THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 40, Issue of October 2, pp. 25889–25892, 1998
Printed in U.S.A.

Tarantula Hemocyanin Shows Phenoloxidase Activity*

(Received for publication, May 15, 1998, and in revised form, July 9, 1998)

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An enzyme generally catalyzes one well defined reaction with high specificity and efficiency. We report here in contrast that the copper protein hemocyanin of the tarantula *Eurypelma californicum* exhibits two different functions. These occur at the same active site. While hemocyanin usually is an oxygen carrier, its function can be transformed totally to monophenoloxidase and *o*-diphenoloxidase activity after limited proteolysis with trypsin or chymotrypsin. *N*-acetyldopamine (NADA) is more effectively oxidized than L-dopa or dopamine. This irreversible functional switch of tarantula hemocyanin function is limited to the two subunits *b* and *c* of its seven subunit types. A conserved phenylalanine in the hemocyanin molecule acts as a placeholder for other substrates that are phenylalanine derivatives. The proteolytic cleavage removes an N-terminal fragment, including the critical phenylalanine residue, which opens an entrance for substrates. Therefore no new arrangement of the active site, with its two copper atoms and the $\mu - \eta^2{:}\eta^2$ bound O_2 molecule, is necessary to develop the catalytic function.

Although hemocyanins and phenoloxidases, both extracellular proteins in the hemolymph of arthropods, bind oxygen in a $\mu - \eta^2{:}\eta^2$ coordination, their biological functions are very different (1, 2). Hemocyanins serve as oxygen carriers for many chelicerates and crustaceans (3–5). This function is well understood on the basis of the known structure of several hemocyanins (6–9). Phenoloxidases are found in an inactive form in the hemolymph of Crustacea and insects (10–18). After limited proteolysis, the phenoloxidase so produced shows both a monophenoloxidase and an *o*-diphenoloxidase activity. Phenoloxidase is widespread in animals and plants as well as in fungi; it starts the synthesis of melanin, is involved in defense reactions (19–20), and is also crucial for arthropod sclerotization, using *N*-acetyldopamine (NADA)[1] as substrate (21, 22). Although much is known about the biological functions of phenoloxidase, its molecular mechanism and regulation are not well understood because of the lack of any known structure (2, 13).

MATERIALS AND METHODS

Assay for o-Phenoloxidase Activity—*o*-Diphenoloxidase and monophenoloxidase activity were measured spectrophotometrically by recording the formation of dopachrome, with an absorption maximum at 475 nm. Iterative spectra in the region of 300–600 nm were recorded to exclude other by-products. The assay medium contained 4 mM L-dopa in the case of *o*-diphenol oxidation and 2 mM tyrosine in the case of tyrosinase activity, which includes the monophenoloxidase and *o*-diphenoloxidase activities, in 0.1 M potassium phosphate buffer at pH 6.8 at 20 °C. Native polyacrylamide gel electrophoresis (5%) and crossed immuno gel electrophoresis were performed according to Lamy *et al.* (32) and stained by incubation in 4 mM L-dopa, pH 6.8, and 0.1 M potassium phosphate at room temperature. When the protein was to be activated by the staining solution itself, then 1 mg/ml trypsin was also added. After staining, the gels were dried or stored in methanol/acetic acid/water.

Limited Proteolysis—Limited proteolysis was performed with bovine trypsin and bovine chymotrypsin (Sigma) in 0.1 M Tris/HCl buffer, pH 7.8 (in the case of the native 24-mer) and pH 9.6 (in the case of subunits) at room temperature. The ratio between hemocyanin and protease varied between 1:1 and 10:1 (w/w). Proteolysis was terminated by addition of soybean trypsin inhibitor (Sigma).

X-ray Structures and Sequence Comparison—The x-ray structures of subunit II of *Limulus polyphemus* hemocyanin (Protein Data Bank code 1oxy) and the hexameric hemocyanin of *Panulirus interruptus* (Protein Data Bank code 1hcy) were obtained from the Protein Data Bank, Brookhaven National Laboratory. Sequence comparison was performed using DNAstar, based on the clustal method. The various peptide sequences were obtained from the Protein Data Bank as indicated previously (28–30).

Purification of Hemocyanin—The hemocyanin of *Eurypelma californicum* hemolymph was obtained by dorsal puncture of the heart. All samples were immediately diluted 1:2 (v/v) with 0.2 M Tris/HCl, pH 8.0, 10 mM $CaCl_2$, 10 mM $MgCl_2$ to stabilize the protein. The samples were then centrifuged at low speed for 10 min at room temperature using a table centrifuge to remove blood cells. The hemocyanin was purified by gel filtration (TSK-HW 55 F; 0.1 M Tris/HCl, pH 8.0, 5 mM $CaCl_2$, 5 mM $MgCl_2$ at 20 °C). The large leading peak contained purified 24-meric hemocyanin, as verified by UV spectroscopy and two-dimensional immuno gel electrophoresis. 24-meric hemocyanin was dissociated by dialysis against 0.02 M glycine/NaOH buffer, pH 9.6, at 4 °C for 4–5 days. The protein concentration was below 1 mg/ml to avoid reassociation.

RESULTS

The native 24-meric hemocyanin of the tarantula *E. californicum* (23–24) seems incapable of oxidizing *o*-diphenols such as L-dopa and monophenols such as L-tyrosine, even after addition of urea or perchlorate at any concentration. An activity similar to phenoloxidase has recently been observed for the hemocyanins from the crustaceans *Homarus americanus* and *Carcinus maenas* and from the mollusc *Octopus vulgaris* using catechol (25, 26). We have now observed monophenoloxidase activity as well as *o*-diphenoloxidase activity for a hemocyanin from a chelicerate, the tarantula *E. californicum*, after limited proteolysis with serine proteases such as trypsin and chymotrypsin, by following the enzyme-specific formation of dopachrome at 475 nm (Fig. 1, *A* and *B*). Monophenoloxidase activity of activated tarantula hemocyanin starts with a long lag phase that is also characteristic for tyrosinases but not well understood (2, 13). The *o*-diphenoloxidase activity has no lag phase although the active sites are identical for both activities, assessed by means of absorption spectroscopy (2). Compared with the activity of tyrosinase from mushrooms, the activity of hemocyanin is lower by almost two orders of magnitude (Fig. 1*C*). To prove that an oxygen consuming process occurs during phenoloxidase activity, oxygen consumption was followed using

* This work was supported by the Deutsche Forschungsgemeinschaft and the Naturwissenschaftlich-Medizinisches Forschungszentrum of the University Mainz. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviation used is: NADA, *N*-acetyldopamine.

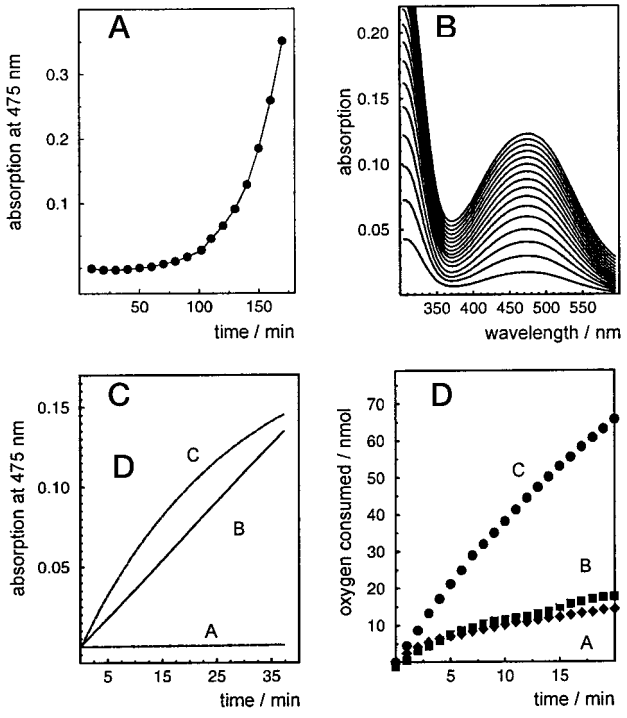

FIG. 1. **Phenoloxidase activity of the 24-meric tarantula hemocyanin.** *Panel A*, time-dependent monophenoloxidase and *o*-diphenoloxidase activity of 24-meric tarantula hemocyanin (1 mg/ml) after tryptic digestion (0.5 mg/ml), recorded at a wavelength of 475 nm. 2 mM tyrosine was used in 0.1 M potassium buffer at pH 6.8 at 20 °C. *Panel B*, time-dependent absorption spectra of the oxidation of 4 mM L-dopa to dopachrome, after activation by tryptic digestion of 24-meric *Eurypelma* hemocyanin. Iterative spectra are taken between 300 and 600 nm every min. The hemocyanin concentration was 1 mg/ml, and the trypsin concentration was 0.5 mg/ml. *Panel C*, time-dependent oxidation of 4 mM L-dopa by trypsin-activated tarantula hemocyanin and mushroom tyrosinase, recording the formation of dopachrome by absorption at 475 nm. *Curve A*, 1 mg/ml native 24-meric hemocyanin, untreated; *curve B*, 1 mg/ml hemocyanin after incubation with 0.1 mg/ml chymotrypsin; *curve C*, 1 μg/ml mushroom tyrosinase; all at 20 °C. *Panel D*, consumption of oxygen after limited chymotryptic digestion of hemocyanin in the presence of L-dopa (*curve A*), dopamine (*curve B*), and NADA (*curve C*). The oxygen consumption was measured with a Clark-type oxygen electrode at 15 °C. The reaction mixtures contained 1 mg/ml hemocyanin and 4 mM of each substrate, respectively.

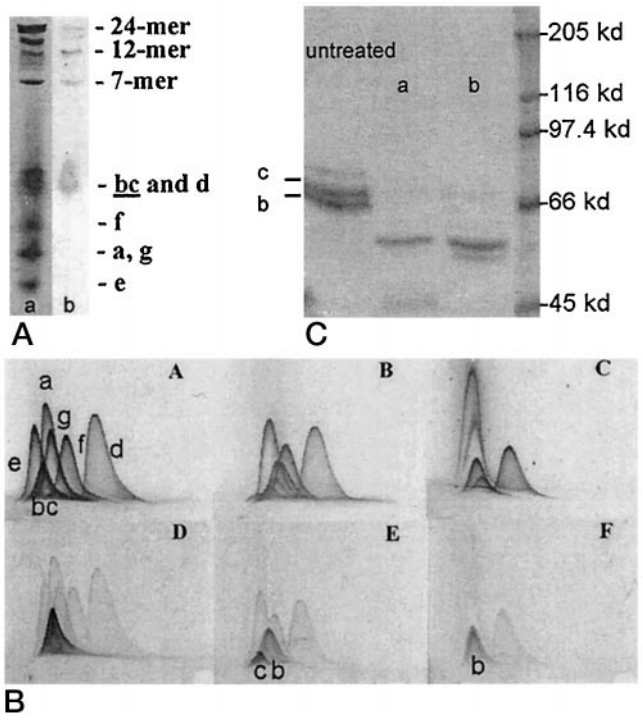

FIG. 2. **Identification of the subunits showing phenoloxidase activity.** *Panel A*, alkaline polyacrylamide gel electrophoresis, pH 8.8, of trypsin-treated 24-meric hemocyanin after staining with Coomassie Brilliant Blue (*lane a*) and after incubation of the gel in a phenoloxidase activity-staining solution (*lane b*). The only bands showing phenoloxidase activity contain the subunit heterodimer *bc*, such as the 24-, 12-, and 7-mer intermediates (24). *Panel B*, crossed immuno gel electrophoresis of tarantula hemocyanin subunit mixture obtained by extended dialysis against alkaline pH 8.8. The samples were incubated for 24 h with different concentrations of urea at 20 °C (from *left* to *right*, 0, 3 M, 4 M). *Sections A, B,* and *C* were stained with Coomassie Brilliant Blue, and *sections D, E,* and *F* were stained for *o*-diphenoloxidase activity by incubation with 4 mM L-dopa and 1 mg/ml trypsin. Only the precipitation band of the heterodimer *bc* shows enzyme activity. At urea concentrations higher than 3 M, a separation of this precipitation band into two bands identified as *b* and *c* can be observed. Both show phenoloxidase activity. *Panel C*, SDS-polyacrylamide gel electrophoresis (10%) of trypsin- (*lane a*) and chymotrypsin-treated (*lane b*) heterodimer *bc*. One major band, $M_r \approx 55{,}000$ is seen in the case of trypsin, and a weak additional band, $M_r \approx 50{,}000$ is observed in the case of chymotrypsin-treated hemocyanin. *c,* molecular weight markers.

protease-treated 24-meric hemocyanin with various *o*-diphenol derivatives such as L-dopa, dopamine, and NADA (Fig. 1*D*). These data represent an absolute measure for comparing the phenoloxidase activity of different substrates. Although dopamine is oxidized at the same rate as L-dopa, NADA is oxidized significantly faster.

The phenoloxidase activity of tarantula hemocyanin is not dependent on the quaternary structure of the 24-mer. When investigated on polyacrylamide gel electrophoresis under conditions where destabilized 24-mers tend to dissociate, pH 8.8, 24-mers are detected as well as various intermediates and monomers (27). The 24-, the 12-, and the 7-mer dissociation intermediates show phenoloxidase activity after incubation with a phenoloxidase-specific activity staining assay (Fig. 2*panel A*). Among the subunits, only the broad band containing the subunit *d* and heterodimer *bc* shows phenoloxidase activity. Using crossed immuno gel electrophoresis (Fig. 2, *panel B*) only the heterodimer *bc* shows phenoloxidase activity. This observation was also confirmed by separating the heterodimer *bc* from the monomeric subunits *a*, *d*, *e*, *f*, and *g* by size-exclusion gel chromatography (TSK HW 55), where again only the heterodimer *bc* showed phenoloxidase activity. We believe that both the *b* and *c* subunits possess enzymic activity. Incubation in 3 and 4 M urea results in a partial dissociation of *b* and *c* from the heterodimer (Fig. 2, *panel B*). At 3 M urea, subunits *b* and *c* are both visible and both show phenoloxidase activity. At higher concentrations of urea, however, all subunits become unstable, in particular subunit *c* becomes undetectable. This exclusive phenoloxidase activity of the heterodimer *bc* of tarantula hemocyanin is observed after limited proteolysis with trypsin as well as with chymotrypsin. Based on sequence alignments (8, 28, 29), including the sequence of subunit *b* (30) (subunit *c* is not sequenced yet), and the x-ray structures of *L. polyphemus* hemocyanin subunit II (chelicerate) and of *P. interruptus* hemocyanin (crustacean) (6–9), several cleavage positions for chymotrypsin and trypsin have been detected that are located on the surface of a chelicerate hemocyanin subunit. According to the sequence numbering of *Limulus* subunit II, trypsin may cleave at Arg-81 (subunit *b*: Arg-68), Lys-130 (subunit *b*: Lys-0117) and Arg-133 (subunit *b*: Arg-120). Chymotrypsin may cleave at Phe-83 (subunit *b*: Tyr-70). The most promising candidate seems to be Arg-120 on subunit *b*, which corresponds to Arg-176 of the closely related prophenoloxidases of the crustacean *Pacifastacus leniusculus* (15). This enzyme, with M_r = 81,000, is activated by trypsin at Arg-176, yielding an active fragment of about 60 kDa. The other tarantula he-

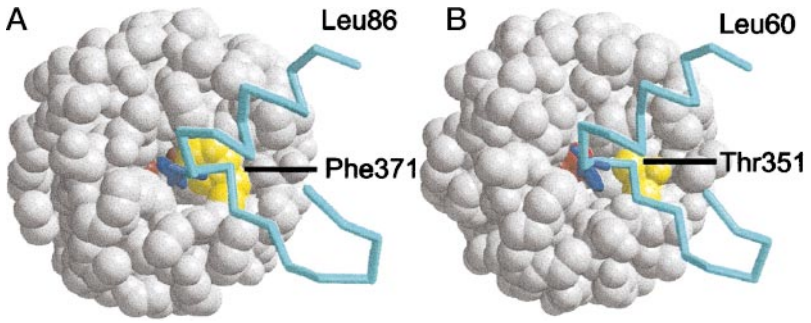

FIG. 3. **Views of the active site of *P. interruptus* (*A*) (Crustacea) and *L. polyphemus* (*B*) (Chelicerata) hemocyanin subunits, after removal of the N-terminal fragments.** The part of the N-terminal fragment (*blue*) with the conserved Phe (*dark blue*) is shown. Its removal opens a substrate channel to the oxygen (*red*) at the active site. In the case of the crustacean hemocyanin, Phe-371 (*yellow*) covers part of this entrance, whereas in case of Chelicerata, hemocyanin free access to the active site is possible because of the smaller Thr-351 (*yellow*).

mocyanin subunits do not have an Arg but a Lys at this position, but surprisingly they are not activated. The prophenoloxidase subunits from the insect *Galleria mellonella* are of similar sizes to *Pacifastacus* prophenoloxidase, with masses of 80 and 83 kDa. After proteolysis with chymotrypsin, two active fragments with 67 and 50 kDa were found. Phenoloxidase-active *Eurypelma* hemocyanin fragments also have comparable masses. SDS-gel electrophoretic analysis reveals that the heterodimer *bc* splits into peptides with two major components of $M_r \approx 50{,}000$ and $M_r \approx 55{,}000$ after cleavage with chymotrypsin and one major component with $M_r \approx 55{,}000$ after cleavage with trypsin (Fig. 2, *panel C*). However, any procedure to obtain a purified pseudo-native 55-kDa fragment with full enzymatic properties has not been successful so far.

DISCUSSION

In the case of tarantula hemocyanin from *E. californicum,* the cleavage of an N-terminal peptide including Phe-49 from the two subunits *b* and *c* seems to open the entrance to the active site, with its copper atoms, for phenolic substrates. This is independent of the association status of the subunits (Fig. 3). This highly conserved phenylalanine in hemocyanins is at a distance of 3.5 Å from the active site and serves as an allosteric trigger during the oxygenation process (8, 9). We think that Phe-49 may also act as a placeholder for phenolic substrates during the time when hemocyanin is acting only as an oxygen carrier, to conserve the hidden function as a phenoloxidase in the native but uncleaved state. Assuming the same orientation of possible substrates for the phenoloxidase activity as Phe-49, the phenyl ring lies almost perpendicular to the Cu-Cu-O_2 plane, with close contact of the ortho position to one of the two oxygen atoms (Fig. 4). In this geometry, the hydroxyl group may bind to the copper atoms to initialize the cleavage of the oxygen molecule (2). The optimal arrangement of the substrate may be sterically hindered by Thr-351 (according to the numbering of *L. polyphemus* hemocyanin). This would explain the low *o*-phenoloxidase activity of hemocyanin with respect to other phenoloxidase and tyrosinase.

Thus, the explanation for the activation of phenoloxidase activity of tarantula hemocyanin by limited proteolysis seems to be based not on a rearrangement of the active site but by providing a free access for various substrates to the active site, following removal of the nonreactive substrate analogue Phe-49. The greater accessibility of the active site of mushroom tyrosinase compared with hemocyanin can also be deduced from kinetic removal of the copper atoms by cyanide in both proteins (31). This hypothesis about a free entrance is supported by a recent study (25) which shows that access for catechol to the active site is potentiated by the presence of salts of the Hofmeister series such as high concentrations of perchlorate. The salts increase the low phenoloxidase activity of crustacean hemocyanins (*H. americanus* and *C. maenas*) by a factor of two (25). However, we have not been able to induce any significant phenoloxidase activity in the crustacean hemocyanins from *P. interruptus* or *H. americanus* by proteolysis, using tyrosine or L-dopa as substrates. This difference in the hemocyanins from Crustacea and Chelicerata may be explained on the basis of their x-ray structures (8, 9). Although the active sites are highly conserved, Thr-351 belonging to the active site of *Limulus* hemocyanin is replaced by the larger Phe-371 in *Panulirus* hemocyanin (Fig. 3), which sterically hinders any substrate larger than oxygen from coming close to the active site, even after removal of the N-terminal fragment. Thr-351 is conserved in Chelicerata hemocyanins. In the case of *Eurypelma* hemocyanin, however, a reason for the exclusive phenoloxidase activity of subunits *b* and *c* remains unknown.

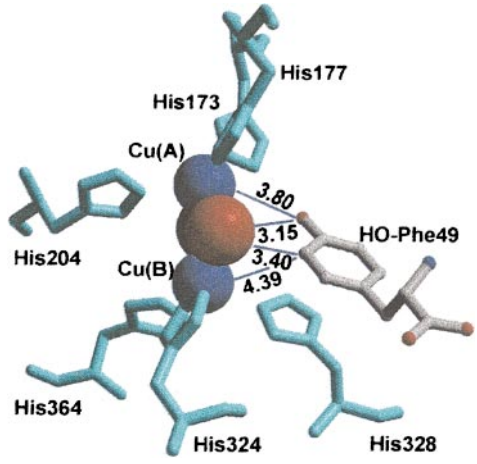

FIG. 4. **The orientation of a hypothetical substrate HO-Phe-49 replacing Phe-49 at the oxygen binding site.** The hemocyanin subunit II (*L. polyphemus*) is used (9). The two copper atoms (*blue*) are complexed by six histidines. The dioxygen (*red*) is bound in a $\mu - \eta^2{:}\eta^2$ coordination. A hypothetical hydroxyl group is added to Phe-49 to design a hypothetical tyrosine. Note that Phe-49 as well as the HO-Phe-49 are closer to the dioxygen and Cu(A) than to Cu(B).

We may make three significant deductions from our results. First, our findings may explain the high conservation of quaternary structure of the 24-meric tarantula hemocyanin throughout evolution in this ancient animal. Subunits *b* and *c* bridge the two structurally identical 2 × 6-meric half-molecules (23). In the case of crustacean hemocyanins, these linker subunit types are not present: only 6-mers and 2 × 6-mers are found in the hemolymph of modern arthropods (3–5). In addition, species of two large groups among arthropods, Crustacea and Insecta, have evolved prophenoloxidases, most probably by gene duplication of hemocyanins, which is indicated by the close structural relationship between phenoloxidase and hemo-

cyanin (14, 15), and consecutive optimization of a physiologically beneficial phenoloxidase activity.

Second, evolution seems to have stabilized a double function in the hemocyanin from the ancient tarantula *Eurypelma californicum*. This is a further example in contrast to the dogma: one gene—one protein—one function with respect to the active site. We suggest that the *Eurypelma* hemocyanin can develop two different functions consecutively using the same active site. During normal life, the hemocyanins serve as oxygen carriers. At a crucial moment of the life of arthropods, *i.e.* the growing phase, hemocyanin function may switch to phenoloxidase activity by limited *in vivo* proteolysis. Such phenoloxidase activity is known to be necessary to harden the exoskeleton rapidly after molting (*i.e.* within minutes to hours) for protection against predators. The "new function" of hemocyanin may contribute toward catalyzing the first steps in the sclerotization process, using NADA as the main starter substrate, as is the case for many insect phenoloxidases (21, 22). This hypothesis may be supported by the more efficient oxidation rate of NADA compared with L-dopa or dopamine (Fig. 1). The enzymatic activity is low, but it may be compensated for by the extremely high concentration of hemocyanin in the hemolymph of the tarantula, ranging between 5 and 120 g/liter. It should be mentioned that we were not able to detect other phenoloxidase activity in the hemolymph of tarantula under our experimental conditions. We tested fresh hemolymph with intact cells and crude but cell-free hemolymph by gel-electrophoresis but not spectroscopically because of the high turbidity of the samples. However, we did not look for prophenoloxidase activity within any cells. Thus, we cannot exclude the existence of any prophenoloxidase either in the blood cells or in other tissue cells. Experiments to demonstrate the physiological relevance of our experiments *in vivo* are in progress.

Third, prophenoloxidases have been identified in the hemocytes of insects and a crustacean but have not been looked for in chelicerates yet (18). Nevertheless, as cheliceratean hemocyanin are also synthesized in the cytoplasm of hemocytes in contrast to crustacean hemocyanins, which are synthesized in cells of the hepatopancreas (5), we could make the following very tentative hypothesis. In ancestral arthropods, hemocyanin was synthesized in hemocytes and had both functions, the oxygen transport and the oxidation of aromatic compounds after proteolytic cleavage. This ancestral state is still found in the chelicerates. But after divergence of chelicerates from other arthropods like crustaceans and insects, a gene duplication has occurred leading to crustacean hemocyanins and hexamerins (28, 29). These hemocyanins no longer possess the capability to become phenoloxidases by proteolytic cleavage and become secondary proteins synthesized elsewhere in the body. The other line has lost the capability to form hexamers and to transport oxygen and has become the prophenoloxidases identified presently in insects and crustaceans.

Finally, these observations suggest a system whereby one may examine the structural basis of phenoloxidase activity because no crystal structure of any phenoloxidase is available.

Acknowledgments—We thank Drs. F. Tuczek, J. Beintema, and N. Terwilliger for intensive discussions and Dr. K. Miller for reading the manuscript and help with the English language.

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J. Biol. Chem. 1998, 273:25889-25892.
doi: 10.1074/jbc.273.40.25889