

Equilibrium and kinetic studies of oxygen binding to fragments of *Lymnaea stagnalis* (freshwater snail) haemocyanin obtained by proteolytic digestion

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Functional fragments of the haemocyanin from the gastropod mollusc *Lymnaea stagnalis* (freshwater snail) were obtained by partial digestion with trypsin and plasmin. The fragments were purified by ion-exchange chromatography and characterized by detergent/polyacrylamide-gel electrophoresis and crossed immunoelectrophoresis. Three types of single-functional unit fragment were isolated from the trypsin digest, and two immunologically distinct three-functional unit fragments and a single-functional unit fragment were isolated from the plasmin digest. The O₂-binding behaviour of the fragments was investigated by equilibrium and kinetic methods. Over the pH range 7.0–8.2, in the presence of 10–20 mM-CaCl₂, all of the single-functional unit fragments displayed non-co-operative O₂ binding and showed no evidence of a Bohr or a salt effect. A Hill coefficient of less than 1.0 was obtained with one of the two three-functional unit fragments studied, whereas both of these fragments displayed a Bohr effect. Functional heterogeneity of the fragments was indicated by the variation in the O₂ affinity, the p_{50} (partial pressure of O₂ at half saturation) ranging between 0.26 and 0.77 kPa (approx. 2–6 mmHg). Stopped-flow data reflected the O₂ equilibrium behaviour. Thus there was a fall in the value of the O₂ dissociation rate constant from approx. 15 to 1 s⁻¹ in parallel with the increase in O₂ affinity.

Haemocyanins are the multisubunit high-molecular-weight copper-containing oxygen-transporting proteins of many arthropods and molluscs (Wood, 1980). The active site of the protein is capable of reversibly binding one molecule of O₂ per two copper atoms (Lontie & Gielens, 1979). Arthropodan haemocyanins contain one O₂-binding site per subunit polypeptide and are composed of 6, 12, 24 or 48 subunits of mol.wt. 70 000–95 000 per native molecule, depending on the species. Molluscan haemocyanins, in contrast, have markedly different structures, being composed of twenty 400 000–450 000 Da subunit polypeptides assembled in a cylindrical arrangement (Berger *et al.*, 1977). Each of the subunit polypeptides contains eight O₂-binding sites as a string of more or less globular functional units, which were formerly named 'domains' (Gielens *et al.*, 1981).

Despite differences in their structures, the functional properties of arthropodan and molluscan haemocyanins are qualitatively very similar. Homo-

tropic interactions resulting from the influence of one O₂-binding site upon neighbouring sites give rise to co-operative O₂ binding, which is modulated by allosteric effectors such as protons, alkali-metal ions and some anions (Zolla *et al.*, 1978). The presence of such large numbers of O₂-binding sites in the native molecule, e.g. 160 in mollusc haemocyanin, raises an intriguing question of whether the co-operative interactions involve the entire protein, or whether functionally independent allosteric units exist within the molecule. In the case of molluscan haemocyanins, problems of this type can be investigated, since the molecules can be made to dissociate partly or completely to the individual subunit polypeptide chains (Gullick *et al.*, 1979) and can also be dissected into functional fragments of various sizes, e.g. single-functional unit, two-functional unit etc. (Lontie *et al.*, 1973; Gullick *et al.*, 1979).

In a previous paper (Dawson & Wood, 1982) we reported the equilibrium and kinetic behaviour of O₂ binding to the whole or native haemocyanin molecule of the freshwater snail *Lymnaea stagnalis* under different conditions of pH, CaCl₂ and NaCl concentration. We have now extended these studies to

Abbreviations used: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; IgG, immunoglobulin G; iPr₂P-F, di-isopropyl phosphorofluoridate.

the investigation of the O₂-binding behaviour of various fragments of this haemocyanin obtained by proteolytic digestion.

Material and methods

Isolation and partial dissociation of the haemocyanin

Lymnaea haemocyanin was isolated and purified as described previously, (Dawson & Wood, 1982). Partial dissociation to one-tenth haemocyanin molecules was achieved by increasing the pH to 8.2, with removal of Ca²⁺ by means of EDTA.

Proteolytic digestion and separation of products

Trypsin and porcine plasmin were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

One-tenth haemocyanin molecules in 10 mM-Tris/HCl buffer, pH 8.2, containing 50 mM-NaCl, were digested with plasmin (3%, w/w) for 90 min at room temperature. The digestion was terminated by the addition of a molar excess of iPr₂P-F. The digest solution was equilibrated in 10 mM-Tris/HCl buffer, pH 7.5, and was fractionated on a column of DEAE-Sepharose CL-6B equilibrated with the same buffer, a linear NaCl gradient (0–0.5 M) being used. Digestion of the whole molecules with trypsin [3% (w/w)] was carried out in 15 mM-Tris/HCl buffer, pH 8.2, containing 15 mM-CaCl₂, for 2 h at room temperature. The digestion was terminated by the addition of a molar excess of iPr₂P-F. Digestion of native *Lymnaea* haemocyanin by this procedure results in the removal of the so-called 'collar' observed by electron microscopy (Mellema & Klug, 1972), and the formation of tubular structures, the so-called 'tubes', by indefinite end-to-end polymerization of the remaining cylindrical 'wall' structures (Wood, 1977). The digest solution was separated into collar and tube fractions by ultracentrifugation for 2 h at 40000 rev./min in a Beckman L2-65B instrument using a type 50Ti rotor. The pellet (containing the tubes) was equilibrated with 10 mM-Tris/HCl buffer, pH 8.2, in the absence of Ca²⁺, which causes dissociation into wall fragments. Collar and wall fragments were fractionated separately by ion-exchange chromatography on columns of DEAE-Sepharose CL-6B equilibrated with 10 mM-Tris/HCl buffer, pH 8.2, by a stepwise addition of NaCl (0–0.4 M).

Characterization of the digestion products

The digestion products were analysed by SDS/polyacrylamide-gel electrophoresis on 1 mm-thick slabs formed of a 4–16% gradient of polyacrylamide in the buffer system of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R250, and molecular weights were estimated from mobilities by comparison with proteins of known molecular weight.

Crossed immunoelectrophoresis was performed on the isolated *Lymnaea* fragments under conditions described by Booth *et al.* (1978), by using antibodies raised to whole *Lymnaea* haemocyanin (Wood & Gullick, 1981).

O₂-binding experiments

O₂-equilibrium curves were determined as previously described (Dawson & Wood, 1982) by monitoring absorbance changes at the oxy-protein peak wavelength (~346 nm) using the tonometric (Riggs & Wolbach, 1956) and diffusion-chamber (Sick & Gersonde, 1969) techniques. Kinetic measurements of O₂ dissociation were made using the stopped-flow technique (Chance, 1940) with an instrument designed and built in the Department of Biophysics of Leeds University. The rate of dissociation of O₂ from the oxygenated fragments was measured by mixing the air-equilibrated protein with an equal volume of buffer containing sodium dithionite (1 mg/ml). The time course of the reaction was monitored by measuring the signal change at 400 nm. In all cases a signal change corresponding to a decrease in absorbance with time was observed with at least 90% of the total signal change corresponding to a single exponential process. The apparent first-order rate constant of the reaction ($k_{app.}$) was assumed to be equivalent to the dissociation rate constant (k_{off}) and was obtained from the slope of a plot of log (absorbance change) against time.

Results

Separation and identification of the plasmin digestion products

Digestion of one-tenth haemocyanin molecules with plasmin produced three major fractions separable by ion-exchange chromatography. The fractions are denoted P1, P2 and P3 in the ion-exchange separation profile shown in Fig. 1. Samples from each fraction were analysed by SDS/polyacrylamide-gel electrophoresis. Fraction P1 contained a single-functional unit fragment with apparent mol.wt 50000 (approx.) (denoted 1DWP). Fraction P2 contained a three-functional unit fragment (mol.wt. 150000 approx.) and was almost completely electrophoretically homogenous (denoted 3DWP). Fraction P3 also contained a three-functional unit fragment that had a slightly greater mobility on SDS/polyacrylamide-gel electrophoresis (denoted 3DWPL), with a small amount of single- and two-functional unit fragments. Fractions P1 and P2 were further analysed by crossed immunoelectrophoresis. Fig. 2 shows the immunological reaction of these fractions to antiserum raised to whole *Lymnaea* haemocyanin. Fig. 2(a) shows the reaction to fraction P2, which consists of a single major

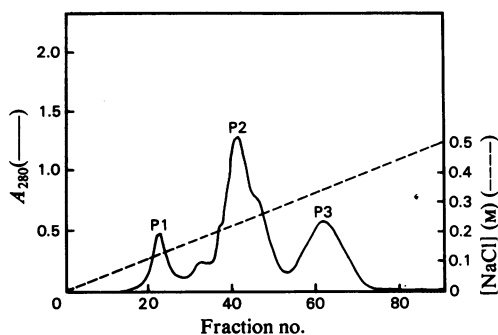


Fig. 1. Ion-exchange chromatography of the plasmin fragments on DEAE-Sepharose CL-6B

The column (1.2 cm × 20 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, was loaded with ~120 mg of protein and was eluted in 8 ml fractions with a linear gradient of NaCl (----).

immunological component, the three-functional unit fragment 3DWPH, with a small amount of some other fragment. The immunological reaction to fraction P1 is shown in Fig. 2(b) and shows it to consist of one component corresponding to a single-functional unit 1DWP. A mixture of fractions P1 and P2 produced completely separate immunological peaks (Fig. 2c), indicating that the two components are distinct.

Separation and identification of the trypsin digestion products

Digestion of whole haemocyanin molecules with trypsin initially produced two fractions, which were separated by ultracentrifugation. The pellet contained the tubular aggregates, which were dissociated and separated into three major fractions by ion-exchange chromatography. These fractions are denoted T1, T2 and T3 in the ion-exchange separation profile shown in Fig. 3. The supernatant obtained from the initial ultracentrifugation step contained the collar fragments, which were separated into two fractions by an ion-exchange chromatography procedure similar to that used to separate the wall fragments. The collar fragments are denoted by C1 and C2 in the separation profile shown in Fig. 4. Fraction samples from the two separations were analysed by SDS/polyacrylamide-gel electrophoresis. Fractions T1 and T2 were heterogeneous electrophoretically and were not investigated further in the present work. Fraction T3 contained a single electrophoretic band corresponding to a single-functional unit with apparent mol.wt. 50000 approx. (denoted 1DWT). Fraction C1 contained two main bands with apparent mol.wts. 70000 and 50000 approx. (denoted 1DCT). The second collar fraction, C2, emerged as

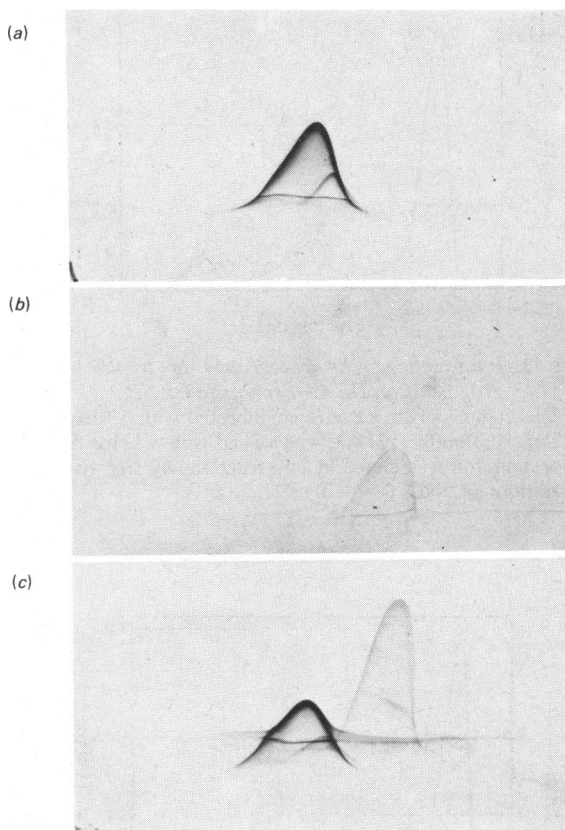


Fig. 2. Crossed immunoelectrophoresis with purified haemocyanin fragments

Fragments were obtained by plasmin digestion as described in the text and after purification run in crossed immunoelectrophoresis against rabbit IgG raised to whole *Lymnaea* haemocyanin. (a) Shows the 'heavy' three-functional unit fragment, which contains a small amount of some other fragment, and (b) shows a single-functional unit. Both of these represent parts of the 'wall' of the haemocyanin molecule. In (c) both were run in tandem crossed immunoelectrophoresis, demonstrating that they are unrelated immunologically. In each case the concentration of anti-*Lymnaea* IgG in the agarose was 1.0 mg/ml. The quantity of protein sample added to the wells was: (a) 2 μl of 0.5 mg/ml, (b) 5 μl of 0.5 mg/ml, and in (c) both of these, i.e. 7 μl total volume.

a doublet of a single-functional unit fragment with mol.wt. of approx. 50000.

Equilibrium O₂-binding experiments

In contrast with the native molecule, the fragments of *Lymnaea* haemocyanin studied so far display non-co-operative O₂-binding, with a Hill coefficient of approx. 1.0, with the *p*₅₀ (partial

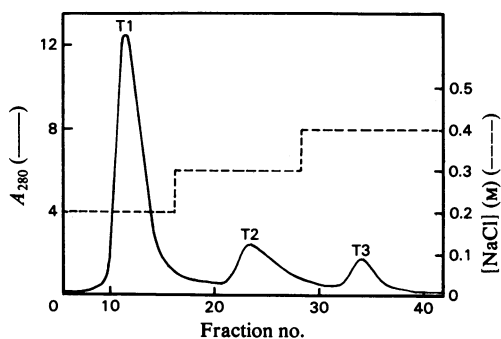


Fig. 3. Ion-exchange chromatography of tryptic wall fragments on DEAE-Sephacel CL-6B

The column (1 cm \times 8 cm), equilibrated with 10 mM-Tris/HCl buffer, pH 8.2, was loaded with \sim 12 mg of protein and was eluted in 2 ml fractions by stepwise addition of NaCl (-----).

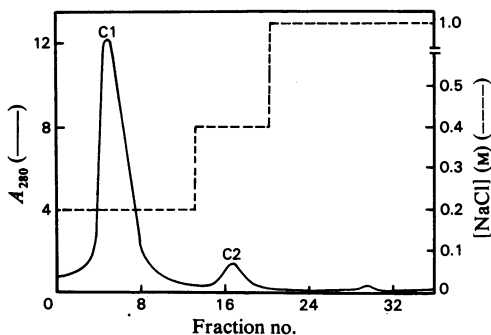


Fig. 4. Ion-exchange chromatography of tryptic collar fragments on DEAE-Sephacel CL-6B

The column (1 cm \times 8 cm), equilibrated with 10 mM-Tris/HCl buffer, pH 8.2, was loaded with \sim 4 mg of protein and was eluted in 2 ml fractions by stepwise addition of NaCl (-----).

pressure of O_2 at half saturation) ranging from about 0.3 to 0.8 kPa. Fig. 5 shows the O_2 -equilibrium data presented in the form of Hill plots and shows that the O_2 affinity of each fragment approaches that of the high-affinity R-state of the native protein. Furthermore, the single-functional unit fragments of *Lymnaea* haemocyanin studied so far show no evidence of a Bohr or a salt effect. However, over the pH range 7.2–8.2, a Bohr effect was observed with both of the three-functional unit fragments. Fig. 6 shows Hill plots for the heavier three-functional unit fragment 3DWPH at pH 6.8, 7.2 and 8.2. In contrast, the effect of changing $CaCl_2$ concentration on the O_2 -binding curves of these fragments was

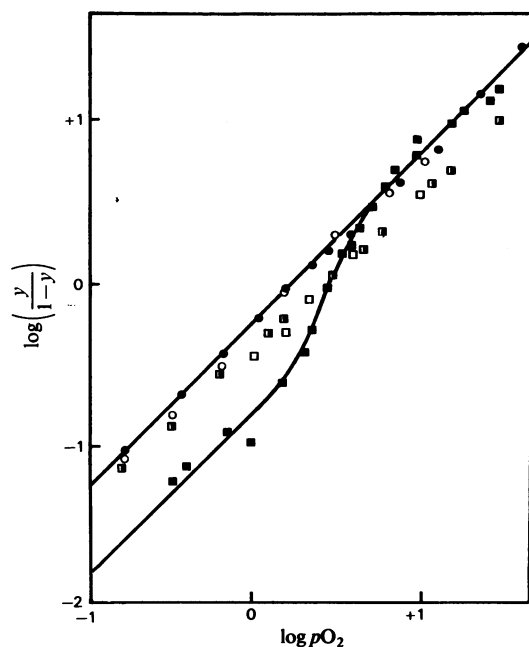


Fig. 5. Hill plot of O_2 equilibrium for native *L. stagnalis* haemocyanin and some of its fragments in the presence of 10 mM- $CaCl_2$ /10 mM-Hepes buffer, pH 7.2

Protein condition (see Table 1): \blacksquare , N; \square , 3DWPL; \blacksquare , 3DWPH; \circ , 1DCT; \bullet , 1DWT. The temperature was 20°C and the protein concentration was approx. 1 mg/ml.

negligible (see, e.g., Fig. 7). The results of the equilibrium studies are presented in Table 1. Both of the three-functional unit fragments studied exhibit a lower O_2 affinity than do any of the single-functional unit fragments, and under certain conditions the heavier of the two three-functional unit fragments 3DWPH exhibits a Hill coefficient somewhat less than 1.0.

Kinetic O_2 -binding experiments

Stopped-flow reaction curves of O_2 dissociation from native *Lymnaea* haemocyanin and some of its fragments are shown in Fig. 8. O_2 dissociation rate constants were determined from the reaction curves as previously described (Dawson & Wood, 1982), and are presented with the equilibrium binding data in Table 1. The dissociation rate constant varied from approx. $31 s^{-1}$ for the native protein, and approx. $14 s^{-1}$ for the 'heavy' three-functional unit fragment 3DWPH to a value of about $3 s^{-1}$ for the single-functional unit fragments. The changes in O_2 affinity observed in the equilibrium measurements are clearly reflected in the kinetic binding data, with an increase in O_2 affinity

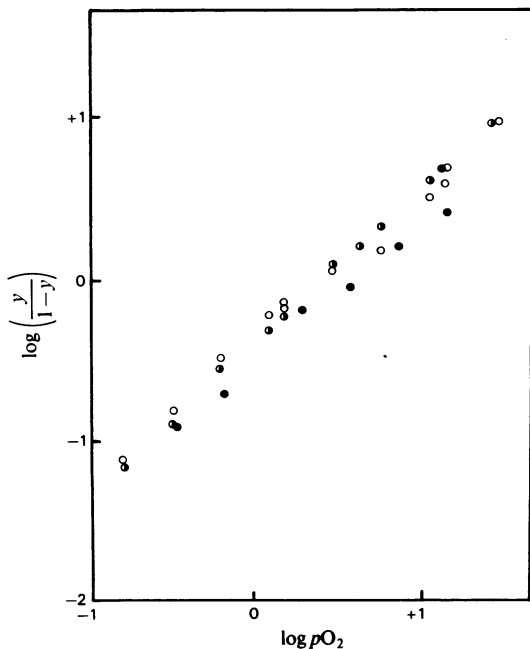


Fig. 6. Hill plot of O₂ equilibrium for a three-functional unit wall fragment (3DWPH) of *L. stagnalis* haemocyanin in the presence of 10 mM-CaCl₂/10 mM-Hepes buffer, pH 6.8 (○), pH 7.2 (◐) or pH 8.2 (●)

The temperature was 20°C and the protein concentration was approx. 1 mg/ml.

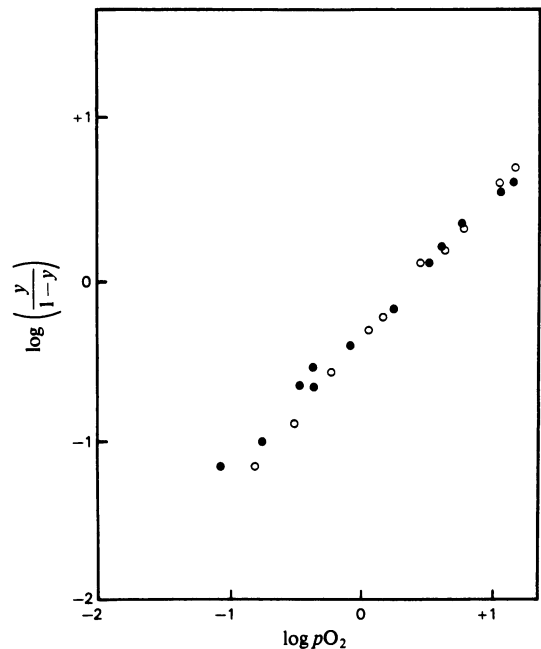


Fig. 7. Hill plot of O₂ equilibrium for a three-functional unit wall fragment (3DWPH) of *L. stagnalis* haemocyanin in the presence of 10 mM-Hepes buffer, pH 7.2, and 10 mM-(○) or 20 mM-(●) CaCl₂

The temperature was 20°C and the protein concentration was approx. 1 mg/ml.

Table 1. Equilibrium and kinetic O₂-binding data for *L. stagnalis* haemocyanin and its fragments

The conditions were as follows: buffer, 10 mM-Hepes; protein concentration, ~1 mg/ml; temperature, 20°C. Protein condition: N, native; 1DWT, tryptic one-functional unit (1-F.U.) 'wall' fragment; 1DWP, plasmin 1-F.U. 'wall' fragment; 1DCT, tryptic 1-F.U. 'collar' fragment; 3DWPL, plasmin 3-F.U. 'wall' fragment (light); 3DWPH, plasmin 3-F.U. 'wall' fragment (heavy). p_{50} is the partial pressure of O₂ at half saturation; h_{max} is the Hill coefficient at half saturation; k_{off} is the dissociation rate constant.

Protein condition	pH	[CaCl ₂] (mM)	p_{50}		h_{max}	k_{off} (s ⁻¹)
			(kPa)	(mmHg)		
N	7.0	10	~0.53	~4	~2	30.85
1DWT	7.0	10	0.27	2.0	0.96	1.22
1DWP	7.0	10	—	—	—	5.51
1DCT	7.0	10	0.26	1.95	1.07	3.19
3DWPL	7.0	10	0.38	2.88	1.13	—
3DWPL	8.2	10	0.55	4.17	1.15	—
3DWPH	7.0	10	0.42	3.16	0.97	14.97
3DWPH	8.2	10	0.77	5.75	0.78	13.03
3DWPH	7.0	20	0.42	3.16	1.02	14.85

accompanied by a decrease in the value of the O₂ dissociation rate constant. These data also show some kinetic heterogeneity between different single-functional unit fragments, but the kinetic origin of the Bohr effect with the three-functional unit fragments has not been established.

Discussion

The native *Lymnaea* haemocyanin molecule binds O₂ co-operatively with a normal Bohr effect and exhibits allosteric modulation by H⁺ and other ions. It is very unlikely that co-operative O₂ binding involves interaction between all 160 O₂-binding sites of the native molecule, and there is probably an 'allosteric unit' composed of a minimum number of interacting O₂-binding centres. In a previous paper (Dawson & Wood, 1982) we reported a computer-fitting procedure that was used to estimate values of r , the number of interacting O₂-binding centres, from equilibrium O₂-binding data. For native *Lymnaea* haemocyanin under conditions approximating to those *in vivo*, i.e. pH 7.5 and 5 mM-CaCl₂, we obtained a value of 5.15 for r , which

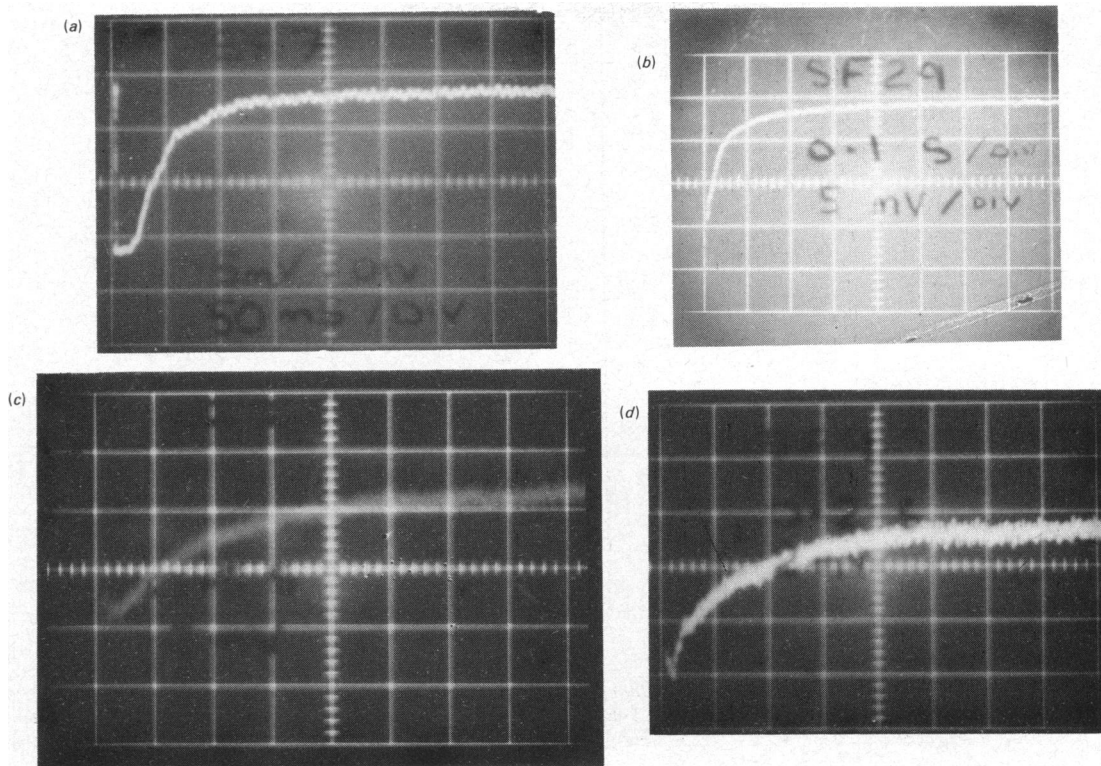


Fig. 8. Stopped-flow traces for reaction of native *L. stagnalis* haemocyanin and some of its fragments with dithionite (a) Native protein. Conditions: buffer, 10 mM-Hepes (pH 7.0)/10 mM- CaCl_2 ; final protein concentration was 0.5 mg/ml; temperature, 20°C; horizontal axis, 0.05 s/division. (b) Fragment 3DWPH (see Table 1). Conditions: buffer, 10 mM-Hepes (pH 7.0)/20 mM- CaCl_2 ; final protein concentration was ~0.5 mg/ml; temperature, 17°C; horizontal axis, 0.1 s/division. (c) Fragment 1DWT (see Table 1). Conditions: buffer, 10 mM-Hepes (pH 7.0)/10 mM- CaCl_2 ; final protein concentration was ~0.5 mg/ml; temperature, 20°C; horizontal axis, 0.5 s/division. (d) Fragment 1DCT (see Table 1). Conditions: buffer, 10 mM-Hepes (pH 7.0)/10 mM- CaCl_2 ; final protein concentration was 0.31 mg/ml; temperature, 20°C; horizontal axis, 0.2 s/division. For each experiment the protein and dithionite solutions were prepared from the same buffer. In each case the initial concentration of dithionite was 1.0 mg/ml and the monitoring wavelength was 400 nm.

suggests that the allosteric unit of this particular haemocyanin molecule is composed of approximately five O_2 -binding units. However, it is not possible to say at present whether the allosteric unit resides in, say, the individual subunit polypeptide chain (=eight functional units) or is formed by the juxtaposition of functional units from different subunits. In the present work we have studied the equilibrium and kinetic O_2 -binding behaviour of fragments obtained from *L. stagnalis* haemocyanin. Evidence from previous work on the proteolytic digestion of *L. stagnalis* haemocyanin (Gullick *et al.*, 1979, 1981) supported the proposal that the two single-functional unit fragments initially cleaved by trypsin correspond to the C-terminal 'collar' functional units, designated *g* and *h* of the original *a-h* subunit polypeptide chain. The other single-func-

tional unit fragment isolated in the present work is believed to correspond to unit *d*, the heavier three-functional unit fragment to units *a-b-c* and the lighter three-functional unit fragment to units *e-f-g* of the polypeptide chain respectively (see Gullick *et al.*, 1981).

O_2 -binding studies indicate functional heterogeneity between the different *Lymnaea* fragments. The single-functional unit fragments bound O_2 non-co-operatively with a Hill coefficient very close to 1.0, but showed variation in their O_2 affinities. Moreover, the O_2 -binding characteristics of these fragments were not affected by changes in the pH or CaCl_2 concentration. Both of the three-functional unit fragments (3DWPH and 3DWPL) displayed a lower O_2 affinity than did any of the single-functional unit fragments, and were also insensitive to changes

in CaCl₂ concentration. By equilibrium measurement, only the three-functional unit fragments (3DWPH and 3DWPL) showed a Bohr effect. Interestingly, the Bohr effect exhibited by these fragments is the reverse of the normal Bohr effect displayed by the native molecule. Moreover, at pH 8.2 the 'heavy' three-functional unit fragment exhibited a Hill coefficient significantly less than 1.0. Some workers have suggested that a Hill coefficient of less than 1.0 indicates the presence of more than one kind of O₂-binding species (Bonaventura *et al.*, 1977); however, this phenomenon may also be explained by intersite interaction within a single species, which is manifested as negative co-operativity. On the whole, the kinetic binding data reflected the equilibrium measurements of O₂ binding to the fragments of *Lymnaea* haemocyanin studied so far.

Reports of investigations of O₂ binding to fragments of molluscan haemocyanins are rare. However, in all reported cases, functional heterogeneity has been observed. Bonaventura *et al.* (1977) investigated the O₂-binding properties of functional units isolated from subtilisin digests of six molluscan haemocyanins. These workers found considerable variation in the O₂ affinity of different fragments of haemocyanin within one species, and between fragments obtained from different molluscan species. Values of the Hill coefficient were generally close to 1.0, but were frequently less than 1.0. Bohr effects were sometimes normal, sometimes reverse and sometimes negligible in functional units obtained from a haemocyanin that normally would display a strong Bohr effect. Moreover, some of the molluscan fragments studied showed sensitivity to the presence of 3 M-NaCl. Bonaventura *et al.* (1977) also investigated the O₂ dissociation kinetics of some of the molluscan fragments, using the stopped-flow technique. Large variations in the rates of O₂ dissociation were found (4–98 s⁻¹), with the k_{off} varying by a factor of 2 with *Nautilus pompilius* fragments to a factor of 20 with *Octopus* sp. fragments. Furthermore, these workers found that the differences in k_{off} did not parallel the differences in O₂ affinity observed in the equilibrium measurements, and hence suggested that there might also be significant differences in the O₂ association rate constant (k_{on}). More recently, van der Laan *et al.* (1981) investigated the equilibrium O₂-binding behaviour of wall and collar fragments of *Helix pomatia* β -haemocyanin. The three wall fragments investigated showed no significant difference in O₂ affinity and no obvious Bohr or salt effects. In contrast, the two collar fragments had different O₂ affinities, which were affected by pH and to some extent by the presence of CaCl₂ (see also Torensma *et al.*, 1981). In another paper the same workers and others (Torensma *et al.*, 1980) reported further

studies of O₂ binding to well-characterized tryptic wall fragments of *Helix pomatia* β -haemocyanin. A marked reverse Bohr effect was observed with the three-functional unit fragment *a-b-c* and with the single unit *c*, whereas the other fragments studied displayed little or no Bohr effect. A Hill coefficient of less than 1.0 was obtained with the *a-b-c* fragment, which was attributed to the different O₂ affinities of the constituent functional units. The p_{50} values obtained for the *a-b-c* fragment of 2.9 mmHg at pH 7, and 5.5 mmHg at pH 8.2, are in very good agreement with those obtained for the equivalent *Lymnaea* fragment (3DWPH) in the present work (Table 1).

The present O₂-binding studies confirm functional heterogeneity of the various fragments obtained by limited proteolysis of the haemocyanin from *Lymnaea stagnalis*, but it is impossible to say at present to what extent this functional heterogeneity of the fragments is responsible for the allosteric effects exhibited by the native molecule.

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