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HETEROGENEOUS BINDING OF OXYGEN AND CARBON MONOXIDE TO DISSOCIATED MOLLUSCAN HEMOCYANIN

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Functional heterogeneity in O_2 or CO binding of sites of dissociated molluscan hemocyanin polypeptide chains (*Helix pomatia* and *Octopus vulgaris*) has been estimated by an analysis of accurate noncooperative binding curves. Three types of experiments were performed: pure O_2 or CO binding, competitive displacement of one ligand by the other, and simultaneous removal of both gases from protein partially saturated with O_2 and CO. The data were analyzed in terms of a model which has two fractions of sites with different properties for O_2 and CO. The relative proportion of the different binding sites and their affinity constant values were found by the combined use of the three different procedures. All species show a marked functional heterogeneity of sites for O_2 binding, while for CO binding it has been observed only in the case of *H. pomatia* β-hemocyanin. Moreover, in all three molluscan hemocyanins examined, the two classes of O_2-binding sites, although present in different proportions within the polypeptide chains, display similar affinity constant values. The data reported show a good consistency with results obtained using digested and isolated domains, providing confidence in the analytical procedure used. From comparison of the O_2/CO affinity ratios (K_{O_2}/K_{CO}) of each class it may be suggested that the difference in O_2 affinity of two kinds of binding sites is related to a different local structure of the active sites. The results, moreover, unequivocally confirm that binding and displacement of two gaseous ligands to hemocyanin occur by a simple competitive mechanism, although the binding site is structurally complex and the two ligands are bound with different geometries.

1. Introduction

Hemocyanins are multisubunit respiratory proteins contained in the hemolymph of arthropods and molluscs. Fundamental differences exist in the molecular architecture and functional properties between the two subphyla [1]. Arthropodal hemocyanins are composed of more than one type of subunit (each containing one binding site), which in vivo forms aggregates of 6, 12, 24 and 48 monomers. In some species single subunits can easily be isolated and characterized; a marked heterogeneity has often been observed [2].

In contrast to arthropods, the structure of molluscan hemocyanins is more complicated. Electron micrographs of *Helix pomatia* α- and β-hemocyanin reveal that the native molecules are cylindrical in shape [3], and can be dissociated into fragments (1/2, 1/10 and 1/20 units) by increasing pH and removing divalent cations [4,5]. The smallest unit (1/20) consists of a linear chain of eight domains, each containing one O_2- or CO-binding site [6–8]. The eight functional domains are linked by covalent linkage and may be separated only by enzymatic digestion. It is of general interest to establish whether the functional properties of the domains are similar or different and if some heterogeneity exists between polypeptide chains (chain heterogeneity). In the case of *Helix* β-hemocyanin separation of the domains by proteolysis reveals functional and structural differences between the eight domains [9–11]. This

result was expected since O_2 binding of the eight-site polypeptide chains of *H. pomatia* α- and β-hemocyanin is characterized by a Hill coefficient of 0.82 and 0.90, respectively, indicating nonequivalent binding sites [5,12]. Functional and structural heterogeneity of the separate domains has been observed for a wide variety of molluscan hemocyanins [13,14]. Obviously it is uncertain whether the O_2 affinity of the domains separated after digestion reflects that displayed within the intact polypeptide chain.

In this paper a variety of precise O_2- and CO-binding procedures have been applied to three molluscan hemocyanins. Computer analysis of the data has been used to reveal the degree of functional heterogeneity as well as the affinity constants of different binding sites for O_2 and CO. The simultaneous use of two ligands (O_2 and CO) allowed us to obtain information on the stoichiometry and mechanism of the competitive binding. The procedure used is not dependent upon enzymatic digestion, fractionation and functional characterization of isolated fragments. The experimental and data analysis techniques which have been applied to the noncooperative systems addressed in this paper can hopefully be exploited to examine details of the effect of subunit functional heterogeneity in the cooperative process.

2. Materials and methods

H. pomatia (α and β components) and *Octopus vulgaris* hemocyanins were isolated, stored and regenerated as previously described [4,15,16]. *Panulirus interruptus* was prepared and stored as reported [17]. Prior to each set of experiments, molluscan hemocyanin was regenerated by treatment with hydroxylamine [18]. The dissociated material from molluscan and arthropodal hemocyanins was obtained by dialysing the hemocyanins at high pH in the absence of calcium. It may be recalled that the α and β components are both fully dissociated into 1/10 molecules at pH 8.1 and 8.3 and into 1/20 molecules only above pH 9.0 and 10.0, respectively [4,5]. The state of aggregation of the hemocyanin was checked with a Beckman Spinco analytical ultracentrifuge.

O_2-binding equilibrium measurements were carried out with a thin-layer apparatus as already described equipped with an O_2 electrode [19]. The cell was thermostatted at 25°C. The time dependence of the optical absorption was recorded at 340 nm with a Cary 219 spectrophotometer. The change in absorbance was determined for the equilibrated state, which was achieved in 5–20 min depending on the degree of O_2 saturation. The barometric pressure was measured during calibration of the electrode and during binding experiments. Corrections were made for the vapor pressure of water.

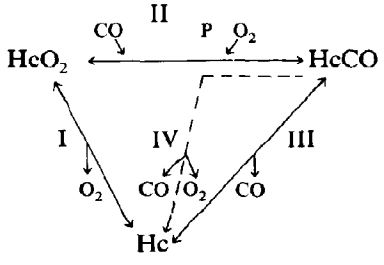
CO binding equilibria of dissociated *H. pomatia* α- and β-hemocyanins were measured with a thin-layer Gill apparatus adapted for fluorimetric measurements in a Perkin Elmer fluorimeter [20]. The excitation wavelength was 295 nm, while the emission intensity changes upon addition of CO were followed at 540 nm.

The O_2-CO competition experiments were performed with the same apparatus. In this experiment the cell is initially loaded with pure O_2 and then repeated dilutions with CO are made. The total pressure was maintained at 1 atm. The presence of bound O_2 is monitored by observing the absorbance change at 340 nm. Once the protein was CO saturated, the displacement of CO by O_2 was carried out filling the stopcock bore with water-saturated O_2 and reversing the procedure described above. Fully oxygenated hemocyanin spectra were recorded before and after each replacement experiment in order to determine the general stability of the sample and the layer. In view of the similar affinity of *H. pomatia* α- and β-hemocyanins for O_2 and CO, the stopcock bore was reduced in order to add smaller amounts of CO at each step.

A third procedure was used to examine the ligand binding of hemocyanin partially saturated with O_2 and CO, while maintaining the ratio of partial pressures fixed. This type of procedure is called a 'diagonal experiment'. Pure CO was added to oxyhemocyanin until the desired intermediate degree of O_2-CO saturation was produced. The O_2 pressure was determined with the O_2 oxygen electrode and the CO pressure was calculated by the difference from the total pressure.

Then the partial pressures of CO and O_2 were reduced in a precise stepwise manner by using the dilution valve filled with nitrogen. The optical absorption at 340 nm was followed to determine the degree of O_2 saturation as a function of O_2 and CO pressures.

The three procedures used are depicted in scheme 1, which shows the three derivatives of hemocyanin as unliganded (Hc), fully oxygenated (HcO_2), and fully carbon monoxygenated (HcCO). The usual CO, O_2, and displacement binding curves are generated by the reactions given by the sides of the triangle (I–III); the diagonal experiment is shown as a line from the base of the triangle to the apex.



Scheme 1. Reaction scheme for determining O_2 and CO binding to hemocyanin. Paths I and III represent O_2 and CO dissociation equilibria, respectively. Path II represents the competition between CO and O_2. Path IV indicates the diagonal experiment starting at a selected degree of O_2-CO saturation (point P).

3. Binding curve analysis

Since the experimental measurements consist of changes in optical properties due to precise stepwise changes in ligand partial pressures, the usual methods of displaying the results (such as Hill plots) are unsuitable. Thus, the data are presented along with the theoretical fitted curves in a graphical form where the change in optical property is plotted vs. the number of steps, characterized by the partial pressure at the end of the step. The pressure values at each step can be determined by a dilution factor (D) which varies for each type of experiment and which can be readily determined. The mathematical expressions used to determine the partial pressure at each step (p_i) of the gas diluted and/or of the gas added are:

$$p_i^{\mathrm{Dil}} = p_0 D^i \qquad p_i^{\mathrm{Add}} = p_0(1 - D^i)$$

where p_0 is the initial pressure of either O_2 or CO (depending on the experiment); D the dilution factor, which is determined independently for each cell; and i the number of steps. The specific values of p_0 and D for each experiment are reported in the figure legends.

The system is described by either one or two classes of independent and nonequivalent sites, which can bind either CO or O_2. In the latter case, the fraction of sites bound to O_2 for the i-th step ($\bar{Y}_i$), at a given O_2 partial pressure ($p_i^{O_2}$), is then given as follows:

$$\bar{Y}_i^{O_2} = \alpha_1 \cdot \frac{K_1^{O_2} p_i^{O_2}}{1 + K_1^{O_2} p_i^{O_2} + K_1^{CO} p_i^{CO}} + \alpha_2 \cdot \frac{K_2^{O_2} p_i^{O_2}}{1 + K_2^{O_2} p_i^{O_2} + K^{CO} p_i^{CO}} \qquad (1)$$

where α_1 and α_2 denote the relative fraction of sites 1 and 2 ($\alpha_1 + \alpha_2 = 1$); $K_1^{O_2}$, K_1^{CO}, $K_2^{O_2}$ and K_2^{CO} are the respective binding constants for O_2 and CO; $p_i^{O_2}$ and p_i^{CO} denote the partial pressures of the two gases (when both are present) at step i.

The equivalent expression for the degree of CO binding is found by replacing the terms $K_1^{O_2} p_i^{O_2}$ and $K_2^{O_2} p_i^{O_2}$ by $K_1^{CO} p_i^{CO}$ and $K_2^{CO} p_i^{CO}$. The relation to the change in the desired optical parameter ΔT_i is given by the difference in binding between the i and $(i-1)$-th steps, times the overall change in the optical parameter, denoted by T_0:

$$\Delta T_i = T_0 [\bar{Y}_i - \bar{Y}_{i-1}]$$

Here $\bar{Y}_i$ and $\bar{Y}_{i-1}$ indicate the degree of saturation at two successive steps, either for O_2 or CO as appropriate for the particular experiment. Each data point is expressed by a change in the observed optical effect ΔT_i for the set of partial pressures p_i^{CO}, $p_i^{O_2}$, p_{i-1}^{CO} and $p_{i-1}^{O_2}$.

Analysis of the experimental data was accomplished using a standard nonlinear least-square procedure [21], by which the best-fit values for α_1, $K_1^{O_2}$, K_1^{CO}, $K_2^{O_2}$, K_2^{CO} and T_0 may be determined.

The standard error of a point, σ, was used to choose the best-fit values. In the data-fitting analysis the weighting was assumed to be equal for all data points. The analysis of individual data sets places a certain emphasis on the determination of particular parameters. In pure O_2- or CO-binding experiments the binding constants of only one ligand can be determined. In such experiments there are two parameters less in the fitting process and thus the remaining parameters, in particular the degree of heterogeneity, can be estimated with the highest certainty.

4. Results

The simplest system we chose to examine was a purified subunit (fraction I) of *Panulirus* hemocyanin. Here there is no functional heterogeneity and the analysis of the different binding experiments should provide the best test of the experimental methodology. In fig. 1 we show the results for (a) oxygen binding, (b) the displacement of oxygen with CO, and (c) a 'diagonal' experiment (in which the hemocyanin initially saturated with O_2 at 746 Torr was converted to the partially saturated form with O_2 and CO upon addition of two steps of CO (56% of the sites were saturated with O_2 and 44% with CO) and was then subjected to stepwise reduction in the partial pressures of both ligands by dilution with N_2). For the latter experiment all species, namely, Hc, HcO_2 and HcCO, are populated to a sufficient extent so that accurate binding parameters can be determined. The general consistency of the single-site model used to analyze these experiments is perhaps best illustrated by the excellent agreement between the theoretical lines and the experimental points for the three different types of experiments as shown in fig. 2. The parameters of fitting are given in table 1. The observed affinities correspond to values found by other workers [22]. Since the proposed model describes the data precisely, this is important proof that the stoichiometry of the O_2-CO reaction is 1:1.

Similar experiments on the mixture of the subunits of *Panulirus* were conducted in order to assess what level of heterogeneity may be detected

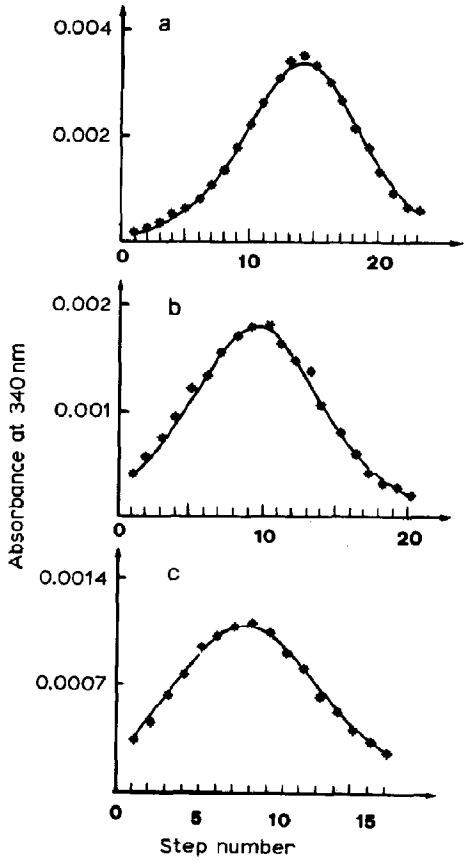


Fig. 1. Ligand binding results for solution of purified monomers, fraction I. O_2 binding, O_2-CO competition and diagonal experiments are represented in a, b and c, respectively. The data are given in terms of the change in O_2 optical absorbance at 340 nm following stepwise dilution of the O_2 partial pressure or/and upon addition of CO. For pure ligand binding and displacement experiments, the initial O_2 partial pressure was 746 Torr and the stepwise dilution factor 0.71. For diagonal experiment stepwise dilution of both gases by N_2 was carried out on protein with 56% of the sites saturated with O_2 and 44% with CO and at partial pressures of $p_{O_2} = 36$ Torr and $p_{CO} = 710$ Torr, respectively; the dilution factor was 0.71. The solid line represents the best fit by least-squares analysis based on eq. 1 fixing the fraction of sites at $\alpha_1 = 1$. Experimental condition: pH 9.5 in 0.1 M ETAM at 15°C.

by this analytical procedure. In this case, it is known that the three subunits isolated bind O_2 at 15°C with very similar affinities ($p_{1/2}$ 5.9, 6.4 and 5.1 Torr, respectively) [23]; comparable informa-

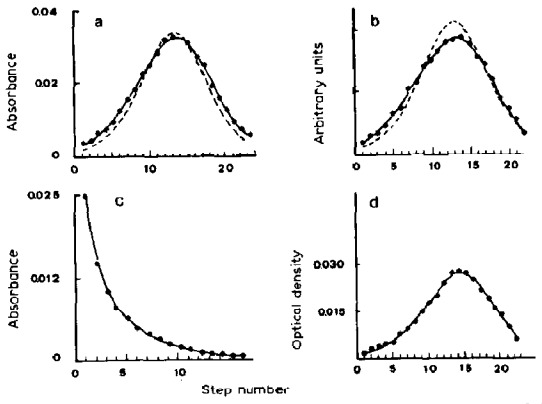


Fig. 2. Ligand binding results for solution of dissociated β-hemocyanin for *H. pomatia* at pH 8.5. O_2 and CO binding, O_2-CO competition and diagonal experiments are represented in a, b, c and d, respectively. The data are given in terms of the change in optical absorbance or fluorescence intensity caused by stepwise dilution of the ligand partial pressure and/or upon addition of CO. The initial partial pressure (p_0) of each gas for the O_2- or CO-binding experiments was 605 Torr and the stepwise dilution factor (D) 0.706, while for O_2-CO competition $p = 605$ Torr and $D = 0.783$. In the diagonal experiment, stepwise dilution of both gases by N_2 was carried out on protein with 55% of sites saturated with O_2 and 45% with CO; the partial pressure of O_2 and CO was $p_{O_2} = 137.9$ Torr and $p_{CO} = 487.1$ Torr, respectively. The solid lines were obtained from least-squares analysis based on eq. 1 while the dashed lines in a and b represent the best fit obtained assuming only one O_2- and CO-affinity constant for all sites. Experimental conditions: pH 8.5 in 0.1 M Tris-HCl at 25°C.

tion on CO binding is not available, given the low CO affinity of the mixture. The results of the O_2-binding analysis of the mixture are also shown in table 1. It may be observed that when the difference in $p_{1/2}$ of two different molecular species is less than about 50%, the heterogeneity cannot be well resolved.

The test case applied to arthropodal hemocyanin gives confidence in approaching the analysis of the polypeptide chains containing several domains as in molluscan hemocyanins. Typical results for (a) O_2 binding, (b) CO binding, (c) displacement and (d) diagonal experiments for *H. pomatia* β-hemocyanin are shown in fig. 2. The dashed lines represent the best fit obtained assuming only one O_2- and CO-affinity constant, while the full line represents the best fit assuming two different kinds of binding sites. The fit to the latter model gives a standard error of a point 3–10-times smaller than the single-site case.

Results for O_2 and displacement of CO by O_2 for dissociated *O. vulgaris* hemocyanin are shown in fig. 3. In both measurements the curves are strongly asymmetric. Here again the dashed line represents the best fit obtained assuming only one O_2- and CO-affinity constant, while the continuous line represents the best fit assuming two kinds of binding sites for both gases. It is evident that

Table 1

O_2- and CO-affinity constants for monomeric fraction I and dissociated *P. interruptus* hemocyanin at pH 9.5

Species	Process	Binding constants ($Torr^{-1}$)		Fraction of sites (α_1)	Total optical changes (T_0)	Point error of fit (σ)
		K^{O_2}	K^{CO}			
Monomer (fraction 1)	pure O_2 binding	0.132 (+0.004)		1	0.098 (+0.098)	1.6×10^{-4}
	displacement by CO	0.113 (+0.010)	0.004 (+0.011)	1	0.082 (+0.003)	3×10^{-4}
	diagonal [a]	0.145 (+0.008)	0.006 (+0.0004)	1	0.096 (+0.002)	1.3×10^{-4}
	multifit	0.145 (+0.060)	0.006 (+0.002)	1		2.4×10^{-4} 4.6×10^{-4} 5.3×10^{-4}
P. interruptus (mixture)	pure O_2 binding	0.153 (+0.004)		1	0.108 (+0.0006)	1.6×10^{-4}

[a] From protein with 56% of sites saturated with O_2 and 45% with CO and at initial partial pressures $p_{O_2} = 36$ Torr and $p_{CO} = 710$ Torr, respectively.

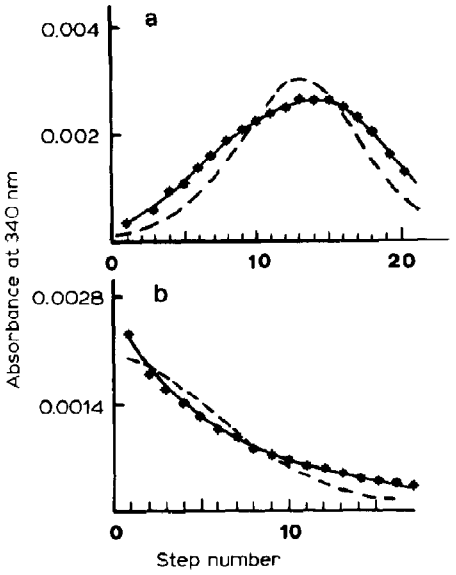


Fig. 3. Ligand-binding results for solution of dissociated *O. vulgaris* hemocyanin at pH 8.6. O_2 binding and CO competition experiments are presented in a and b, respectively. The initial pressure in both experiments was 746 Torr and the dilution factor was 0.71. The solid lines were obtained from least-squares analysis based on eq. 1, while the dashed lines represent the best fit obtained assuming only one O_2- and CO-affinity constant for all sites. Experimental conditions: pH 8.6 in 0.1 M Tris-HCl at 25°C.

the fit to the model with two kinds of sites gives significantly better results.

The characteristic parameters and indications of the quality of the fit for the experiments on the three molluscan hemocyanins are summarized in tables 2–4.

The overall measure of precision of the fitting process is given by the standard error of a point, which in all experiments is approx. 10^{-4} units absorbance for $\Delta T_i \approx 10^{-2}$. The small magnitude of the standard error of a point is gratifying, since it is comparable to the expected resolution and noise of the Cary 219 spectrophotometer. The O_2- and CO-affinity constant values obtained from two opposite displacements (O_2 by CO or vice versa) are similar and agree with the values obtained from direct binding of O_2 or CO. This

Table 2

O_2- and CO-affinity constants and relative proportion of the two classes of binding sites determined in the polypeptide chain (1/10 form) of *H. pomatia* β component at pH 8.5

Parameters listed without errors were held fixed in the fitting procedures.

Process	Binding constants ($Torr^{-1}$)				Fraction of sites (α_1)	Total optical changes (T_0)	Point error of fit (σ)
	$K_1^{O_2}$	$K_2^{O_2}$	K_1^{CO}	K_2^{CO}			
Pure binding							
CO [b]			0.033 (±0.033)	0.208 (±0.040)	0.29 (±0.10)	277 (±8)	0.51
O_2	0.049 (±0.010)	0.214 (±0.020)			0.32 (±0.060)	0.184 (±0.001)	1.3×10^{-4}
Displacement							
by CO	0.049	0.214	0.021 (±0.002)	0.430 (±0.010)	0.32	0.09	1.2×10^{-4}
by O_2	0.049	0.214	0.024 (±0.001)	0.380 (±0.010)	0.32	0.09	7.6×10^{-5}
Diagonal [a]	0.049	0.214	0.050 (±0.005)	0.300 (±0.020)	0.32	0.089	9.0×10^{-5}

[a] From protein with 55% of sites satured with O_2 and 45% with CO and at initial partial pressures $p_{O_2} = 137$ Torr and $p_{CO} = 487$ Torr, respectively.

[b] Determined by fluorescence at 540 nm.

Table 3

O_2- and CO-affinity constants and relative proportion of the two classes of binding sites in *H. pomatia* α component polypeptide chain

Parameters listed without errors were held fixed in the fitting procedures.

Process	pH [b]	Binding constants ($Torr^{-1}$)				Fraction of sites (α_1)	Total optical changes (T_0)	Point error of fit (σ)
		$K_1^{O_2}$	$K_2^{O_2}$	K_1^{CO}	K_2^{CO}			
Pure O_2 binding	8.1	0.071 (±0.003)	0.339 (±0.027)			0.50 (±0.03)	0.230 (±0.006)	4×10^{-4}
	8.6	0.064 (±0.022)	0.391 (±0.151)			0.43 (±0.15)	0.280 (±0.009)	5×10^{-4}
	9.5	0.040 (±0.008)	0.267 (±0.06)			0.47 (±0.08)	0.181 (±0.004)	2×10^{-4}
Displacement by CO	9.5	0.040	0.267	0.150 (±0.01)	0.170 (±0.01)	0.50	0.140 (±0.001)	2×10^{-4}
by O_2	9.5	0.032 (±0.003)	0.278 (±0.013)	0.110 (±0.01)	0.152 (±0.007)	0.48 (±0.02)	0.135 (±0.003)	1.5×10^{-4}
Diagonal [a]	9.5	0.040	0.267	0.230 (±0.10)	0.080 (±0.06)	0.50	0.061	1.2×10^{-4}

[a] From protein with 55% of sites with O_2 and 45% with CO and at partial pressures $p_{O_2} = 391$ Torr and $p_{CO} = 234$ Torr.

[b] In the absence of calcium *H. pomatia* α component is in the 1/10 form at pH 8.1 and in the 1/20 form at pH 9.5, while a mixture of both is present at pH 8.5.

consistency emphasizes the adequacy of a simple competition to describe the data.

In the case of *H. pomatia* β-hemocyanin, two kinds of binding sites are determined for both O_2 and CO; they differ by a factor 4 for O_2 and 10 for CO and the relative proportion of higher-affin-

Table 4

O_2- and CO-affinity constants and relative proportion of the two classes of binding sites determined in the polypeptide chain of *O. vulgaris* hemocyanin

Parameters listed without errors were held fixed in the fitting procedures.

Process	Binding constants ($Torr^{-1}$)				Fraction of sites (α_1)	Total optical changes (T_0)	Point error of fit (σ)
	$K_1^{O_2}$	$K_2^{O_2}$	K_1^{CO}	K_2^{CO}			
Pure O_2 binding	0.024 (±0.002)	0.239 (±0.018)			0.38 (±0.030)	0.255 (±0.004)	2.5×10^{-4}
Displacement by O_2	0.024	0.239	0.321 (±0.080	0.258 (±0.020)	0.40	0.260	3.3×10^{-4}
by CO	0.024	0.239	0.290 (±0.030)	0.350 (±0.010)	0.40	0.255	5.6×10^{-4}

ity sites is $\alpha_1 = 0.3$ for both gases. In the case of the α component, two different kinds of binding sites in equimolar amounts are observed for O_2 while binding of CO seems to be homogeneous. Similarly, *O. vulgaris* hemocyanin shows two binding sites for O_2 which differ by a factor 10 and in the absence of heterogeneity for CO binding. For this protein, given the high affinity for CO as compared to O_2, analysis of diagonal experiments is unsatisfactory.

5. Discussion

During the last decade, several pieces of evidence have indicated that hemocyanins are composed of more than one type of polypeptide chain [1]. While functional and structural subunit heterogeneity is well documented for arthropodal hemocyanins, due to the ease of isolating single subunits, quantitative information on molluscan hemocyanins is more limited. In this subphylum, isolation of the domain has been achieved by enzymatic digestion of the polypeptide chain [11,13,14] which, however, may lead to structural perturbations and thus changes in the functional properties. Alternatively, by use of precise binding studies we have found that it is possible to obtain detailed information on the heterogeneity and binding properties of molluscan hemocyanin polypeptide chains.

The basic assumption in the analysis of the results reported above is that the dissociated molecules (1/10 and 1/20 species of *H. pomatia* and *O. vulgaris* hemocyanin) lack homotropic interactions in the binding of both O_2 and CO. This assumption is validated by theoretical fits obtained with such a model in the present paper. A second assumption used in the analysis is that competitive stoichiometric binding of CO and O_2 applies in the case of hemocyanins with a ratio 1:1. Finally, a strict linearity between chemical reaction and change in optical property is valid throughout the range of saturations. The latter two assumptions are consistent with previous data [24,25].

Several types of experimental procedures, described in detail in section 2, were used to probe the heterogeneous binding properties of CO and O_2 under a variety of conditions where the concentration of different ligated forms could be varied over a wide range. The combination of methods involving high-precision determination of binding and ligand activities was found to be of crucial importance in resolving the parameters that describe each system. With regard to the quality of the results, it should again be noted that the standard error of a point obtained in the fitting process is comparable to the resolution and noise of the instrument used and that advantage is taken from the type of plot employed, which is more suitable than the Hill plot.

In the simple case of purified fraction I from *P. interruptus* hemocyanin, the estimates of precision of the resolved parameters, along with the point errors (table 1), provide a general idea of the effectiveness of the combined analysis of the various types of experiments in producing consistent binding parameters. Experiments with fraction I, moreover, verify unequivocally the competitive replacement of one O_2 by one CO.

With this background, the same procedures have been applied to the more interesting complex multisubunit chains found in the molluscan hemocyanins. The results could not be fitted with single binding constants for O_2 and CO, and required the introduction of two classes of binding sites present in comparable amounts. As might be expected, resolution beyond two classes of binding sites was found not to improve the fit, presumably due to the introduction of more parameters than can be resolved. The two types of binding sites bind O_2 and CO independently, and competitively; the binding constant values determined for the hemocyanin examined are summarized in table 1.

The absence of a functional heterogeneity found in *P. interruptus* hemocyanin has also been observed in other arthropodal hemocyanins [25,27–29]. Thus, except in some particular cases [30,31], the subunits of most arthropodal hemocyanins show structural heterogeneity (by electrophoresis, etc. [2]) but do not differ significantly in their O_2-binding properties. In contrast, O_2 and CO binding to molluscan hemocyanins requires at least two functionally different types of binding

sites within and/or between the polypeptide chain. This conclusion may be correlated with independent functional, spectroscopic and biochemical observation. In the case of the β component of *H. pomatia*, circular dichroism spectra of the proteolytic fragments indicated the presence of two classes of copper sites [32]. Similarly, the O_2-affinity constant values reported for tryptic fragments from β-hemocyanin indicate the presence of two groups of binding sites, one of five or six domains showing higher O_2 affinity ($K = 0.2–0.25$ Torr^{-1}) and the other of two or three domains with lower affinity ($K = 0.06–0.7$ Torr^{-1}) [9–11]. These values conform to results given in table 3. It should be recalled that the β component at pH 8.5 is present in the 1/10 form and that no microheterogeneity between polypeptide chains (1/20 molecules) has been observed.

In the case of *H. pomatia* α-hemocyanin, two groups of binding sites, in approximately equimolar amounts, have been determined for O_2 binding, while no significant heterogeneity was found for CO binding. In this hemocyanin the parameter values are insensitive to pH from 8.1 to 9.5. Recalling that *H. pomatia* α-hemocyanin exists in the 1/10 species at pH 8.1 and in the 1/20 species at pH 9.5, this is an indication that the heterogeneity in O_2 binding is independent of quaternary structure. A number of recent studies have provided information about chemical heterogeneity in *H. pomatia* α-hemocyanin. A study of the kinetics of digestion [14] indicated that hemocyanin consists of at least two kinds of subunits, which differ in their proteolytic susceptibility and occur in equimolar amounts. O_2 binding of these isolated proteolytic fragments reveals two fractions with different O_2 affinity [33]. Plasminolysis of the 1/10 molecule at pH 8.2 and characterization of the fragments indicated the presence of two types of polypeptide chains associated into the 1/10 molecules [34]. A similar chain heterogeneity has also been observed in the molluscan hemocyanins of *Murex f.* and *Nautilus p.* [35,36].

In the case of *O. vulgaris* hemocyanin, the heterogeneity in oxygen binding is even more marked with strongly asymmetric binding curves. Oddly, CO binding does not show any heterogeneity, as is also observed for the α component of *H. pomatia*. Proteolytic digestion of *O. vulgaris* hemocyanin in a fragment of 50 kDa (corresponding to the single domain) gives two distinct bands on SDS electrophoresis whose relative proportion is 40 and 60% (Salvato, unpublished data). The same proportion was found for our O_2 data on the intact chain.

These new accurate data indicate that the molluscans examined display a higher functional heterogeneity of domains for O_2 binding than for CO binding. This might be correlated with the different mode of binding of these two gaseous ligands at the active site of hemocyanin. In fact, it is known that O_2 binds as a bridge between the two copper atoms [37], while CO binds to only one of two coppers [38]. Thus, O_2 binding is expected to demand a more stringent stereochemistry with respect to the distance between the copper atoms in a site. The affinity ratios (K_{O_2}/K_{CO}) for the two classes of binding sites are significantly different in all three molluscan hemocyanins (table 1). This ratio is independent of factors which affect the absolute affinity of both ligands in a similar way [26]. Thus, it may be suggested that the different affinity determined for the two classes of binding sites is related to a different local structure. Examination of the data, moreover, reveals that in all three molluscan hemocyanins, the two classes of binding sites, although present in different proportions, display similar oxygen affinity constant values. This evidence is in line with the phylogenetic hypothesis [1] that in the evolution of molluscan hemocyanins, the first step seems to have been a massive gene replication, to produce the tandem gene arrays necessary to code for a polypeptide chain made of multiple domains. Possibly, the relative amount of binding sites with low or high O_2 affinity constant occurs at different levels of development in different molluscan hemocyanins.

In summary, it has been shown that the use of two ligands, O_2 and CO, in conjunction with three different types of binding experiments allows resolution of the functional properties of domains present within multisubunit proteins. The functional heterogeneity determined in three molluscs is more marked for O_2 binding than CO and this reflects the greater sensitivity of O_2 to different local structures.

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