The reaction of nitrogen monoxide with the haemocyanins of the crayfish *Astacus leptodactylus* and the snail *Helix pomatia**

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The rate of the reaction of *Astacus leptodactylus* methaemocyanin with NO follows the Henderson–Hasselbalch equation with a pK_a of 5.85, suggesting that one imidazole ligand of Cu was exchanged for NO. The reaction is blocked by F^- as a bridging ligand. The same imidazole residue might be responsible for the decomposition of nitrosylhaemocyanin, $[Cu^I NO^+ Cu^{II}]$, with an unlocated binding site for NO, into methaemocyanin and NO, as the rate increase with pH. NO could react preferentially with Cu_A of *Helix pomatia* methaemocyanin, $Cu_A^{II} Cu_B^{II}$, as it possibly has only two histidine ligands instead of the three of Cu_A in *Astacus* haemocyanin. This difference might explain the higher formation rate and the much greater stability of *Helix* nitrosylhaemocyanin. The fast reaction is governed by a pK_a of 6.80, probably of a bridging μ -aquo ligand. With F^- or a μ -hydroxo bridging ligand a low reaction rate was still observed, in contrast with *Astacus* methaemocyanin. *Helix* nitrosylhaemocyanin was transformed by N_3^- into methaemocyanin with the liberation of N_2 and N_2O . This methaemocyanin could almost quantitatively be regenerated with H_2O_2 . *Helix* nitrosylhaemocyanin was only partially regenerated by a direct treatment with H_2O_2 and almost quantitatively by $HONH_2$ in a similar fairly fast reaction, followed by a much slower one.

INTRODUCTION

Haemocyanins (Hcs) are O_2 carriers in Arthropoda (Crustacea and Chelicerata) and in Mollusca. Their binuclear copper sites, $Cu^I Cu^I$ in DeoxyHc and $Cu^{II} O_2^{2-} Cu^{II}$ in OxyHc, are e.p.r.-silent (Préaux & Gieles, 1984). MetHc, $Cu^{II} Cu^{II}$, of the Roman snail *Helix pomatia* is usually e.p.r.-silent too, but binuclear e.p.r. signals were observed at pH 5.0 in the presence of 0.2 M-sodium acetate, and they were enhanced by the presence of 0.2 M-NaCl (Deleersnijder *et al.*, 1983). These signals have been attributed to a small fraction of the copper sites, as a result of experiments carried out with the MetHc of the whelk *Busycon canaliculatum*, which lacked them (Wilcox *et al.*, 1984). With *Helix* MetHc, however, in 0.1 M-sodium acetate buffer, pH 5.0, containing 0.2 M-NaCl, the e.p.r. signal at $g = 4$ was eliminated almost quantitatively by titration with H_2O_2 , with the concomitant appearance of the OxyHc spectrum (Witters *et al.*, 1981). These binuclear e.p.r. signals can thus be attributed to a μ -acetato derivative of *Helix* MetHc. Acetate buffers were therefore replaced by Mes or succinate buffers when we wanted to avoid these signals.

A green derivative has been obtained by an anaerobic treatment in alkaline medium of *Helix* DeoxyHc with NO in the presence of urea. It appeared to be quite stable, since it resisted dialysis for 10–15 days (Dhéré & Schneider, 1919). The treatment of the DeoxyHc of the crab *Cancer magister*, especially at low pH, likewise produced a green derivative, which showed a mono-

nuclear e.p.r. signal at $g = 2$ (Simo, 1966). *Helix* Hc, treated with nitrite, with nitrite plus ascorbate, or anaerobically with NO, also yielded a mononuclear e.p.r. signal at $g = 2$, which was attributed to the oxidation of one Cu(I) by NO (Schoot Uiterkamp, 1972). The binuclear e.p.r. signals at $g = 2$ and at $g = 4$ (seven-line signal), observed there in the treatment with NO, were interpreted as being due to the presence of traces of air in the sample, responsible for the formation of nitrite from NO (Van der Deen & Hoving, 1977). *Helix* DeoxyHc on treatment with NO yielded MetHc and N_2O in a fast reaction. In a further slow reaction with NO under strictly anaerobic conditions MetHc was transformed into NitrosylHc, $[Cu^I NO^+ Cu^{II}]$, with the characteristic mononuclear e.p.r. signal at $g = 2$. (The vertical bars indicate that the order of the copper atoms was not established.) Moreover MetHc yielded reversibly a nitrite derivative with a binuclear e.p.r. signal at $g = 4$ with seven hyperfine lines (Verplaetse *et al.*, 1979).

The product obtained on treating *Busycon* Hc with nitrite and ascorbate was interpreted as a nitrite derivative of Semi-MetHc, $[Cu^I Cu^{II}]$, called ‘half-met- NO_2^- ’ by the authors (Himmelwright *et al.*, 1979). This assumption is disproved by the following data for *Helix* NitrosylHc. It is quite stable on dialysis (Dhéré & Schneider, 1919; Schoot Uiterkamp, 1972; Verplaetse *et al.*, 1979), in contrast with the nitrite derivative of MetHc (Verplaetse *et al.*, 1979). The mononuclear e.p.r. signal shows four $A_{||}$ lines, whereas seven lines would have been expected at $g = 2$ with a stable nitrite derivative of Semi-MetHc.

Abbreviation used: Hc, haemocyanin.

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Nitrite is, moreover, incompatible with Cu(I), as it readily yielded MetHc in the reaction with the Hc of the crayfish *Astacus leptodactylus* (Tahon *et al.*, 1988).

There are striking differences in the structure of arthropodan and molluscan Hcs. The subunits of arthropodan Hcs (M_r 75000) correspond to the functional unit with one O₂-binding site (Cu_ACu_B). They are associated into hexamers and further mainly into bis-, tetrakis- and octakis-hexamers (Préaux & Gielens, 1984). In the Phylum of the Mollusca the subunits of gastropodan Hcs (M_r approx. 450000) are composed of eight functional units (M_r approx. 55000) with one active site each (Cu_ACu_B). Usually 20 subunits are helically associated into hollow cylinders (Préaux & Gielens, 1984).

Differences in the reactivity of the active sites have regularly been observed between arthropodan, especially crustacean, and molluscan Hcs.

The histidine ligands of copper were identified by X-ray diffraction at 0.32 nm resolution for the Hc of a crustacean, the spiny lobster *Panulirus interruptus* (Gaykema *et al.*, 1984). The sequences of crustacean and cheliceratan Hcs show a pronounced homology around these histidine ligands in their second domain, which contains the active site (Linzen *et al.*, 1985). The three histidine ligands of Cu_B in arthropodan Hcs were also observed in the sequence of functional unit *d* of *Helix* β_c -Hc, His-175, His-179 and His-206 (Drexel *et al.*, 1987), of the C-terminal functional unit of the giant Pacific octopus *Octopus doylei* Hc (Lang, 1988), and of the tyrosinases of the bacterium *Streptomyces glaucescens* and the fungus *Neurospora crassa* (Huber *et al.*, 1985), of the mouse (Shibahara *et al.*, 1986), and of man and of another tyrosinase of the mouse (Kwon *et al.*, 1988). The three histidine ligands of Cu_A in arthropodan Hcs, on the contrary, were not found in functional unit *d* of *Helix* β_c -Hc, in the C-terminal functional unit of *Octopus* Hc, or in the mentioned tyrosinases.

The hypothesis that Cu_A in molluscan Hcs has only two histidine ligands or two histidine ligands plus a weaker one allowed an interpretation of the regeneration of *Helix* MetHc with hydroxyurea, in contrast with *Astacus* MetHc, which did not react (Van Hoof *et al.*, 1988). Such a difference in co-ordination is also consistent with the action of cyanide. With *Astacus* Hc the two copper atoms were removed at the same rate, whereas for *Helix* α -Hc, on the whole, the first copper atom was removed 15-fold faster than the second one (D. Groeseneken, R. Witters & R. Lontie, unpublished work). This hypothesis also provides an interpretation for the differences in the formation and stability of *Astacus* and *Helix* NitrosylHcs reported here.

MATERIALS AND METHODS

Astacus Hc was obtained as described by Tahon *et al.* (1988). MetHc was prepared by the reaction for 24 h at pH 5.70 of OxyHc with NaNO₂ in a molar ratio to copper, *R*, of 110 (Witters *et al.*, 1986).

Helix Hc was precipitated from the haemolymph with (NH₄)₂SO₄ at pH 5.30 as described by Heirwegh *et al.* (1961) and redissolved in sodium acetate/acetic acid buffer, pH 5.70 and 10.1. From this solution β_c -Hc was precipitated by dialysis at low ionic strength as described by Gielens *et al.* (1975). The remaining supernatant, which contained the α_D - and α_N -Hc components, about 83% of total Hc (Lontie, 1983), was used for the

experiments with *Helix* Hc unless mentioned otherwise. MetHc was prepared by the reaction of OxyHc in 0.10 M-sodium acetate/0.05 M-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 acetate buffer, pH 5.70 (Witters & Lontie, 1975).

Limited trypsinolysis of the β_c -Hc of *Helix*, dissociated into one-tenth molecules, yielded fragments *a-c* and *ef* and functional units *d, g* and *h₂* (the last as a dimer). They were separated by chromatography on Ultrogel AcA 34 (L'Industrie Biologique Française, Villeneuve-la-Garenne, France), DEAE-Sephacrose CL-6B and concanavalin A-Sephacrose (Pharmacia, Uppsala, Sweden) (Gielens *et al.*, 1980; Préaux *et al.*, 1981; Finotto *et al.*, 1988). A limited subtilisin treatment of fragment *ef* liberated the functional units, which were separated on concanavalin A-Sephacrose (Finotto *et al.*, 1988).

In the preparation of NitrosylHc O₂ was eliminated with N₂ (A28; L'Air Liquide Belge, Liège, Belgium) for 2 h. The reaction vessel was then flushed for 10 min with NO (N30; L'Air Liquide Belge) and kept for a given time under 101 kPa NO, which was thereupon removed with N₂ for 15 min. NO was washed with 1 M-NaOH and with the appropriate buffer (Verplaetse *et al.*, 1979).

The buffers (10.1) sodium acetate/acetic acid, succinic acid/NaOH, Mes/NaOH, Na₂HPO₄/NaH₂PO₄, Tris/HClO₄, Na₂B₄O₇/HClO₄ and Na₂B₄O₇ were used. In the experiments as a function of pH the Hc solutions were dialysed against the appropriate buffers.

Hc concentrations were determined in Na₂B₄O₇ buffer, pH 9.20, in a Perkin-Elmer 554 spectrophotometer by using $A_{1\text{ cm}, 278}^{1\%}$ 12.36 for *Astacus* and 14.16 for *Helix* Hc. The amount of OxyHc was estimated from the absorbance of the Cu-O₂ band with $A_{1\text{ cm}, 338}^{1\%}$ 2.16 for *Astacus* OxyHc at pH 5.70 and $A_{1\text{ cm}, 346}^{1\%}$ 3.45 for *Helix* OxyHc at pH 9.20.

The c.d. spectra were recorded at room temperature in a Cary 61 spectropolarimeter (Cary, Monrovia, CA, U.S.A.). Cells with 1 cm path-length were used with protein concentrations of about 5 mg/ml for scanning between 300 and 600 nm. The full range setting was 0.02° ellipticity for NitrosylHc solutions and 0.1° for solutions with large amounts of OxyHc. The ellipticities were converted into molar values $\Delta\epsilon$ (M⁻¹·cm⁻¹) per mol of active sites. The contribution of residual OxyHc was suppressed by the addition of Na₂SO₃ (20 mg/ml) to the NitrosylHc solutions in Na₂B₄O₇/HClO₄ buffer, pH 8.20. The small contribution of the protein moiety to the c.d. signals was eliminated by subtraction of the value for MetHc also treated with Na₂SO₃.

Cu was determined by atomic absorption in a Perkin-Elmer 372 spectrophotometer.

The gaseous products of the reaction of NitrosylHc with NaN₃ were analysed by means of a modified Micromass type 8-80 mass spectrometer (Vacuum Generators, Hastings, Sussex, U.K.) and the e.p.r. measurements with a Varian E-109 spectrometer (Palo Alto, CA, U.S.A.) performed as described previously (Tahon *et al.*, 1988).

RESULTS AND DISCUSSION

Characterization of NitrosylHc

The reaction of NO with *Helix* MetHc yielded a mononuclear e.p.r. signal ($g_{\parallel} = 2.31$, $A_{\parallel} = 0.0133$ cm⁻¹, $g_{\perp} = 2.07$), which indicated the binding of a single NO to

Fig. 1. C.d. spectra of *Helix* MetHc (traces A and C) and NitrosylHc (traces B and D) in Na₂B₄O₇/HClO₄ buffer, pH 8.20, measured under air respectively before and after addition of an excess of Na₂SO₃ (20 mg/ml)

the active site: [Cu^INO⁺Cu^{II}] (Verplaetse *et al.*, 1979). (A plausible binding of NO⁺ to Cu_A in *Helix* NitrosylHc is justified below.) The e.p.r. signal corresponded to about 50% of the Cu in Hc.

The green *Helix* NitrosylHc showed only weak absorption bands in the visible region, apparently due to *d-d* transitions of Cu(II), and a somewhat stronger band at 336 nm ($A_{1\text{cm},336}^{1\%}$ 0.400, ϵ approx. 2200 M⁻¹·cm⁻¹, expressed per functional unit with M_r 55000), probably a weak metal-to-ligand charge-transfer band of Cu^INO⁺. The c.d. spectrum of the preparation showed an additional negative band at 336 nm, characteristic for NitrosylHc (Fig. 1). The residual negative band at 346 nm of small amounts of OxyHc in the preparations to be measured under air was eliminated by the addition of solid Na₂SO₃ (20 mg/ml), which did not affect the NitrosylHc spectrum. According to the spectra, there was less OxyHc in the NitrosylHc preparation than in the original MetHc solution, the remaining DeoxyHc being further oxidized to MetHc during the treatment with NO.

Helix NitrosylHc can thus be quantified by the intensity of the e.p.r. signal at $g = 2$ or by the negative c.d. band at 336 nm ($\Delta\epsilon = -3.60 \text{ M}^{-1}\cdot\text{cm}^{-1}$, expressed per mol of active sites). For *Astacus* NitrosylHc the c.d. band was measured at 320 nm ($\Delta\epsilon = -4.40 \text{ M}^{-1}\cdot\text{cm}^{-1}$, also expressed per mol of active sites). With *Astacus* Hc the c.d. protein band at 280 nm was negative (Nickerson & Van Holde, 1971), in contrast with the positive band of *Helix* Hc (Fig. 1).

Rate and pH-dependence of the reaction of NO with *Astacus* MetHc

The kinetics of the formation at room temperature of NitrosylHc, monitored in Mes/NaOH buffer, pH 5.70, by e.p.r. (Fig. 2) and c.d., yielded a pseudo-first-order rate constant k_1 of $58 \times 10^{-6} \text{ s}^{-1}$.

Fig. 2. E.p.r. signal at $g = 2$, measured at 133 K as a function of time, of *Astacus* MetHc (23.5 mg/ml, 0.56 mM-Cu) treated with 101 kPa NO at room temperature in Mes/NaOH buffer, pH 5.70

Trace A, MetHc; trace B, after 30 min reaction; trace C, after 60 min reaction; trace D, after 4 h reaction; trace E, after 68 h reaction. Receiver gain was 10000.

Fig. 3. Percentage as a function of pH of NitrosylHc, estimated by c.d., after 4 h reaction of MetHcs of *Astacus* (○) and *Helix* (●) with 101 kPa NO at room temperature

The curves were calculated by the Henderson-Hasselbalch equation with a pK_a of respectively 5.85 and 6.80.

The percentage of NitrosylHc, calculated from the corrected c.d. signal at 320 nm after 4 h reaction of NO with MetHc, plotted against pH followed the Henderson-Hasselbalch equation with a pK_a value of 5.85 (Fig. 3). The percentage of NitrosylHc obtained at pH 5.70 after 18 h reaction of NO with MetHc (Fig. 2, trace E) provided the extreme value at low pH, which could not be

determined directly owing to the instability of the Hc. No NitrosylHc was formed at pH 8.20.

The formation of NitrosylHc was completely inhibited by 0.2 M-NaF in a succinic acid/NaOH buffer, pH 5.00 and 10.1. NO thus did not react with a Cu(II) pair strongly coupled by superexchange with F⁻.

A pK_a of 6.50 had been observed for the binding of nitrite to the active site of *Astacus* MetHc and attributed to the deprotonation of a bridging μ -aquo ligand, which can be displaced by nitrite, in contrast with a μ -hydroxo ligand (Tahon *et al.*, 1988). The pK_a of 5.85, observed in the reaction of NO with *Astacus* MetHc, might, however, belong to the weakest bound imidazole (im) ligand of copper. Its protonation could liberate a co-ordination site for NO, $im_2Cu^+NO^+$, inspired by our model for the Cu_A site of *Helix* Hc. The μ -aquo ligand moves thereby entirely to the other copper atom, $H_2OCu^{II}im_3$. Such a type-2 Cu(II) with a mononuclear e.p.r. signal does not react with NO, as shown with bovine superoxide dismutase (Gorren *et al.*, 1987). No choice can yet be made between Cu_A and Cu_B .

This hypothesis can also provide a partial explanation for the observed instability of *Astacus* NitrosylHc.

Instability of *Astacus* NitrosylHc

In contrast with the known stability of *Helix* NitrosylHc, a decrease of the *Astacus* [NitrosylHc] was observed at pH 6.68 and a greater one at pH 8.20 after 45 min dialysis against the indicated buffers (Table 1). The influence of pH on the decomposition rate suggests that the mentioned imidazole residue, when deprotonated, could promote the decomposition of NitrosylHc into MetHc and NO, which then reacts further with the dissolved O₂.

But even in Mes/NaOH buffer, pH 5.70, the [NitrosylHc] was decreased to about 50% of its initial value after 30 h. No c.d. signal of NitrosylHc was detected anymore after 24 h dialysis against a sodium acetate/ acetic acid buffer, pH 5.70. In contrast with Mes, acetate seems able to reach the active site, probably as acetic acid, since a weak binuclear e.p.r. signal at $g = 2$ was observed with MetHc in the presence of 0.2 M-sodium acetate at pH 5.00. Acetate could thus displace NO from NitrosylHc. The absence of a mononuclear e.p.r. signal at $g = 2$ indicates that no Semi-MetHc was formed and that NO was accordingly liberated from MetHc.

pH-dependence and kinetics of the reaction of NO with *Helix* α -MetHc

The percentage of NitrosylHc, calculated from the corrected c.d. signals at 336 nm after 4 h reaction of NO with MetHc, plotted against pH also followed the Henderson-Hasselbalch equation, but with a pK_a of 6.80 (Fig. 3). By comparison with the pK_a value of 6.50, observed for the binding of nitrite to *Astacus* MetHc (Tahon *et al.*, 1988), it might also be attributed to a μ -aquo bridging ligand of the copper pair. In contrast with *Astacus* MetHc, there was, however, still a slow reaction of NO at the higher pH values.

The pseudo-first-order rate constants for the reaction of NO with *Helix* α -MetHc and the corresponding percentages of MetHc were determined for five pH values from the corrected c.d. data at 336 nm (Table 2). The higher value at low pH and the lower one at high pH were respectively attributed to the presence of a μ -aquo and of a μ -hydroxo bridging ligand at the active site. The

Table 1. Stability of NitrosylHcs of *Astacus* (19.9 mg/ml, 0.47 mM-Cu) and *Helix* (14.2 mg/ml, 0.51 mM-Cu) at room temperature, estimated by c.d. measurements of preparations in Mes/NaOH buffer, pH 5.70, dialysed for 45 min against the indicated buffer

Buffer (I 0.1)	pH	NitrosylHc (%)	
		<i>Astacus</i>	<i>Helix</i>
Mes/NaOH	5.70	103	100
Na ₂ HPO ₄ /NaH ₂ PO ₄	6.68	83	96
Na ₂ B ₄ O ₇ /HClO ₄	8.20	70	99

Table 2. Rate constants k_1 and the percentages, obtained by kinetic analysis of the pseudo-first-order reactions, at increasing pH values of *Helix* MetHc (20 mg/ml, 0.72 mM-Cu) yielding NitrosylHc under 101 kPa NO at room temperature

Buffer (I 0.1)	pH	$10^5 \times k_1$ (s ⁻¹)	NitrosylHc (%)
Succinic acid/NaOH	5.00	67	98
Succinic acid/NaOH	5.70	58	97
Na ₂ HPO ₄ /NaH ₂ PO ₄	6.88	92	40
Na ₂ HPO ₄ /NaH ₂ PO ₄	6.88	4.5	62
Na ₂ B ₄ O ₇ /HClO ₄	8.20	4.3	90
Na ₂ B ₄ O ₇	9.20	3.5	94

addition in succinic acid/NaOH buffer, pH 5.00, of 0.2 M-NaF ($R = 250$) decreased the rate constant k_1 from 67×10^{-5} to 5.2×10^{-5} s⁻¹, which indicates that F⁻ as a bridging ligand behaves like OH⁻, stabilizing the active site.

The two parallel pseudo-first-order rate constants, observed at pH 6.88, pointed to heterogeneity of the active sites, as the equilibrium between a μ -aquo and a μ -hydroxo ligand on a given active site is reached extremely rapidly. The reaction of NO was therefore investigated with a fragment and with functional units of β_C -Hc, which were separated after limited proteolysis (see the Materials and methods section). With *Helix* α -Hc the presence of two different polypeptide chains (De Sadeleer *et al.*, 1982) hampers the separation of the functional units considerably.

pH-dependence of the reaction of NO with a fragment and with functional units of *Helix* β_C -DeoxyHc

In a first comparison of the reaction rates the samples were dialysed against 20 mM-EDTA/3 mM-NaN₃ in 0.1 M-NH₄HCO₃ in order to remove loose copper. After a further exhaustive dialysis against Na₂HPO₄/NaH₂PO₄ buffer, pH 6.68, they were treated anaerobically at room temperature with NO and the e.p.r. and c.d. signals, characteristic for NitrosylHc, were measured after 16 h. Fragment $a-c$, functional unit e , α -Hc and β_C -Hc were transformed for about 82% in NitrosylHc, functional units d and f for 100% and functional units g and h for only about 61%. The fragment and the functional units were checked after this treatment by SDS/polyacrylamide-gel electrophoresis. No further degradation of the primary structure was observed.

Fig. 4. Percentages as a function of pH of *Helix* NitrosylHc, estimated by c.d., after 4 h reaction at room temperature of DeoxyHc with 101 kPa NO

●, Total α -Hc (Fig. 3); □, functional unit *d*; ■, functional unit *f*; ▲, functional unit *g*; △, functional unit *h*. The curves (continuous lines) were calculated by the Henderson–Hasselbalch equation with a pK_a of respectively 6.25, 6.80 and 7.50. The broken curve was estimated from the Henderson–Hasselbalch equations for the functional units of β_c -Hc and their proportions.

α -Hc was used as a model for fragment *a–c* and for functional unit *e*, as they all formed NitrosylHc at the same rate. The pH-dependence was then estimated from the c.d. spectra after 4 h reaction with NO for the functional units *d*, *f*, *g* and *h* and for α -Hc (Fig. 4). The pH-dependence of the reaction of β_c -Hc, calculated from that of the constituents and from their proportions, did not differ appreciably from the actual Henderson–Hasselbalch equation (Fig. 4). The two reaction rates, observed at pH 6.88 (Table 2), are thus due to the heterogeneity of the functional units causing differences in the pK_a values of the μ -aquo ligand of their active sites in MetHc.

Reaction of azide with *Helix* NitrosylHc

Solutions (40 mg/ml, 1.45 mM-Cu) of NitrosylHc and of a MetHc blank were adjusted to pH 5.00 by dialysis against succinic acid/NaOH buffer, NaN₃ was added to a concentration of 300 mM ($R \approx 200$). After 24 h reaction at room temperature the N₃[−] was removed in less than 1 h by elution on a Sephadex G-25 column (25 cm × 3 cm) with Na₂B₄O₇/HClO₄ buffer, pH 8.20, in order to avoid the c.d. and e.p.r. signals of the N₃[−] derivative of MetHc. The reaction with N₃[−] completely suppressed the c.d. signal of NitrosylHc, which remained constant in a blank experiment without N₃[−]. The mononuclear e.p.r. signal at $g = 2$ of the NitrosylHc treated with N₃[−], which can be ascribed to broken copper pairs, equalled that of the MetHc blank (15% of the Cu) (Fig. 5). The reaction of NitrosylHc with N₃[−] yielded MetHc, as no additional mononuclear e.p.r. signal (possibly of Semi-MetHc) was detected, nor was any OxyHc observed, which would have been formed from DeoxyHc. This MetHc could

Fig. 5. E.p.r. signals at 133 K of *Helix* NitrosylHc (traces *A* and *C*) and MetHc (traces *B* and *D*) in Na₂B₄O₇/HClO₄ buffer, pH 8.20, respectively before and after treatment for 24 h at room temperature with 300 mM-NaN₃ in succinic acid/NaOH buffer, pH 5.00

Buffer change and removal of N₃[−] were performed by column chromatography on Sephadex G-25. [Hc] = 40 mg/ml, 1.45 mM-Cu. Receiver gain was 4000.

almost completely be regenerated by a treatment with H₂O₂ ($R = 10$) in Na₂B₄O₇/HClO₄ buffer, pH 8.20, as shown by absorbance and c.d. measurements at 346 nm.

The gaseous products of the reaction were determined by m.s. in order to establish its stoichiometry (Table 3). Only the signals of N₂⁺, HN₃⁺ and N₂O⁺ were considered, as there was a fragmentation of HN₃ and of N₂O. The fragmentation of HN₃ yields 30%, 20% and 10% of the HN₃⁺ signal ($m/z = 43$) at respectively $m/z = 28$, 29 and 42. Similarly with N₂O 11% and 31% of the N₂O⁺ signal ($m/z = 44$) are expected at respectively $m/z = 28$ and 30 (Cornu & Massot, 1975). The corrected m.s. data thus indicate that only N₂ and N₂O were formed, according to the probable reactions:

The sensitivity for N₂ (9.1 nA/μPa) and for N₂O (15.5 nA/μPa) was estimated from the determined value for NO (10.9 nA/μPa), taking into account the ionization cross-sections (Cornu & Massot, 1975). Per 4 mol of Cu (1.70 mol of active sites, as 15% of them were damaged) 2.33 mol of N₂ and 1.10 mol of N₂O were detected instead of the expected values of respectively 2.55 and 0.85 mol.

The active sites were not appreciably damaged, although the N₃[·] radical seems a plausible intermediate. The rate constant for the reaction of N₃[·] with histidine at pH 7.8, $k_2 < 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Land & Prütz, 1979), is actually

Table 3. Determination by m.s. of the gaseous products of the reaction for 24 h at room temperature of *Helix* NitrosylHc (42.9 mg/ml, 1.55 mM-Cu) and MetHc (41.7 mg/ml, 1.51 mM-Cu) with 300 mM- NaN_3 ($R \approx 200$) in succinic acid/ NaOH buffer, pH 5.00, under 101 kPa He

m/z	Molecular ion	Signal origin	Signal intensity (nA)		
			NitrosylHc	MetHc	Difference
28	N_2^+	Experimental	8.21	3.35	
		Net amount*	6.17	0.12	6.05
29	HN_2^+	20 % $\text{HN}_3^+ \dagger$	0.50	0.44	(0.06)
30	NO^+	31 % $\text{N}_2\text{O}^+ \dagger$	1.80	0.30	(1.50)
32	O_2^+	Air	0.08	0.50	(-0.42)
42	N_3^+	10 % $\text{HN}_3^+ \dagger$	0.30	0.31	(-0.01)
43	HN_3^+	HN_3	3.00	3.13	(-0.13)
44	N_2O^+	N_2O	7.60	2.71	4.89

* N_2 signal corrected for contributions originating from traces of air, HN_3 (30 % of the HN_3^+ signal) and N_2O (11 % of the N_2O^+ signal).

$\dagger$ Theoretical value.

Fig. 6. Percentage of *Helix* OxyHc, estimated from the absorbance at 346 nm, as a function of time during the regeneration of NitrosylHc (1.00 mg/ml, 36 μM -Cu) in $\text{Na}_2\text{B}_4\text{O}_7/\text{HClO}_4$ buffer, pH 8.20

○, Regeneration with 0.36 mM- H_2O_2 ; ●, regeneration with 0.90 mM- HONH_2 .

much smaller than the dimerization constant of N_3^- , $9 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Hayon & Simic, 1970).

These data cannot be reconciled with the transformation by N_3^- of the so-called 'half-met- NO_2^- ' into 'half-met- N_3^- ' (Himmelwright *et al.*, 1979).

Regeneration of *Helix* NitrosylHc with H_2O_2 and HONH_2

Van der Deen & Hoving (1977) have reported the regeneration of *Helix* NitrosylHc with HONH_2 to functional Hc. After the treatment of *Helix* NitrosylHc with N_3^- a nearly quantitative regeneration was obtained with H_2O_2 ($R = 10$), in accordance with the mentioned

Fig. 7. E.p.r. signals at 133 K of *Helix* NitrosylHc (20 mg/ml, 0.72 mM-Cu) after regeneration for 66 h at room temperature in sodium acetate/acetic acid buffer, pH 5.70

Trace A, NitrosylHc; trace B, after regeneration with 18 mM- H_2O_2 ; trace C, after regeneration with 18 mM- HONH_2 . Receiver gain was 10000.

formation of MetHc. In a direct treatment of NitrosylHc in $\text{Na}_2\text{B}_4\text{O}_7/\text{HClO}_4$ buffer, pH 8.20, H_2O_2 ($R = 10$) yielded, however, only a partial regeneration, and HONH_2 ($R = 25$) an almost complete one, produced in a similar rather fast step, followed by a slow one (Fig. 6).

A solution of NitrosylHc (20 mg/ml, 0.72 mM-Cu) in sodium acetate/acetic acid buffer, pH 5.70, regenerated with H_2O_2 ($R = 25$) for 66 h, contained 53 % OxyHc and 41 % NitrosylHc, estimated respectively by absorbance and c.d. measurements and by c.d. measurements. After a similar treatment with HONH_2 ($R = 25$) the preparation contained 94 % OxyHc and no NitrosylHc any

more. The residual, apparently large, e.p.r. signal of NitrosylHc, partially regenerated with H_2O_2 , was due to about 40 % NitrosylHc and 13 % broken Cu pairs (Fig. 7, trace B), and the small e.p.r. signal of NitrosylHc, regenerated with HONH_2 , corresponded to about 5 % of the Cu (Fig. 7, trace C).

The regenerated NitrosylHc preparations showed a normal O_2 binding, when measured at 23 °C under non-co-operative conditions in $\text{Na}_2\text{B}_4\text{O}_7/\text{HClO}_4$ buffer, pH 8.20. The p_{50} value amounted to 4.6, 4.9 and 4.7 mmHg for samples regenerated respectively with HONH_2 , H_2O_2 and H_2O_2 followed by HONH_2 against 4.8 mmHg for the native Hc.

The reaction of H_2O_2 with NitrosylHc involves the formation of MetHc, which is then further transformed into OxyHc, and the plausible liberation of NO_2 , which disproportionates in solution into NO_2^- and NO_3^- :

NO_2 does not appreciably react below pH 6.0 with the most reactive protein residues (Prütz *et al.*, 1985). A one-electron oxidation of H_2O_2 to $\text{O}_2^{\cdot -}$ and a one-electron reduction to $\text{H}_2\text{O} + \cdot\text{OH}$ are not allowed by the oxidation-reduction potentials involved (Wood, 1988). The latter reaction would, moreover, partially damage the active sites:

The anaerobic regeneration of NitrosylHc by HONH_2 yields DeoxyHc:

The partial regeneration of NitrosylHc by H_2O_2 cannot be explained by the exhaustion of H_2O_2 by the catalase activity of Hc or by the destruction of active sites. For after a second treatment with H_2O_2 ($R = 25$) for 66 h 57 % OxyHc and 41 % NitrosylHc were found, and in a further treatment with HONH_2 ($R = 25$) for 66 h the 40 % NitrosylHc was regenerated and 90 % OxyHc was reached. The partial regeneration of NitrosylHc is not due either to heterogeneity of the active sites. Preparations of functional, units *d* (15.8 mg/ml, 0.57 mM-Cu) and *g* (10.2 mg/ml, 0.37 mM-Cu) of *Helix* β_C -Hc contained respectively 27 and 30 % NitrosylHc and 69 and 66 % OxyHc after 18 h regeneration with H_2O_2 ($R = 25$) in sodium succinate/NaOH buffer, pH 5.00. These solutions, treated thereupon for 66 h with HONH_2 ($R = 25$) after a thorough dialysis against the buffer, contained respectively 91 and 90 % OxyHc.

The fast step in the regeneration (Fig. 6) could correspond to a direct interaction of H_2O_2 or HONH_2 with Cu_A , and the slow one, which does not occur with H_2O_2 , could correspond to a reaction of HONH_2 with the bound NO^+ . This interpretation would imply the presence of two different orientations of the ligands of Cu_A in *Helix* NitrosylHc, although no spectral heterogeneity was observed. There were also two reaction rates in the transformation at pH 5.00 and 4 °C of *Helix* NitrosylHc into MetHc with N_3^- , when followed by the determination of nitrite after chromatography on Sephadex G-25 and decomposition of the remaining NitrosylHc

(A.-M. Vandamme, W. Deleersnijder, R. Witters & R. Lontie, unpublished work).

CONCLUSIONS

In the treatment of *Astacus* MetHc with NO the pK_a of 5.85, which determines the slow formation of NitrosylHc, could indicate that the reaction of NO with Cu(II) depends on the release and protonation of the weakest bound imidazole residue. The binding site of NO could not be located [$\text{Cu}^I \text{NO}^+ \text{Cu}^{II}$]. The reaction was blocked by F^- , probably as a bridging ligand between Cu_A and Cu_B . The instability of *Astacus* NitrosylHc increases with pH and might be attributed to the deprotonation of the mentioned imidazole residue, which could again displace the bound NO.

In the reaction of *Helix* MetHc with NO a difference in the co-ordination of Cu_A , with possibly only two imidazole ligands instead of the three for Cu_A in *Astacus* Hc, could be responsible for the almost 12-fold faster formation of NitrosylHc ($\text{Cu}_A^I \cdot \text{NO}^+ \text{Cu}_B^{II}$) and for its much greater stability. Even with a bridging ligand between Cu_A and Cu_B such as F^- or OH^- (pK_a of 6.80 for the μ -aquo ligand) there remained a slow formation of NitrosylHc. The two rates observed in the reaction with NO of *Helix* α -MetHc at a pH near the pK_a value are due to heterogeneity of the active sites in the functional units.

A nearly quantitative regeneration of *Helix* NitrosylHc was obtained with HONH_2 and, after a treatment with N_3^- , with H_2O_2 . The direct regeneration with H_2O_2 was only partial and cannot be explained by exhaustion of H_2O_2 , by a destruction of active sites, or by their heterogeneity. This partial regeneration and the two reaction rates observed with HONH_2 and with N_3^- suggest at least two different orientations of the ligands of Cu_A in NitrosylHc.

These data also provide further arguments against the formulation of NitrosylHc as a nitrite derivative of Semi-MetHc (Himmelwright *et al.*, 1979). The reaction of NO with MetHc could only yield this derivative by the reduction of Cu(II) to Cu(I) and the oxidation of NO to NO_2^- . This would imply that the latter were strongly bound to the copper pair, which is not consistent with the four $A_{||}$ lines in e.p.r. The stability of *Helix* NitrosylHc was confirmed by its resistance to the elution with buffer on a Sephadex G-25 column.

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