FUNCTIONAL PROPERTIES OF THE ISOLATED DOMAINS OF *HELIX POMATIA* β_c-HEMOCYANIN

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1. Introduction

Hemocyanins are large copper-containing proteins. They serve as oxygen carriers in many arthropods and molluscs and display allosteric behavior.

The hemocyanin of the mollusc *Helix pomatia* has a relative molecular mass of 9×10^6. It 75% dissociated into half molecules in 1 M NaCl or KCl in the stability region; this fraction was called α-hemocyanin. The fraction which does not dissociate under these conditions was called β-hemocyanin [1] consisting of a β_c-component which crystallizes at pH 5.3, I 10 mM, and of a β_s-component which remains soluble [2,3]. On raising the pH the components dissociate into half molecules, which yield further tenth and twentieth molecules.

The polypeptide chain of β_c-hemocyanin consists of 8 globular domains, like beads on a string, as can be seen from electron micrographs [4]. These functional domains, designated a–h, were isolated after limited proteolysis of tenth molecules with several enzymes [5]. They contain 2 copper atoms and have a relative molecular mass of 55 000 on average. Limited trypsinolysis of the undissociated cylindrical molecules removed the collar, leaving hollow cylinders which polymerized into tubes [6].

Oxygen binding by tubes proceeded with pronounced cooperativity. The slope of the high-affinity state was significantly <1, which indicated heterogeneity of the oxygen-binding sites. Similarly tenth molecules of β_c-hemocyanin of *H. pomatia* showed a Hill coefficient, h, of 0.9 under non-cooperative conditions at pH 8.1 [7].

Therefore the functional domains of wall fragments and of the polypeptide chain of β_c-hemocyanin of *H. pomatia* were isolated and their oxygen binding was determined at different pH values.

2. Experimental

H. pomatia β_c-hemocyanin and the tryptic fragments obtained on tenth molecules were isolated according to [2]. Tubular polymers were prepared by the method [6], as modified in [8]. Separation of the tubular polymers from the collar fraction and isolation of the tryptic wall fragments, consisting of 1, 2, 3, 4 and 6 domains, were performed according to [8]. The 3-domain fragment was split with subtilisin as in [9].

Oxygen binding was measured according to [7]. Any 'aged' active sites possibly formed were previously regenerated with hydrogen peroxide or hydroxylamine [10]. All reagents were analytical grade.

3. Results and discussion

Trypsinolysis of dissociated tubes comprising the 6- and 4-domain fragments yielded fragments consisting of 1, 2 and 3 domains. We were unable to separate the 4-domain from the 3-domain fragment, but further trypsinolysis of the mixture resulted in the appearance of the 1-domain fragment and an increased quantity of the 3-domain fragment. Comparison of these wall fragments by SDS–polyacrylamide gel electrophoresis with the well-characterized fragments

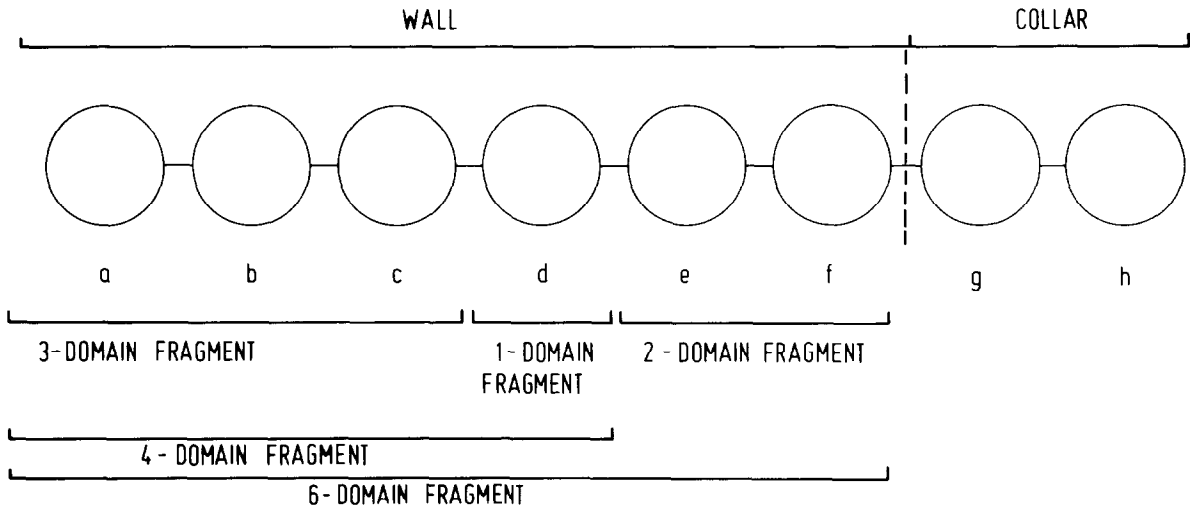

Fig.1. Sequence of the proteolytic fragments in the polypeptide chain of β_C-hemocyanin of *H. pomatia*. Domains a–h are represented by spheres. The amino-terminal end of the polypeptide chain is located in domain a. The fragments obtained after dissociation of the tubes are indicated.

obtained after proteolysis of tenth molecules, which were located in the polypeptide chain [11], allowed the alignment of the fragments (fig.1).

The oxygen affinity of the wall domains a–d, and of the fragment with domains e,f was measured as a function of pH (fig.2). These fragments bound oxygen with $h \approx 1$, in contrast with the 3-domain fragment a–c (table 1). Domain c showed a marked negative Bohr effect, while the other domains displayed no Bohr effect or a very small one.

The low Hill coefficient of fragment a–c seems thus to result from the differences in the oxygen affinity of the constituent domains: b with low, a with average, and c with relatively high affinity. Could the negative Bohr effect of domain c be the result of association of this domain at lower pH?

Domain g of the collar showed also low oxygen affinity (table 1) like domain b of the wall (fig.2). The C-terminal domain h was present as a dimer in borate–HCl buffer (pH 8.2) [11], which could be responsible for the lower Hill coefficient as a result of negative cooperativity.

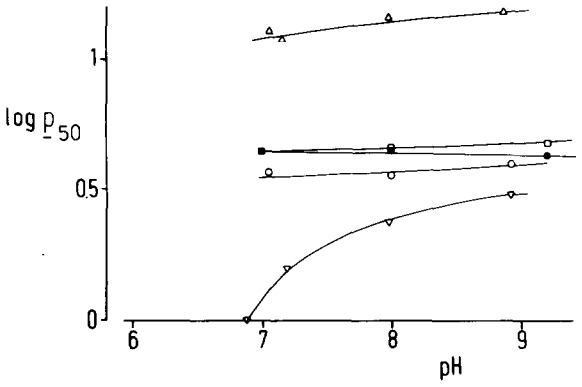

Fig.2. The oxygen affinity, expressed as log p_{50}, of the functional fragments of the wall of β_C-hemocyanin of *H. pomatia* as a function of pH in the following buffers: pH 6–7, 20 mM bis–Tris–HCl; pH 8–9, 20 mM Tris–HCl; pH >9, 20 mM ethanolamine–HCl. Fragments with domain a (○), b (△), c (▽), d (●), and with domains e,f (□).

Table 1
Oxygen-binding parameters of the a–c fragment of the wall and of the tryptic fragments of tenth molecules of β_C-hemocyanin of *H. pomatia*

Domain(s)	pH	p_{50}[a] (mmHg)	h[b]
a–c	7.0[c]	2.9	0.76
	8.4	5.2	0.76
	9.5	4.9	0.76
a–c	8.2[d]	5.5	0.81
d	8.2	4.2	0.92
e,f	8.2	4.6	0.93
g	8.2	8.2	0.98
h_2	8.2	4.8	0.87

[a] Oxygen pressure at which 50% of the binding sites are occupied with oxygen
[b] Slope of the oxygen-binding curve at 50% saturation
[c] The buffers are given in the legend of fig.2
[d] Measured in borate–HCl buffer (pH 8.2), I 0.1 M

These oxygen binding studies confirm that the domains isolated from the polypeptide chain of β_c-hemocyanin of *H. pomatia* by limited proteolysis are functional and that they show definite heterogeneity in their oxygen affinity.

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