475 nm. The condition of the chemically unstable substrate was checked spectroscopically. Substrates DL-dopa, dopamine, and catechol were purchased from Sigma.

Kinetic Measurements—The time course of the phenoloxidase activity of the hemocyanins was followed by monitoring the formation of dopachrome and its derivatives by the absorbance at 475 nm. The substrate was added to the hemocyanin solution in 0.2 M phosphate buffer, pH 7.5. The reaction was then initiated by injection of a concentrated SRS stock solution to a final SDS concentration of 0.085 ± 0.005% and mixing briefly. The dead time was less than 10 s. Substrates and reaction mixtures were maintained in the dark because of their light sensitivity. The condition of the chemically unstable substrates was checked spectroscopically. Substrates DL-dopa, dopamine, and catechol were purchased from Sigma.

Results and Discussion

Phenoloxidase Activity of Native Oligomeric Hemocyanins—Two of the four peaks of L. polyphemus hemolymph separated by BioGel A5m chromatography (Fig. 1) showed phenoloxidase activity when subjected to pH 7.4 PAGE and then incubated in the presence of dopamine and SDS (Fig. 2). These same peaks, A and B, had 280/340-nm absorbance ratios of 4.2, indicative of copper oxygen-binding proteins. Peak B corresponded to the native 8-hexamer hemocyanin with a molecular mass of 3.6 MDa, whereas peak A was a higher molecular mass aggregate of hemocyanin (7.3 MDa) as determined by light scattering.

(data not shown). The two lower molecular mass peaks, C and D, showed neither 340-nm absorbance nor phenoloxidase activity. To determine whether other hemolymph proteins besides hemocyanin exhibit phenoloxidase activity, we tested the unpurified hemolymph with the same assay for phenoloxidase activity. Only those bands containing hemocyanin were stained by the activity assay.

We examined whether observed phenoloxidase activity of the structurally closely related 4 × 6-mer hemocyanin from E. californicum, another chelicerate, could also be induced by SDS. When intact 8 × 6-mer L. polyphemus (peak B) and 4 × 6-mer E. californicum hemocyanins were assayed by pH 7.4 PAGE, both hemocyanins showed phenoloxidase activity (Fig. 2). As in the case of L. polyphemus, no bands other than the hemocyanin bands showed phenoloxidase activity in E. californicum hemolymph (data not shown). The absence of a unique phenoloxidase protein in chelicerate hemolymph is in agreement with the literature (10). Thus it is unclear what enzyme participates in vivo in the essential biological processes, sclerotization of the cuticle after molting and the immune response, in chelicerates.

To compare the phenoloxidase activities of chelicerate hemocyanins with other hemocyanins, we tested the activity of crustacean (C. magister) and molluscan (Helix aspersa) hemocyanins by pH 7.4 PAGE (Fig. 2). The C. magister hemolymph contained a slowly migrating molecule with strong phenoloxidase activity (30). Purified 25 S hemocyanin of C. magister also
showed phenoloxidase activity but only after extended incubation with dopamine and SDS. The quaternary structure of the subunit structure of the crustacean hemocyanin, compared with chelicerate hemocyanin, may hinder the access of subunits to the active site sterically. Other studies on crustacean hemolymph have reported the presence of a hemocyte prophylloid that can be converted into a hemocyanin (31–33). Here, we report the presence of two different hemolymph molecules with phenoloxidase activity, one is hemocyanin.

The activity of crustacean hemocyanin is rather weak in comparison to the slowly migrating phenoloxidase (Fig. 2).

Qualitative spectroscopic studies confirmed the results of the phenoloxidase activity staining of the PAGE (Fig. 3). Hemocyanins of L. polyphemus and E. californicum (31–33) and L. polyphemus (34) have shown phenoloxidase activity only with dopamine and catechol and lacked activity with L-tyrosine. No activity was observed when the above assay was applied using L-tyrosine as a substrate to test the monophenoloxidase activity of these hemocyanins. Thus, these hemocyanins seem to behave like catechol oxidases when activated by SDS.

**Phenoloxidase Activity of the Hemocyanin Monomers**—When the hemocyanins were dissociated into monomers, subjected to pH 8.9 PAGE, and tested for phenoloxidase activity, not all subunit types showed a reaction (Fig. 4). Three of the eight bands of dissociated L. polyphemus hemocyanin appeared to be capable of O-diphenol oxidase activity. Several authors have detected heterogeneous subunit types of Limulus hemocyanin (25, 34–39). Subunits LpII, IIIa, IIIb, and IV are present in four copies; subunit I is present in three copies; subunit V and VI are present in two copies; and subunit IIa is present in one copy (40). Using the nomenclature of Brenowitz et al. (38), the Limulus hemocyanin bands showing phenoloxidase activity are assigned to subunits LpII, II', and II" (all three belong to the same subunit I), V, and VI. The quaternary structure of the 4 × 6-mer half-molecule of L. polyphemus hemocyanin (41).

In *E. californicum* hemocyanin, only subunits b and c showed SDS-activated O-diphenol oxidase activity (Fig. 4). The latter two subunits also showed phenoloxidase activity after activation by limited proteolysis (24). Two of the four subunits from *L. polyphemus* that exhibit phenoloxidase activity, LpV and LpVI, and subunits b and c from *E. californicum* hemocyanin have the same topological positions within the quaternary structure and can even be exchanged without losing the quaternary structure or the functional properties of the oligomeric hemocyanins (Fig. 5) (42). These subunits, located in the center of the oligomer, bridge the four hexamers in *E. californicum* hemocyanin and both of the four-hexamer half-molecules of *L. polyphemus* hemocyanin (43, 44).

In the dissociated 2–6-mer hemocyanin of *C. magister*, all subunits exhibit some phenoloxidase activity with subunits IV and V giving the strongest reaction. *H. aspersa* hemocyanin was included as a positive control and demonstrates strong phenoloxidase activity on both pH 7.4 and pH 8.9 PAGE. This finding confirms the catechol oxidase activity of molluscan hemocyanin as observed previously (45, 46).

**Are Hemocyanins Phenoloxidases in Vivo?**—Could these
observations be of physiological importance for the animals? In the case of the horseshoe crab *L. polyphemus*, no *O*-diphenoloxidase activity in hemolymph cells has been observed so far. The defense mechanism has been described to be similar to other arthropods, however, and all parts of the immune response cascade are found in the hemolymph (1, 10). Nellaiappan and Sugumaran (12) recently detected an *O*-diphenoloxidase with a molecular mass of ~70 kDa in the hemolymph of *L. polyphemus*. For several reasons, we suggest that hemocyanin is responsible for this enzymatic activity. First, the observed molecular mass is comparable with hemocyanin that hemocyanin is capable of phenoloxidase activity when activated by SDS. Our hypothesis is supported by the lack of any significant of the phenoloxidase activity of chelicerate and crustacean hemocyanins has been linked to prophenoloxidase activation through non-enzymatic interactions of specific coagulation factors with hemocyanin (26). This leads to the functional conversion of hemocyanin to phenoloxidase, most probably because of a conformational change of the hemocyanin. In both *L. polyphemus* and *L. polyphemus* *E. californicum* when centrifuged hemocytes were tested with the same assay (data not shown). Therefore, we think that the *O*-diphenoloxidase activity of the purified protein in *L. polyphemus* observed by Nellaiappan and Sugumaran (12) is exhibited by hemocyanin. The physiological significance of the phenoloxidase activity of chelicerate and crustacean hemocyanins has recently been addressed. In the horseshoe crab, *T. tridentatus*, the coagulation cascade has been linked to prophenoloxidase activation through non-enzymatic interactions of specific coagulation factors with hemocyanin (26). This leads to the functional conversion of hemocyanin to phenoloxidase, most probably because of a conformational change of the hemocyanin. In both *L. polyphemus* and *L. polyphemus* *E. californicum* when centrifuged hemocytes were tested with the same assay (data not shown). Therefore, we think that the *O*-diphenoloxidase activity of the purified protein in *L. polyphemus* observed by Nellaiappan and Sugumaran (12) is exhibited by hemocyanin. The physiological significance of the phenoloxidase activity of chelicerate and crustacean hemocyanins has recently been addressed. In the horseshoe crab, *T. tridentatus*, the coagulation cascade has been linked to prophenoloxidase activation through non-enzymatic interactions of specific coagulation factors with hemocyanin (26). This leads to the functional conversion of hemocyanin to phenoloxidase, most probably because of a conformational change of the hemocyanin.