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Structure and function of the hemocyanin from a semi-terrestrial crab, *Ocypode quadrata*

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Summary. Structural and functional studies of the hemocyanin of the semi-terrestrial ghost crab, *Ocypode quadrata*, demonstrate a variety of differences in comparison to the hemocyanin of aquatic crabs. These differences may be related to the terrestrial habit of this crab. Unlike aquatic crabs, the major (56%) blood component is the hexamer; the remaining 44% is dodecamer. The hexamers and dodecamers are not in rapid equilibrium. Electrophoretic analysis of the subunit composition indicates three major components referred to as 1, 3, and 4, and one minor component referred to as component 2. These components, although electrophoretically distinct, are alike immunologically. Components 1 and 2 are essentially absent from purified hexamers, whereas they compose $^1/_3$ of the subunits in dodecamers. These results suggest that they are involved in linking hexamers to form dodecamers, and that two, rather than one, subunits are involved in the bridge. Oxygen-binding measurements show a higher degree of cooperativity, and a much reduced allosteric effect of L-lactate on the dialyzed hemocyanin as compared to the hemocyanin of aquatic crabs. Exercise rapidly induces a large drop in hemolymph pH (0.5 units) and a corresponding increase in lactate concentrations (to 10 m*M*).

Introduction

Hemocyanins are the large, extracellular oxygen-transport proteins that are found in arthropods and molluscs and that reversibly bind oxygen at a bi-nuclear copper site. The state of knowledge of hemocyanin has been reviewed in a number of recent publications (Van Holde and Miller 1982; Wood 1983; Ellerton et al. 1983; Mangum 1983a; Linzen 1986; Markl 1986). Based on these reviews, a number of generalizations can be made about both the structural and functional properties of crab hemocyanins. The hemocyanins are present in crab hemolymph predominantly in the form of dodecamers (Markl et al. 1979; Ellerton et al. 1983; Johnson et al. 1984). In cases where electrophoretic heterogeneity of the subunits has been examined by using high-resolution polyacrylamide gel electrophoresis, typically four to seven bands are found (Markl et al. 1979).

Complete dissociation to the subunit level is often not achieved even at high pH in the absence of calcium (Markl et al. 1979), but may require the addition of denaturants such as urea (Herskovits et al. 1981). When returned to pH 7 to 8 in the presence of calcium, these hemocyanins generally reassociate to the level of the hexamer. Reassembly is often blocked, however, at the hexameric level; and only a small quantity of reassembled dodecamers can be obtained (Herskovits et al. 1981; Carpenter and Van Holde 1973; Ellerton et al. 1983).

The oxygen-binding properties of crab hemocyanins are generally characterized by a high degree of cooperativity, a large Bohr effect, an increased affinity in the presence of calcium ions (Mangum 1983a), and, as demonstrated in recent years, an increased affinity in the presence of L-lactate (Truchot 1980; Graham et al. 1983; Johnson et al. 1984). The magnitude of the lactate effect in a variety of crab species, and the case for the existence of unidentifed effectors of hemocyanin oxygen affinity, is summarized by Bridges and Morris (1986).

Many of our present conclusions, however, are largely based on extrapolations from the few

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aquatic species for which both careful structural and functional studies exist. The generality of these extrapolations can be tested by the careful examination of hemocyanins from other crab species. In particular, studies of species that are phylogenetically or environmentally distant are most likely to show exceptions to these generalizations. It was on this basis that investigations were undertaken on some aspects of the structure and function of hemocyanin from the semi-terrestrial crab, *Ocypode quadrata*. The results obtained are compared with those reported for aquatic crabs, and in particular, with the results of our previous study on hemocyanin of the blue crab, *Callinectes sapidus* (Johnson et al. 1984) In the course of this study a number of properties that distinguish the hemocyanin of *O. quadrata* from that of *C. sapidus* and other previously studied aquatic crabs have been observed and are reported here.

Materials and methods

Ghost crabs, *O. quadrata* were captured at night on Bird Shoal, a small island near the Duke University Marine Laboratory at Beaufort, NC. Hemolymph was obtained from an infrabranchial sinus at the base of a walking leg. Hemocyanin was isolated from the hemolymph as previously described (Johnson et al. 1984). Removal of loosely bound endogenous effectors was achieved by dialyzing the sample for at least 24 h against a buffer appropriate to the particular experiment (see below).

The hexameric and dodecameric components of *O. quadrata* hemocyanin were separated by chromatography through Fractogel 55-S (EM Science). A 2.5 by 95 cm column was eluted at a flow rate of approximately 70 ml/h with 50 m*M* Tris-HCl buffer, pH 7.5, ionic strength 0.13, 10 m*M* $CaCl_2$. Sample load was 7 ml of protein solution at a concentration of approximately 15 mg/ml.

The proportions of hexamers and dodecamers under different conditions were quantified by analytical ultracentrifugation of the samples in a Beckman Model E centrifuge equipped with scanner optics. The sedimentation coefficients were calculated from the movement of the midpoint of the boundary of the absorbance tracings. The values were corrected from those obtained in the above buffer to $s_{20,w}$ using standard procedures and the assumption that $v=0.725$ (Carpenter and Van Holde 1971). The percentages of each component were calculated from the relative heights of the plateaus of the absorbance tracings and were corrected for radial dilution.

Subunit composition was studied by gel electrophoresis on 16 cm, 7.5% polyacrylamide gels. A discontinuous buffer system with a resolving gel at pH 8.9 was used. Gels were stained with 0.1% Coomassie Blue G-250 in 25% trichloroacetic acid. Relative protein concentrations in each band were obtained by scanning the stained gels at 565 nm with a Gilford model 2520 gel scanner. The area under each peak was measured with a digitizing tablet connected to an Apple II Plus microcomputer. Two-dimensional immunoelectrophoresis was performed as described by Weeke (1973).

Oxygen-equilibrium experiments were performed at 20 °C in a modified Imai cell (Imai 1981; Johnson 1984) The buffer solution for these experiments was artificial sea water (423 m*M* NaCl, 9 m*M* KCl, 23 m*M* $MgCl_2$, 26 m*M* $MgSO_4$, 9.3 m*M* $CaCl_2$) buffered with 50 m*M* Tris. The pH was adjusted with HCl.

Data from the oxygen-equilibrium curves were fit to equation 1, which characterizes the concerted

$$y=\frac{\alpha(1+\alpha)^{n-1}+Lc\alpha(1+c\alpha)^n}{(1+\alpha)^n+L(1+c\alpha)^n} \quad (1)$$

Monod-Wyman-Changeux model (Monod et al. 1965). In this equation $c=P_{50_R}/P_{50_T}$ and $\alpha=P_{O_2}/P_{50_T}$, where P_{50_r} is the oxygen affinity of the R-state and P_{50_t} is the oxygen affinity of the *T*-state. $L=[T]/[R]$, where $[T]$ and $[R]$ are the concentrations of the protein in the *T*-state and *R*-state, respectively, in the absence of oxygen, and n is the number of interacting sites. The equation was fit to the data with a non-linear least-squares regression program (Johnson et al. 1981).

Hemolymph pH was measured on crabs captured at night and kept in individual containers in the laboratory until the next evening. Hemolymph lactate levels were measured on a separate set of crabs kept in the same manner. Measurements on crabs reported as resting were from crabs removed from their containers and sampled with a minimum of disturbance. Crabs were exercised by forcing them to run until they would not run anymore. A set of crabs was also analyzed for hemolymph lactate concentrations in the field. Only crabs that were caught with a minimum of disturbance were used for the field lactate analysis. The field hemolymph samples were kept on ice until they were returned to the laboratory for analysis (approximately 2 h later).

Pre-branchial blood pH in the crabs was determined in resting and exercised crabs by removing and immediately injecting an aliquot of blood directly into a Radiometer capillary pH electrode. The pH was measured at 20 °C, the approximate temperature of field and laboratory animals.

L-lactate levels in hemolymph were determined with a Sigma Chemical Co., No. 826-A, diagnostic kit. The pH of the glycine-hydrazine solution was lowered to 9.0, and EDTA was added to a final concentration of 12 m*M* to avoid interference by copper released during precipitation of the hemocyanin (Graham et al. 1983; Engel and Jones 1978).

Results

Structure

The elution profile of *O. quadrata* hemocyanin from a gel filtration column is shown in Fig. 1. The leading peak was identified by analytical ultracentrifugation as being 25 S hemocyanin (dodecamers) and the trailing peak as 16 S hemocyanin (hexamers). Integrations of the area under each peak indicate that dodecameric hemocyanin constitutes only 44% of the native hemocyanin and that 56% was present as hexamer. The same composition was obtained by analysis of the scan profiles obtained by analytical ultracentrifugation of the unfractionated hemocyanin.

Ultracentrifugation of the isolated hexameric and dodecameric components showed no tendency for the dodecamer to dissociate to hexamers, or for the hexamers to associate to dodecamers. Thus the native composition does not represent a rapid equilibrium between the two molecular species.

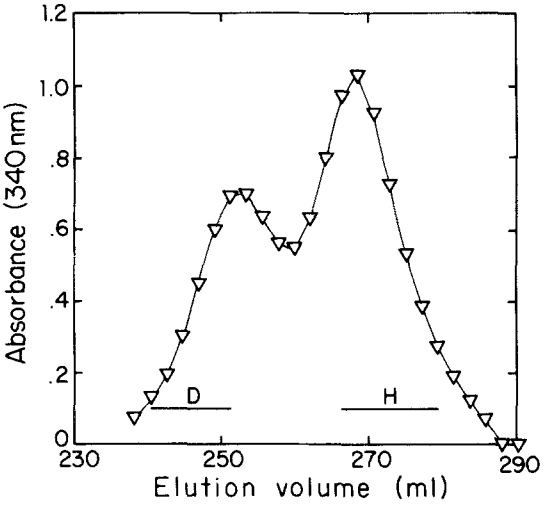

Fig. 1. Elution pattern of native *O. quadrata* hemocyanin on Fractogel 55-S gel chromatography column. Labelled lines indicate fractions pooled for hexamers (*H*) and dodecamers (*D*)

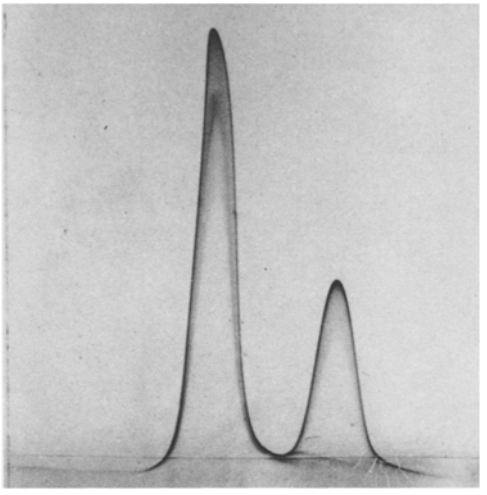

Fig. 3. Two-dimensional immunoelectrophoresis of dissociated *Ocypode* hemocyanin against rabbit anti-*Ocypode* antiserum. Anode is on the left. Peaks $^{3}/_{4}$ are on the left, $^{1}/_{2}$ on the right

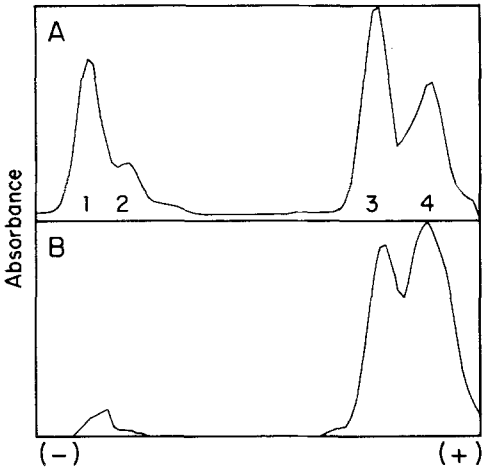

Fig. 2. Densitometric scans of polyacrylamide electrophoresis gel of dissociated dodecameric (**A**) and hexameric (**B**) hemocyanin from *O. quadrata*. Only the portion of the gel containing presumptive subunits has been scanned. The combined area under the scan of zones 1 and 2 is 6% of the total in hexamers, and 34% of the total in dodecamers. Zones 3 and 4 account for 94% of the area in hexamers and 66% of the area in dodecamers

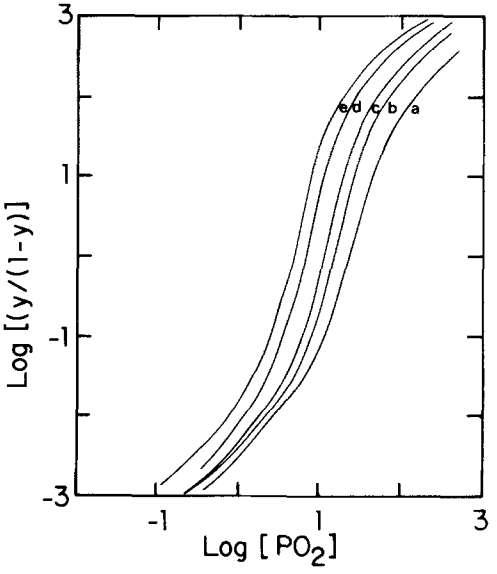

Fig. 4. Hill plots of oxygen binding by *O. quadrata* hemocyanin. *a* pH 7.150; *b* pH 7.432; *c* pH 7.570; *d* pH 7.881; *e* pH 8.093. All in artificial seawater (see methods)

The native mixture and the hexameric and dodecameric components were separately dialyzed against a buffer of 50 m*M* Tris-HCl, pH 8.9, with 10 m*M* EDTA. Under these conditions both the dodecameric and hexameric components dissociate completely to the subunit level (5S as shown by analytical ultracentrifugation). The dissociated subunits were then dialyzed for 24 h against 50 m*M* Tris-HCl, pH 7.5, I=0.1, with 10 m*M* $CaCl_2$. Subunits obtained from the native mixture reassociate to a mixture of 20% dodecamer and 80% hexamer. Subunits obtained from the hexameric fraction reassociated completely to the hexameric state. Subunits obtained from the dodecameric fraction reassociated to a mixture of 49% dodecamers and 51% hexamers.

Isolated hexamers and dodecamers were dissociated to the monomeric level and examined for heterogeneity with polyacrylamide gel electrophoresis. Fig. 2 shows the densitometric scan of the bands from this gel. Three major peaks and one minor peak are seen. Electrophoretic bands 1 and 2 are essentially absent from the hexameric hemocyanin. The percentage areas given in Fig. 2 are

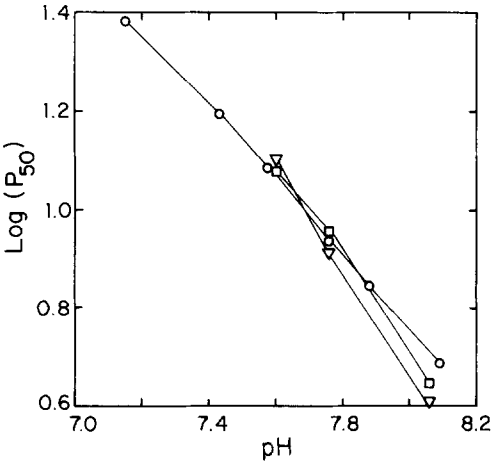

Fig. 5. Bohr effect of *O. quadrata* hemocyanin. Dodecamers (□), hexamers (▽), and the native mixture (○). All in artificial seawater (see methods). Data from three hemocyanin preparations

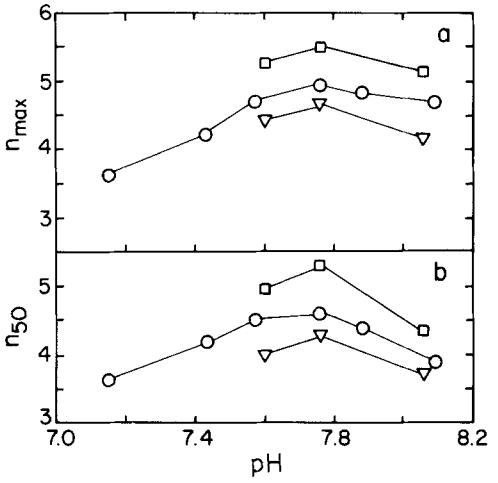

Fig. 6. The maximal slope of Hill plot (**a**) and the slope of the Hill plot at 50% saturation (**b**) as a function of pH. Dodecamers (□), hexamers (▽), and the native mixture (○). All in artificial seawater (see methods). The data is from the same experiments as that of Fig. 5

consistent with bands 1 and 2 contributing four of the twelve subunits in the dodecameric hemocyanin.

The profile of a two-dimensional immunoelectrophoresis of dissociated *Ocypode* hemocyanin is shown in Fig. 3. It can be seen that the peaks are fused, indicating immunological identity of the subunits.

Oxygen binding

Hill plots of oxygen-equilibrium curves of native ghost crab hemocyanin measured in the Imai apparatus are shown in Fig. 4. Each curve consists of 100 to 200 original points smoothed to approximately 30–40. The shift in the upper and lower asymptotes of the Hill plot suggest that the affinity of both the hypothetical *R* and *T* states are increased with increasing pH.

The effect of pH on the oxygen affinity is shown in Fig. 5. The Bohr effect ($\Delta \log P_{50}/\Delta pH$) is -0.74. Cooperative interactions between the oxygen-binding sites are clearly affected by pH. Fig. 6a shows the maximal Hill coefficient (n_{max}) and Fig. 6b the Hill coefficient at half-saturation (n_{50}) as a function of pH. Cooperativity is maximal at approximately pH 7.7.

The oxygen-binding properties of the isolated hexamers and dodecamers were also studied. The affinity of dodecamers is similar to that of the hexamers; however the cooperativity of dodecamers is higher (Fig. 6). The oxygen-binding curves for the isolated hexamers and dodecamers, measured at pH 7.76 in artificial seawater, were fit to equation 1 as described in the methods section. The best fit values are reported in Table 1. These values indicate that the number of interacting sites is greater in the dodecameric hemocyanin.

Table 1. Parameters of the MWC model describing oxygen binding by the hexameric and dodecameric components of *Ocypode* hemocyanin

	$\log P_{50_R}$	$\log P_{50_T}$	log L	*n*
Hexamer	−0.37	2.12	6.5	5.1
	(−0.43, −0.31)	(2.08, 2.16)	(6.3, 6.8)	(4.9, 5.4)
Dodecamer	−0.32	2.17	7.6	6.0
	(−0.38, −0.27)	(2.13, 2.23)	(7.2, 8.1)	(5.5, 6.6)

Artificial seawater (see methods) at pH 7.76. Numbers in parentheses represent 65% confidence intervals

Table 2. Effect of lactate on oxygen affinity and cooperativity of *O. quadrata* hemocyanin

	P_{50}	P_{med}	n_{50}	n_{max}
Control	12.2±0.2	11.8±0.2	4.5±0.05	4.69±0.03 ($n=3$)
10 m*M* L-lactate	10.8±0.1	10.4±0.1	4.03±0.005	4.45±0.02 ($n=3$)

Artificial seawater (see methods) at pH 7.57. All values are statistically different from control. ($P<0.05$. Student's *t*-test)

The oxygen affinity of the hemocyanin of *Ocypode* was determined in the presence and abcence of 10 m*M* L-lactate. As can be seen from Table 2, L-lactate has only a small effect on the oxygen affinity and cooperativity of *O. quadrata* hemo-

Table 3. Hemolymph pH and L-lactate concentration in ghost crabs

	Field	Resting	Active
pH	nd	7.87 ± 0.03 ($n=4$)	7.35 ± 0.04 ($n=5$)
L-lactate (mM)	1.1 ± 0.3 ($n=5$)	0.56 ± 0.07 ($n=4$)	10.4 ± 1.4 ($n=5$)

nd not determined. Variances are all significantly different (F-test). Mean values for active crabs are significantly different from field and resting crabs, while mean values from resting and field crabs are not significantly different from each other

cyanin, when measured at pH 7.57 in artificial sea water.

In-vivo pH and L-*lactate*

The pH's and lactate concentrations in the hemolymph of resting and exercised crabs are shown in Table 3. Hemolymph lactate in field crabs was also measured. Crabs were exercised by chasing them on land for approximately 2 min. They were, however, apparently fatigued after about the first 30 sec of running, and moved thereafter only with much encouragement. After two min of exercise, stumbling of the crabs was common. Hemolymph lactate levels rise, and pH levels fall significantly after this short period of exercise. The lactate level of crabs bled in the field is not significantly different from that of undisturbed crabs in the laboratory, although the variability was greater.

Discussion

Structure

One remarkable structural feature that distinguishes the hemocyanin of *Ocypode quadrata* from that of other crabs is the presence of the hexamers as the major hemocyanin component in the hemolymph. The only other similar cases reported in the literature are from two Amazon River crabs (Bonaventura et al. 1979).

It is now generally accepted, based on results from a variety of hemocyanin-containing species, that the formation of dodecamers is generally dependent on specific subunit types that are involved in linking hexamers to form dodecamers. Specific interactions between the linkers in each hexamer serve to hold the hexamers together. These interactions may be covalent, as in the disulfide bonds formed between the linkers in the spider, *Cupiennius* (Markl 1980) and the crayfish, *Cherax* (Jeffrey et al. 1978), or non-covalent as found for example in *Limulus* (Johnson and Yphantis 1978) or *Ligia* (Terwilliger 1982). Within the crustacea, the hexamer-hexamer bridge has generally been found to consist of one pair of linking subunits, with one subunit of the pair in each hexamer (Jeffrey et al. 1978; Terwilliger 1982; Johnson et al. 1984). Recently, however, Bijlholt and van Bruggen (1986) have described a model of *Squilla* hemocyanin in which two pairs of linking subunits are present.

The unusual mixture of aggregation states found in ghost crab hemocyanin could result from a number of factors. The explanation that seems most probable is that, as in *Squilla* hemocyanin (Bijlholt and van Bruggen 1986), the bridge between hexamers requires two linkers in each hexamer instead of one. If this is true, then the same number of linkers would form only half as many dodecamers (i.e., 44% as observed, rather than 88%). The essential absence of subunits 1 and 2 from hexamers suggests that they are the linkers. Additionally, reassociation to dodecamers occurs only when subunits 1 and 2 are present. The fact that they constitute two out of every six subunits in dodecamers suggests that this possibility is indeed the correct one.

An alternate possibility is that insufficient quantities of the linking subunit are synthesized. However, if the subunits of zones 1 and 2 are the linkers and, as in other crabs, one linking subunit is present in each hexamer, then there is sufficient quantity present to form 86% dodecamers. This value would conform to the levels of dodecamers generally found in crab hemolymph. With the assumption of one linking subunit per hexamer it seems unlikely, therefore, that a low level of linker can account for the decreased amount of dodecamer relative to that found in other crab hemocyanins.

A final possibility is that the assembly process leads to many hexamers which contain linking subunits but do not assemble to dodecamers. Such anomalous hexamers have been observed during reassembly experiments with other crustacean hemocyanins (Terwilliger 1982). There is a small quantity of the presumptive linker in purified *O. quadrata* hexamers, suggesting that 'anomalous' assembly may occasionally occur, but it is insufficient to explain the low quantity of dodecamers.

The immunological identity of the four subunits of *Ocypode* hemocyanin also contrasts with the pattern seen in other crabs. In other species the subunits can be divided into two immunologically distinct categories (Markl and Kempter

1981). These are at times denoted the alpha (or conservative) subunits, and the beta (or variable) subunits. Stöcker (1984) has shown that the *O. quadrata* subunits correspond to the alpha type. He suggests that the ghost crabs have lost the beta subunit that is present in other crabs. The extensive review of Markl (1986) should be consulted for a discussion of the phylogenetic implications of the immunologic analysis of arthropod hemocyanin subunits.

Oxygen binding

The Bohr effect (−0.74) measured in this study is comparable to that (−0.78) reported by Burnett (1979) in his study of ghost crab hemocyanin. My measurements indicate that the hemocyanin has a higher affinity for oxygen than that reported by Burnett (1979). This difference is at least in part explained by the 5 °C lower temperature used in this study.

Cooperative interactions among the oxygen-binding sites, as measured by the Hill coefficient, are higher in this study than previously reported. This may in part be due to the greater precision obtained in the use of the Imai apparatus in this study. Mangum (1980) has reported *O. quadrata* hemocyanin cooperativity as being independent of pH. In the preparations used here this is clearly not the case. Cooperativity is maximal at about pH 7.7. These differences may also be the consequence of the experimental technique used. Precise oxygen binding curves, as generated in the Imai apparatus, demonstrate that the n value is not constant over the entire curve, even within the 30–70% saturation range commonly used. The Hill coefficients reported here are based on numerical analysis of the oxygen equilibrium curve at 50% saturation.

Cooperative interactions between the oxygen-binding sites are greater in *O. quadrata* dodecamers than those found in a number of other crustacean hemocyanins (Ellerton et al. 1983). A number of studies have indicated that hexameric and dodecameric components from the same species show similar levels of cooperativity (Arisaka and van Holde 1979; Brouwer et al. 1982; Jeffrey and Treacy 1980). It has been suggested that the functional set of interacting sites in crustacean hemocyanins is the hexamer (van Holde and Miller 1982). The cooperativity of *O. quadrata* dodecamers is greater than that of the hexamers. This difference does not, however, clearly implicate interactions between hexamers. A theoretical curve, based on the two-state model for allosteric transitions (Monod et al. 1965) and fit to the data from a curve showing maximum cooperativity indicates that the number of interacting sites is between 5.5 and 6.5. The existence of more than one pair of inter-hexamer subunits acting as linkers may enhance the possibility of allosteric interactions being communicated across the hexamer-hexamer interface. However, the number of interacting sites clearly does not correspond to that of a dodecamer. Alternatively, the pair of linkers in each hexamer may lead to increased interaction between the trimers within hexamers thereby minimizing the role of hybrid states as postulated for other crustacean hemocyanins (Arisaka and van Holde 1979; Brouwer et al. 1978; Johnson 1984). Further experimental and theoretical analysis of binding curves obtained under varied conditions of allosteric effectors will be useful to confirm and elucidate the significance of this result.

The log P_{50} of *O. quadrata* hemocyanin is decreased by 0.05 after addition of 10 m*M* L-lactate. The log P_{50} of the hemocyanin of *Cancer pagurus* (Truchot 1980), *Cancer magister* (Graham et al. 1983) and *Callinectes sapidus* (Johnson et al. 1984) are decreased by approximately 0.3 after addition of 10 m*M* L-lactate. Thus, the lactate effect in vitro is present in *O. quadrata* but much reduced relative to these other crab species.

In vivo pH and lactate

A significant amount of L-lactate does accumulate during forced exercise of this crab. A lactate level of 10 m*M* is reached after only 2 min of exercise. The lactate level (1 m*M*) in hemolymph of crabs during normal nocturnal activity is not significantly greater than that in crabs at 'rest' in the laboratory. Carbon dioxide may also accumulate during exercise. While the latter may also contribute to the lowered pH in the blood (and may indeed directly affect the hemocyanin), a large portion of this acidification of the hemolymph is probably due to metabolic production of lactic acid. Post-exercise acidosis in the land crab, *Cardisoma carnifex* is largely metabolic; and the metabolic component is totally due to the entry of lactate anions (7.5 m*M*) and protons, in equal amounts, into the hemolymph (Wood and Randall 1981). In *Gecarcinus lateralis*, where the accumulation of lactate is much less (1.5 m*M* after exercise), the metabolic acidosis is only partly due to lactate (Smatresk et al. 1979).

The lowered pH observed after exercise will significantly reduce the oxygen affinity of the hemocyanin. Using P_{50}s and pre- and post-branchial

P_{O_2}s from Burnett (1979), one can calculate that post-branchial hemocyanin will be less than 50% saturated with oxygen after a 0.5 unit drop in pH induced by exercise. If post-branchial P_{O_2} falls during exercise in *O. quadrata* as it does in *Cardisoma carnifex* (Wood and Randall 1979), then the saturation of post-branchial hemocyanin will be even less. The small effect of L-lactate on the oxygen affinity is insufficient to counterbalance the large reduction of affinity induced by the lowered pH. Thus, the exercise-induced acidosis in the ghost crab will lead to a serious impairment of oxygenation of the blood at the gills. While other factors of the ghost crab respiratory system may be involved, the lack of a significant compensating lactate effect is a factor that may be responsible for the rapid fatigue of ghost crabs during exercise noted in this and earlier studies (Herreid 1981). Conversely, in the tissues, the reduced lactate effect ensures efficient unloading of hemocyanin oxygen stores during this rapid sprinting behaviour. Recently Morris and Bridges (1985) have demonstrated that the oxygen affinity of dialyzed hemocyanin of the related ghost crab, *Ocypode saratan* is also less sensitive to L-lactate as compared to that of other crabs. However, the oxygen affinity of hemocyanin in undialysed hemolymph exhibited a larger response to L-lactate addition. Clearly, further studies on *Ocypode quadrata* hemocyanin are necessary to confirm this effect and elucidate the molecular mechanism for this unusual response if it is present.

Conclusion

Are the features of structure and function of ghost crab hemocyanin that distinguish it from other crabs related to the terrestrial life of this crab? *O. quadrata* is more closely related to *Callinectes sapidus* than either is to *Cancer magister*, the latter being in the section Cancridea and the former two in the section Brachyrhyncha (Bowman and Abele 1982). The structural and functional properties of *O. quadrata* hemocyanin, however, distinguish it from either of these aquatic crabs. The differences may, therefore, indeed be related to the terrestrial habitat.

How are the features of ghost crab hemocyanin related to the terrestrial habitat? First, the rationale for the evolution of hemocyanins that assemble into polymers is related both to the possibility of cooperative oxygen binding allowed by subunit interactions, to the reduction of blood viscosity, and to the reduction of colloid osmotic pressure exerted by the subunits (Snyder and Mangum 1982). The latter explanation may be particularly important in assembly above the level of the hexamer in crabs. If the colloid osmotic pressure is greater than the hydrostatic pressure, fluid balance between various body compartments will be disturbed. Aquatic crabs, such as *Callinectes sapidus* operate with hemocyanin concentrations that border on the maximum allowed according to this argument. As shown by Snyder and Mangum (1982) the assembly of subunits into polymers reduces the total colloid osmotic pressure. *O. quadrata*, if it possesses a higher hydrostatic pressure as do other semi-terrestrial crabs (Cameron and Mecklenburg 1973), may be freed from this particular evolutionary pressure, and thus a reduction in the relative concentration of dodecamers may not be opposed by selective processes. Indeed, as demonstrated by Burnett (1979), the ghost crab does have a much higher hemocyanin concentration than do aquatic crabs.

Second, the nature of the gills of terrestrial crabs results in a relatively low post-branchial P_{O_2}. Oxygen must be loaded and unloaded over a very narrow range of P_{O_2}. Thus the relatively high level of cooperativity probably facilitates oxygen transport in this crab.

Finally, Mangum (1983b) has postulated that the presence of a large normal Bohr effect and a metabolic acidosis during hypoxia are the determinants of the presence of a lactate effect. Several examples of the interaction of the lactate effect and the Bohr effect are discussed by McMahon (1986). His in vivo measurements demonstrate the important role of lactate in maintaining sufficient oxygen affinity to load oxygen at the gill during exercise induced acidosis. Ghost crabs have the Bohr effect and undergo a large metabolic acidosis during exercise, yet the lactate effect is small. Ghost crabs are generally active near their burrows and the shoreline (Wolcott 1978; personal observations). It is unlikely that they are frequently more than 30 sec from the refuge of water or burrow. With a need for bursting, rather than sustained, high levels of activity, there is probably selective pressure for the reduction of the lactate effect. This is in contrast to the situation of the migratory blue crab which has a strong lactate effect (Johnson et al. 1984). The blue crab can sustain high locomotory activity for long periods of time in waters which may be low in oxygen (Booth et al. 1982; Mangum and Towle 1977). Thus, the hemocyanin of the ghost crab has a number of features that appear to be related to its semi-terrestrial habit. Whether these features are common to other essentially terrestrial crabs remains to be seen.

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