THE PRESERVATION OF HAEMOCYANIN UNDER CARBON MONOXIDE

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1. Introduction

The carbon monoxide combining capacity of haemocyanin has been in dispute for a long time. Root [1] concludes that binding occurs with *Limulus* haemocyanin from the solubility increase in serum of carbon monoxide by the presence of haemocyanin, and from the shift to the right of the oxygen dissociation curve. Rawlinson [2], on the contrary, does not find a difference in solubility of carbon monoxide between water and a solution of *Palinurus vulgaris* haemocyanin and consequently assumes no combination. Using ^{14}C-labelled carbon monoxide, Vanneste and Mason [3] could demonstrate the binding of carbon monoxide by *Cancer magister* haemocyanin.

We shall prove the formation of a compound with *Helix pomatia* haemocyanin by studying the competition between carbon monoxide and oxygen for the copper groups, and by measuring the solubility of carbon monoxide in a buffer with and without haemocyanin. During storage haemocyanin undergoes an ageing process, consisting in a decrease of the absorbance at 346 nm [4] and a conversion of a sigmoidal to a hyperbolic oxygen dissociation curve. We shall try to prevent this transformation by storage under carbon monoxide, a method currently used for haemoglobin [5].

2. Materials and methods

2.1. *Haemocyanin*

Helix pomatia haemocyanin is prepared according to Heirwegh et al. [6].

2.2. *Competition between carbon monoxide and oxygen*

The competition for the copper groups is investigated by equilibration of haemocyanin solutions in a tonometer with given mixtures of carbon monoxide and oxygen, their composition being determinated with the Oxygen Analyzer (Model E2, Beckman, Fullerton, California, USA). The absorbance at 346 nm is measured in a Beckman DU spectrophotometer (München, Germany).

2.3. *Solubility of carbon monoxide*

The solubility of gases in aqueous solutions can be determined by means of the gas chromatograph (Model C, Type ATC/f, Carlo Erba, Milano, Italy), fitted for this purpose with an extraction chamber, that can be brought in the stream of the hydrogen carrier gas. This chamber contains a solution consisting of 0.1 M HCl, 0.2 M KSCN, and a few drops of an antifoaming agent. A given volume (about 0.4 ml) of the solution under investigation is injected. Calibration is made by injection of distilled water, saturated at constant temperature with carbon monoxide at atmospheric pressure.

2.4. *Storage of haemocyanin solutions*

A set of haemocyanin solutions (80 g/l, 0.1 M sodium acetate buffer, pH 5.7) is saturated with carbon monoxide and stored in sealed tubes at 4°C in the dark. Every month one tube is opened, the contents are saturated with air. After dilution with half-saturated disodium tetraborate, pH 9.2, in order to reduce light-scattering [6], the absorbance is measured at 278 and 346 nm. From these values

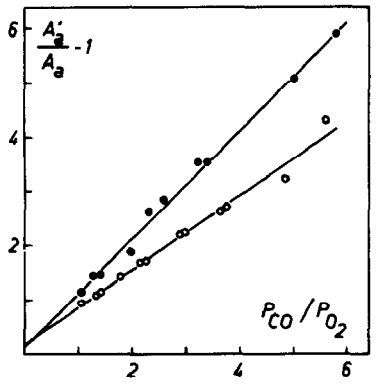

Fig. 1. The competition for haemocyanin between carbon monoxide and oxygen at pH 5.7 (o——o) and 9.2 (•——•), protein concentration 1 g/l, 22°C.

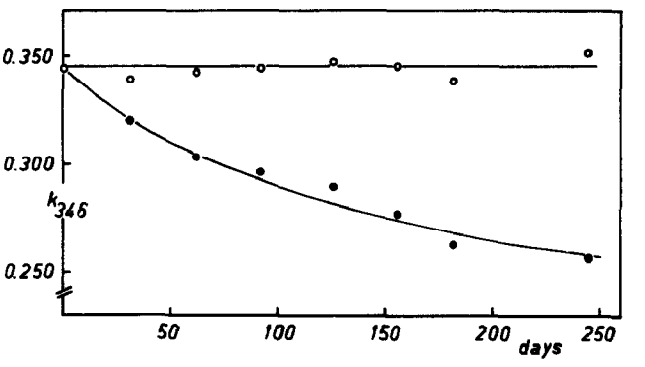

Fig. 2. The absorbance (1 g/l, 1 cm) at 346 nm as a function of time for haemocyanin solutions stored at 4°C at pH 5.7 under carbon monoxide (o——o) and at the air (•——•).

k_{346} (the absorbance for a haemocyanin concentration of 1 g/l and a path length of 1 cm) is calculated. The same experiments were carried out with haemocyanin solutions stored at the air.

2.5. *Oxygen dissociation curves*

Fresh haemocyanin gives a sigmoidal oxygen dissociation curve in the presence of 6 mM Ca^{2+} at a pH between 7.8 and 8.2. During ageing there is a gradual change to a hyperbolic curve.

The solution in a tonometer according to Pantin and Hogben [7] is completely deoxygenated until the absorbance at 346 nm remains constant. The subtraction of the residual absorbance from k_{346} yields k_a values. After each increase of the oxygen pressure, the solution is equilibrated for 20 min in an air thermostat, and the absorbance measured at 346 nm. The value for complete saturation is obtained with pure oxygen.

3. Results and discussion

Supposing all binding sites (S) identical and independent, the competition can be represented as follows:

$$S\text{-}O_2 + CO \overset{K}{\rightleftharpoons} S\text{-}CO + O_2$$

$$\frac{[S\text{-}CO]\,[O_2]}{[S\text{-}O_2]\,[CO]} = K \text{ or } \frac{A'_a}{A_a} - 1 = K\frac{p_{CO}}{p_{O_2}},$$

where A_a represents the absorbance at 346 nm of the oxygenated copper groups and A'_a the value of A_a at complete saturation with oxygen. Experiments were carried out in 0.1 M sodium acetate buffer, pH 5.7, and in half-saturated disodium tetraborate, pH 9.2. The relation between $A'_a/A_a - 1$ and p_{CO}/p_{O_2} is represented by a straight line in both cases (fig. 1). The value of K equals 0.69 at pH 5.7 and 1.00 at pH 9.2.

The amount of carbon monoxide bound to haemocyanin has been determined by gas chromatography. After complete saturation at 17.5°C a volume of 0.395 ml sodium acetate buffer (pH 5.7, μ 0.1) dissolves 450 nmol carbon monoxide. The same experiment in the presence of haemocyanin (80.5 g/l, k_{346} = 0.309) yields 947 nmol. The difference, 497 nmol, corresponds to 95% of the theoretical amount of carbon monoxide bound to haemocyanin.

This property enables us to prevent the ageing of haemocyanin. A series of haemocyanin solutions is treated as mentioned under 2.4. The values of k_{346} on reoxygenation are plotted against time (fig. 2). They remain constant for solutions stored under carbon monoxide, whereas they show the usual dexrease for samples stored at the air.

Oxygen dissociation curves are measured at pH 7.9 in the presence of 6 mM Ca^{2+}. The solutions stored for up to 11 months under carbon monoxide, yield a sigmoid, whereas in the case of air, a hyperbola is obtained (fig. 3).

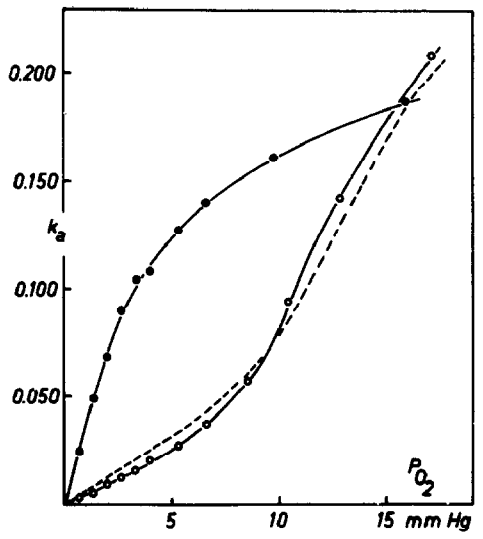

Fig. 3. Oxygen dissociation curves (1 g/l) in the presence of 6 mM Ca^{2+} at pH 7.9 of samples stored for 11 months under carbon monoxide (o——o) and at the air (•——•). The dashed line represents an oxygen dissociation curve for fresh haemocyanin.

4. Summary

A compound is formed between *Helix pomatia* haemocyanin and carbon monoxide. One carbon monoxide replaces one oxygen molecule. From the theoretical amount of carbon monoxide bound to haemocyanin 95% could be recovered. Storage of haemocyanin under carbon monoxide prevents it from ageing, the absorbance at 346 nm on reoxygenation remains constant and the oxygen dissociation curve sigmoidal.

Acknowledgements

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References

[1] R.W.Root, J. Biol. Chem. 104 (1934) 239.
[2] W.A.Rawlinson, Aust. J. Exptl. Biol. Med. Sci. 18 (1940) 131.
[3] W.Vanneste and H.S.Mason, in: The Biochemistry of Copper, eds. J.Peisach, P.Aisen and W.E.Blumberg (Academic Press, New York, London, 1966) p. 465.
[4] K.Heirwegh and R.Lontie, Nature 185 (1960) 854.
[5] D.L.Drabkin, J. Biol. Chem. 164 (1946) 703.
[6] K.Heirwegh, H.Borginon and R.Lontie, Biochim. Biophys. Acta 48 (1961) 517.
[7] C.F.A.Pantin and L.T.Hogben, J. Mar. Biol. Assoc. U.K. (1925) 970.