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Structure, Function, and Assembly in the Hemocyanin System of the Scorpion *Androctonus australis*†

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ABSTRACT: The interactions between structurally and functionally distinct subunit types in the assembly and function of heteropolymers can be subjected to detailed investigations using the high molecular weight copper proteins known as hemocyanins. Hemocyanin of the scorpion *Androctonus australis* is a 34S heteropolymer which contains 24 subunits. Eight distinct types of subunits can be isolated when the oligomer is dissociated. Reassembly can be accomplished by two-step dialysis of an equimolar mixture of the isolated subunits or a similar dialysis of the unfractionated subunit mixture. In both cases the reassembled aggregate is similar to the native 24-mer, as evidenced by electron microscopy, but differs from the native molecule in subunit composition. The functional properties of reassembled forms which lack subunit 3A suggest that this subunit plays an important role in stabilizing a conformation of low oxygen affinity. At pH 7.5 the native molecule binds oxygen with a $P_{1/2}$ of 27 Torr and a high degree of cooperativity. The Hill coefficient is pH sensitive and reaches a maximum value of 9.25 at pH 7.8. Strong pH-dependent homotropic interactions are also evident in the time course of oxygen dissociation. At pH 8.2 the apparent first-order rate constant has an initial value of 2.6 s^{-1} and increases to 20 s^{-1} as the reaction proceeds. Interactions between subunits markedly decrease at low pH where a low affinity form is stabilized. Thus, at pH 6.5 the Hill coefficient is 3.0 and the $P_{1/2}$ is 60.25 Torr. Sodium chloride acts as an allosteric effector that also brings about a stabilization of the low affinity conformation. When the native molecule is dissociated, the unfractionated mixture of subunits shows no cooperativity and $P_{1/2}$ values range from 4.29 to 4.17 Torr in the pH range from 7.5 to 8.9. The kinetics and equilibria of oxygen binding by isolated subunits were studied. Functional diversity at the subunit level is indicated by differences in oxygen affinities, oxygen dissociation rate constants, and sodium chloride sensitivities.

Hemocyanins are high molecular mass copper proteins which reversibly bind oxygen. These proteins are found freely dissolved in the hemolymph of arthropods and mollusks. The hemocyanins of these two phyla are easily distinguished on the basis of their structure. Mollusk hemocyanins are very high molecular mass aggregates having sedimentation coefficients ranging from 60 to 130 S. They can be dissociated into subunits which are uncommonly large polypeptide chains. These polypeptides have multiple oxygen binding domains (Gielens et al., 1977). On the other hand, arthropod hemocyanins are assembled from 70 000 to 95 000 M_r[1] subunits possessing single oxygen binding sites. The native molecules encountered in different arthropod species can be considered to be built by successive dimerizations of building blocks containing six subunits, often designated as hexamers (Schutter et al., 1977; Bonaventura et al., 1977; Bijlholt et al., 1979). Thus, for instance, hexamers are observed in the hemolymph of the shrimp *Penaeus setiferus* (Brouwer et al., 1978a), dodecamers in the freshwater crayfish *Cherax destructor* (Jeffrey et al., 1978), tetracosamers (24 subunits) in the scorpion *Buthus occitanus* (Wibo, 1966), and a 48-mer in the horseshoe crab *Limulus polyphemus* (Van Holde & van Bruggen, 1971). The reversible dissociation of hemocyanin oligomers into halves and subfragments has long held the interest of researchers,

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who regarded these proteins as useful tools for analyzing association–dissociation phenomena. The observation of different subunit types in arthropod hemocyanins (Sullivan et al., 1974; Lamy et al., 1977a; Markl et al., 1979; van Eerd & Folkerts, 1980) and, more recently, in mollusk hemocyanins (Brouwer et al., 1978b) has caused these proteins to now be regarded as more complex systems than originally thought. It is now known that different subunit types in some arthropod and mollusk hemocyanins play special roles in the assembly process (Schutter et al., 1977; Bijlholt et al., 1979; Brouwer et al., 1978b). The present study was undertaken to further our understanding of the role of the diverse subunit types in the assembly and function of arthropod hemocyanin oligomers.

The hemocyanin system analyzed in this report is that of the scorpion *Androctonus australis*. The present studies parallel those previously carried out with the hemocyanin system of *Limulus polyphemus* (Sullivan et al., 1974). Preliminary results indicated that the functional properties of *Androctonus* hemocyanin differed significantly from *Limulus* hemocyanin. The scorpion hemocyanin is much more cooperative in oxygen binding and has a pH sensitivity that is opposite that found in hemocyanin of the horseshoe crab. We sought an explanation of these differences by analysis of the scorpion hemocyanin subunits.

Previous studies with *Androctonus* hemocyanin revealed that the native (34 S) form can be dissociated into six electrophoretically resolvable fractions by extensive dialysis against a pH 7.5 buffer containing 1 M urea. Crossed immunoelectrophoresis revealed eight subunit types within these six fractions, and procedures for isolating the eight subunit types have been described (Lamy et al., 1979). The results presented here concern the functional properties of native *Androctonus* hemocyanin and its subunits. In addition, reconstituted oligomers were subjected to structural and functional analysis, and we present a comparison of their properties with those of native *Androctonus* oligomers.

Material and Methods

The hemocyanin present in the hemolymph of the Tunisian scorpion, *Androctonus australis garzonii* (Goyffon & Lamy, 1973), was purified by repeated gel filtration on Bio-Gel A-5m in 50 mM Tris-HCl and 10 mM $CaCl_2$, pH 7.5. It is designated as "whole hemocyanin". Subunits of eight distinct types were isolated as described elsewhere (Lamy et al., 1979). In reassociation experiments, subunits 3C and 5B were used in the form of the heterodimer (fraction 1). Fraction 1 and the monomeric subunits 2, 3A, 3B, 4, 5A, and 6 are all antigenically pure when tested by crossed immunoelectrophoresis against a rabbit antiserum specific for the unfractionated mixture of dissociation products. The mixture of dissociation products is referred to as "whole stripped hemocyanin".

Oxygen equilibria were performed at 20 °C using a tonometric method (Riggs et al., 1956). Spectral changes were measured using a Cary 14 spectrophotometer. Values for percent saturation with oxygen were determined at 340 nm. The rate of dissociation of oxygen from oxyhemocyanin was measured by rapid mixing of air-equilibrated protein solutions with buffers containing dithionite in a Gibson-Durrum stopped-flow apparatus. Data collection and analysis were accomplished via an analog to digital converter (DASAR, American Instrument Co.) coupled to a PDP 11/34 computer (Digital Equipment Corp.). All experiments of oxygen binding or oxygen dissociation were carried out in 50 mM Tris or Bistris buffer made 0.1 M in ionic strength by NaCl. Tris was used at and above pH 8 and Bistris was used below pH 8. In all experiments with whole hemocyanin, 10 mM $CaCl_2$ was present to prevent dissociation of the molecule into subunits.

Sedimentation velocity experiments were carried out at 20 °C using a Beckman Spinco Model E analytical ultracentrifuge with mechanical speed control and schlieren optics.

The procedure followed in reassembly experiments was a modified version of the two-step method of reassembly by calcium described by Schutter et al. (1977). An equimolar mixture of isolated monomeric (2, 3A, 3B, 4, 5A, and 6) and dimeric (fraction 1) subunits was concentrated by vacuum dialysis to a total protein concentration of approximately 20 mg/mL. The concentrated mixture was first dialyzed against a 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM EDTA for 48 h (first step), and then against the same buffer without EDTA and with 5 mM $CaCl_2$ and 10 mM $MgCl_2$ (second step). The preparations were then submitted to preparative thin-layer gel filtration using an apparatus from Pharmacia Fine Chemicals. About 5 mg was deposited in a horizontal line. The migration was in the second step buffer in Sephadex G-200 superfine at room temperature, at an angle of 15°. After separation, two narrow replicas (1 cm in width) were made on both sides of the gels, and the spots were detected with coomassie blue R, scraped from the gel, and eluted by the second step buffer. The eluate from each spot was concentrated by vacuum dialysis and analyzed for function (oxygen binding and rate of oxygen dissociation) and for subunit composition. The subunit analysis was carried out by the technique of crossed immunoelectrophoresis as described by Weeke (1973) after redissociation of the molecule in a 50 mM Tris-HCl buffer containing 1 M urea and 10 mM EDTA. The stability of the hemocyanin aggregates used in the functional studies was confirmed by analytical thin-layer gel chromatography.

Electron microscopy of the native and reconstructed hemocyanin aggregates was carried out by a previously described method (Lamy et al., 1977b).

Results

Functional Properties of Androctonus Hemocyanin. Oxygen equilibrium studies were done as a function of pH with whole (34 S) *Androctonus* hemocyanin. The hemocyanin is cooperative and exhibits a pronounced positive Bohr effect, as shown in Figure 1. At pH 6.5, the material is half saturated at an oxygen pressure of 60.25 Torr, and Hill plots give slopes of about 3.0. As the pH is increased, there is an increase in oxygen affinity, accompanied by a sharp increase in the degree of cooperativity. Figure 2 shows Hill plots of oxygen binding at pH 7.5 and 8.5 that illustrate the high degree of cooperativity that characterizes the binding of oxygen to this hemocyanin. Between pH 7.5 and 8.5, the slopes of Hill plots of oxygen binding are significantly greater than 6, and a maximum of 9.25 is observed at pH 7.8. This high degree of cooperativity implies that the number of interacting subunits must be more than 6.

Ultracentrifuge experiments performed at pH 7.0 and 8.5, in the presence of 10 mM calcium, showed a homogeneously sedimenting band with a sedimentation coefficient of 34 S for oxyhemocyanin of *Androctonus*. At pH 8.5 the same sedimentation behavior was observed for deoxyhemocyanin. The high cooperativity in oxygen binding is thus not attributable to oxygen-linked changes in the state of aggregation.

The cooperative nature of oxygen binding by native scorpion hemocyanin is reflected in strongly autocatalytic time courses of oxygen dissociation. The oxygen dissociation process was monitored by following the absorption changes which accompany rapid mixing of oxygenated hemocyanin with a buffer containing sodium dithionite. The apparent first-order rate

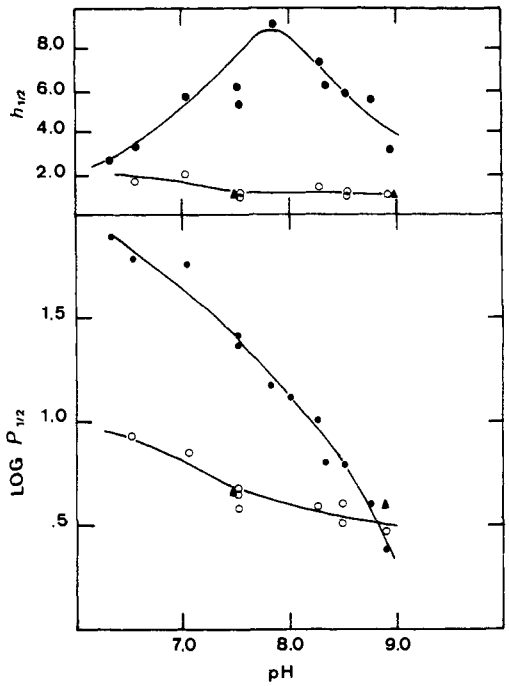

FIGURE 1: pH dependence of Hill coefficient and oxygen pressure at half-saturation for whole and whole stripped hemocyanin: (closed circles) whole hemocyanin; (open circles) whole stripped hemocyanin.; (closed triangles) predicted values of whole stripped hemocyanin affinity calculated from $h_{1/2}$ and $P_{1/2}$ of isolated subunits and from the weight average subunit composition of whole hemocyanin.

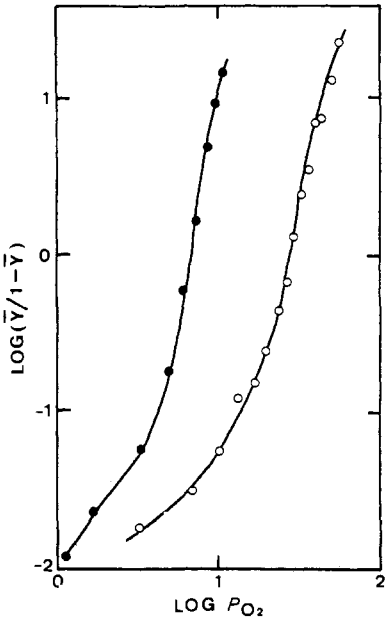

FIGURE 2: Hill plots of oxygen binding by whole hemocyanin at pH 7.5 (open circles) and pH 8.5 (closed circles).

constant for the initial phase of oxygen dissociation was about 2.5 s^{-1} in the pH range from 8.25 to 9.0. Below pH 8.25, there is an appreciable increase in the initial rate of the oxygen dissociation process. The final rate of the oxygen dissociation process is generally much faster than the initial rate, although, as illustrated in Figure 3, the magnitude of the difference is strongly pH dependent. Even at pH 7, where the degree of cooperativity is substantially reduced, the presence of cooperative interactions exerts an influence on the oxygen dissociation process that is manifest in a strongly autocatalytic time course of oxygen dissociation. The data obtained are summarized in Table I.

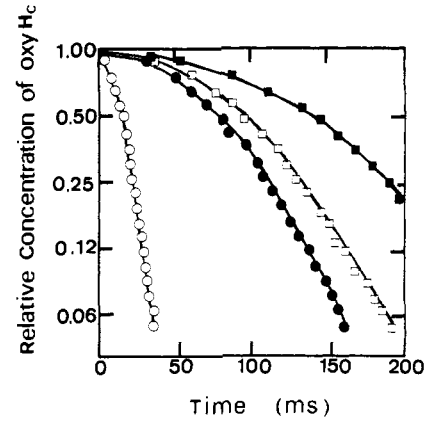

FIGURE 3: Time course of deoxygenation reaction of whole *Androctonus australis* oxyhemocyanin at different pH values in the presence of dithionite. Results are expressed as relative concentration of the oxy form: (open circles) pH 7.5; (closed circles) pH 8.25; (open squares) pH 8.5; (closed squares) pH 8.75.

Table I: Effect of pH on Oxygen Binding by Isolated Components of *Androctonus* Hemocyanin[a]

components	$P_{1/2}$ (Torr) pH 7.5	pH 8.9
fraction 1	7.3	6.3
subunit 2	2.3	2.8
subunit 3A	5.6	5.4
subunit 3B	5.9	6.6
subunit 4	6.2	4.7
subunit 5A	3.3	3.4
subunit 6	3.9	3.6
whole stripped	4.8	4.2

[a] Values of $P_{1/2}$ of fraction 1, subunits 2, 3A, 3B, 4, 5A, and 6, and whole stripped hemocyanin are presented. The proteins were in 50 mM Bistris or Tris buffers (I = 0.1 M) at 20 °C.

The concentration of NaCl present with the protein also influences the oxygen dissociation kinetics: at pH 8.25, with 0.1 M NaCl, the oxygen dissociation process has an initial rate of 2.6 s^{-1} and accelerates to 20 s^{-1}. Increasing the NaCl concentration to 3.54 M is accompanied by an increase in both the initial and final rates of oxygen dissociation, to 5.5 and 34 s^{-1}, respectively. The last line of Table II refers to a sample containing 3.54 M NaCl, which was then diluted by addition of salt-free buffer. The time course observed when this sample was mixed with a buffer of equal ionic strength that contained dithionite showed that the effects induced by NaCl are reversible. The addition of NaCl is analogous to a decrease in pH in that both treatments appear to decrease the oxygen affinity of the whole molecule.

Functional Properties of Isolated Subunits and of the Unfractionated Mixture of Subunits. Table I presents a summary of oxygen binding data for *Androctonus* hemocyanin, subunits 2, 3A, 3B, 4, 5A, and 6, and for the dimeric component, fraction 1. There are significant differences in the intrinsic oxygen affinities of these components. Inspection of Table II shows that the isolated components show very little pH dependence. Fraction 1, although dimeric, does not bind oxygen cooperatively.

Table III summarizes the kinetics of oxygen dissociation from the isolated components of *Androctonus* hemocyanin. As with the equilibria, there are significant differences in the rates of oxygen dissociation of isolated subunits. The time courses of oxygen dissociation are, in most cases, fairly homogeneous. Fraction 1 is exceptional in this regard. Figure 4 shows the time courses of oxygen dissociation from fraction 1, subunit 2, and subunit 3B. The heterogeneous time course

Table II: Apparent First-Order Rate Constants for Oxygen Dissociation from Whole *Androctonus* Hemocyanin as a Function of pH and NaCl[a]

pH	[NaCl] (M)	$k_{initial}$ (s^{-1})	k_{final} (s^{-1})
7.0	0.1	20	70
7.6	0.1	7.7	24.5
8.25	0.1	2.6	20
8.50	0.1	2.5	16.7
8.75	0.1	1.8	12
9.00	0.1	2.2	15
8.25	1.55	5.7	34
8.25	2.66	4.0	33
8.25	3.54	5.5	34
8.25	0.88[b]	4.5	22.5

[a] Rapid mixing experiments were performed with the protein in 50 mM Bistris or Tris and 10 mM $CaCl_2$ (I = 0.1 M) at 20 °C, with a measuring wavelength of 390 nm. [b] Previously 3.54 M.

Table III: Effect of NaCl on the Apparent First-Order Rate Constants for Oxygen Dissociation from Isolated *Androctonus* Hemocyanin Components (I = 0.5 M)[a]

component	k (s^{-1}) with 0.1 M NaCl	k (s^{-1}) with 3.1 M NaCl
fraction 1	22.0	21.0
subunit 2	5.5	22.0
subunit 3A	43.0	42.0
subunit 3B	31.0	58.0
subunit 4	22.0	46.0
subunit 5A	16.0	8.5
subunit 6	8.5	12.0

[a] Fraction 1 and subunits 2, 3A, 3B, 5A, and 6 were in Bistris, pH 7.5 (I = 0.1). Experiments were performed at 20 °C with an observation wavelength of 390 nm.

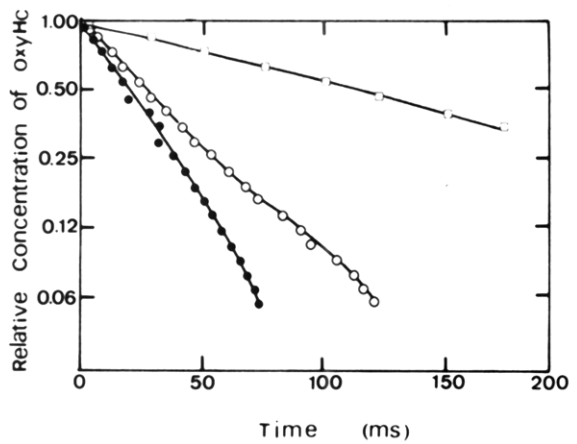

FIGURE 4: Time course of deoxygenation reaction of isolated subunits 2 and 3B and fraction 1 at pH 7.5: (closed circles) subunit 3B; (open circles) fraction 1; (squares) subunit 2.

observed for fraction 1 may be inferred to be due to the fact that it is a heterodimer. The effect of NaCl on the kinetics of oxygen dissociation from the *Androctonus* hemocyanin components was studied. The results are summarized in Table III. It is apparent that NaCl addition increases the rate of oxygen dissociation from subunits 2, 3B, 4, and 6. The rates of oxygen dissociation from these components are 1.5 to 4 times higher when in 3.1 M NaCl. This increase in rate of oxygen dissociation is consistent with decreased oxygen affinity. In contrast, 3.1 M NaCl shows the rate of oxygen dissociation from subunit 5A, and has little effect on fraction 1 and subunit 3A.

Oxygen binding by the unfractionated mixture of components of *Androctonus* hemocyanin was studied as a function of pH. As shown in Figure 1, the whole stripped hemocyanin

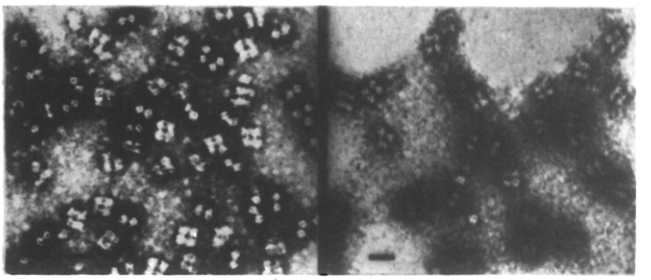

FIGURE 5: Electron microscopic pattern of native and reconstituted hemocyanin: (left) native hemocyanin; (right) reconstituted hemocyanin (excluded spot).

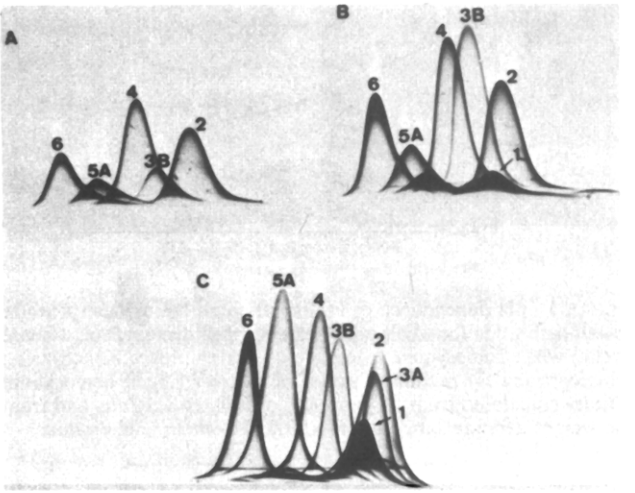

FIGURE 6: Subunit heterogeneity of native hemocyanin and of products obtained by reassembly of an equimolar mixture of isolated subunits: (A) hexameric spot; (B) excluded spot; (C) native hemocyanin. Prior to crossed immunoelectrophoresis, the oligomers were dissociated by an overnight dialysis against 50 mM Tris-HCl buffer, pH 7.5, and 10 mM EDTA. The numbers refer to isolated subunits. The identification of each subunit in the mixture was carried out by a special cross-line immunoelectrophoresis with the isolated subunit in the line.

has a high oxygen affinity with respect to the whole molecule and a greatly reduced Bohr effect. The cooperativity evident at low pH may be due to partial reassociation. Though a similar phenomenon had been reported in some arthropod species, there is presently no direct proof that the mild cooperativity observed at low pH was caused by partial reassociation. Subunits 3A and 2 have been shown to dimerize when the pH was lowered from 8.9 to 7.0, but no parallel increase in cooperativity was observed. At pH 7.5 and 8.5, the mixture exhibits Hill coefficients and half-saturation oxygen pressures as expected from the relative proportions and measured oxygen affinities of noninteracting components.

The kinetics of oxygen dissociation from the whole stripped hemocyanin reflects the functional heterogeneity of the subunits. The apparent first-order rate constant decreases as the reaction proceeds, from 18 to 8 s^{-1} at pH 7.5 and from 17 to 8 s^{-1} at pH 8.5. The insensitivity to pH is consistent with the slight pH sensitivity observed in oxygen equilibria (Figure 1).

Preparation and Analysis of a Reassembled Tetracosamer. The native *Androctonus* hemocyanin molecule contains 24 subunits. A two-step dialysis procedure was followed to promote reassembly of an equimolar mixture of isolated subunits or of the unfractionated subunit mixture. As described under Material and Methods, it is possible to reassemble and isolate *Androctonus* 24-mers by a combination of complementary experimental procedures. Figure 5 presents electron micrographs of native 24-mers and reconstituted

Table IV: Sedimentation Velocity Analysis of the Excluded Spot on Sephadex G-200 SF of the Aggregation Products Resulting from the Reassembly of an Equimolar Mixture of Subunits

component	sedimentation coeff (S)	proportion of each component (%)
1	4.8	2.8
2	14.7	2.2
3	29.9	0.8
4	34.3	77.8
5	46.8	16.3

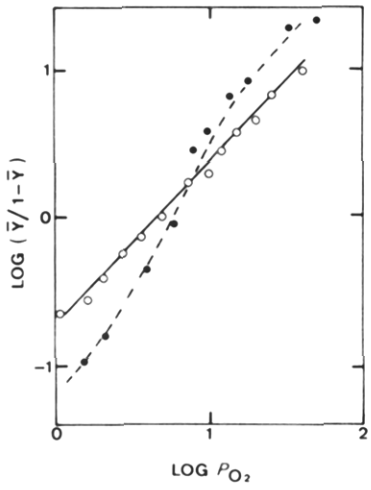

FIGURE 7: Hill plot of oxygen binding at pH 7.5 by hemocyanin reconstructed from isolated subunits (closed circles) and by whole stripped hemocyanin (open circles).

"24-mers" of *Androctonus* hemocyanin. The apparent identity in morphology is not paralleled by identity in subunit composition.

Figure 6 shows crossed immunoelectrophoreses of the subunit mixture resulting from the dissociation of the native 24-mers (C) and the "24-mers" (B) prepared by fractionation of a reassembly mixture by thin-layer gel filtration on Sephadex G-200 SF. The aggregates greater than hexamers are excluded from the gel. The identification of the excluded aggregates as "24-mers" is based on the results of electron microscopy, as shown in Figure 5, and of sedimentation velocity analysis. As shown in Table IV, the excluded spot contained five components, the most abundant of them being by far the 34S "24-mers" (77%). The complete absence of dodecamer from the excluded spot is also a convincing argument that the reassembly is not a simple random aggregation of subunits. The crossed immunoelectrophoresis results shown in Figure 6 (A–C) indicate that subunit 3A is not present in reconstituted "24-mers" and that the concentration of 5A is reduced.

The oxygen binding properties of the reconstituted "24-mers" were studied for comparison with native *Androctonus* hemocyanin molecules. A Hill plot of oxygen binding at pH 7.5 is shown in Figure 7. Half-saturation occurs at an oxygen pressure of 6 Torr, and there is a significant degree of cooperativity as evidenced by a Hill coefficient of 2. Comparison of this behavior with that of oxygen binding by the unfractionated mixture of subunits, also shown in Figure 1, reveals some interesting features. The primary difference between the reconstituted "24-mer" and the mixture of free components lies in the ability of "24-mer" to assume a low affinity conformation. As oxygen is bound, the "24-mer" makes a transition to a high affinity conformation that is of higher affinity than shown by the mixture of free components. An appropriate model for such an oxygen-linked conformational transition is that of Monod, Wyman, & Changeux (1965).

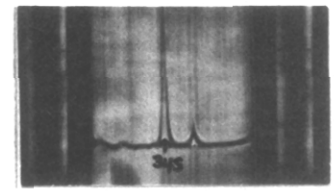

FIGURE 8: Schlieren pattern from ultracentrifuge run of reassembled *Androctonus* hemocyanin.

Comparison of the behavior of the "24-mers" reassembled from the isolated components with the native 24-mer can be qualitatively described within the framework of this model. For this comparison we must draw attention to the curve at pH 7.5 shown in Figure 2 for the native 24-mer. It has a much lower affinity in the initial stages of oxygen binding than does the reconstituted form, implying that its T-state affinity is much lower. Its transition to a high-affinity state is much sharper and its R state, although undefined by the curves of Figure 2, is evidently of higher affinity than that of the reconstituted "24-mers" or of the free hemocyanin components. We will return to the significance of this observation.

The cooperative interactions exhibited by the reconstituted "24-mers" in oxygen equilibria are also evident in the oxygen dissociation kinetics. As in the native 24-mer, the time course of dissociation is autocatalytic. At pH 7.5, the apparent first-order rate constant has an initial value of 5 s^{-1} and increases to 13 s^{-1}. The initial and final rates are, like the R and T states of the reconstituted "24-mers", intermediate between the initial and final rate observed for the native 24-mer.

Discussion

Arthropod hemocyanins exhibit diverse oxygen affinities and varying responses to protons and other allosteric effectors. The distinct functional properties are thought to be adaptive, providing for efficient oxygen uptake and delivery in widely varying environmental conditions and in organisms with differing metabolic demands. The oxygen binding behavior of *Androctonus* hemocyanin is very similar to that reported for another arachnid species, the tarantula *Eurypelma californicum* (Linzen et al., 1977). Both *Androctonus* and *Eurypelma* hemocyanins exhibit positive Bohr effects and remarkably high degrees of cooperativity. Hill's coefficients as high as 6 to 9 are found, and in both proteins the degree of cooperativity is dependent upon pH. The morphological structures of these arachnid hemocyanins involve hexameric building blocks, so that hexamers, dodecamers, and 24-mers are apparent in electron micrographs. With Hill's coefficients of 6 and higher, it is evident that the allosteric unit cannot be the hexamer, but may well be the dodecamer. The dodecamers contain linking components that appear as dimers when the oligomers are dissociated. In both the *Androctonus* and *Eurypelma* hemocyanin systems, the dimeric components are essential for assembly of aggregates larger than hexamers (Lamy et al., 1977a; E. F. J. van Bruggen et al., unpublished experiments). In *Androctonus* hemocyanin, the dimeric component fraction 1 serves as a core to link hexamers (Lamy et al., 1980a,b). This study has shown that fraction 1 of *Androctonus*, although dimeric, is noncooperative in its isolated state. Previous studies indicated that fraction 1 is a heterodimer, made up of two immunologically distinct types of subunits (Lamy et al., 1979). Its kinetic behavior reflects this heterogeneity and does not suggest cooperative interactions between sites.

The arachnids are considered to be closely related to the

horseshoe crabs *Limulus polyphemus* and *Tachypleus tridentatus* and are in the same taxonomic group, the Chelicerata. The morphological characteristics of the horseshoe crab hemocyanins are very similar to those of *Androctonus* and *Eurypelma*, except that the hexameric building blocks in these organisms aggregate to give 8 × 6 ensembles, containing 48 subunits. Variation in functional properties of morphologically similar hemocyanins is illustrated by the fact that in both *Limulus* and *Tachypleus* hemocyanins the Bohr effect is negative, and the Hill coefficient, although pH sensitive, is typically less than 4 (Sullivan et al., 1974; Brouwer et al., 1977; J. Bonaventura, unpublished data). The presence of structurally and functionally diverse subunits in these hemocyanins may provide an explanation for the variation in function observed for the high molecular mass aggregates. It has been established for *Limulus* (Sullivan et al., 1974; Bonaventura et al., 1974), *Tachypleus* (J. Bonaventura, unpublished observations), and *Androctonus* (this report) that the subunits that make up the oligomers differ in their intrinsic oxygen affinities and in their sensitivity to allosteric modulation by sodium chloride. In *Limulus* it is clearly demonstrated that chloride ion is responsible for this sodium chloride effect (Sullivan et al., 1974). In *Androctonus*, however, it has not been definitively established whether chloride or sodium ion is the allosteric modulator of oxygen affinity. Nevertheless, no contrary evidence has presently been accumulated that *Limulus* and *Androctonus* hemocyanins behave differently with respect to the modulation by sodium chloride. The appearance of the reverse Bohr effect in *Limulus* hemocyanin may well be due to the presence of components II and III in the oligomer. These two components of the *Limulus* system are the only ones whose affinities are lowered by addition of chloride. In high NaCl, both components show strong negative Bohr effects. Similarly, *Androctonus* subunits 2, 3B, 4, and 6 show oxygen dissociation kinetics in NaCl, consistent with decreased oxygen affinity. Whole *Androctonus* hemocyanin shows a similar NaCl effect, although some of its subunits (3A and fraction 1) show no NaCl sensitivity, and subunit 6 shows an alteration in oxygen dissociation behavior that suggests increased oxygen affinity in high NaCl. The modulation by sodium chloride seen in *Androctonus* and *Limulus* hemocyanin is not present in all arthropod hemocyanins. In the shrimp *Penaeus*, the hemocyanin shows increased oxygen affinity when NaCl is added (Brouwer et al., 1978a).

The rates of oxygen dissociation from whole *Androctonus* hemocyanin are not only NaCl dependent but also strongly autocatalytic, as shown in Figure 3. Thus, the change in quaternary state from R to T is mirrored by a sharply increasing rate of oxygen dissociation. The oxygen dissociation rate was also found to reflect changes in quaternary state in studies of *Limulus* hemocyanin (Brouwer et al., 1977). The isolated subunits of *Androctonus* hemocyanin have varying oxygen affinities whose oxygen dissociation kinetics vary accordingly. Thus, subunit 2 has the highest oxygen affinity and the lowest rate of oxygen dissociation. The fact that the oxygen dissociation process appears as the dominant kinetic determinant of oxygen affinity in *Androctonus* and other hemocyanins may be relevent to the mechanism of oxygen binding at the bis(copper) site.

The foregoing experiments suggest that the functional diversity of the hemocyanin subunits can be expressed by the function of the oligomers that they form. The mechanics of information exchange within allosteric units may be better understood when the specific locations of the various subunit types are clarified. As recently reported (Lamy et al., 1980b), *Androctonus* hemocyanin contains two classes of subunits. Some are "internal", while others are "external". This conclusion was reached on the basis of a comparison of the immunological precipitations of the free subunits with the precipitations of those subunits within the native 24-mer. Subunits 3A and 3B and fraction 1 are internal in the sense that they are not easily accessible to antibodies in the native oligomer, leading to a weak precipitation. In contrast, subunits 2, 4, 5A, and 6 are external in the sense that the bulk of their antigenic sites (determinants) react readily with antibodies as well in the native 24-mer as in the free state. The fact that subunit 3A is internal in the native 24-mer and absent in the reassembled "24-mers" suggests that its position may be important in the maintenance of in vivo functional properties. Another correlation that can be drawn at this point is that the isolated *Androctonus* subunits fall into two affinity classes: subunits 3A, 3B, 4, and fraction 1 have low oxygen affinities and high rates of oxygen dissociation, while subunits 2, 5A, and 6 have substantially higher oxygen affinities and low rates of oxygen dissociation. With the notable exception of subunit 4, the two affinity classes mirror the differentiation of "internal" and "external" classes in the oligomer.

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Charge-Site Communication in Proteins: Electrostatic Heme Linkage of Azide Binding by Sperm Whale Myoglobin†

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ABSTRACT: The binding of azide ion to sperm whale ferrimyoglobin has been measured at 25 °C over the pH range 4.0–6.0 at ionic strengths 0.001, 0.002, 0.005, and 0.010 M. The pH dependence of the binding constant, k_L, was analyzed in terms of the modified discrete charge electrostatic theory in three ways. First, an "independent-site" model was constructed in which the charged sites that interact with the charged heme iron, and which lose that interaction on neutralization of the iron charge in the azide complex, are considered not to interact with each other. This unrealistic analysis of the heme linkage fails to conform to the experimental results. The subsequent analyses employed the full electrostatic treatment in which each site is taken to interact with each other as well as with the charge borne by the iron. Second, the increase, $\Delta\bar{\nu}$, in binding of hydrogen ions accompanying the azide binding was estimated from the experimental pH dependence of log k_L and compared with that computed in terms of the theoretical predictions of the changes in pK values accompanying binding. The agreement was satisfactory under all conditions studied, with divergence of about 25% at most. Third, the observed pH dependence of log k_L for each ionic strength was described within 0.10 unit in terms of the summed changes in electrostatic free energy for each charge site accompanying the azide binding as sensed at the iron binding site. The contributions attributed to the individual charged groups were most important where their static solvent accessibilities are low. In these terms, the effective electrostatic domain for interactions with the heme site corresponds to nearly the whole of the myoglobin molecule.

The Tanford–Kirkwood discrete-charge electrostatic theory (Tanford & Kirkwood, 1957), as modified by Shire et al. (1974a) allows calculation of the sites. interaction between charged sites on globular proteins as a function of pH and ionic strength. It has been possible to predict the titration curves of other globular proteins, including myoglobin from a number of mammalian species (Botelho et al., 1978), horse cytochrome *c* and human hemoglobin α chain (Matthew et al., 1978), human deoxy- and oxyhemoglobin A (Matthew et al., 1979a,b), and ribonuclease (J. B. Matthew, F. M. Richards, and F. R. N. Gurd, unpublished experiments). Work has also gone into testing the prediction of the hydrogen ion equilibria at individual sites on these molecules (Shire et al., 1974b, 1975; Botelho et al., 1978; Matthew et al., 1979a,b). Extending the application of the theory for sperm whale myoglobin has included prediction of the acid denaturation properties (Friend & Gurd, 1979a), calculation of the stabilizing and destabilizing interactions between specific groups and charge-site clusters as a function of pH and ionic strength (Friend & Gurd, 1979b), and prediction of hydrogen ion dependent conformational changes sensed in the region of the A helix (Friend et al., 1980).

This paper treats the equilibrium binding of azide ion by myoglobin as a sensitive probe of electrostatic interactions between charged sites. The binding at the heme has been selected so as to allow measurements at the functionally active site and because the low static accessibility of the heme pocket (Lee & Richards, 1971) increases the sensitivity to charge-site interactions (Shire et al., 1974a; Friend & Gurd, 1979b). Additionally, ligand binding to the heme is a long recognized property of heme proteins (Theorell & Ehrenberg, 1951) about which there is an abundance of theoretical models (George & Hanania, 1955; Wyman, 1964).

Azide has been used as the ligand in these studies for the following reasons. (1) It has a relatively high association constant, $\sim$100 mM^{-1}, for binding to myoglobin. (2) It forms a low-spin coordination complex with the iron atom, and the resultant cancellation of charge is easily treated by the electrostatic theory. (3) The X-ray crystallographic difference map for azide myoglobin indicates no major structural rearrangement upon azide binding (Stryer et al., 1964; Takano, 1977). (4) ^{1}H NMR (Botelho, 1975) and ^{13}C NMR

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