THE FAST REDUCTION OF *HELIX POMATIA* METHAEMOCYANIN WITH HYDROGEN SULPHIDE

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1. Introduction

Oxygen binds reversibly to the copper of *Helix pomatia* haemocyanin in a ratio of 1 O_2/ 2 Cu, giving rise to absorption bands at 346 and 580 nm [1]. These bands show a continuous decrease when haemocyanin solutions are stored in air over a period of months [2]. This ageing can be prevented by storage of haemocyanin under carbon monoxide [3]. Aged haemocyanin preparations can be regenerated by treatment with hydrogen peroxide, cysteine [4] or hydroxylamine [5]. It was clearly demonstrated that the very slow regeneration with cysteine is mediated by hydrogen peroxide [6]. This paper reports the fast regeneration of aged haemocyanin by treatment with small amounts of sulphide.

2. Materials and methods

2.1. *Haemocyanin*

Haemocyanin was prepared as previously described [7] and allowed to undergo natural ageing during storage in 0.1 M acetate buffer, pH 5.7, in the cold room at 4°C for about 18 months, resulting in a decrease of A_{346} (0.1%, 1 cm) from 0.345 to 0.174. Haemocyanin was artificially aged from A_{346} (0.1%, 1 cm) of 0.345 to 0.072 by keeping it for 48 hr at 37°C in 0.1 M acetate buffer, pH 5.0, in the presence of 0.1 M fluoride, which was afterwards removed by dialysis (R. Witters and R. Lontie, unpublished results). As the same results were obtained with naturally and artificially aged haemocyanin, all the reported experiments were carried out with the latter.

2.2. *Sulphide*

Sulphide solutions were prepared freshly from ammonium sulphide (16% solution, p.a., UCB, Brussels, Belgium). Its concentration was determined by iodometric titration [8], and expressed as 'total' sulphide.

2.3. *Stopped-flow kinetics*

The rapid regeneration at 22°C was followed at 346 nm in the 20 mm cuvette of a stopped-flow spectrophotometer D-110 (Durrum, Palo Alto, California). The time course of the absorbance change was stored in digital form in a Transient Recorder 802 (Biomation, Palo Alto, California), connected to a calculator HP 9810A and a plotter HP 9862A (Hewlett Packard, Loveland, Colorado), for automatic data treatment.

3. Results

Regeneration was performed by mixing equal volumes of aged haemocyanin and sulphide solutions (in the same buffer) in the stopped-flow apparatus. The typical time course of the absorbance change at 346 nm is shown in fig.1a. Plotting the data on a semi-log scale shows that the reaction is strictly first order with respect to aged haemocyanin (fig.1b).

The concentration of sulphide was varied between 0.06 and 0.4 mM in three sets of experiments at pH 5.95 (phosphate buffer, I 0.1), 6.92 (phosphate buffer, I 0.1), and 8.05 (borate-HCl buffer, I 0.1). The linear relationship between log k and log [sulphide] is shown in fig.2. From the slope of these straight lines orders

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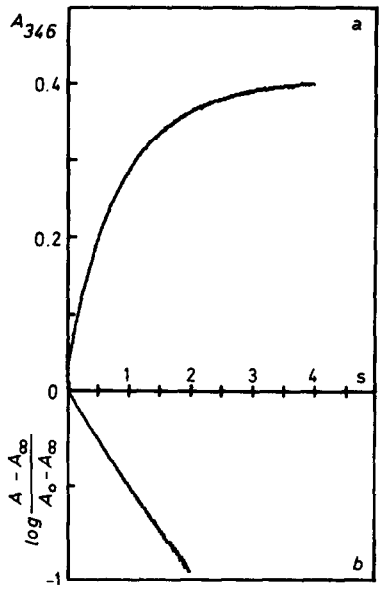

Fig.1. (a) Reduction of *Helix pomatia* methaemocyanin (0.91 g/l, corresponding to 25.7 μM Cu^{2+}, in borate-HCl buffer, pH 8.05, I 0.1) by sulphide (244 μM) as measured at 22°C in the 20 mm cuvette of the stopped-flow spectrophotometer; (b) same data as in a. plotted on a semi-log scale according to first-order kinetics.

of 1.10, 0.97, and 1.41 for sulphide resp. at pH 5.95, 6.92, and 8.05 are calculated. At a given pH the total absorbance change at 346 nm decreases with increasing sulphide concentration, as shown in fig.3. This decrease

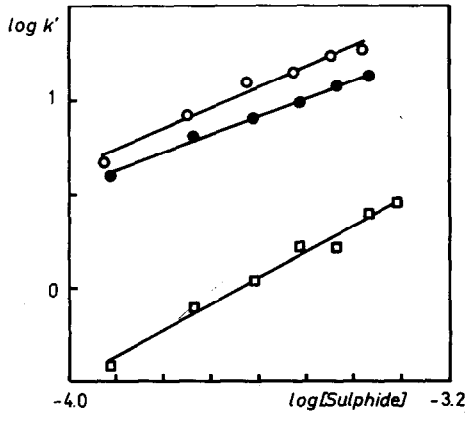

Fig.2. Relationship between pseudo-first-order rate constant (sec^{-1}) and sulphide concentration at pH 5.95 (○), 6.92 (●), and 8.05 (□).

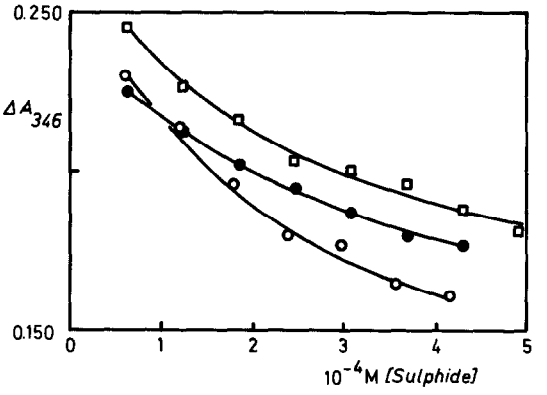

Fig.3. Apparent amount of regeneration, expressed as ΔA_{346} (1 g/l, 1 cm), as a function of sulphide concentration at pH 5.95 (○), 6.92 (●), and 8.05 (□).

is explained by a competition between sulphide and oxygen for binding to the copper groups of the regenerated haemocyanin. Haemocyanin, freshly regenerated with hydrogen peroxide, was therefore mixed with different amounts of sulphide at pH 8.2 (borate–HCl buffer, I 0.1). It was shown that sulphide competes with oxygen for binding to the copper groups in an apparent stoicheiometry of 0.72 H_2S/2 Cu (7 experiments between 0.07 and 0.99 mM sulphide, correlation coefficient = 0.999). A similar conclusion can be drawn from the data of fig.3.

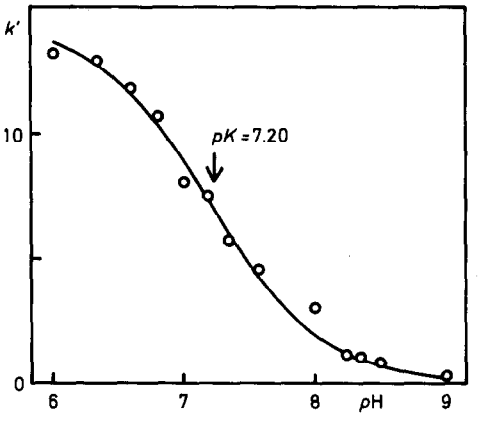

Fig.4. Pseudo-first-order rate constant, k' in sec^{-1}, for the reduction of *Helix pomatia* methaemocyanin (0.91 g/l) by sulphide (239 μM total sulphide) as a function of pH. The curve was drawn according to the Henderson-Hasselbalch equation with pK = 7.20.

At constant sulphide concentration the rate of regeneration decreased with increasing pH. This effect was studied by performing experiments at several pH values between 6 and 9 with 0.239 mM 'total' sulphide. A plot of the pseudo-first-order rate constant as a function of pH has the shape of a titration curve (fig.4). A calculated curve was drawn through the experimental points according to the Henderson-Hasselbalch equation with $pK = 7.20$.

4. Discussion

The experiments reported in this paper are summarized in the following scheme:

$$Cu^{2+} \ldots Cu^{2+} + H_2S \longrightarrow Cu^{+} \ldots Cu^{+} + S_8 \text{ or } HS_n^-$$

$$Cu^{+} \ldots Cu^{+} + O_2 \underset{\text{very fast}}{\rightleftharpoons} Cu^{2+} \ldots Cu^{2+}/O_2^{2-} \quad \text{(abs. at 346 nm)}$$

$$Cu^{+} \ldots Cu^{+} + H_2S \text{ or } HS^- \text{ (excess)} \rightleftharpoons Cu^{+} \ldots Cu^{+}/H_2S \text{ or } HS^-$$

The Cu^{2+} pairs, which are present in aged haemocyanin (C. Gielens and R. Lontie, unpublished results), are reduced by hydrogen sulphide, the resulting Cu^{+} pairs recombine with oxygen very rapidly (in less than 20 ms). The excess sulphide, however, competes reversibly with oxygen for binding to the same groups and thus causes the apparent extent of regeneration to depend on it.

The regeneration of aged haemocyanin involves a group with $pK_a = 7.2$, which is rather close to 6.85, the first pK'_a (at I 0.1) of hydrogen sulphide. A point of uncertainty, however, remains the relative solubility and the pK_a of H_2S in the protein compared to the aqueous solution. It is known that H_2S is much more soluble in hydrophobic media than in water. The apparent competition stoicheiometry of 0.72 H_2S/ 2 Cu could also be due to binding of H_2S in hydrophobic regions of the protein, thus lowering the free concentration of sulphide. This needs, however, further investigation. The somewhat higher order (1.4) at pH 8.05 indicates a mechanism at a higher pH leading to HS_n^-, e.g. the simultaneous reaction of two HS^- leading to HS_2^- instead of one H_2S giving S_8 at near neutral pH.

Acknowledgements

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