The Regeneration with Carbon Monoxide of Gastropodan Met- and Nitrosylhaemocyanins

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Molluscan methaemocyanins can be regenerated with hydrogen peroxide [1], hydroxylamine [2] and traces of hydrogen sulphide [3]. In contrast, the cheliceratan methaemocyanin of *Limulus polyphemus* can only be regenerated with hydrogen sulphide or hydrogen cyanide [4], whereas crustacean methaemocyanins cannot be regenerated. The nitrosylhaemocyanin of *Helix pomatia* has been reported to be regenerated with a large excess of hydroxylamine [5].

In this paper we demonstrate that carbon monoxide is able to regenerate completely the nitrosyl- and methaemocyanins of *H. pomatia* and *Busycon canaliculatum* in a very slow reaction. The reaction is favoured at alkaline pH. The nitrosyl- and methaemocyanins of *L. polyphemus* and *Astacus leptodactylus* are not regenerated by carbon monoxide.

Experimental

Methaemocyanin was obtained by treating oxyhaemocyanin with 100 m*M* potassium fluoride (*H. pomatia*) or 50 m*M* sodium azide (*B. canaliculatum*) for two days at 37 °C in 0.1 *M* sodium acetate buffer pH 5.0 [6]. The methaemocyanins of *L. polyphemus* and *A. leptodactylus* were prepared by anaerobic treatment with hydrogen peroxide [7]. Nitrosylhaemocyanin was obtained by anaerobic treatment of deoxyhaemocyanin solutions with nitrogen monoxide 'N20' (L'Air Liquide Belge, Schelle, Antwerp) for 10 hours, followed by 1 hour flushing with nitrogen 'A28' (L'Air Liquide Belge). Semi-methaemocyanin [Cu(II)Cu(I)] of *H. pomatia* was obtained by dialysis of the nitrosylhaemocyanin for 4 weeks against 0.1 *M* sodium acetate buffer pH 5.0. In this way NO^+ is dispelled from the active site and replaced by acetate.

Electron paramagnetic resonance (EPR) measurements were carried out with an E-109 spectrometer

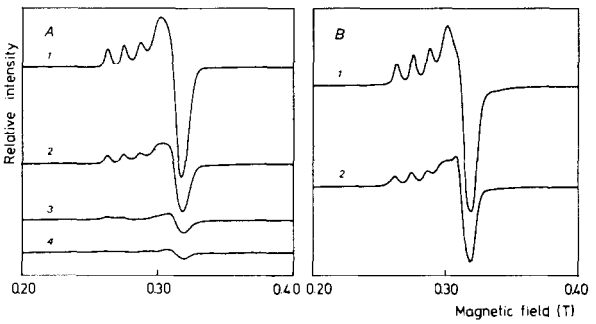

Fig 1. EPR Spectra at 97 K of *Helix pomatia* nitrosyl haemocyanin (34 mg/ml) under 1 bar CO: (A) in 0.1 *M* sodium borate–HCl buffer pH 8.2: time zero (1), after 12 days (2), 41 days (3), and 6 months (4); (B) in 0.1 *M* sodium acetate buffer pH 5.0: time zero (1) and after 6 months (2). Instrument settings: microwave frequency 9.127 GHz, microwave power 30 mW, field modulation amplitude 1 mT, receiver gain 3.2×10^3.

(Varian, Palo Alto, Calif.) at 97 K, microwave frequency 9.127 GHz, field modulation amplitude 1 mT and microwave power 30 mW. Luminescence spectra were recorded with a corrected fluorescence spectrophotometer MPF-44A (Perkin-Elmer, Norwal, Conn.).

CO incubations of the haemocyanin solutions were carried out in stainless steel pressure vessels. Air was first expelled by flushing for 30 min with CO. After adjusting the CO pressure the reaction vessels were stored at room temperature. Carbon monoxide was 'N37' (L'Air Liquide Belge).

Results and Discussion

The green solution of *H. pomatia* nitrosylhaemocyanin in 0.1 *M* sodium borate-HCl buffer pH 8.2 turned colourless upon prolonged storage (4 weeks) under a CO atmosphere (16 bar). After exposure to the air 95% oxyhaemocyanin was obtained. In the course of the reaction all absorption bands between 300 and 900 nm gradually disappeared and there was a progressive increase of emission intensity at 550 nm upon excitation at 285 nm. This phosphorescence was shown to originate from a Cu(I)–carbon monoxide complex by a charge-transfer mechanism [8]. Also, the mononuclear EPR signal of the nitrosylhaemocyanin in 0.1 *M* sodium borate-HCl pH 8.2 was almost completely lost after reaction with CO (Fig. 1A). Similarly, semi-methaemocyanin of *H. pomatia* in 0.1 *M* sodium borate–HCl pH 8.2, stored under 35 bar CO for 6 weeks, yielded 92%

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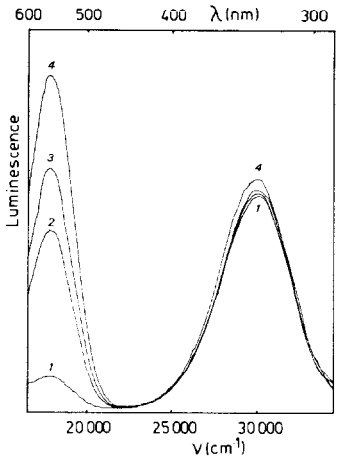

Fig. 2. Luminescence spectra (arbitrary units) of *Helix pomatia* methaemocyanin in 0.1 *M* sodium borate–HCl buffer pH 8.2 under 1 bar CO: time zero (1), after 17 (2), 24 (3) and 31 days (4). Excitation at 285 nm.

oxyhaemocyanin after exposure to the air. Thus the NO^+ ligand is not likely to be involved in the reaction.

Treatment of *H. pomatia* methaemocyanin in 0.1 *M* sodium borate–HCl pH 8.2 with 16 bar CO for 5 weeks, followed by exposure to the air, resulted in an almost complete regeneration (95 to 100%) of the methaemocyanin. Here also a gradual decrease of the weak absorption band and a concomitant increase in the emission intensity at 550 nm was observed (Fig. 2).

These spectral observations indicate that both nitrosyl- and methaemocyanin are reduced by carbon monoxide to a phosphorescent Cu(I)–CO derivative, which is then converted to oxyhaemocyanin by exposure to the air. Carbon monoxide is most likely oxidized to carbon dioxide. The reaction was only slightly accelerated at higher CO pressures (48 bar) and already showed a reasonable rate at 1 bar. The regeneration was, however, much slower at pH 5.0 in 0.1 *M* sodium acetate buffer for both nitrosyl- (Fig. 1B) and methaemocyanin. This pH dependence is the reverse of that observed for regeneration with hydrogen sulphide, excluding a regeneration due to traces of the latter in the CO gas.

The oxygen-binding capacity and cooperativity of CO-regenerated nitrosylhaemocyanin are shown in Fig. 3. From the Hill plot a coefficient h of 3.7 and a p_{50} value of 9.1 mmHg were obtained, to be compared with h = 4.0 and p_{50} = 9.3 mmHg for native *H. pomatia* haemocyanin. For CO-regenerated methaemocyanin identical results were obtained. Hence the difference in cooperativity and affinity is small and it is clear that functional oxyhaemocyanin is obtained. A nearly complete regeneration (90 to 95% oxyhaemocyanin) was also observed for *B. canaliculatum* met- and nitrosylhaemocyanin upon treatment with 16 bar CO in 0.1 *M* sodium borate–HCl buffer pH 8.2 for 6 weeks.

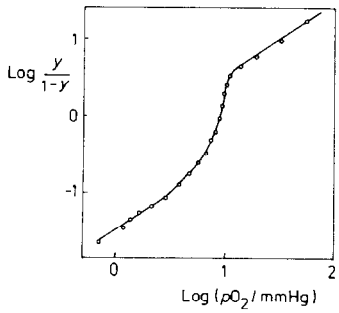

Fig. 3. Hill plot of the oxygen-binding curve of CO-regenerated nitrosylhaemocyanin *(Helix pomatia)* in 0.1 *M* sodium borate–HCl pH 8.2 in the presence of 10 m*M* $CaCl_2$.

Both the met- and nitrosylhaemocyanins of *L. polyphemus* and of *A. leptodactylus* were unreactive to CO. In spite of long incubation times (2 months) and high pressure (50 bar CO), no regeneration was observed.

The regeneration of methaemocyanin by CO can schematically be presented as follows:

$$\mathrm{Cu(II)Cu(II) + 2CO + H_2O \longrightarrow}$$

$$\mathrm{Cu(I)Cu(I)CO + CO_2 + 2H^+}$$

$$\mathrm{Cu(I)Cu(I)CO \rightleftharpoons Cu(I)Cu(I) + CO}$$

$$\mathrm{Cu(I)Cu(I) + O_2 \rightleftharpoons Cu(II)Cu(II)O_2^{2-}} \qquad (1)$$

Byerley and Peters [9] reported the reduction of inorganic Cu(II) to Cu(I) in aqueous solutions by CO at 120 °C and CO pressures from 67 to 1342 bar. They postulated the formation of a CO insertion intermediate $CuC(OH)_2^{2+}$, which, in a rate-determining step, was attacked by a second Cu(II) to yield 2 Cu(I) and CO_2. In view of the apparent need of two Cu(II) it is, however, not clear how CO does reduce nitrosyl haemocyanin or semi-methaemocyanin as these derivatives contain an isolated Cu(II). The recommended storage of molluscan haemocyanin solutions under a CO atmosphere [10] thus does not only prevent ageing but also will regenerate traces of methaemocyanin. The different reactivity of molluscan and arthropodan methaemocyanins towards CO points once again to a profound difference in their active sites.

Recently the conversion of CO to CO_2 by cytochrome *c* oxidase was reported [11], whereby the enzyme acted catalytically and required dioxygen.

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