The reaction of nitrite with the haemocyanin of the Roman snail (*Helix pomatia*)

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The reaction of nitrite at pH 5.0–7.0 with the deoxyhaemocyanin of a mollusc, the Roman snail (*Helix pomatia*), yielded nitrosylhaemocyanin ($\text{Cu}_A^I\text{NO}^+\text{Cu}_B^{II}$), in contrast with the formation of methaemocyanin with the deoxyhaemocyanin of the crustacean *Astacus leptodactylus* (mud crayfish). With *Helix* haemocyanin 1 NO was thereby liberated per active site, as shown by m.s., as against 2 NO with *Astacus* haemocyanin. *Helix* nitrosylhaemocyanin was characterized in c.d. by the negative extremum at 336 nm (Cu_A^INO^+) and by the mononuclear e.p.r. signal at $g = 2$ (Cu_B^{II}). Binuclear e.p.r. signals have been observed after the addition of nitrite to methaemocyanins. With *Astacus* methaemocyanin, no further reaction occurred, whereas with *Helix* methaemocyanin the mononuclear e.p.r. signal, characteristic for nitrosylhaemocyanin gradually appeared. This formation of *Helix* nitrosylhaemocyanin implicates the binding, most likely on Cu_A^I , of a second nitrite besides a bridging nitrite, so that a dismutation into NO and NO_2 can occur there. A further dismutation of NO_2 yields nitrite and nitrate. The formation of the latter was demonstrated by Raman spectrometry. The reaction rate of *Helix* methaemocyanin with nitrite decreased with increasing pH according to the Henderson–Hasselbalch equation with a pK_a value of 6.77, attributed to a μ -aquo bridging ligand, which can be exchanged for nitrite, in equilibrium with a μ -hydroxo ligand which cannot. These data also favour the formulation of the final reaction product as nitrosylhaemocyanin instead of semi-methaemocyanin, with or without bound nitrite.

INTRODUCTION

Haemocyanins (Hcs) are O_2 carriers in arthropods (Crustacea and Chelicerata) and in molluscs. The subunit of arthropodan Hcs (M_r 75000) contains one binuclear copper site: $\text{Cu}_A^I\text{Cu}_B^I$ in deoxyHc, and $\text{Cu}_A^I\text{O}_2^{2-}\text{Cu}_B^{II}$ in oxyHc. With molluscan Hcs the gastropodan subunit ($M_r \approx 450000$) is constituted of eight functional units with one binuclear copper site each (Préaux & Gielens, 1984). The derivatives with strongly coupled Cu(II) pairs, oxyHc and usually metHc, are e.p.r.-silent. The metHc of the Roman snail *Helix pomatia* yielded binuclear e.p.r. signals at pH 5.0–5.7 in the presence of nitrite (Van der Deen & Hoving, 1977; Verplaetse *et al.*, 1979) and of acetate (Deleersnijder *et al.*, 1983).

The copper ligands have been identified by X-ray diffraction at 0.32 nm resolution for the deoxyHc of the spiny lobster (*Panulirus interruptus*): three histidine residues each for Cu_A and for Cu_B (Gaykema *et al.*, 1984). The three-histidine ligands of Cu_B are also observed in the sequence of the functional unit *d* of the β_c -Hc of *Helix*, but no sequence with the Cu_A site was found (Drexel *et al.*, 1987). The presumption that Cu_A in *Helix* Hc is only bound to two histidine residues, or to two histidine residues plus a weaker ligand, offers an interpretation for the difference in reactivity with crustacean Hcs. Hydroxyurea, which regenerated *Helix* metHc, did not react with the metHc of the freshwater crayfish *Astacus leptodactylus* (Van Hoof *et al.*, 1988). The reaction rate of NO, which was higher with *Helix* than with *Astacus* metHc, and the concomitant greater stability of *Helix* nitrosylHc point also to the presence of an easily accessible co-ordination site in *Helix* Hc, most likely on Cu_A . The pH-

dependence of the formation of nitrosylHc in the reaction of *Astacus* deoxyHc with NO was interpreted by the detachment and protonation of one imidazole ligand. The deprotonation of this imidazole residue might inversely be responsible for the lower stability of *Astacus* nitrosylHc (Tahon *et al.*, 1989b).

The reaction of nitrite with *Astacus* deoxyHc yielded an undamaged metHc, as it could almost quantitatively be regenerated by an anaerobic treatment with hydroxylamine. Binding of nitrite to the active site of metHc was observed by e.p.r., but no further reaction (Tahon *et al.*, 1988). The oxidation–reduction potential, $E'_0 = 0.60$ V at pH 5.0 [with sign inversion from Latimer (1952)], enables nitrite to oxidize Cu(I) to Cu(II) with the liberation of 2 NO per active site, which were observed by m.s.:

NitrosylHc was formed, in contrast, when *Helix* deoxyHc was treated with nitrite at pH 5.0:

This reaction had been attributed to the oxidation of one Cu(I) in the active site by Schoot Uiterkamp (1972). A very high E'_0 would be required for the remaining Cu(I) in order to resist oxidation:

The amount of NO liberated in the reaction was determined by m.s. The [NO] in the nitrite solution at pH 5.7 was similarly checked and found to be far too low to be effective. The reaction with nitrite had been attributed by Spira & Solomon (1983) to

Abbreviation used: Hc, haemocyanin.

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small amounts of NO formed by the decomposition of HNO_2 ($\text{p}K_a = 3.4$):

Moreover, the reaction of nitrite with *Helix* metHc under air slowly yielded nitrosylHc, characterized by c.d. and e.p.r. A direct formation of semi-metHc by the oxidation of nitrite:

would also require a very high E'_0 for Cu(II), owing to the E'_0 having a value of 0.88 V at pH values above 3.4 for the system $\text{N}_2\text{O}_4/\text{NO}_2^-$ [with sign inversion from Latimer (1952)]. The binding of a second nitrite, most likely on Cu_A^{II} with an accessible co-ordination site, besides a bridging nitrite, would permit the formation there of NO, which immediately yields nitrosylHc (as there was no interference from the dioxygen of the air):

The oxidation-reduction potentials, $E'_0 = 0.60$ V at pH 5.0 and 0.88 V at a pH above 3.4 for the systems $\text{NO}_2^-/\text{NO}^+$ and $\text{N}_2\text{O}_4/\text{NO}_2^-$ respectively, might not seem to favour the dismutation of NO_2^- there. They implicate, however, standard conditions, and the partial pressures of NO and NO_2 are far below unity. NO can, moreover, directly bind to Cu_A^{II} , yielding nitrosylHc. NO_2 does not appreciably react below pH 7.0, even with the more reactive protein residues like tyrosine (Prütz *et al.*, 1985), so that it can leave the active site, dimerize and disproportionate in solution:

The nitrate formed in the reaction of nitrite with the active sites of metHc was determined in the nitrite solutions by Raman spectrometry as described by Brooker & Irish (1968).

Additional direct evidence for the presence of NO^+ at the active sites is provided here by the determination by the Griess-Ilosvay reagent of the nitrite obtained after acid decomposition of nitrosylHc.

MATERIALS AND METHODS

Total Hc was precipitated from the haemolymph with $(\text{NH}_4)_2\text{SO}_4$ as described by Heirwegh *et al.* (1961). The precipitate was dissolved in 0.10 M-sodium acetate/10 mM-acetic acid buffer, pH 5.7, and dialysed against this buffer in order to remove $(\text{NH}_4)_2\text{SO}_4$. By a further 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 (Lontie, 1983), was dialysed against 0.11 M-sodium acetate buffer, pH 5.7, and stored at 4 °C. This Hc solution (about 20 mg/ml) was the main one used for the experiments unless mentioned otherwise. More concentrated solutions for e.p.r. measurements were obtained by preparative ultracentrifugation at 27500 rev./min for 3 h at 4 °C in a Spinco Model L ultracentrifuge (rotor type 30).

Helix metHc was prepared by the reaction of oxyHc in 0.10 M-sodium acetate/50 mM-acetic acid buffer, pH 5.0, with 0.1 M-NaF at 37 °C for 2 days. Excess fluoride was removed by dialysis against the 0.11 M-sodium acetate buffer, pH 5.7 (Witters & Lontie, 1975). DeoxyHc was obtained by expelling O_2 from an oxyHc solution with N_2 (A28; L'Air Liquide Belge, Liège, Belgium) for 2 h.

The following buffer systems (I0.1) were used for the experiments: sodium acetate/acetic acid, succinic acid/NaOH, $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, $\text{Na}_2\text{B}_4\text{O}_7/\text{HClO}_4$ and $\text{Na}_2\text{B}_4\text{O}_7$. The chemicals were of analytical grade: NaNO_2 was from Union Chimique Belge (Brussels, Belgium) and KNO_3 was from Baker (Phillipsburg, NJ, U.S.A.).

The protein concentration and the amount of oxyHc for solutions in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ buffer, pH 9.2, in equilibrium under air were determined in a Perkin-Elmer 554 spectrophotometer by using an $A_{1\text{cm}, 278}^{1\%}$ value of 14.16 for *Helix* Hc and an $A_{1\text{cm}, 346}^{1\%}$ value of 3.45 for the Cu-O₂ absorption band.

The experiments and measurements were carried out at room temperature, except the e.p.r. measurements, which were made at 133 K.

Excess nitrite was removed after a given time by dialysis against the appropriate buffer or by chromatography on a column (25 cm × 3 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden), eluted with $\text{Na}_2\text{B}_4\text{O}_7/\text{HClO}_4$ buffer, pH 8.2.

NitrosylHc was characterized by c.d. measurements at 336 and by e.p.r. measurements at $g = 2$ as described by Tahn *et al.* (1989b). The c.d. spectra between 300 and 600 nm were recorded with a Cary 61 spectropolarimeter (Cary, Monrovia, CA, U.S.A.). The contribution of residual oxyHc to the negative band of nitrosylHc at 336 nm was suppressed by adding solid Na_2SO_3 (20 mg/ml) to the preparation in order to remove dioxygen from the solution. The e.p.r. spectra were recorded with a Varian (Palo Alto, CA, U.S.A.) E-109 spectrometer, and the gaseous products in the reaction of nitrite with deoxyHc were analysed by means of a modified Micromass type 8-80 mass spectrometer (Vacuum Generators, Hastings, Sussex, U.K.) as described by Tahn *et al.* (1988).

The Hc solutions for Raman spectrometry were deproteinized by ultrafiltration on Pellicon membranes (Millipore, Bedford, MA, U.S.A.); the filtrate was concentrated by a limited evaporation in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). The NO_2^- blank and the NO_3^- standard were similarly concentrated. The Raman spectra were recorded on a Coderg (actually DILOR, Lille, France) T800 spectrometer, equipped with a Spectra Physics (San Jose, CA, U.S.A.) Ar⁺ laser model 164. The laser line at 514.5 nm was used at a power of 800 mW. The spectral slit width varied from 2.8 to 3.4 cm^{-1} . Solutions were measured in a small rotating glass cell (2800 rev./min). Coupling of the spectrometer with a Tracor Northern (Middleton, WI, U.S.A.) Multichannel Analyzer TN-1750 allowed a 6-fold accumulation of the weak ν_1 (NO_3^-) Raman band at $\Delta\nu = 1050 \text{ cm}^{-1}$. For the standard solutions the intensities of this band were obtained by planimetry as well as from the peak height (h). Both methods yielded comparable results for the ratio $[\text{NO}_3^-]/[\text{NO}_2^-]$. This enabled the use of the peak height to estimate the intensity of ν_1 in the protein-free solution, which showed a rather strong deviation of the baseline, probably due to fluorescence. The NO_3^- signal was calibrated by standard addition. After raising the $[\text{NO}_3^-]$ by 0.52 mM, h increased from 11 to 28 mm (the solutions before and after the addition of NO_3^- were concentrated from 20.8 to 165 and 160 mM- NO_2^- respectively). The $[\text{NO}_2^-]$ was determined from the absorbance at 354 nm. The 20.8 mM- NO_2^- solution thus contained 0.33 mM- NO_3^- .

The nitrite, obtained after decomposition of nitrosylHc in acid medium, was determined with a modified Griess-Ilosvay reagent after the free nitrite had been removed as mentioned above. A 200 μl volume of the nitrosylHc solution was added to a mixture of 1 ml of 34 mM-sulphanilic acid ('pro analysi'; Merck, Darmstadt, Germany) in 0.67 M-HCl and 2 ml of acetic acid; after 30 min, 1 ml of a solution of 34 mM-N-naphthylethylenediamine dihydrochloride ('pro analysi'; Union Chimique Belge) in 0.88 M-

HCl was added and the absorbance was measured at 550 nm. A calibration curve was established with NaNO_2 in the presence of the same amount of Hc as in the unknown.

RESULTS AND DISCUSSION

Reaction of nitrite with *Helix* deoxyHc

A solution of *Helix* deoxyHc (24.9 mg/ml; 0.90 mM-Cu) was treated for 30 min with NaNO_2 in a molar ratio to Cu, R , of 100 in sodium acetate buffer, pH 5.0. The mononuclear e.p.r. signal at $g = 2$ amounted to nearly 48 % of the copper and showed the formation of more than 95 % nitrosylHc. The complete lack of a seven-line binuclear e.p.r. signal at $g = 4$, characteristic for the binding of NO_2^- to metHc (Verplaetse *et al.*, 1979), confirmed the absence of metHc. After removal of the excess nitrite on a Sephadex G-25 column, the c.d. band at 336 nm likewise indicated the presence of over 95 % nitrosylHc.

The reaction was checked with the isolated functional units of *Helix* β_c -Hc. They were treated for 2 h under N_2 at room temperature with NaNO_2 ($R = 100$) in a $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 6.7. After removal of the excess nitrite on a Sephadex G-25 column, the c.d. and e.p.r. measurements showed their quantitative transformation into nitrosylHc, with the exception of functional units g and h , which were only transformed to the extent of 68 and 41 % respectively (Tahon *et al.*, 1989a).

After 2 h reaction in a closed vessel of NaNO_2 with *Helix* deoxyHc at pH 5.7, the gaseous reaction products were determined by m.s. against a buffer blank (Table 1). A significant signal was only detected for NO^+ . From a sensitivity for NO of 18.6 nA/ μPa a molar fraction of 774×10^{-6} was obtained, which corresponded to 4.22 μmol of $\text{NO}/8.90 \mu\text{mol}$ of Cu.

Whereas the reaction of nitrite with *Astacus* deoxyHc yielded metHc, nitrosylHc was formed with *Helix* deoxyHc with a pseudo-first-order rate constant, k_1 , of $2.56 \times 10^{-3} \text{ s}^{-1}$ at pH 5.0 and room temperature, as shown by the appearance of the c.d. band at 336 nm and of the mononuclear e.p.r. signal. Only half the amount of NO , obtained in the reaction of nitrite with *Astacus* deoxyHc, was detected with *Helix* deoxyHc according to eqn. (2).

Reaction of nitrite with *Helix* metHc

Helix metHc yielded rapidly a broad binuclear e.p.r. signal at $g = 2$ and a seven-line binuclear signal at $g = 4$ after the addition at room temperature of NaNO_2 at pH 5.0. In contrast with the nitrite derivative of *Astacus* metHc, which showed no further reaction (Tahon *et al.*, 1988), the derivative of *Helix* metHc slowly developed, in addition, the mononuclear e.p.r. signal of

Fig. 1. E.p.r. signal at $g = 2$, measured at 133 K as a function of time, in the reaction at room temperature of 13.0 mM- NaNO_2 with *Helix* metHc (18.0 mg/ml; 0.65 mM-Cu) in succinic acid/ NaOH buffer, pH 5.0

Trace A, blank; trace B, after 1 min reaction; trace C, after 24 h reaction; trace D, after 48 h reaction. The receiver gain was 10000.

nitrosylHc (Fig. 1). In $\text{Na}_2\text{B}_4\text{O}_7/\text{HClO}_4$ buffer, pH 8.2, by contrast, the addition of 20.6 mM- NaNO_2 ($R = 20$) to *Helix* metHc (28.5 mg/ml; 1.03 mM-Cu) only yielded a weak e.p.r. signal at $g = 2$, which did not increase after 24 h or at a 10-fold higher NaNO_2 concentration.

In order to measure the pH-dependence of the reaction, metHc solutions were dialysed against a set of buffers and treated with NaNO_2 ($R = 20$). After 4 h reaction at room temperature the excess nitrite was removed by dialysis. The c.d. spectrum at the higher pH values did not differ from that of metHc. At the lower pH values, about 60 % nitrosylHc was detected (Fig. 2), an amount which increased to almost 100 % after 96 h in the presence of NaNO_2 . The decrease as a function of pH of the percentage of *Helix* nitrosylHc followed the Henderson-Hasselbalch equation with a pK_a value of 6.77 (Fig. 3). Like the pK_a value of 6.50 observed for the decrease in the binding of NO_2^- to *Astacus* metHc (Tahon *et al.*, 1988), it may also be attributed to a μ -aquo ligand which can be exchanged for NO_2^- , in contrast with a μ -hydroxo ligand, which cannot. The rate of the reaction of *Helix* metHc with 101 kPa NO decreased similarly, according to the Henderson-Hasselbalch equation, with a pK_a value of 6.80 (Fig. 3). The interpretation of the prevention of the binding of a bridging NO_2^- by a μ -hydroxo ligand was confirmed by the addition at a low pH of F^- , which also completely inhibited the reaction of NO_2^- with *Helix* metHc. The e.p.r. signal at $g = 2$ of *Helix* metHc, treated for 18 h with 10 mM- NaNO_2 in the presence of 0.2 M- NaF at pH 5.0 (Fig. 4, trace B) did not differ significantly from that of the blank (Fig. 4, trace A). After removal of NO_2^- and F^- by dialysis (Fig. 4, trace C), the metHc treated again with 10 mM- NaNO_2 showed, after 18 h reaction, a binuclear and a mononuclear e.p.r. signal at $g = 2$ (Fig. 4, trace D). The binuclear signal disappeared in the presence of 0.2 M- NaF , whereas the mononuclear nitrosylHc signal remained, which corresponded to 75 % of the active sites (Fig. 4, trace E).

Table 1. Signal intensities, determined by m.s. after 2 h reaction in sodium acetate/acetic acid buffer, pH 5.7, of 10.0 ml of *Helix* deoxyHc (24.5 mg/ml; 0.89 mM-Cu) and of the buffer blank in the presence of 92 mM- NaNO_2 under 101 kPa He at room temperature

m/z	Molecular ion	Signal intensity (nA)		
		DeoxyHc	Buffer	Difference
28	N_2^+	0.43	0.71	(-0.28)
30	NO^+	7.24	1.56	
		6.91*	1.16*	5.75
32	O_2^+	0.02	0.11	(-0.09)
44	N_2O^+	0.90	1.30	(-0.40)
46	NO_2^+	0.02	0.00	(0.02)

* Corrected for fragmentation of N_2O^+ (31 %) and NO_2^+ (270 %) (Cornu & Massot, 1975).

Fig. 2. C.d. spectra of *Helix* metHc (5.0 mg/ml; 0.18 mM-Cu) treated with NaNO_2 ($R = 20$) for 4 h at room temperature at increasing pH values

Trace A, pH 5.00; trace B, pH 5.70; trace C, pH 6.25; trace D, pH 6.75; trace E, pH 7.20; trace F, pH 7.70; trace G, pH 8.20; trace H, pH 9.20.

The $\text{p}K_a$ of 6.77 of the pH-dependence of the formation of nitrosylHc by the action of nitrite on *Helix* metHc already indicates that this reaction cannot be due to the traces of NO , formed in the nitrite solutions by the decomposition of HNO_2 according to eqns. (4) and (5), as postulated by Spira & Solomon (1983). The inhibition by F^- (Fig. 4) suggests a dismutation of NO_2^- at the active sites of metHc rather than in solution. But as F^- could also have reduced the rate of the reaction of NO with metHc, the $[\text{NO}]$ in a nitrite solution was determined by m.s. and the reaction allowed to proceed at a lower $[\text{NO}]$. In 85 mM- NO_2^- under 101 kPa He in sodium acetate buffer, pH 5.70, the $[\text{NO}]$ merely amounted to 0.25 μM and to 0.20 μM in the same buffer

Fig. 3. Percentage, as a function of pH, of the nitrosylHc obtained after 4 h reaction of *Helix* metHc at room temperature

●, Percentage in the reaction with NaNO_2 ($R = 20$), calculated from the ellipticity at 336 nm in Fig. 2; ○, percentage in the reaction with 101 kPa NO [Fig. 3 of Tahon *et al.* (1989b)]. The curves were calculated by the Henderson-Hasselbalch equation with $\text{p}K_a$ values of, respectively, 6.77 and 6.80.

Fig. 4. E.p.r. signal at $g = 2$, measured at 133 K, of *Helix* metHc (13.8 mg/ml; 0.50 mM-Cu) in succinic acid/ NaOH buffer, pH 5.0

Trace A, in the presence of 0.2 M- NaF ; trace B, solution A treated for 18 h with NaNO_2 ($R = 20$); trace C, solution B after dialysis against the succinate buffer; trace D, solution C treated for 18 h with NaNO_2 ($R = 20$); trace E, solution D treated for 18 h with 0.2 M- NaF . The receiver gain was 10000.

after 2 h in the presence of metHc (24.5 mg/ml; 0.89 mM-Cu). On treating deoxyHc (22.5 mg/ml; 0.81 mM-Cu) for 4 h at room temperature in acetate buffer, pH 5.70, with a solution of 20 μM - NO (equilibrated with 101 kPa He containing 1.03% NO) the [oxyHc], after removal of NO and reoxygenation, was decreased by only 11%. The traces of NO in nitrite solutions could thus not account for the formation of nitrosylHc.

The dismutation of NO_2^- was demonstrated by the determination of NO_3^- by Raman spectrometry. *Helix* metHc (59.7 mg/ml; 2.16 mM-Cu) was treated for 4 days at room temperature with 20.8 mM- NaNO_2 in 50 mM-ammonium acetate buffer, pH 5.6. By reaction with metHc (eqn. 8) the $[\text{NO}_2^-]$ was reduced by $(2.16 \times 3/4)$ mM to 19.2 mM, as 1 NO_2^- was recovered by the dismutation of 2 NO_2^- (eqn. 10), resulting from the reaction of 4 NO_2^- with metHc (eqn. 8). The $[\text{NO}_3^-]$ rose from 0.33 mM in the NO_2^- blank, treated similarly for 4 days, to 0.92 mM ($h = 19$ mM after concentrating the solution from 19.2 to 93 mM- NO_2^-). This increase by 0.59 mM is in fair agreement with the expected value of 0.54 mM- NO_3^- .

Structure of the copper site of nitrosylHc

The interpretation of nitrosylHc as a semi-metHc with or without bound nitrite, by Himmelwright *et al.* (1979) and Hwang & Solomon (1982) and by Salvato *et al.* (1989) respectively, is not consistent with its formation. The oxidation of only one Cu(I) per active site on treating *Astacus* and *Helix* deoxyHc with NO and *Helix* deoxyHc with nitrite (eqn. 3) would require a very high E_0 for the remaining Cu(I). The formation of nitrosylHc on treating *Astacus* and *Helix* metHc with NO (Tahon *et al.*, 1989b) and its slow formation in the reaction of *Helix* metHc with nitrite cannot be explained by the reduction of one Cu(II) by NO and by NO_2^- (eqn. 6), for this would imply an oxidation of NO to NO_2^- and of NO_2^- to NO_2 .

Salvato *et al.* (1989) accepted the instability of a nitrite derivative and postulated semi-metHc without bound nitrite, as they could find only 10% of the expected amount of nitrite (with

1 NO⁺ per copper site) after acid decomposition of a purified nitrosylHc of *Octopus vulgaris* (the common octopus). They attributed this low value to the presence of nitrite esters, claiming that there was no binding of NO at the copper sites. In our experiments, however, a much higher value was consistently found. The nitrite, determined with the Griess–Ilosvay reagent after decomposition of *Helix* nitrosylHc by acid, amounted to 60–70% of the theoretical value. The presence of acetic acid in our reagent kept the protein in solution. Only 25% of the theoretical amount of nitrite was detected by using the method of Salvato *et al.* (1989), which might have been due to the lower ratio of sulphanilic acid to Hc.

The fast decrease in the amount of liberated nitrite on treating nitrosylHc with azide or thiocyanate, like its slow decrease in the presence of acetate (acetic acid), chloride or borate (boric acid) (Vandamme *et al.*, 1990), cannot be explained, furthermore, by the presence of nitrite esters. By these nitrite determinations it was also shown that *Helix* nitrosylHc resisted dialysis against phosphate buffer, pH 6.0, for over 50 days.

The presence of NO, rather than of Cu¹NO⁺ according to the e.p.r. data, in the copper sites of *Helix* nitrosylHc, was already suggested by the liberation of only 1 NO per active site in the reaction of NO₂⁻ with deoxyHc against 2 NO with *Astacus* deoxyHc, which yielded metHc (Tahon *et al.*, 1988). This presence of NO was confirmed by the production of N₂ and N₂O in the reaction of N₃⁻ with *Helix* nitrosylHc (Tahon *et al.*, 1989b) and here by the nitrite determinations with the Griess–Ilosvay reagent after acid decomposition of nitrosylHc.

CONCLUSIONS

The amount of NO liberated in the reaction of NO₂⁻ with *Helix* deoxyHc at pH 5.7 was only half that found with *Astacus* deoxyHc, in accordance with the concomitant formation of nitrosylHc from metHc.

Helix metHc was slowly transformed into nitrosylHc by the dismutation of 2 NO₂⁻ at the active site, with the eventual production in solution of 1 NO₃⁻/4 Cu, in contrast with *Astacus* metHc, which only formed a nitrite derivative. Such a reaction of NO₂⁻ cannot very likely yield a nitrite derivative of semi-metHc.

These differences in reactivity between *Helix* and *Astacus* Hcs with NO₂⁻, like the regeneration of *Helix* metHc by hydroxyurea, in contrast with *Astacus* metHc, might be linked to the coordination of Cu_A and Cu_X with possibly only two and with three histidine ligands respectively. This hypothesis is also supported by the comparison of the sequences of tyrosinases and of the functional units of molluscan Hcs with those of the subunits of arthropodan Hcs (Préaux *et al.*, 1988).

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