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Structure and Function of the Carboxyl-Terminal Oxygen-Binding Domain from the Subunit of *Octopus dofleini* Hemocyanin[†]

Karen I. Miller,[*,‡] K. E. van Holde,[‡] Arazdordi Toumadje,[‡] W. C. Johnson, Jr.,[‡] and Josette Lamy[§]

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, and Laboratoire de Biochimie, Faculté de Pharmacie, Université Francois Rabelais, 37032 Tours Cedex, France

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ABSTRACT: The C-terminal domain, Od-1, of the 7-domain subunit of *Octopus dofleini* hemocyanin has been prepared by partial trypsinolysis followed by ion-exchange chromatography. It binds oxygen reversibly and is homogeneous in molecular weight. Its physical properties have been compared with those of the subunit. The domain molecular weight is found by sedimentation equilibrium to be 4.7×10^4, in excellent agreement with the result recently obtained in our laboratory from cDNA sequencing of this domain [Lang, W. H. (1988) *Biochemistry* (preceding paper in this issue)]. It has a sedimentation coefficient of 3.8 S. Both the molecular weight and sedimentation coefficient are consistent with the domain constituting approximately one-seventh of the M_r 3.5×10^5 subunit. Its amino acid composition and carbohydrate content differ significantly from that of the whole subunit, confirming the heterogeneity in domains previously established on an immunological basis. Circular dichroism predicts similar secondary structure for the domain and subunit. The domain does not self-associate in the presence of Mg^{2+} but does bind to the whole molecule in a ratio of approximately 1 domain/subunit. The oxygen affinity of this domain is quite low. It shows intrinsic magnesium and Bohr effects similar to those of the whole molecule but of greatly reduced magnitude.

Hemocyanins are high molecular weight copper proteins responsible for oxygen transport in arthropods and molluscs. They are composed, in the case of arthropods, of $\sim$70 000-dalton subunits each binding one oxygen, arranged into hexamers and then into higher order structures with molecular weights as high as 3 million. Although early studies on the properties of the subunits were complicated by problems of subunit heterogeneity, it has been possible to obtain fairly detailed information on structure and function of the hemocyanins of several arthropods. Details of structure now known include amino acid sequences for several subunits as well as precise subunit localization within the whole molecules of *Limulus* and *Androctonus* (Lamy et al., 1981, 1985, 1987) and *Eurypelma* (Markl et al., 1981; Linzen et al., 1985) hemocyanins and 3.4-Å resolution X-ray crystallography on the subunit of *Panulirus* hemocyanin (Gaykema et al., 1984). Detailed functional studies have been performed on *Eurypelma* hemocyanin as well (Savel et al., 1986; Markl et al., 1986).

Molluscan hemocyanins, on the other hand, are composed of much larger subunits, each containing seven or eight oxygen-binding domains of about 50 000 daltons. Here, too, subunit heterogeneity has been a problem, especially with gastropod hemocyanins. *Helix pomatia* hemocyanin, for example, contains two types of subunits, termed α and β, which assemble in stoichiometrically complex ways to form whole molecules (Gielens et al., 1973; Brouwer et al., 1977; Verschueren et al., 1981). Each subunit contains eight immunologically distinct domains, of which one, domain d from the βc subunit, has now been purified and used to determine the amino acid sequence (Drexel et al., 1987). This domain was not obtained intact, however, but in the form of two disulfide-bridged fragments. So although the sequence has been determined, it has not been possible to obtain much purified domain in a functionally intact condition. Thus, the many studies that might relate structure and function of this domain to the parent molecule have not been possible.

The properties of cephalopod hemocyanins have made them better subjects for such investigations. *Octopus dofleini* hemocyanin has significant advantages as a model molluscan hemocyanin. The parent molecule is a 51S decamer composed of *identical* 11S subunits, each containing seven immunologically distinct oxygen-binding domains (Lamy et al., 1987). The dissociation and reassociation of the molecule is fully reversible upon removal and readdition of divalent cations (Miller & van Holde, 1982). The order of the domains is now almost completely known (Lamy et al., 1987). In the accompanying paper (Lang, 1988), cloning of the cDNA corresponding to the *O. dofleini* hemocyanin subunit is described, and the sequence of the C-terminal domain, Od-1 (Lamy et al., 1987), is presented.

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[‡] Oregon State University.

[§] Université Francois Rabelais.

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Lamy et al. (1987) have shown that Od-1 can be obtained by limited tryptic cleavage of the subunit. For the study described in this paper a sufficient quantity of this domain has been purified to permit detailed structural and functional analysis.

There have been two previous studies on proteolytic cleavage products of *Octopus* hemocyanin. Bonaventura et al. (1977) studied oxygen binding of partially purified chromatographic fractions of subtilisin digests of *O. dofleini* hemocyanin. Ricchelli et al. (1986) examined a single domain produced by more extensive tryptic digestion of *Octopus vulgaris* hemocyanin. In neither case is the identity of the domain(s) in question, the purity, or the location within the parent molecule known.

MATERIALS AND METHODS

Purification of Domain Od-1. Dissociated *O. dofleini* hemocyanin was submitted to trypsinolysis (Sigma Type XI, DPCC treated, code T-1005) overnight at 37 °C in a 0.05 M ammonium carbonate buffer, pH 8.0, with an enzyme to substrate ratio of 1/500.

The Od-1 domain was purified by ion-exchange chromatography on Mono Q HR 10/10 in a 0.05 M Tris[1] buffer, pH 8.9, with 0.01 M EDTA. Elution was carried out in a 0–0.5 M NaCl gradient. This procedure produced a roughly homogeneous fraction containing $\simeq 5\%$ impurities. Impurities were removed by immunoaffinity according to a previously reported method (Lamy et al., 1979a,b).

Amino Acid and Carbohydrate Analyses. The amino acid composition of the whole subunit was determined by the method of Heinrikson and Meredith (1984). Reverse-phase high-performance liquid chromatography using precolumn derivatization with phenyl isothiocyanate was employed to generate phenylthiocarbamyl derivatives. The analyzer was an IBM HPLC with an IBM C18 column. Sample size was 0.01 nmol. Samples were prepared as described previously (Lamy et al., 1987).

Carbohydrate analysis was performed by Dr. J. P. Kamerling using techniques described previously (van Kuik et al., 1985).

Determination of Protein Concentrations. Domain Od-1 concentration was determined by the guanidine hydrochloride method of Mulvey et al. (1974) and Elwell et al. (1977). Lyophilized protein was dissolved in double-distilled water and dialyzed against 0.01 M sodium phosphate buffer, pH 7.7. The concentration of unfolded protein in 5.7 M guanidine hydrochloride (Schwarz/Mann) was computed from the absorption of Trp, Tyr, and Cys at 280 nm since the number of these residues and their extinction coefficient at 280 nm are known. From the absorption of the same native protein solution (without guanidine hydrochloride) at 190 nm, the extinction coefficient for Od-1 was determined to be 9.735×10^3 M^{-1} cm^{-1}.

The extinction coefficient at 280 nm of the 11S subunit was determined by drying to constant weight under vacuum at 98 ± 2 °C. From these results, an extinction coefficient at 190 nm was determined for use in CD studies.

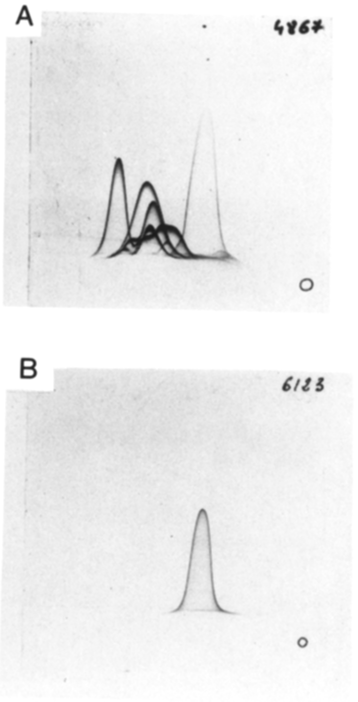

FIGURE 1: Demonstration of identity and purity of Od-1 preparation by crossed immunoelectrophoresis. (A) Deposit hole contains *Octopus* hemocyanin after digestion with trypsin. Antiserum is antidissociated *Octopus* hemocyanin. (B) Deposit hole contains pure Od-1 (previously identified by reaction with antibodies specific to each domain). Antiserum is antidissociated *Octopus* hemocyanin.

Circular Dichroism Measurements and Analysis. CD measurements were made over the 178–260-nm range by using a vacuum ultraviolet (VUV) CD spectrophotometer (Johnson, 1971) and protein solutions of 1 mg/mL in 0.01 M sodium phosphate, pH 7.7. Spectra, expressed as $\Delta\epsilon$, were digitized at 2-nm intervals, yielding vectors with 42 points. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $\Delta\epsilon(290.5) = 2.36$. To avoid absorption artifacts, the total optical density of the cell, solvent, and sample never exceeded 1.0 over the range of the CD spectrum. All spectra were measured at 20 °C with a 20-s time constant and a scan rate of 1 nm/min.

Buffer Solutions. Sedimentation and oxygen-binding experiments were carried out by using 0.1 M HEPES or 0.1 I Tris buffers with either 10 mM EDTA or varying amounts of $MgCl_2$.

Sedimentation Experiments. Sedimentation velocity and sedimentation equilibrium experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. Wavelengths used were either 345 or 280 nm, depending on the concentration of the solution. Most experiments were performed in the vicinity of 20 °C and corrected to standard conditions ($s_{20,w}$). Sedimentation equilibrium experiments were done at $\sim$5 °C in order to decrease the likelihood of proteolytic degradation during the course of the experiment.

Oxygen binding experiments were performed by using the tonometric method described previously (Miller & van Holde, 1974; Miller, 1985). Adequate binding curves could be obtained using as little as 1 mL of a solution of Od-1 containing slightly more than 1 mg/mL protein.

RESULTS AND DISCUSSION

Purification of Od-1 hemocyanin by partial tryptic digestion of $\sim$500-mg subunits followed by ion-exchange chromatography yielded 25 mg of protein that gave a single band of $\sim$50K daltons on SDS gels in the presence of β-mercaptoethanol (not shown). It was immunologically pure and identifiable as the C-terminal domain Od-1 by immunological methods. Figure 1A shows immunoelectrophoresis of whole tryptic digest against antibody to the whole subunit. Several

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

Table I: Comparison of Amino Acid Composition of *Octopus* Hemocyanin Subunit and C-Terminal Domain from That Subunit

	domain 1			
	no. of residues (from cDNA sequence)	mol % of total residues	subunit mol % (from amino acid analysis)	% difference, SU vs Od-1
Ala	27	6.77	7.4	+9.3
Cys[a]	8	2.01	(2.0)	
Asp	31	7.77 (+4.76)	10.6	−15.4[b]
Glu	19	4.76 (+3.01)	11.2	+44.1[b]
Phe	23	5.76	5.6	−2.8
Gly	25	6.27	6.6	+5.3
His	24	6.02	3.0	−50.2
Ile	21	5.26	4.8	+8.7
Lys	23	5.76	3.3	−42.7
Leu	33	8.27	9.5	+14.9
Met	6	1.50	2.5	+66.7
Asn	19	4.76		
Pro	17	4.26	6.1	+43.2
Gln	12	3.01		
Arg	13	3.26	3.8	+16.6
Ser	22	5.51	6.6	+19.8
Thr	33	8.27	5.8	−29.9
Val	19	4.76	5.6	+17.6
Trp[a]	7	1.75	(1.8)	
Tyr	17	4.26	3.7	−13.1
total	399	100.00	100.0	

[a] Both Cys and Trp were destroyed during hydrolysis. In order to calculate the fractions of other amino acids, these were assumed to be the same in the subunit as in Od-1. [b] For Od-1 the sum of fractions Asp + Asn and Glu + Gln was used to compare with the amino acid analysis of the subunit since there is conversion of Asn/Gln to the Asp/Glu form during hydrolysis.

peaks are present, corresponding to the seven domains. Figure 1B shows the purified Od-1 (previously identified by reaction with antibodies to the individual domains). There is no evidence of contaminating material from other domains. Its absorbance spectrum showed a peak at 280 nm and a second peak at 345 nm, typical of molluscan hemocyanins. Od-1 demonstrated reversible oxygen binding, implying normal functional properties. It had a fairly sharp, symmetrical sedimentation boundary which suggested that it was homogeneous in size (see below for confirmation of homogeneity). These results indicated that the protein was not significantly damaged structurally or functionally by the purification process and could safely be compared with the subunit from which it was prepared.

Amino Acid Composition and Carbohydrate Content. The amino acid analysis of the subunit shows significant differences in relative amounts of several amino acids when compared with the numbers of amino acids determined for Od-1 on the basis of the sequence of the cDNA (Lang, 1988). This comparison is summarized in Table I. Six amino acids are more than 25% different, and of these, two, His and Met, are more than 50% different. We know from previous studies (Lamy et al., 1987) that the seven domains are immunologically distinct. None of the domains cross reacts significantly with any of the other six, and an antiserum against Od-1 does not precipitate the various fragments that do not contain Od-1. Thus, significant differences in amino acid sequence between domains are expected. Indeed, we have observed some differences in fragmentary sequences from several domains (Lamy et al., 1987). However, this much difference in amino acid *composition* between Od-1 and the subunit may be of special significance. Domain Od-1, because of its position at the C-terminal end of the subunit, must play a different role in the assembly of the whole molecule than domains that could fit only into the wall of the cylindrical 51S structure and hence might be expected to differ in both sequence and composition.

Table II: Carbohydrate Content of *Octopus* Hemocyanin Subunit and Its C-Terminal Domain

carbohydrate	subunit (nmol of sugar/mg of protein)	Od-1 (nmol of sugar/mg of protein)
fucose	2.1	27.6
mannose	92.4	77.5
galactose		+
N-acetylglucosamine	27.1	47.0
N-acetylgalactosamine		29.7
% (w/w)	2.3	3.6

The carbohydrate content of the subunit and Od-1 is presented in Table II. The overall carbohydrate content of the subunit closely resembles that reported for *O. vulgaris* hemocyanin by Albergoni et al. (1972). However, comparison of the carbohydrates of Od-1 with those in the whole subunit reveals distinct differences. There is substantially more fucose and *N*-acetylglucosamine in Od-1. It is puzzling that *N*-acetylgalactosamine can be found *only* in Od-1. Even if it were present only in this domain, it should still be detected (at one-seventh this level) in the whole subunit. The discrepancy cannot be accounted for by microheterogeneity in the hemocyanin, for both the whole subunit and the Od-1 sample submitted for analysis came from the same blood sample taken from one *Octopus*. The overall carbohydrate constant of *O. dofleini* hemocyanin is found to be 2.3%, whereas Od-1 has a content of 3.6%. This means that the average percent carbohydrate in the remaining domains must be approximately 2.1%. This does not, of course, imply a uniform carbohydrate content in each of these remaining domains. We have, for example, observed a quite extensive carbohydrate content in Od-1, the N-terminal domain, which complicated early N-terminal amino acid sequencing efforts (Lamy et al., 1987). Differing carbohydrate content as well as sequence differences could contribute significantly to the immunological differences seen between the seven domains.

Molecular Weight and Physical Characterization of Domain Od-1 and the Whole Subunit. Knowing both numbers of each amino acid [from Lang (1988)] and the weight percent carbohydrate, it is possible to calculate the exact molecular weight of Od-1. The protein weight is 45 316; with 3.6% carbohydrate the resultant total weight is thus 46 947.

We have also carried out sedimentation equilibrium studies of purified Od-1, with two purposes in mind. First, we wished to ascertain that this proteolytic fragment represented the whole of domain Od-1. Second, we wished to know whether or not it existed as a monomer in solution. Sedimentation equilibrium experiments gave linear ln c vs r^2 plots (data not shown). Thus, the preparation appeared to be homogeneous. Calculation of the molecular weight of the domain from sedimentation equilibrium requires a value for the partial specific volume, $\bar{v}$. Since we know the exact amino acid composition, as well as the carbohydrate content, it should be possible to make an accurate estimate of $\bar{v}$. The calculation of partial specific volume from amino acid composition has been discussed by Zamyatnin (1972), who gives a list of "preferred" values for the amino acid residue calculation. However, comparison of tables in the Zamyatnin paper shows that these values tend to give systematically low values for $\bar{v}$ as compared with experimental results, whereas the values used by McMeekin and Marshall (1952) give more evenly distributed positive and negative deviations from experimental values. Therefore, we have chosen to use the latter. To correct for the carbohydrate present, we have assumed it to have the

Table III: Physical Parameters of *O. dofleini* Hemocyanin

	51S	11S	Od-1
mol wt	3.5×10^6	3.5×10^5	4.695×10^4
partial specific volume (cm^3/g)	0.730	0.730	0.726
sedimentation coefficient ($s^0_{20,w}$) (svedberg)	51	11.1	3.8
f/f_0	1.60	1.58	1.25
ϵ_{280} (mg^{-1} cm^2)	1.394[a]	1.356[b]	
ϵ_{190} (M^{-1} cm^{-1}		10695[c]	9735[b]

[a] Corrected for increase in OD_{280} due to scattering by whole molecule. [b] Measured directly. [c] Derived from ϵ_{280}.

partial specific volume of sucrose (0.618 cm^3/g) (Gosting & Morris, 1949). On this basis, we calculate $\bar{v} = 0.726$ for subunit Od-1. This yields, from the sedimentation equilibrium experiments, M_r 47 300. This close agreement with the sequence value is surely fortuitous, considering the possible errors in both $\bar{v}$ and the molecular weight determination, but the result clearly shows that Od-1 exists in dilute buffer solution as a monomeric protein, without discernible tendency to associate, and that it represents the entire domain as defined by the sequencing studies.

The availability of amino acid composition and carbohydrate content for the whole subunit allows us to make an estimate of $\bar{v}$ for this molecule. We calculate $\bar{v} = 0.730$ cm^3/g. This value is somewhat lower than that used in our earlier calculations of *O. dofleini* hemocyanin molecular weights (0.738 cm^3/g). In the absence of other information, we had relied on a very old experimental value for *O. vulgaris* hemocyanin (0.740; Svedberg & Eriksson, 1932) and an (erroneous) calculation from the amino acid composition of this protein. Making the correction for the new $\bar{v}$ value, we find 349 000 for molecular weight of the subunit and 3 480 000 for the whole protein from the data of Miller and van Holde (1982).

The ratio of subunit weight to the weight of Od-1 is 7.4. A value of exactly 7 is not necessarily to be expected, since the domains are clearly not identical, but the result indicates that Od-1 is comparable in size to the average domain.

The sedimentation coefficient of Od-1, extrapolated to zero concentration, is $s^0_{20,w} = 3.8S$ (Table III). Knowing both s and molecular weight allows us to calculate the frictional coefficient f/f_0 for the various aggregation states. For Od-1 we find $f/f_0 = 1.25$, a value typical of globular proteins. If we assume a hydration of 0.5 cm^3 of H_2O/cm^3 of protein (typical of globular proteins), the particle volume increases by a factor of 1.5 and $(f/f_0)_{hyd} = (1.5)^{1/3} = 1.15$. Then $(f/f_0)_{shape} = 1.25/1.15 = 1.09$. This indicates that the domain is very close to spherical ($f/f_0 = 1$) and is consistent with electron micrographs of isolated domains (Lamy et al., 1987).

Electron microscopic pictures of the subunit (Lamy et al., 1987) suggest a loose string of spherical domains. For a rigid structure composed of n spherical subunits, the ratio of the sedimentation coefficient of the assembly (S_n) to that of a subunit (S_1) is given by (van Holde, 1975)

$$\frac{S_n}{S_1} = 1 + \frac{R}{n}\sum_i\sum_j\frac{1}{R_{ij}} \tag{1}$$

where R_{ij} is the distance between centers of subunits i and j, each of (hydrated) radius R. If the beads are assumed to be in contact, the second term in eq 1 becomes independent of actual bead dimensions, since each R_{ij} can be expressed as some geometrical factor times R. Using $S_1 = 3.8S$, we have calculated the expected sedimentation coefficients for various rigid arrays, as shown in Figure 2. Clearly, neither an extended nor a compact structure accounts for the observed S, but a loosely jointed form, like that observed in the electron microscope, should. Unfortunately, there exists no simple method for prediction of the sedimentation coefficient of such structures. However, it seems reasonable that a flexible molecule would have an average sedimentation coefficient between the values of 9.8 and 12.0 and thus close to the observed value of 11.1. Our conclusion is that the subunit in solution must look rather like the particles observed in the electron microscope. Consequently, some ordering of the subunit structure must be necessary during association to form the whole 51S molecule.

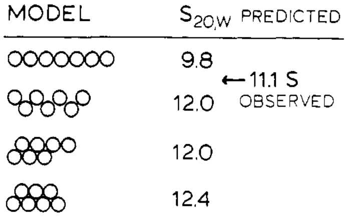

FIGURE 2: Predicted sedimentation coefficients for several possible arrangements of the 7-domain structure of the subunit of *Octopus* hemocyanin.

A summary of the physical parameters determined for the whole protein, the subunit, and the domain is given in Table III.

Secondary Structure of Domain Od-1 and the Subunit. The CD data were analyzed for secondary structure by using the method of Hennessey and Johnson (1981) as modified by the statistical technique called "variable selection" (Mosteller & Tukey, 1977). The Hennessey and Johnson method utilizes the CD spectra of proteins with known secondary structure in analyzing the CD of a protein with unknown structure. A CD spectrum will depend upon many factors: α-helix, parallel and antiparallel β-sheet, the various types of β-turn, aromatic side chains, prosthetic groups, etc. On the other hand, spectra measured from 260 to 178 nm have an information content corresponding to only five such factors. Variable selection reduces the information content necessary for a good analysis by removing reference proteins that have contributions to the CD spectra from sources not found in the protein to be analyzed. Since we do not know a priori which proteins should be removed from the basis set, calculations are performed for all subsets. The fractions of the secondary structures are not constrained to be positive, nor is their sum constrained to unity. The following criteria are used to select solutions from among the subsets: (1) The sum of fractions for predicted secondary structures (α-helix, parallel and antiparallel β-sheet, β-turn, and others) must be in the range 0.9–1.10. (2) No single fraction should have a value less than −0.05. (3) The root-mean-square residual should be less than 0.22 $\Delta\epsilon$ unit. This method has recently been evaluated by predicting the secondary structure of proteins with known secondary structure (Manavalan & Johnson, 1987).

The CD spectra of the hemocyanin subunit and Od-1 are shown in Figure 3. They are characterized by two negative minima at about 208 and 220 nm and one positive peak at 192 nm. The curve crosses the base line from negative to positive around 202 nm and again from positive to negative around 182 nm. The shapes of these CD spectra are characteristic of proteins with both α and β structure (Manavalan & Johnson, 1983). The CD of the subunit has a more intense band at 208 than at 220 nm, and this is one of the characteristics of $\alpha + \beta$ type proteins. The CD from Od-1 protein has negative bands of equivalent intensity, and the intensity of the entire CD spectrum is significantly reduced. Analysis of the CD for the subunit using the variable selection procedure

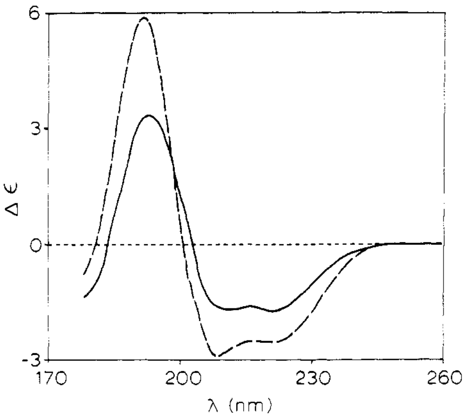

FIGURE 3: Circular dichroism of *Octopus* subunit (dashed line) and Od-1 (solid line) in 0.01 M sodium phosphate, pH 7.7.

Table IV: Percent Secondary Structure from CD Analysis of Hemocyanin Subunit and Domain 1

structure[a]	subunit[b]	domain 1[b]
H	0.23 ± 0.01	0.19 ± 0.01
A	0.21 ± 0.02	0.18 ± 0.02
P	0.09 ± 0.01	0.11 ± 0.01
T	0.14 ± 0.02	0.12 ± 0.01
O	0.26 ± 0.02	0.32 ± 0.01
total	0.93	0.92

[a] H, α-helix; A, antiparallel β-sheet; P, parallel β-sheet; T, β-turn; O, other structure. [b] ± standard deviation for combination meeting the specified criteria.

generated 120 combinations that satisfied the three criteria when 2 of the 22 reference proteins were removed. Similarly, analysis of Od-1 by this method generated 30 combinations that met the specified criteria when 6 proteins were removed. The CD predictions are an average of all combinations that met the criteria, and the results with their standard deviations are collected in Table IV.

The subunit and Od-1 are predicted to contain very similar secondary structures in spite of the fact that their CD spectra are quite different. β-sheet is predicted to be in the parallel form, which is normally found only in α/β proteins, as well as the antiparallel form. The secondary structure of Od-1 has also been predicted from its amino acid sequence (Lang, 1988) by using the method of Chou and Fasman (1978). This calculation predicted 27% α-helix, 26% β-sheet (antiparallel + parallel), and 22% β-turn and showed alternating α and β regions characteristic of the α/β type tertiary folding. In analogy with the subunit, *Eco*RI endonuclease (Manavalan et al., 1984) has a CD with a shape characteristic of α + β proteins but with an analysis showing some parallel β-sheet that is characteristic of α/β proteins. X-ray diffraction of *Eco*RI endonuclease–DNA complex (McClarin et al., 1986) shows an interesting structure that is a mixture of α + β and α/β folding. Thymidylate synthetase also shows a mixture of α + β and α/β folding (Hardy et al., 1987). It appears that the hemocyanin subunit and Od-1 belong to this subclass of tertiary structure that contains both α + β and α/β type folding.

Interaction of the Domain with Whole Hemocyanin. It has long been known that adding even small amounts of divalent cations promotes association of hemocyanin subunits to form whole molecules. Preliminary studies of this association reaction in *Octopus* hemocyanin suggest that magnesium binds to the subunit, triggering an "activating" process, or series of processes, perhaps involving conformational changes in the subunit. This is followed by a rate-limiting dimerization and fast polymerization to the decamer (van Holde & Miller, 1986). If this model is correct, it implies that the magnesium-mediated interactions are not between one subunit and its neighbors (which together form the wall of the hollow cylinder of the whole molecule) but rather between domains within each subunit. It was of interest, therefore, to learn whether magnesium ions could cause aggregation of Od-1. Both sedimentation velocity and sedimentation equilibrium in the presence of 50 mM Mg^{2+} give no evidence for self-association of Od-1 upon addition of magnesium (data not shown).

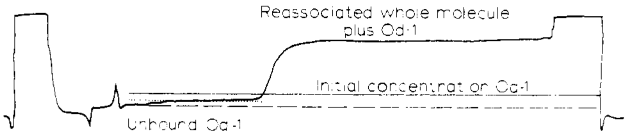

FIGURE 4: Scanner trace of a mixture of *Octopus* subunits and Od-1, allowed to reassociate in the presence of 50 mM Mg^{2+}, demonstrating that reassociation occurs but with some incorporation of Od-1. The solid horizontal line represents the initial concentration of Od-1, and the dotted line represents the observed concentration of free Od-1.

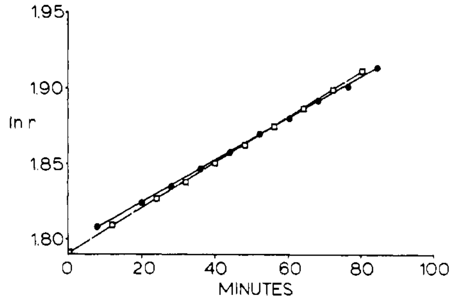

FIGURE 5: Determination of sedimentation coefficients for a mixture of subunits and Od-1 (●) and a control containing only subunits (□) allowed to reassociate in the presence of Mg^{2+}. With Od-1 $S = 44.2$, without Od-1 $S = 47.5$.

However, it remained possible that Od-1 might bind to *other* domains, either within the subunit or on adjacent subunits. In order to determine whether Od-1 bound to subunits and whether in doing so it prevented reassociation to whole molecules, we performed the following experiment. Od-1 was mixed together with subunits in sufficient concentration that there was an excess of Od-1, on a molar basis, compared to subunits, the actual ratio being 4 parts subunit protein (based on OD_{280}) to 1 part Od-1 protein. To this mixture was added 4 M $MgCl_2$ to make the final mixture 50 mM in magnesium. The buffer was 0.1 M HEPES, pH 7.45. The mixture was allowed to stand overnight to permit maximum reassociation. The scan of this mixture is presented in Figure 4. Two things are obvious at first glance: (1) The mixture *does* reassociate to form whole molecules, and (2) there is less free Od-1 than the mixture originally contained, implying that some is bound to the whole molecule. Figure 5 shows the determination of sedimentation coefficients for the mixture and a control reassociation mixture minus Od-1. For the native molecule at this concentration, the value is 47.5S; with the addition of Od-1 it *decreases* to 44.2S. The 7% decrease in sedimentation coefficient cannot be accounted for by an increase in solution viscosity caused by the addition of Od-1. At the Od-1 concentration used (0.5 mg/mL), the relative viscosity of the Od-1 solution is estimated to be about 1.002. The observed change in sedimentation coefficient is well outside experimental error; the samples were run together, which eliminates temperature or rotor speed errors, and the data are quite precise (Figure 5). This decrease in sedimentation coefficient with the addition of Od-1 must then indicate that Od-1 is bound in such a way that the structure of the whole molecule is expanded, leading

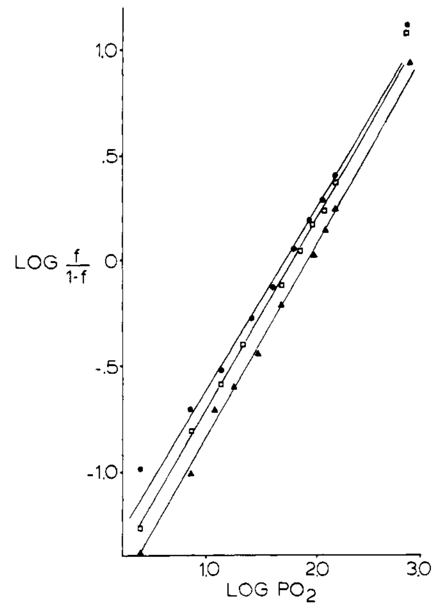

FIGURE 6: Effect of magnesium on oxygen binding by *Octopus* Od-1. Binding curves were obtained at 20 °C in 0.1 M HEPES, pH 7.65: (●) 100 mM $MgCl_2$; (□) 50 mM $MgCl_2$; (▲) 10 mM EDTA.

to a less compact (though heavier) protein, which sediments more slowly. On the basis of the known amounts of subunit and Od-1 and the plateau heights of the two boundaries (corrected for radial dilution), it is possible to estimate that roughly seven Od-1 are bound per whole molecule. This estimation is by its nature not highly accurate, but it suggests that approximately 1 domain is bound per subunit, which would correspond to 10 per whole molecule. Nothing is known at present about the nature of the interaction that permits this binding, except that it occurs in the presence of magnesium. It could be either specific binding to a location determined by the normal interactions of Od-1 or more nonspecific binding to a variety of sites on the whole molecule.

Oxygen-Binding Properties of the Domain. Od-1 has oxygen-binding properties that resemble those of whole hemocyanin, although it naturally lacks the cooperativity in binding. Figure 6 shows three binding curves demonstrating the effect of $MgCl_2$. Previous experiments with *Octopus* hemocyanin have not demonstrated a significant effect of chloride on oxygen affinity. Magnesium increases oxygen affinity of Od-1, although to a lesser extent than is seen in whole molecules. The Hill coefficient is 1, as expected, for all three curves. The oxygen affinity for the isolated domain is quite low. At pH 7.65 with 50 mM Mg^{2+} the p_{50} for whole molecules is 11 mmHg, whereas it is 50 mmHg for Od-1. It is possible to compare the magnitude of the magnesium effects for the two forms of the molecule, despite their differing affinities, by defining the magnesium effect as $\Delta \log p_{50}/\Delta\ [\mathrm{M}]\mathrm{Mg}^{2+}$. On this basis the magnesium effect for whole molecules (6.25) is 2.5 times greater than for Od-1 (2.5).

The Bohr effect is shown in Figure 7, which reveals a considerably lower oxygen affinity for Od-1 than is observed for either the subunit or the whole molecule at all pH values. The Bohr effect was measured for Od-1 and whole molecules in the presence of physiological levels of magnesium. Of course the subunit only exists in the absence of divalent cations, so it was not possible to examine all three forms under identical conditions. Nevertheless, what *is* significant is the presence of a Bohr effect in all three levels of structure. The differences in affinity are most pronounced at high pH, leading to a decrease in the slope of the Bohr effect from ($\Delta \log p_{50}/\Delta$ pH) = −1.7 for the whole molecule to −0.3 for Od-1. Before we conclude that association alone is responsible for the enhanced Bohr effect in whole molecules, it will be necessary to examine the oxygen binding of the other domains. There is heterogeneity of function within the subunit (Miller, 1985), and thus the Bohr effect of the subunit must be an average of the individual Bohr effects of the seven domains, in addition to whatever modifications are imposed on this average by association. Bonaventura et al. (1977) described widely varying Bohr and salt effects for partially purified mixtures of domains from subtilisin digests of *O. dofleini* hemocyanin.

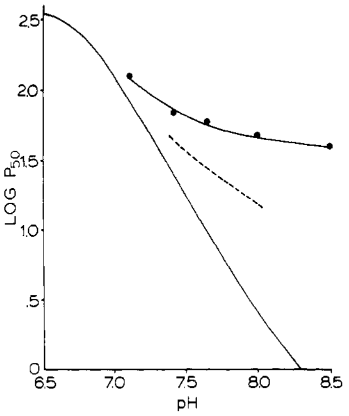

FIGURE 7: Bohr effect of three forms of *Octopus* hemocyanin. Buffer was 0.1 M HEPES plus 10 mM EDTA for subunits, 50 mM $MgCl_2$ for Od-1, and physiological saline for whole hemocyanin. Whole molecule (solid line) and subunit (dashed line) data are from Miller and van Holde (1985). Od-1 data are solid circles.

Ricchelli et al. (1986) examined a domain of *O. vulgaris* hemocyanin prepared by tryptic digestion followed by a purification procedure (Ricchelli & Zatta, 1985) that yields two slightly different 50000-dalton components after ion-exchange chromatography. One of these was characterized by oxygen binding and fluorescence techniques and proved to have a *higher* oxygen affinity than native hemocyanin at low pH but similar affinity at high pH. It differs in this respect from Od-1. There is no way to tell which domain they were examining without immunological evidence, but their results and ours are consistent with the widely varying results for different fractions described by Bonaventura et al. (1977). In addition, Gielens et al. (1986) purified functional fragments of *O. vulgaris* hemocyanin and observed some variation in the extinction coefficient at 346 nm, which they ascribed to differences in active site. In this study we have shown the existence of a reduced normal Bohr effect and magnesium effect for the highly purified C-terminal domain of *O. dofleini* hemocyanin. Clearly, these effects are an intrinsic property of this isolated domain, and the remaining domains almost certainly have different Bohr effects; but it is equally clear from Figure 7 that association of subunits into the whole molecule greatly enhances the Bohr effect. In future, as we are able to examine the individual oxygen-binding properties for other domains, we may find the "nesting" theory, proposed by Wyman (1972) and discussed more recently for *Eurypelma* hemocyanin by Decker et al. (1986), to be a useful model. They define nesting as the influence of macromolecular conformation on the ligand-binding properties of the composite subunits. This is possibly true of the development of cooperative binding in *Octopus* hemocyanin, and it may well be true of Bohr and magnesium effects as well.

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Registry No. Oxygen, 7782-44-7; magnesium, 7439-95-4.

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