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Oligomer Composition and Oxygen Binding of the Hemocyanin of the Blue Crab *Callinectes sapidus*

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Abstract. In the blue crab, the ratio of hexamers to dodecamers of the O_2 carrier hemocyanin varies in natural populations. Isolated dodecamers have a lower O_2 affinity and greater cooperativity than isolated hexamers. The difference in O_2 binding can also be resolved in native mixtures that differ in polymer composition. A high content of dodecamers in native mixtures is, in fact, correlated with the presence of an invariant polypeptide chain that is believed to link two hexamers to form dodecamers. On the other hand, the content of a variable chain that has been postulated to play a role in hexamer pairing is correlated with a *low* content of dodecamers. The variable, but not the invariant, monomers can be present in levels so low that they must not be represented in all dodecamers in the blood.

Introduction

The hemocyanin (Hc) of the blue crab *Callinectes sapidus* Rathbun, like that of most crustaceans, exists in the blood as a mixture of hexamers (1×6) and dodecamers (2×6). The two oligomers are stable aggregates, not members of a chemical equilibrium. Oligomerization is important in optimizing the physical properties of the blood (Snyder and Mangum, 1982; Mangum, 1986) but its respiratory significance, if any, is not known. While both Brouwer *et al.* (1982) and Johnson *et al.* (1984) mentioned that hexamers and dodecamers do not differ in O_2 binding, neither reported the data. Brouwer (pers. comm.) has kindly communicated his opinion that the finding was not definitive because the two oligomers were poorly separated in their experiments by atmospheric pressure permeation gel chromatography.

In *C. sapidus*, the Hc oligomers are built of five to six different polypeptide chains, four of which (in our terminology, Nos. 1, 3, 5, and 6) exhibit considerable quantitative variability in natural populations. In the laboratory, changes in the concentrations of two to three of the four (Nos. 3, 5, and 6) can be induced by prolonged hypoxia or a change in acclimation salinity. Accompanying the alterations in monomeric subunit composition are highly adaptive changes in intrinsic O_2 affinity, which can be specifically attributed to the levels of chains 3 and 6 (Mason *et al.*, 1983; Mangum and Rainer, 1988; Rainer, 1989; deFur *et al.*, 1990).

The nature of the hexamers that do not form dodecamers, but remain as such in the blood, is unclear. Hamlin and Fish (1977) described them as comprising "as much as" 20% of the total material in their purified Hc preparations. Brouwer *et al.* (1982) and Johnson *et al.* (1984) mentioned similar figures. Herskovits *et al.* (1981), however, specified a range of 30–50% and raised the possibility of a seasonal change. Recently, we have found that the ratio does in fact vary in nature (Greaves *et al.*, 1991).

On the basis of a combination of electrophoretic and immunological properties, Markl (1986, for review) has classified the monomeric chains of the arthropod Hcs into four categories that differ in their interspecific variability and their putative role in the assembly of the native polymers. The chain (No. 4) that is believed to serve as the dodecamer-former in *C. sapidus* (Stöcker *et al.*, 1988) is one of the two essentially invariant ones in our ongoing sample, which presently totals about 1000 individuals (Rainer, 1989; Mangum, 1990). Densitometric scans shown by Johnson *et al.* (1984), however, indicate that

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the concentration of chain 4 was at least as high in a hexameric fraction as in a dodecameric fraction. Moreover, their hexameric fraction lacked all but perhaps trace quantities of chains 5 and 6, two of the most variable ones in nature. They suggested that these chains, presumably instead of chain 4, play a role in dodecamer formation.

The way in which the variable chains influence O_2 binding is presently unclear. Those that do not play an important role in hexamer pairing (*e.g.*, No. 3) may have simple and direct effects on O_2 affinity. Chains that do participate in hexamer pairing, however, could exert their influence indirectly if O_2 binding of the two oligomers differs.

We attempted to circumvent the problem of poor separation at atmospheric pressure by developing a protocol for HPLC, which proved to achieve clean separation (Greaves *et al.*, 1992). In the present investigation we used the procedure 1) to examine the respiratory properties of the two fractions and 2) to correlate the ratio of $2 \times 6{:}1 \times 6$ aggregates in native mixtures with their subunit composition and O_2 binding. We also tried, without success, to compare the respiratory properties of native hexamers with those of hexamers prepared as dissociation products of dodecamers.

Materials and Methods

Large adult males (≥80 g wet wt) were obtained from either the seaside (Eastern Shore of Virginia at Wachapreague) or estuarine (York R.) populations studied earlier (Mason *et al.*, 1983; Mangum and Rainer, 1988; Rainer, 1989). Blood samples were taken from the infrabranchial sinuses and declotted as previously described. In the present investigation the samples were also filtered at 0.22 μm for injection into the HPLC system.

HPLC

HPLC was carried out on a Perkin Elmer Series 4 system equipped with one or two (connected in series) Ultrahydrogel 1000 size exclusion columns (7.8 × 300 mm; Waters Associates). The mobile phase was a saline containing 300 mmol l^{-1} NaCl, 25 mmol l^{-1} $MgCl_2$, and 10 mmol l^{-1} $CaCl_2$; this saline was also filtered at 0.22 μm immediately prior to use. The Perkin Elmer Model LC-95 variable wavelength UV absorbance detector was set at 337 and 280 nm. Further details of the protocol were described by Greaves *et al.* (1992); an example of the separation is shown as Figure 1.

O_2 binding

These measurements were performed on the fractions separated by HPLC, after reconcentration by membrane centrifugation (Centricon 30), or on unfractionated aliquots of the same samples analyzed by HPLC, following dialysis against 0.05 mol l^{-1} Tris maleate buffered saline (specified above). Because reconcentration is a lengthy process, aliquots of the isolated oligomers were examined as soon as the Hc levels reached an absorbance at 337 nm of 0.25–0.58 (2–3 h centrifugation), using a tonometric procedure suitable for dilute samples. Precision mixed, humidified gases were passed across rapidly shaken samples at constant temperature and atmospheric pressure, and the changes in absorbance at 337 nm determined (procedure described in detail by Mason *et al.*, 1983) with a Mervyn Roy 501 spectrophotometer. Rather than dissociate the remainder of the available material for electrophoresis, we chose to use the cell respiration procedure (Mangum and Lykkeboe, 1979) to document more extensively the preliminary findings of the tonometry by performing repeated O_2 binding measurements on the two intact oligomers. The procedure required further reconcentration for a total of 7–8 h. Native mixtures of the two oligomers, aliquots of which were analysed by electrophoresis, were also examined using this procedure. These samples were dialyzed overnight against buffered saline.

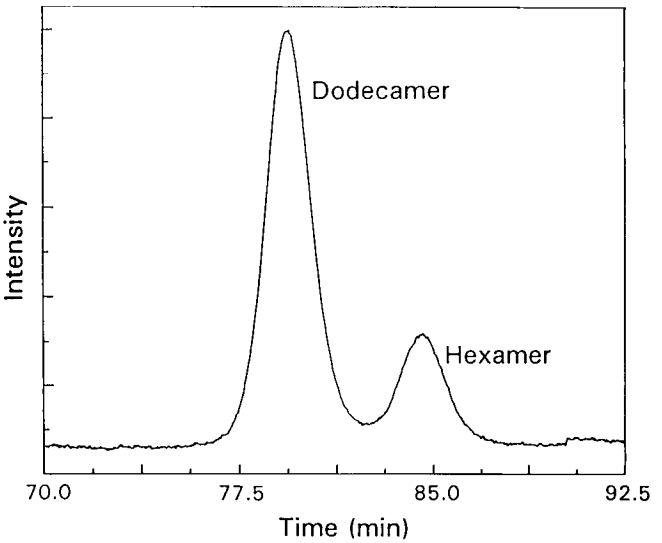

Figure 1. An example of the separation of oligomers obtained by HPLC. Absorbance was monitored at 337 nm.

Electrophoresis

Samples were dialyzed overnight against 50 mmol l^{-1} Tris HCl (pH 8.9) containing 10 mmol l^{-1} EDTA to dissociate the native oligomers into their monomeric subunits. Alkaline dissociation electrophoresis was carried out, as described by Mangum and Rainer (1988). The gels (12%) were scanned with a Shimadzu densitometer.

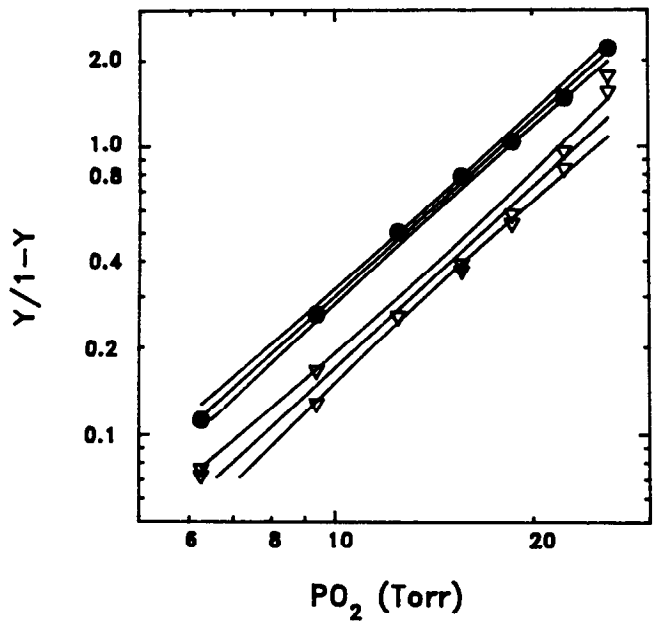

Figure 2. Hill plots of O_2 binding properties of isolated hexamers and dodecamers. 25°C, 0.05 mol l^{-1} Tris buffered saline containing 10 mmol l^{-1} $CaCl_2$, 25 mmol l^{-1} $MgCl_2$, and 300 mmol l^{-1} NaCl. Tonometric data for one aliquot of hexamers (circles, pH 7.49) and two of dodecamers (triangles, pH 7.52). Lines are regression lines and their 95% confidence intervals.

Results

HPLC

We did not observe the material larger than the about 900 kD dodecamers that had been reported by both Hamlin and Fish (1977) and Herskovits *et al.* (1981). Because this material (which has a sedimentation coefficient of 33S) should have a molecular weight in excess of the separation range of our gel, it should have appeared as a shoulder on the peak of the dodecameric fraction. Moreover, as pointed out earlier (Greaves *et al.*, 1992), the ratios of the 280:337 nm areas, obtained for the two oligomer fractions and for the samples from different individuals, were statistically indistinguishable, despite the variation in contents of the two oligomers described below. The optical evidence, then, indicates that no material other than Hc was present and that the condition of the active site of the two oligomers does not differ.

O_2 binding

The results obtained in three tonometric determinations suggest that isolated dodecamers have a significantly lower O_2 affinity than isolated hexamers (Fig. 2), despite a slightly higher experimental pH. The 95% confidence intervals do not overlap even though the fit of regression lines to the data for dodecamers is poor because, like many native mixtures of crustacean Hcs, they show an increase in cooperativity at higher oxygenation states. Because of the poor fit, cooperativity (n_{50}) was estimated in Figure 3 from regression analysis of only the points (n = 6) in Figure 2 that exceed 30% HcO_2; the available data in this region suggest that dodecamers have a significantly ($P < .015$) higher cooperativity than hexamers. The cell respiration procedure, with which a larger number of determinations was made (Fig. 3), yielded very similar results: the hexameric fraction has a significantly higher O_2 affinity ($P = .0325$ according to Student's t test) and lower cooperativity ($P = .0495$). Neither mean value for O_2 affinity of the isolated oligomers differs significantly from the intermediate value for the native, unfractionated mixture ($P = .25$ for the dodecamers and .12 for the hexamers) although, following the dilution, fractionation and reconcentration, cooperativity had clearly fallen in both cases ($P < .0005$). The value of n_{50} for the native mixture, which had not been diluted, fractionated, and reconcentrated, is typical of this species under the experimental conditions

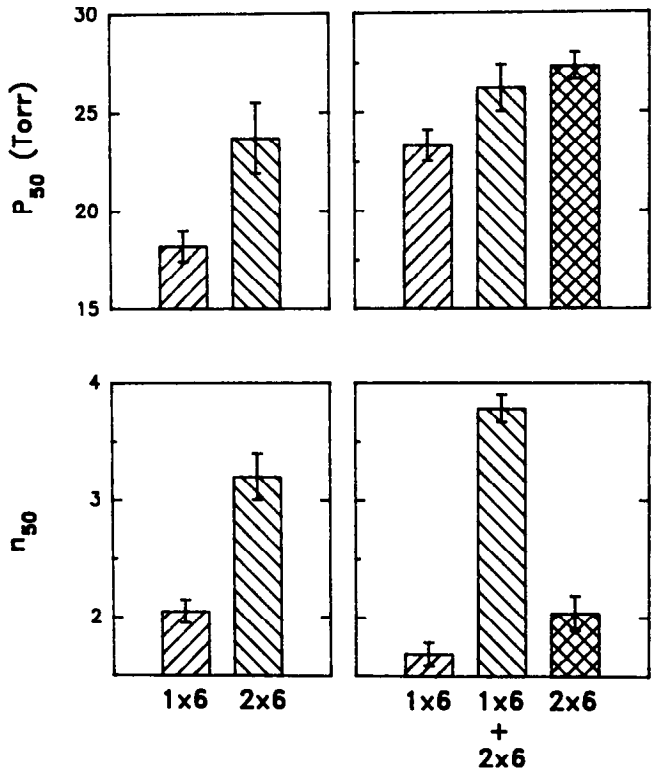

Figure 3. O_2 binding of isolated hexamers and dodecamers. 25°C. Panels at left show tonometric data (pH 7.49–7.52) from Figure 1, with error as 95% confidence intervals. Panels at right show cell respiration data for 6 replicate measurements of isolated hexamers (pH 7.40), dodecamers (pH 7.40), and the dialyzed but unfractionated (77% dodecamers) sample (pH 7.38) from which the two fractions were prepared. Buffered saline as in Figure 1. Mean values ± S.E.

employed here (*e.g.*, Mason *et al.*, 1983; Mangum and Rainer, 1988).

The O_2 binding properties of two undiluted and unfractionated samples with a high content of dodecamers (Fig. 4) are indistinguishable from one another (according to Student's *t* test, $P = .2$ for both P_{50} and n_{50}; $n = 6$); similarly, two with a low content of dodecamers had indistinguishable O_2 binding properties ($P > .4$; $n = 6$). The O_2 affinity of each pair differs significantly from that of the alternative pair ($P = .004$); specifically, a high content of dodecamers is accompanied by a lower affinity. The cooperativity values, which are well within the range usually observed in this species, are also significantly greater in the sample with the higher dodecamer content ($P = .005$).

We attempted to create artificial hexamers by freezing and thawing (11× over a two-day period) unreconcentrated dodecamers. Following membrane centrifugation, all O_2 binding activity had disappeared in this fraction, as well as that containing native hexamers. We then examined the polymer composition and O_2 binding of the remainder of the unfractionated and never diluted sample from which the two fractions had been prepared, which had also been frozen and thawed 11 times. Prior to freezing, 77% of this sample consisted of dodecamers, and it had the O_2 binding properties shown in Figure 2. Following freezing and thawing, 76% consisted of dodecamers, and it exhibited an indistinguishable O_2 affinity and cooperativity ($P = .63$).

Subunit composition

The monomer and oligomer compositions of samples from 13 individuals collected in the summer from the seaside population are shown in Table I. After first transforming the percentages to a normal distribution (arcsin), we examined the relationship between the quantities of each chain and the content of dodecamers by linear regression analysis. There is no indication of a correlation between the variation in levels of chains 1, 2, 3, or 6 and the content of dodecamers ($r = -.103$ to $.421$; $P > .10$). However, dodecamer content is highly and directly correlated with relative content of chain 4 ($r = .601$; $P = .02$) (Fig. 5). Both of the two invariant chains (2 and 4), unlike variable ones, are present in sufficient quantities to be represented in each hexamer of a 2 × 6 pair. The correlation with dodecamer content is not improved, however, by summing the relative contents of the chains 2 and 4 ($r = .341$; $P > .10$). Dodecamer content is highly but inversely correlated with the levels of chain 5 ($r = -.653$; $P = .01$).

The numerical relationship between the % composition values for the two parameters is also of interest. Any chain present in less than the figure [(its % composition)/(% dodecamers) = $^1/_{12}$th or] 8.3% must not be represented in all dodecamers. All four variable (but neither of the invariant) chains can fall into this category, some trivially and probably not outside of the error of densitometry, but some by a large margin (Table I).

Table I

Subunit and oligomer composition of hemocyanins from 13 individuals of Callinectes sapidus

	% Total peak area*						
No.	1	2	3	4	5	6	% 2 × 6-mers
1	8.4	27.2	*5.4*	20.0	26.2	12.7	65.5
2	23.4	28.2	7.0	24.8	9.3	7.0	70.6
3	10.8	22.2	9.8	25.4	14.9	16.9	74.4
4	9.7	29.0	6.8	32.7	12.2	9.6	78.3
5	17.3	19.6	19.5	23.4	*5.3*	14.9	79.6
6	12.5	31.8	11.0	23.9	8.3	12.3	81.1
7	9.3	24.7	15.2	26.8	12.0	11.9	82.5
8	*5.8*	20.0	10.8	34.6	12.1	16.7	84.4
9	9.2	32.3	7.1	34.1	*4.0*	13.3	84.4
10	23.0	23.8	10.0	28.7	9.0	*5.8*	84.9
11	17.4	21.0	9.7	29.4	10.2	12.3	85.1
12	*5.1*	29.8	9.1	31.1	10.4	14.3	86.8
13	*5.4*	24.5	13.5	25.5	*6.1*	15.2	88.0

* Figures less than $^1/_{12}$th of the dodecameric fraction shown in italics.

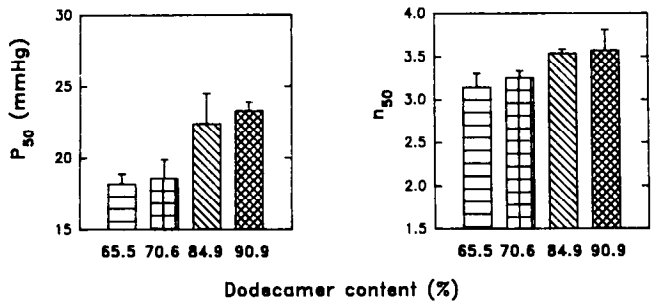

Figure 4. O_2 binding properties (cell respiration procedure) of native Hcs with different contents of dodecamers. 25°C, pH 7.50. Buffered saline as in Figure 1. Mean ± S.E. (n = 6).

Discussion

Morris (1988) systematically investigated the effects of freezing on crustacean Hcs. Repeated bouts of freezing and thawing resulted in progressive dissociation of dodecamers to hexamers, although to a different degree in the four species examined, and an attendant decrease in cooperativity. As noted by Morris (1988), much of our own experience concurs with his: thawed crustacean Hcs often show impaired cooperativity (see also Reese, 1989). Because freezing (at least using high concentrations and freezing for only a brief period) had no dis-

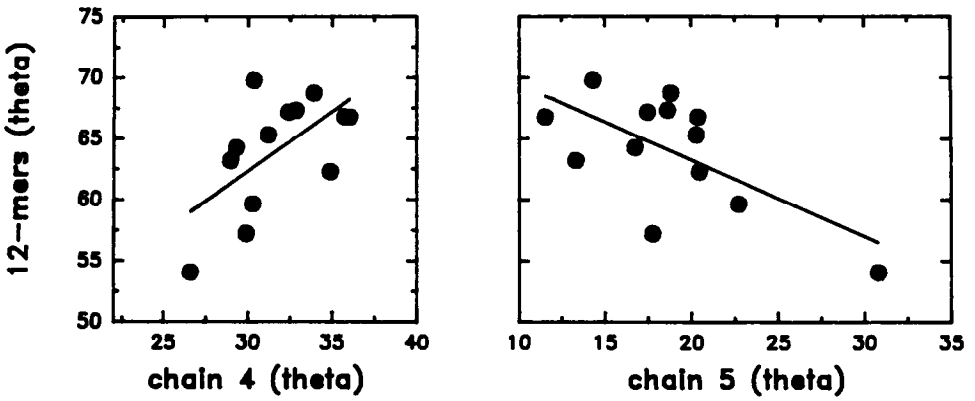

Figure 5. The relationship between the fractional composition of dodecamers and chains 4 and 5 of *Callinecles sapidus* Hc, expressed as arcsin transformed percentages. Lines are fitted regression lines.

cernible effect on *C. sapidus* Hc, we can only conclude that it is more stable than those examined by Morris (1988).

The present data provide further information on the natural variability of the two native oligomers in this species. They provide no evidence that chain 6 plays a role in the pairing of hexamers. However, our data strongly support the inference of Stöcker *et al.* (1988) that high levels of chain 4 promote dodecamer formation. Moreover, they do not support the conclusion reached by Johnson *et al.* (1984) that chain 5 promotes dodecamer formation.

We should note that, in the present sample, chain 1 appears to be more variable than 3 or 6 and almost as variable as 5. Although we have noticed the variability of this chain in much larger samples (Rainer, 1989), we have not previously found nearly as much variability, and we suggest that the present sample is misleading because of its small size. In addition, we have no evidence that chain 1 responds to an environmental stimulus or that it influences respiratory properties. The high and constant levels of chain 2 are intriguing because this monomer is not known to play a role in oligomerization. According to Markl's classification, it is an interspecifically conservative type of chain, but chain 3 (Stocker *et al.*, 1988), which is highly variable intraspecifically, is also classified as conservative interspecifically.

Our findings demonstrate different O_2 binding properties in the two native oligomers: dodecamers, the predominant aggregate in the blood, have a lower O_2 affinity and greater cooperativity than hexamers. The difference is detectable in both isolated fractions and native mixtures of different proportions of the two. The variable chain No. 5, which is not known to influence O_2 affinity directly, could have an indirect influence via inhibition of hexamer pairing. On the other hand, the variable chains 3 and 6, which influence O_2 affinity, are not correlated with dodecamer content. The most likely inference at present is that their respiratory effect is simple and direct.

Finally, Johnson *et al.* (1984) concluded that the levels of chains 5 and 6 indicate that they comprise $1/6$th of the dodecamers. The present data indicate that they (as well as chain 1) may occur in either considerably higher or lower levels, which suggests heterogeneity of the dodecamers in the blood. In fact, some heterogeneity would be expected if the changes in subunit composition that are responsible for the observed adaptability of respiratory properties of the Hc are physiologically labile.

Acknowledgments

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