The reaction of hydroxyurea with oxyhaemocyanin and methaemocyanin of the crayfish *Astacus leptodactylus* and the snail *Helix pomatia*

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The reaction of hydroxyurea with the oxyhaemocyanins of *Astacus leptodactylus* and *Helix pomatia* yielded methaemocyanins which could be regenerated with hydroxylamine. Hydroxyurea did not react with *Astacus* methaemocyanin, but quantitatively regenerated *Helix* methaemocyanin under N₂. The reaction of hydroxyurea with *Helix* haemocyanin at pH 5.7 under air thus led to a steady state, with an oxyhaemocyanin/methaemocyanin ratio of 2.05:1.

INTRODUCTION

For the haemocyanin (Hc) of the crayfish *Astacus leptodactylus*, each subunit (*M_r* 75000) corresponds to a functional unit (Cu^I_ACu^I_B in deoxyHc, Cu^{II}_AO₂²⁻-Cu^{II}_B in oxyHc, Cu^{II}_ACu^{II}_B with at least one bridging ligand in metHc). The subunit of snail (*Helix pomatia*) Hc is constituted of eight functional units (average *M_r* 55000) with similar active sites (Préaux & Gielen, 1984).

MetHc of *Astacus*, prepared by treatment of deoxyHc with nitrite at pH 5.7, could almost fully be regenerated at pH 5.7 with hydroxylamine under N₂ and for about 70% under air, whereas the metHc obtained with H₂O₂ (Felsenfeld & Printz, 1959) could not be regenerated with hydroxylamine under air and for about 47% under N₂ (Tahon *et al.*, 1988). The lower regeneration by hydroxylamine under air was attributed to traces of H₂O₂ formed in its autoxidation.

Helix metHc could not be obtained by treating deoxyHc with nitrite, as this metHc was rapidly transformed into nitrosylHc (Cu^INO⁺Cu^{II}_B) (Verplaetse *et al.*, 1979). The metHc, prepared by expelling peroxide from oxyHc by a treatment with fluoride, was quantitatively regenerated by H₂O₂ (Heirwegh *et al.*, 1965) and by hydroxylamine (Lontie & Witters, 1966). *Helix* Hc shows a moderate catalase activity (Witters *et al.*, 1988), so that there was no secondary reaction with hydroxylamine under air.

The formation of *Astacus* metHc by the action of nitrite occurred in two one-electron steps, as nitrite was reduced to NO (Tahon *et al.*, 1988). A one-electron reduction of H₂O₂ by Cu(I) in *Astacus* deoxyHc could lead to the formation of the hydroxyl radical (OH[•]):

or rather to a transient Cu(III) (Baxendale *et al.*, 1965):

capable of damaging the active site.

Hydroxyurea, a powerful radical scavenger (Georgieff, 1971), was added to H₂O₂ in the preparation of *Astacus* metHc in order to try to inactivate the OH[•] radicals. It did not prevent the destruction of active sites. The blank experiments, however, yielded the following unexpected results.

MATERIALS AND METHODS

Astacus Hc was prepared as described by Tahon *et al.* (1988). The lipoproteins and glycoproteins, present in the haemolymph, were precipitated on dialysis against distilled water. The Hc was stored at 4 °C in 0.10 M-sodium acetate/0.01 M-acetic acid buffer, pH 5.7.

Total *Helix* Hc was prepared as described by Heirwegh *et al.* (1961) and solubilized in 0.11 M-sodium acetate buffer, pH 5.7. On dialysis at 4 °C against 10 mM-sodium acetate/1.9 mM-acetic acid buffer, pH 5.4, β_c-Hc was precipitated. The supernatant, which contained the α_D- and α_N-Hc components, about 83% of total Hc (Lontie, 1983), was stored at 4 °C in 0.11 M-sodium acetate buffer, pH 5.7, after dialysis against this buffer. This preparation was used for the experiments with *Helix* oxyHc after regeneration with small amounts of H₂O₂ (Heirwegh *et al.*, 1965).

Astacus MetHc was prepared under N₂ (A28; L'Air Liquide Belge, Liège, Belgium) by a treatment with NaNO₂ (NaNO₂/Cu molar ratio 100:1) (Tahon *et al.*, 1988).

Helix metHc was obtained by titrating oxyHc with 0.1 M-NaF at 37 °C for 2 days in 0.10 M-sodium acetate/0.05 M-acetic acid buffer, pH 5.0. Excess fluoride was removed by dialysis (Witters & Lontie, 1975).

The main experiments were carried out with hydroxyurea from UCB (Brussels, Belgium) with m.p. 133–136 °C. Control experiments were performed with chromatographically homogeneous *N*-hydroxyurea from Boehringer (Mannheim, Germany) with m.p. 140 °C. The high m.p. is characteristic for the isomer represented in eqn. (3) below (Davies & Spiers, 1959). All other chemicals were of reagent grade.

Abbreviation used: Hc, haemocyanin.

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The experiments were carried out at room temperature in 0.11 M-sodium acetate buffer, pH 5.7. The absorbances were measured in a Perkin-Elmer 554 spectrophotometer. The samples were re-equilibrated in air after the experiments under N₂ or O₂ in order to measure the Cu-O₂ absorption band at 338 nm (*Astacus*) or at 346 nm (*Helix*), which is proportional to the amount of oxyHc. E.p.r. spectra were recorded as described by Tahon *et al.* (1988).

RESULTS AND DISCUSSION

Reaction of hydroxyurea with *Helix* methaemocyanin

Hydroxyurea regenerated almost quantitatively *Helix* MetHc at pH 5.7 under N₂ (Fig. 1), which indicated that it is able to reach the active site like hydroxylamine. The lower regeneration under air follows from the formation of metHc in the reaction of hydroxyurea with oxyHc, whereby a steady state was reached (see below). Control experiments with hydroxyurea from Boehringer yielded the same results.

Although hydroxylamine regenerated both *Helix* and *Astacus* metHcs, albeit the latter at a lower rate, hydroxyurea did not regenerate *Astacus* metHc (Fig. 1). Some 2–3% of residual oxyHc was observed in the metHc preparation measured under air.

The possibility of reducing Cu^{II}Cu^{II} by hydroxyurea in *Helix* metHc in contrast with *Astacus* metHc might be linked to differences in the ligands of Cu_A. In arthropodan deoxyHcs, each copper ion is bound to three histidine residues, as shown by X-ray diffraction at 3.2 nm resolution for the deoxyHc of the spiny lobster *Panulirus interruptus* (Volbeda & Hol, 1986). These histidine residues are conserved in the amino acid sequences known for arthropodan Hcs, including that of subunit *b* of *Astacus* Hc (Soeter *et al.*, 1986). The amino acid sequence of functional unit *d* of the β_C-Hc of *Helix* showed only the homology of one possible copper-binding site (His-175, His-179 and His-206) with the Cu_B site of arthropodan Hcs (Drexel *et al.*, 1987). The other copper ion might be linked to only two histidine residues or to two histidine residues plus another more easily displaced residue (Lontie & Gielsens, 1979). It is this copper site in *Helix* metHc that could react with

Fig. 1. Percentage of oxyHc as a function of time after the addition of hydroxyurea to metHc in 0.11 M-sodium acetate buffer, pH 5.7, at room temperature

Abbreviations: A.I., *Astacus* metHc prepared with nitrite; H.p., *Helix* metHc prepared with fluoride. O, [Hydroxyurea] = 20 mM under air and [Hc] = 1 mg/ml; ●, [hydroxyurea] = 100 mM under 101 kPa of N₂ and [Hc] = 15 mg/ml. The samples were re-equilibrated in the air before the absorbance measurements were made.

Reaction of hydroxyurea with oxyhaemocyanin

The reaction of 20 mM-hydroxyurea with *Astacus* oxyHc at pH 5.7 yielded a metHc (Fig. 2), which was undamaged, as it could be regenerated by hydroxylamine, like the metHc obtained on treating *Astacus* deoxyHc with NO (Witters *et al.*, 1986) or with nitrite (Tahon *et al.*, 1988).

Hydroxyurea is co-planar with the exception of the hydroxy-group hydrogen atom (Kjøller Larsen & Jerslev, 1966) and can form a highly resonance-stabilized radical in accordance with the captodative effect (Viehe *et al.*, 1985). Hydroxyurea could thus attack the peroxide in the active site and be oxidized to the radical with the structure established by Fox & Smith (1968):

hydroxyurea. The metHc of the chelicerate *Limulus polyphemus* (horseshoe crab) was also not regenerated by hydroxyurea, which is in agreement with the conservation of the histidine ligands of copper in arthropodan Hcs (Linzen *et al.*, 1985).

This difference in the copper ligands could also explain the transformation of *Helix* oxyHc into metHc by a treatment with azide or fluoride at pH 5.0, presumably by HN₃ or HF according to the pH-dependence, whereas *Limulus* oxyHc was not affected (Witters & Lontie, 1975). Similarly the metHcs of *Helix* and of *Busycon canaliculatum* (common whelk) were very slowly regenerated by CO, in contrast with *Astacus* metHc, which was not (Deleersnijder *et al.*, 1983).

The hydroxyurea radical would then immediately be oxidized further by the OH[•] radical or by the transient Cu(III) (eqn. 2), so that the active sites are not damaged. The *g* = 2 e.p.r. signal (20.8 ± 3.5% of the copper), due to broken copper pairs, remained constant during this treatment as a function of time.

The identical reaction rate under 101 kPa of O₂ and under air (Fig. 2) indicated a direct reaction of hydroxyurea with the oxygenated active sites. The initial percentages of oxyHc were 98.3 and 92.3% respectively, calculated from the *p*₅₀ value, whereas the concentration of an intermediate, formed in the oxidation of hydroxyurea under O₂ and under air, would have been lower under air.

MetHc was also formed with *Helix* oxyHc (Fig. 2,

Fig. 2. Percentage of residual oxyHc as a function of time after the addition of hydroxyurea to oxyHc in 0.11 M-sodium acetate buffer, pH 5.7, at room temperature

Abbreviations: *A.I.*, *Astacus* oxyHc; *H.p.*, *Helix* oxyHc. $\circ$, [Hydroxyurea] = 20 mM under air and [Hc] = 1 mg/ml; $\triangle$, [hydroxyurea] = 100 mM under air and [Hc] = 15 mg/ml; $\square$, [hydroxyurea] = 100 mM under 101 kPa of O_2 and [Hc] = 15 mg/ml.

upper curve), but a steady state was reached as the metHc was regenerated by hydroxyurea:

$$k[\text{OxyHc}][\text{hydroxyurea}] = k'[\text{metHc}][\text{hydroxyurea}] \quad (4)$$

with $k' = 2.05 k$.

Limulus oxyHc, in contrast with *Astacus* and with *Helix* oxyHc, did not react with hydroxyurea.

Hydroxyurea did not protect *Astacus* Hc in the reaction with H_2O_2 , but unexpectedly transformed *Astacus* oxyHc at pH 5.7 into an undamaged metHc, as it could completely be regenerated with hydroxylamine under N_2 . Hydroxyurea did not regenerate *Astacus* metHc, nor did it react with the oxyHc and metHc of *Limulus*. Hydroxyurea also transformed *Helix* oxyHc into metHc, which it did, moreover, regenerate, so that a steady state was reached for the ratio oxyHc/metHc.

These reactions illustrate the differences in the active sites, especially between crustacean (e.g. *Astacus*) and molluscan (e.g. *Helix*) Hcs.

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