THE FORMATION OF *HELIX POMATIA* METHAEMOCYANIN ACCELERATED BY AZIDE AND FLUORIDE

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1. Introduction

Haemocyanin, the oxygen-carrying pigment from the haemolymph of the mollusc *Helix pomatia*, shows two copper–oxygen absorption bands with a maximum at 346 and at 580 nm. These bands decrease slowly on storage [1]. They reach about half their original value in one year and can be regenerated with hydrogen peroxide [2] and with hydrogen sulphide [3].

A considerable decrease of the copper bands after a few weeks, observed in the separation of α- and β-haemocyanin in the presence of 1 M NaCl at pH 5.7, incited us to investigate the influence of halogenides. Similarly the abnormal bleaching of the colour of fresh haemocyanin by the addition of 3 mM sodium azide as a preservative led us to try the action of azide (C. Gielens, unpublished observation).

2. Materials and methods

H. pomatia haemocyanin was prepared according to Heirwegh et al. [4] and stored at 4°C in 0.1 M acetate buffer, pH 5.7. Protein concentrations were measured in a Beckman DU spectrophotometer at 278 nm: $A(1\%, 1\text{ cm}) = 14.16$ at pH 9.2.

Limulus polyphemus haemolymph was centrifugated to remove coagulated material and dialysed against distilled water, the precipitate was removed by centrifugation. The supernatant was dialysed against 0.1 M acetate buffer pH 5.7 and stored at 4°C.

The absorption measurements were carried out with a Cary 16 spectrophotometer (Monrovia, Calif., USA) equipped with a sample changer thermostatted at 37°C and a recorder interface accessory. Circular-dichroic spectra from 300 to 800 nm were recorded on a Cary 61 spectropolarimeter using stoppered cells of 0.2 and 1 cm pathlength, thermostatted at 20°C. The circular-dichroic spectra from 600 to 1000 nm were recorded at the Laboratoire de Spectroscopie biomoléculaire, Institut de Biologie moléculaire de la Faculté des Sciences de Paris on an instrument of their own design. The molar circular dichroism $\Delta\epsilon$ ($1\ \text{mole}^{-1}\ \text{cm}^{-1}$) is expressed per mole of Cu. The spectra recorded in wavelengths λ (nm) are plotted in wavenumbers σ (cm^{-1}).

3. Results

Preliminary experiments with azide and fluoride showed the decrease of the copper bands to diminish with increasing pH. As the dissociation into halves

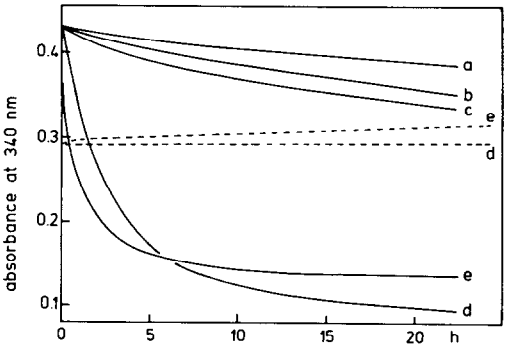

Fig.1. Decrease at 340 nm of the copper band of *Helix pomatia* oxyhaemocyanin (0.1%, 1 cm) (——) at 37°C in 0.1 M sodium acetate buffer pH 4.90 (a) and in the same buffer in the presence of 0.1 M potassium bromide (b), sodium chloride (c), potassium fluoride (d) and sodium azide (e). Similar experiments with *Limulus polyphemus* oxyhaemocyanin (----) in the presence of 0.1 M potassium fluoride (d) and 25 mM sodium azide (e).

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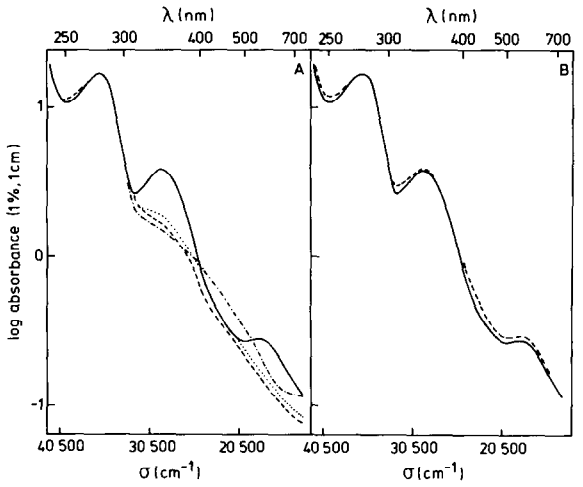

Fig.2. (A) Absorption spectrum of *Helix pomatia* oxyhaemocyanin (——), of oxyhaemocyanin treated at 37°C for 48 h at pH 5.0 with 0.1 M potassium fluoride (----) or 25 mM sodium azide (. . . .) after removal of the reagents by dialysis. Spectrum of the latter solution in the presence of 0.1 M sodium azide at pH 5.0 (·····). (B) Absorption spectrum after reduction with hydrogen peroxide of the solutions treated with azide or fluoride (----), oxyhaemocyanin blank (——).

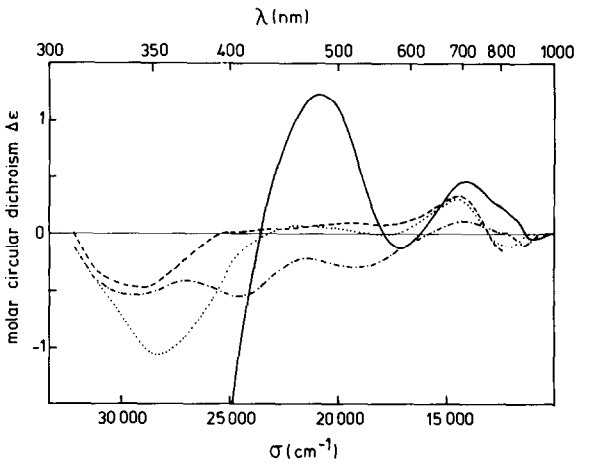

Fig.3. Molar circular dichroism Δε (1 mole^{-1} cm^{-1}) of the solutions presented on fig.1A: oxyhaemocyanin (——), oxyhaemocyanin treated with 0.1 M potassium fluoride (----) or 25 mM azide (. . . .) after removal of the reagents by dialysis. the latter solution in the presence of 0.1 M sodium azide (·····).

of *H. pomatia* haemocyanin already starts at pH 4.75 and proceeds to tenths on further lowering the pH [5], a pH of 4.90 (0.1 M sodium acetate buffer) was chosen in order to study the decrease of the copper band at 340 nm (the maximum of the copper band in the presence of light scattering) (fig.1). The decrease was small in acetic acid–acetate buffer, there was a definite influence of the addition of 0.1 M bromide and 0.1 M chloride. The effect of 0.1 M fluoride was striking: after 1 day the copper–oxygen bands had nearly completely vanished, the remaining absorbance approaching the residual value due to the light scattering of haemocyanin. The decrease of the absorbance with 0.1 M azide was very fast, but seemed more complex owing to the development of a brownish colour. The copper band disappeared faster initially than with fluoride, the final absorption spectrum did not show a pronounced new band (fig.2A). The brown colour disappeared when azide was removed by dialysis. The copper bands, which did not reappear on removal of azide or fluoride by dialysis, were recovered by treatment with hydrogen peroxide in a ten-fold excess with respect to the copper present in haemocyanin (fig.2B). With hydrogen sulphide a complete reduction in a few seconds of methaemocyanin, prepared by the fluoride treatment, was observed [3].

Circular-dichroic spectra of *H. pomatia* methaemocyanin formed by the action of azide and fluoride on oxyhaemocyanin were recorded from 300 to 1000 nm (fig.3). The residual copper band at 346 nm appeared more clearly for both preparations than in the absorption spectrum. By the addition of azide several new bands were found corresponding to the increase in absorbance between 300 and 750 nm.

The extent of the reaction with azide and fluoride increased on lowering the pH, the results for azide are presented on fig.4. Logarithmic plots of these absorbances against time are linear for fluoride concentrations below 50 mM and for azide concentrations below 5 mM. The pseudo first-order constants are given in table 1. At higher concentrations of these reagents the logarithmic plots deviate from linearity.

Experiments with the oxyhaemocyanin of the arthropod *L- polyphemus* did not show any influence at 37°C of 0.1 M potassium fluoride nor 25 mM sodium azide in 0.1 M sodium acetate buffer pH 4.90 (fig.1). Under similar conditions there was no formation of methaemocyanin of *H. pomatia* when the reac-

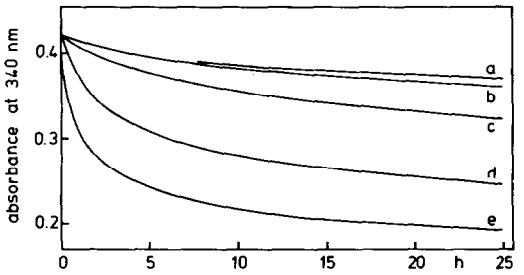

Fig.4. Influence of the pH on the decrease at 340 nm of the copper band of *Helix pomatia* oxyhaemocyanin (0.1%, 1cm) at 37°C in the presence of 25 mM azide in 0.1 M buffers: borate, pH 9.22 (a), Tris–HCl, pH 8.00 (b), phosphate, pH 6.89 (c), sodium acetate, pH 5.80 (d) and pH 5.01 (e).

tion was carried out under nitrogen. Obviously only the oxygenated form of *H. pomatia* haemocyanin reacted with azide or fluoride.

4. Discussion

The spectrum of oxyhaemocyanin points to the presence of 1 O_2^{2-} and 2 Cu^{2+} per active site [6]. The action of azide or fluoride on the oxyhaemocyanin of *H. pomatia* (fig.1) could thus correspond to a displacement of peroxide. It seems very similar to the effect of these anions on oxyhaemerythrin [7] and analogous to the displacement of superoxide by these anions in oxyhaemoglobin [8].

The formation of methaemocyanin (the presence of a Cu^{2+} pair per active site) seems in accordance with the weak positive transition at 700 nm, observed in circular dichroism after removal of the reagents (fig.3), and the possibility of regeneration of the copper bands with traces of hydrogen peroxide (fig. 2B) or hydrogen sulphide [3]. The weak new bands observed after the addition of 0.1 M azide to the treated and dialysed solutions (fig.2A and 3) provide another indication for the presence of Cu^{2+}.

The influence of the pH on the rate of the reaction with azide (fig.4) is reminiscent of the action of hydrogen sulphide [3], where the reaction rate yielded a pK of 7.20 against 6.85, the value of the first pK of hydrogen sulphide. The pK value of hydrogen azide and hydrogen sulphide are 4.76 and 3.2 respectively.

The absence of a reaction of azide or fluoride with the oxyhaemocyanin of *L. polyphemus* emphasizes

Table 1
Pseudo first-order rate constants k' for the reaction of azide and fluoride with *Helix pomatia* oxyhaemocyanin

Azide (mM)	k' (h^{-1})	Fluoride (mM)	k' (h^{-1})
1	0.22	10	0.11
2	0.23	20	0.14
3	0.26	50	0.25

The reactions were carried out at 37°C in 0.1 M acetate buffer pH 4.90. Haemocyanin–copper concentration was 90 μM.

the differences between the active site of arthropodan and molluscan haemocyanin, suggesting for the former a μ-peroxo structure as proposed by Bannister and Wood [9]. The rather easy displacement of peroxide by azide or fluoride for a molluscan haemocyanin points to a lateral binding of peroxide with possibly a bridging ligand according to the model suggested by Fager and Alben [10] for the binding of carbon monoxide by haemocyanin. An imidazole anion would explain the observed coupling of the copper atoms.

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