Nitrite and Nitric Oxide Treatment of *Helix pomatia* Hemocyanin: Single and Double Oxidation of the Active Site†

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ABSTRACT: The reaction of nitrite and nitric oxide with *Helix pomatia* hemocyanin has been studied. One or both of the two copper ions in the active site can be oxidized, depending upon reaction conditions. The single oxidation of the oxygen binding site can be reversed by reduction with hydroxylamine, and the oxygen binding properties of the protein are simultaneously restored. The experiments, including electron paramagnetic resonance, indicate that nitric oxide is not a ligand of copper in the singly oxidized active site and that the oxidized copper ion is coupled to at least two nitrogen atoms of amino acid residues. The doubly oxidized protein can be reduced to a singly oxidized one with ascorbic acid or hydroxylamine; the latter reagent is again able to reduce the singly oxidized state and to restore the oxygen binding properties.

Hemocyanins are copper proteins of high molecular weight which function as the oxygen carriers in the hemolymph of molluscs and arthropods. These nonheme proteins reversibly bind oxygen with a stoichiometry of one oxygen molecule for every two copper atoms. The protein, colored blue when oxygenated, becomes colorless when deoxygenated; it is generally recognized that the copper in deoxyhemocyanin is entirely in the cuprous state (Van Holde and Van Bruggen, 1971; Lontie and Vanquickenborne, 1974).

The fact that the magnetic susceptibility of oxyhemocyanin is low over a large temperature range has been ascribed to strong antiferromagnetic exchange coupling between a pair of cupric ions in the active site (Moss et al., 1973; Solomon et al., 1976). Recently resonance Raman studies have shown that oxygen in oxygenated hemocyanins is bound as O_2^{2-} (Loehr et al., 1974; Freedman et al., 1976).

Neither oxygenated nor deoxygenated fresh hemocyanins exhibit EPR[1] signals that account for more than a few percent of the available copper in the protein (Van Holde and Van Bruggen, 1971; Schoot Uiterkamp, 1973). However, Schoot Uiterkamp (1972) and Schoot Uiterkamp and Mason (1973) reported EPR signals from nitrite and nitric oxide treated molluscan (*Helix pomatia*) and arthropod (*Cancer magister*) hemocyanins which accounted for about 50% of the available copper. They interpreted the EPR signal of deoxygenated hemocyanin that had been incubated with a solution containing either sodium nitrite with or without ascorbic acid, or NO gas with ascorbic acid, as being due to isolated, mononuclear Cu(II) ions. If the hemocyanin solution was incubated with NO alone, the mononuclear EPR spectrum was still observed, but there was also a broader resonance at $g = 2$ and a weak signal at $g = 4$. These signals were interpreted as being due to dipole–dipole interactions between the two Cu(II) ions in the active site. Computer simulation of the EPR spectrum showed that the Cu(II)–Cu(II) distance was about 6 Å (Schoot Uiterkamp et al., 1974).

From simulations of the mononuclear EPR spectra, it has been concluded that the mononuclear Cu(II) sites have a rhombic distortion. Simulations including superhyperfine coupling indicated that the ligands of the Cu(II) ions included at least two nitrogen atoms (Schoot Uiterkamp et al., 1974). The presence of ^{63}Cu and ^{65}Cu isotopes in copper (in natural abundance 69% and 31%, respectively) increases the line width of the EPR spectra, because the magnetic moments of these two isotopes are slightly different (cf. for example, Taylor and Coleman, 1971). We attempted to determine reliably the number of nitrogen ligands by performing experiments with hemocyanin samples enriched in ^{63}Cu.

In a first attempt to understand the chemistry of the nitric oxide and nitrite reaction with hemocyanin, we investigated whether one of the nitrogen ligands of the mononuclear Cu(II) ions originates from nitric oxide by performing experiments with nitrite enriched in ^{15}N (nuclear spins of ^{14}N and ^{15}N are 1 and 0.5, respectively). We also investigated the reversibility of the reactions with nitrite and nitric oxide. At the same time we performed experiments to determine the role of ascorbic acid in these reactions.

Materials and Methods

Chemicals. All chemicals were reagent grade and most of them were used without further purification. ^{63}Cu (99% enriched) powder and $Na^{15}NO_2$ (95% enriched) were acquired from Rohstoff-Einfuhr GmbH (Düsseldorf, West Germany). Sodium nitrite was purified by recrystallization. ^{63}Cu powder was used without further purification in the preparation of a Cu(I)–acetonitrile complex. The copper powder was dissolved in sulfuric acid and Cu_2O was prepared from the resulting copper sulfate solution with Fehling's reagent using glucose as a reductant (Vogel, 1961). The cuprous acetonitrile complex was synthesized from Cu_2O according to Lontie et al. (1965).

Preparation of Hemocyanin. α-Hemocyanin was isolated from Roman snails (*Helix pomatia*) and stored according to Heirwegh et al. (1961) as modified by Konings et al. (1969) and Siezen and Van Driel (1973).

Determination of Hemocyanin Concentration. Hemocyanin concentrations were determined as described previously by Konings et al. (1969). It was assumed that the extinction coefficient of hemocyanin at 278 nm did not change significantly upon preparation of apohemocyanin or nitrite and nitric oxide treated hemocyanin.

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[1] Abbreviations used: EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid.

Oxygen Binding of Hemocyanin. Oxygen binding curves were measured according to Konings et al. (1969). Hill coefficients and p_{50} values were calculated from oxygen binding curves determined in Tris buffers (ionic strength 0.1, pH 8.2) which were 10 mM in $CaCl_2$. The oxygen binding capacities (ΔA_{340}^{1cm} per mg of hemocyanin/mL) were monitored in 0.1 M phosphate buffers at pH 7.0.

Enrichment of Hemocyanin in ^{63}Cu. Apohemocyanin was prepared according to a slightly modified method of Konings et al. (1969). The copper was removed by dialysis against a buffer containing 10 mM KCN, 10 mM $CaCl_2$, and 0.1 M sodium borate (pH 8.2) at 20 °C during 6 h. The apoprotein preparations obtained were exhaustively dialyzed against Tris buffer, pH 7.1 (ionic strength 0.1), at 4 °C. Copper contents were measured with a Perkin-Elmer 403 atomic absorption spectrophotometer. Apohemocyanin analyzed in this way contained about 20% of the original copper. The reconstitution was performed with a fourfold excess of the cuprous acetonitrile complex in a Tris buffer, pH 7.1 (ionic strength 0.1), according to Konings et al. (1969). Copper contents were again measured by atomic absorption spectroscopy and values up to 90% of reconstitution were obtained.

Preparation of Nitrite and Nitric Oxide Treated Hemocyanin and Its Regeneration. Before the hemocyanin solutions were used in this preparation, traces of aged hemocyanin were regenerated by an overnight incubation in 0.1 M phosphate buffer, pH 7.0, at 4 °C with NH_2OH in a molar ratio to copper of 10 (Lontie and Witters, 1966). After the incubation the excess of NH_2OH was removed by exhaustive dialysis against 0.1 M phosphate buffer (pH 7.0) at 4 °C for 24 h. Mononuclear EPR spectra were obtained from *Helix pomatia* hemocyanin samples, which were prepared in 0.1 M phosphate buffer, pH 7.0, according to Schoot Uiterkamp et al. (1974). Unless stated otherwise, a 10-fold excess of $NaNO_2$ and 40-fold excess of ascorbic acid (excess calculated with respect to copper concentration) were used in these preparations. The regeneration of these oxidized hemocyanin samples was performed in the same way as the regeneration of aged hemocyanins, except that the NH_2OH:Cu ratio was varied over a large range. In cases where the NH_2OH:Cu molar ratio was above 200, the regeneration was no longer performed by the addition of NH_2OH, but by dialysis against aqueous solutions of NH_2OH·HCl brought to pH 7.0 with NaOH. Experiments were repeated several times with good reproducibility.

Electron Paramagnetic Resonance. EPR spectra were recorded with a Varian E-4 spectrometer equipped with an E-257 variable temperature accessory. The EPR spectrometer was operated at a microwave frequency of 9.24 GHz. EPR intensities were calculated from computer-programmed double integration of the signals, using a copper standard solution, which was 358 μM in $CuSO_4$·$5H_2O$ and 3580 μM in EDTA. If the unknown sample and the copper standard had significantly different g values, a correction for differences in the transition probabilities was made (Aasa and Vänngård, 1970, 1975). The EPR signal of the dipolar coupled cupric system was obtained by manual subtraction of the mononuclear signal from the mixture of mononuclear and binuclear EPR signals (Schoot Uiterkamp, 1972). In the calculations of the percentage of dipolar coupled copper pairs in hemocyanin, we assumed that the EPR intensity of a binuclear complex was twice that of a mononuclear complex with the same concentration and g values.[2]

Simulation of EPR Spectra. Simulations of EPR spectra were performed as earlier described (Schoot Uiterkamp et al., 1974). To investigate the effect of a varying ^{63}Cu:^{65}Cu ratio on the line width of the EPR spectra, the mononuclear EPR simulating computer program was slightly modified. The A values of the ^{63}Cu were taken as starting point, whereas the A values of the ^{65}Cu were calculated from A (^{63}Cu) by multiplying with the magnetic moment ratio of both isotopes. The programs were written in Fortran IV and the computations were carried out on a CDC Cyber 74-16 computer at the University of Groningen.

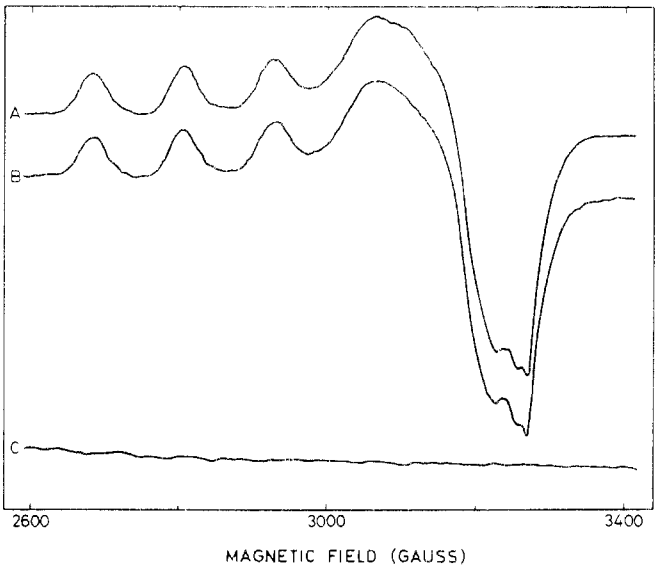

FIGURE 1: EPR spectra around $g = 2$. Protein concentration, 33 mg/mL; temperature, 143 K; microwave power, 10 mW; frequency, 9.24 GHz; modulation amplitude, 4 G; scanning rate, 2 G/s; time constant, 3 s. (A) *Helix pomatia* hemocyanin incubated with $Na^{14}NO_2$; (B) *Helix pomatia* hemocyanin incubated with $Na^{15}NO_2$; (C) cavity and dewar.

Results

The mononuclear EPR spectrum of *Helix pomatia* hemocyanin, treated with $NaNO_2$ in the presence of ascorbic acid, is shown in Figure 1A. Double integration shows that the spectrum accounts for about 45% of the available copper. In the perpendicular region partly resolved superhyperfine structure with a coupling constant of about 15 G, normal for nitrogen ligands, can be clearly seen. The samples of native hemocyanin sometimes exhibit an EPR signal that accounts for about 1% of the available copper in the protein (Schoot Uiterkamp, 1973). If present, this small percentage of cupric ions causes an additional line broadening in the perpendicular region, which can partly reduce the resolution of the superhyperfine structure.

Figure 1C, which depicts the EPR signal of the Dewar and cavity, shows that this superhyperfine structure is completely caused by the hemocyanin sample.

In order to determine reliably the number of superhyperfine lines, our hemocyanin samples were enriched in ^{63}Cu. No sharpening of the superhyperfine lines was measured (not shown), although the ^{63}Cu:^{65}Cu ratio was increased from 2.2 to 10.4 in our samples. A higher enrichment could in principle

[2] EPR signal simulations of Schoot Uiterkamp et al. (1974) show, neglecting exchange coupling, that the Cu-Cu distance of the dipolar coupled copper pair in *Helix pomatia* nitric oxide treated hemocyanin is ~6 Å. Calculations, using these simulation computer programs and the other parameters for this dipolar coupled cupric pair in this hemocyanin sample, show that the total $\Delta M = 1$ transition probability remains constant in the range from ~5 Å to infinite distances between the two copper ions. Neglecting exchange coupling, which seems reasonable as judged from the EPR temperature dependence study (Schoot Uiterkamp et al., 1974), we therefore estimate the ratio in EPR intensity between a binuclear dipolar coupled complex and a mononuclear complex as a factor two.

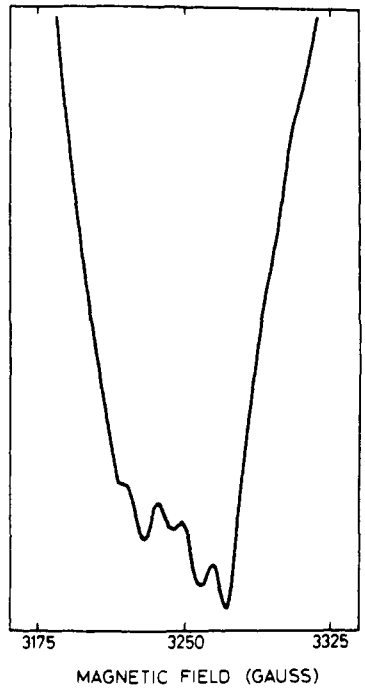

FIGURE 2: Perpendicular region of an EPR spectrum of nitric oxide treated deoxyhemocyanin (38 mg/mL) in the presence of 40-fold excess of ascorbic acid (0.1 M phosphate buffer, pH 7.0); incubation for 12 min in ice. Temperature, 143 K; microwave power, 10 mW; frequency, 9.24 GHz; modulation amplitude, 4 G; scanning rate, 2 G/s; time constant, 3 s.

be obtained, but when we performed computer simulations, using the parameters as described by Schoot Uiterkamp et al. (1974), of a mononuclear spectrum with a copper isotope ratio varying from 2.2 to 99, no effects were seen on the line widths in the parallel and perpendicular region of such a spectrum. Therefore we did not try to raise the copper isotope ratio above 10.4.

Figure 1B shows the spectrum of hemocyanin treated with $Na^{15}NO_2$ in the presence of ascorbic acid. The superhyperfine patterns of the spectra in Figures 1A and 1B are identical, whereas the nuclear spins of ^{14}N and ^{15}N are 1 and 0.5, respectively. Therefore it can be concluded that there is no magnetic coupling of copper with the nitrogen atom originating from nitrite. The same conclusion is valid for nitric oxide, because it has been shown (Schoot Uiterkamp, 1972) that NO_2^- and NO yield identical products.

Accurate simulations of the superhyperfine structure, using a program including superhyperfine coupling of the copper ion with two or more nitrogen atoms with a nuclear spin $I = 1$, were not successful. This was due to the mutual dependency in the simulation of the large line widths and hyperfine splitting constants of copper, and the superhyperfine splitting constants of nitrogen, all in the perpendicular region of the spectrum. Nevertheless, the superhyperfine pattern of at least five lines in the perpendicular region (Figure 2) can now be explained by assuming magnetic coupling between copper and at least two nitrogen ligands, not originating from nitric oxide.

The EPR signal intensity of nitrite treated hemocyanin did not decrease upon exhaustive dialysis against 0.1 M phosphate buffer, pH 7.0, which is in agreement with earlier observations of Schoot Uiterkamp (1972). If a similar oxidized hemocyanin sample was prepared by the reaction of NO gas with a solution of deoxyhemocyanin containing ascorbic acid (Schoot Uiterkamp, 1972), an EPR spectrum identical with the one in Figure 1A was obtained. The intensity of this spectrum did not diminish when the sample was evacuated and flushed several times with N_2 to remove NO. Therefore the reactions, both with nitrite and with NO, seemed to give rise to an irreversibly oxidized hemocyanin sample, which was no longer capable of binding oxygen, as determined by measuring ΔA_{346} (see Table I).

TABLE I: Regeneration of Nitrite-Treated Hemocyanin (18 mg/mL) with Hydroxylamine.

Ratio of NH_2OH and Cu	% EPR detectable Cu	% oxygen binding capacity[a]
0	38	12
2.2	22	53
108	17	60
$\lesssim$690[b]	5	84
$\lesssim$2750[b]	2	91

[a] The percentage oxygen binding capacity was calculated from the comparison between the ΔA_{346}^{1cm} (mg of hemocyanin)$^{-1}$ mL^{-1} of the regenerated samples and the ΔA_{346}^{1cm} of the native protein, which was determined to be 0.339 in a 0.1 M phosphate buffer at pH 7.0. [b] Ratio estimated from regeneration dialysis of an oxidized hemocyanin solution against 0.5 and 2.0 M hydroxylamine buffers, respectively, as described in Materials and Methods.

We were able to reduce the nitric oxide and nitrite treated hemocyanin samples by incubating them with NH_2OH. In Table I the effect of a 16-h incubation with NH_2OH, followed by dialysis against 0.1 M phosphate buffer, pH 7.0, on the EPR intensity and oxygen binding capacity of nitrite treated hemocyanin samples, is given for a typical series of experiments. The reduction reaction with hydroxylamine seems to be very slow. The increase in oxygen binding capacity corresponds almost completely with the decrease of mononuclear EPR intensity. These tabulated experiments give a clear indication that, upon oxidation of one copper per active site (single oxidation), the complete oxygen binding capacity disappears.

In Figure 3 the effect of a nitrite treatment followed by incubation with hydroxylamine on the oxygen binding capacity and cooperativity of hemocyanin is shown. For native hemocyanin (Figure 3A) a Hill coefficient (n_H) of 4.0 and a p_{O_2} value (p_{50}) of 9.3 mmHg were calculated (cf. Van Driel, 1973) at 50% saturation. Dialysis of native hemocyanin (23 mg/mL) against 2.0 M hydroxylamine buffer (pH 7.0) for 16 h resulted in a n_H of 3.7 and a p_{50} value of 8.6 mmHg, as can be seen in Figure 3B. The n_H and p_{50} values of nitrite-treated hemocyanin (18 mg/mL, ~40% mononuclear EPR intensity), which was subsequently dialyzed against a 2 M hydroxylamine buffer, were 3.4 and 11 mmHg, respectively (Figure 3C). The changes in cooperativity and affinity are small and thus the single oxidation with nitrite, followed by the reduction with hydroxylamine, can be considered as reversible.

The incubation of hemocyanin with a 10-fold excess of $NaNO_2$ and a 40-fold excess of ascorbic acid in 0.1 M phosphate buffer, pH 7.0, gave, as stated before, EPR spectra, which accounted for ~45% of the available copper. If under the same experimental conditions the incubation was performed with a 10-fold excess of sodium nitrite, but without ascorbic acid, the EPR intensity accounted only for ~20% of the available copper. Higher percentages up to ~45% were obtained when, for example, an incubation with a 40-fold excess of sodium nitrite was performed under the same experimental conditions.

If deoxyhemocyanin (23 mg/mL) was incubated with nitric oxide for 24 hours at room temperature under strict anaerobic

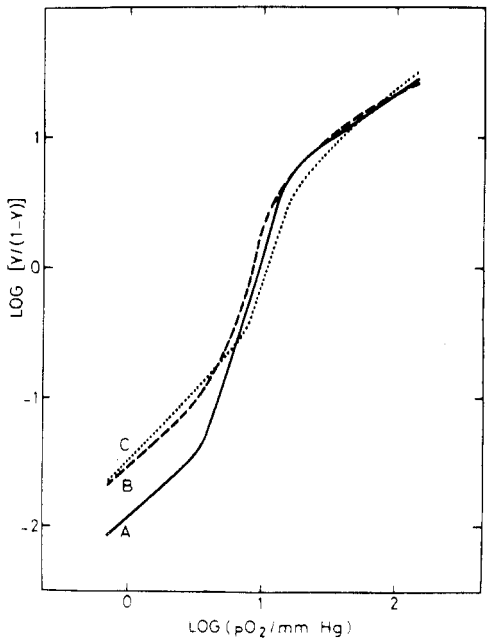

FIGURE 3: Hill plots of oxygen-binding curves in Tris buffer, pH 8.2, ionic strength 0.1, in the presence of 10 mM $CaCl_2$. For clarity these plots are only shown as best fits without indicating positions of the experimental points. (A) (—) native hemocyanin; (B) (- - -) native hemocyanin dialyzed against a 2 M hydroxylamine buffer. (C) (···) native hemocyanin incubated with sodium nitrite, followed by dialysis against 2 M hydroxylamine buffer.

TABLE II: Percentages of Singly and Doubly Oxidized Oxygen Binding Sites in Hemocyanin.

	% oxidized oxygen binding sites		
Sample of Figure 4[a]	Singly oxidized[b]	Doubly oxidized[c]	Total
A	80	0	80
B	14	64	78
C	30	45	75
D	84	0	84
F	21	0	21

[a] The description of the samples can be found in the respective figure captions and in the text. [b] This percentage resembles the percentage of mononuclear EPR detectable copper, multiplied by a factor 2 in order to get the percentage of singly oxidized active sites. [c] This percentage resembles the percentage of EPR detectable dipolar coupled cupric pairs in hemocyanin.

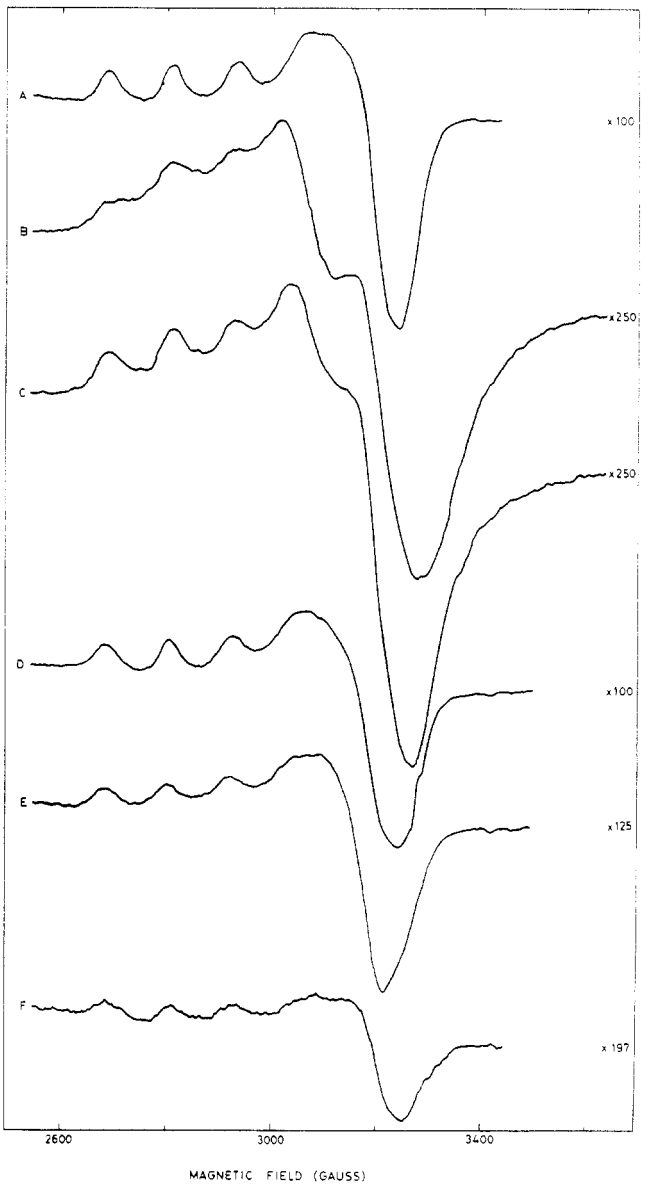

FIGURE 4: EPR spectra of the reaction products of nitric oxide with *Helix pomatia* hemocyanin around $g = 2$. Temperature 173 K; microwave power, 10 mW; frequency, 9.24 GHz; modulation amplitude, 10 G; scanning rate, 4 G/s; time constant, 3 s. Relative receiver gains are indicated in the figure. (A) Nitric oxide reaction with deoxyhemocyanin under anaerobic conditions; (B) sample of A in the presence of a slight amount of oxygen; (C) sample of B after 48 h at room temperature; (D) sample of B after 48 h of incubation with a 40-fold excess of ascorbic acid; (E) sample of B after 48 h of incubation with a 100-fold excess of NH_2OH; (F) sample of D after 72 h of incubation with a 10-fold excess of NH_2OH.

conditions in the absence of ascorbic acid in 0.1 M phosphate buffer (pH 7.0), only an EPR spectrum of mononuclear sites (Figure 4A) was obtained. During this long incubation with NO, no precipitation at all could be observed. Of the oxygen binding sites of the sample prepared in this way, 80% were singly oxidized (see Table II).

We were unable to oxidize both copper ions in the active site under strict anaerobic conditions at pH 7.0 (Schoot Uiterkamp, 1972; Schoot Uiterkamp and Mason, 1973). However, if we allowed a slight amount of air into the sample of Figure 4A and mixed it for about half a minute with the hemocyanin solution in contact with NO gas, after which the sample was immediately evacuated and flushed several times with N_2 (the whole procedure being carried out at 0 °C), an EPR spectrum of dipolar coupled cupric ions in the active site was obtained, as is shown by the broad resonance at $g = 2$ in Figure 4B (cf. Schoot Uiterkamp, 1972). Superimposed on the broad resonance, a mononuclear signal can still be seen. Table II shows that the total number of singly and doubly oxidized active sites remained constant. Comparison of Figures 4A and 4B (see also Table II) shows that, upon treatment with NO in the presence of a slight amount of oxygen, most of the singly oxidized sites in anaerobic nitric oxide treated hemocyanin become doubly oxidized.

If the sample of Figure 4B was kept for 48 h at room temperature, an EPR spectrum as depicted in Figure 4C was obtained. As these figures and Table II show, the amount of mononuclear oxidized copper sites increased, whereas the percentage of dipolar coupled Cu(II) sites decreased to the same extent.

If the sample of Figure 4B was incubated for 48 h at 4 °C with a 40-fold excess of ascorbic acid, a spectrum of only singly oxidized hemocyanin resulted (Figure 4D). The total number of oxidized oxygen binding sites remained nearly constant (Table II).

In Figure 4E an EPR spectrum of the sample of Figure 4B, which was incubated with a 100-fold excess of hydroxylamine during 48 h at 4 °C, is shown. The spectrum shows that all doubly oxidized sites and part of the singly oxidized sites have been reduced.

If the sample that had been incubated with ascorbic acid (Figure 4D) was equilibrated for 3 days with a 10-fold excess of hydroxylamine at 4 °C, most of the singly oxidized sites were reduced as well (Figure 4F and Table II). The oxygen binding capacity, as judged from the ΔA_{346}, was restored for about 75%, which is in close agreement with the remaining 21% singly oxidized active sites of this sample. When more hydroxylamine was used, EPR signals with intensities close to that of native hemocyanin (not shown) were obtained.

Discussion

The mononuclear EPR spectra of hemocyanin, treated with nitric oxide or nitrite in the presence of ascorbic acid, were identical. This is in agreement with earlier observations (Schoot Uiterkamp, 1972). In the nitrite treatment, NO can arise from the reactions

$$3NO_2^- + 2H^+ \rightarrow NO_3^- + 2NO + H_2O \qquad (1)$$

or

$$3H^+ + AH^- + 2NO_2^- \rightarrow A + 2NO + 2H_2O \qquad (2)$$

where AH^- is singly protonated ascorbic acid (Dahn et al., 1960; Schoot Uiterkamp, 1972, 1973).

Under the same experimental conditions, a nitrite treatment without ascorbic acid resulted in about half as much singly oxidized active sites as in experiments with ascorbic acid. In addition almost all oxygen binding sites could be singly oxidized by raising the excess of sodium nitrite. Our experiments therefore show that reactions 1 and 2 are both involved in the production of NO from nitrite at pH 7.0.

Our attempts to determine reliably the number of nitrogen ligands by performing experiments with hemocyanin samples enriched in ^{63}Cu were not successful. Computer simulations with parameters as described by Schoot Uiterkamp et al. (1974) established that successful experiments might not be expected in our case, where in the perpendicular region the line widths are large and the copper hyperfine splittings zero or very small. Replacing copper by pure ^{63}Cu should only give improved results in a limited number of cases (Vänngård, 1972).

In any case the experiments indicate that there should be magnetic coupling of copper with at least two nitrogen atoms. Because our observations (Figure 1) show that there is no magnetic coupling between the nitrogen atom of nitric oxide and copper, this implies that there should be coupling between copper and at least two nitrogen atoms of amino acids in the active site. This result is in good agreement with evidence obtained from acid–base titrations of molluscan and arthropod hemocyanins which showed the existence of histidines in the active site of hemocyanin (Salvato et al., 1974; Engelborghs et al., 1976). Photooxidation experiments with light irradiation at the absorption band in the near ultraviolet also point to histidine residues in the active site (Wood and Bannister, 1968; Tallandini et al., 1975).

In the reaction of nitrite and nitric oxide with hemocyanin, NO did not seem to bind reversibly to copper. Dialysis or repeated evacuations, respectively, were not able to diminish the EPR intensity and to restore the oxygen binding properties. If nitric oxide would bind as $Cu(II)NO^-$, as suggested by Schoot Uiterkamp (1972), the irreversibility of the nitric oxide reaction with hemocyanins should indicate a strong interaction between copper and nitric oxide. The nitrogen isotope experiments clearly show that there is no strong interaction between copper and the nitrogen atom of nitric oxide. Therefore, if nitric oxide were a ligand of copper in a singly oxidized active site in hemocyanin, the only remaining possibility would be that the oxygen atom is the ligand.

In the absence of any well-known copper–NO complexes, comparisons can only be made with other metal nitrosyl complexes (Enemark and Feltham, 1974; Eisenberg and Meyer, 1975). These comparisons make it unrealistic that nitric oxide coordinates with the oxygen atom to copper.[3] Therefore it seems that nitric oxide in this case only oxidizes copper, but does not form a ligand. For comparison we want to note that the reversibility of the reduction reaction of NO with oxidized ceruloplasmin and laccase has indicated that nitric oxide is only a ligand of type-1 copper in ceruloplasmin, but not in laccase (Wever et al., 1973; Rotilio et al., 1975).

The absence of a NO infrared stretching vibration in nitric oxide treated hemocyanin that was singly oxidized (H. van der Deen, J. C. Maxwell, and W. S. Caughey, unpublished data) supports the conclusion that NO is not a ligand of copper in this protein. An attempt to obtain resonance Raman spectra of nitrite treated hemocyanin also failed, although this could partly be due to the fluorescence and to the absence of intense charge transfer bands in the visible region in these samples (H. van der Deen and T. M. Loehr, unpublished data, cited by Freedman et al., 1976). If there would be a strong binding between oxygen of nitric oxide and copper, the probability of finding a NO vibration with one or both techniques should certainly be present. Our experiments therefore indicate that nitric oxide does not form a stable complex with copper, but acts as an oxidant and decays afterward in solution to irreversible products.

The experiments summarized in Table I are a confirmation of the earlier suggested oxidation of one of the coppers in the active site by nitrite in the presence of ascorbic acid (Schoot Uiterkamp, 1972). The oxygen binding capacity can be restored by reduction of this singly oxidized active site with hydroxylamine. Because the reaction products of the oxidation with nitric oxide are not known and hydroxylamine is capable of several redox reactions (Latimer, 1952), it is almost impossible to give an estimation of the standard redox potential of this singly oxidized active site. Figure 3 shows that not only the oxygen binding capacity is restored, but that also the cooperativity of the oxygen binding is almost normal after regeneration. So the single oxidation of the active site and its regeneration is a reversible oxidation–reduction process.

Figure 4 and Table II show that we were unable to oxidize both copper ions in the oxygen binding site under strict anaerobic conditions as described by Schoot Uiterkamp (1972) and Schoot Uiterkamp and Mason (1973). Only in the presence of a slight amount of oxygen were we able to oxidize both copper ions in the active site, as calculated on the basis of reasonable assumptions.[2] Whether the second copper in the

[3] In metal complexes, carbon monoxide coordinates with the carbon to the central metal atom, whereas it has been suggested that carbon monoxide in hemocyanin and hemoglobin coordinates with oxygen to copper and iron, respectively (Fager and Alben, 1972; Alben and Caughey, 1968). Nitric oxide was established to coordinate with nitrogen to iron in hemoglobin (cf. Kon, 1968).

active site is oxidized by NO_2 or by reactions involving oxygen at the active site remains the subject of further investigation.

The doubly oxidized active site can be reduced to a singly oxidized one with ascorbic acid. Figure 4D shows that the function of ascorbic acid in the preparation of singly oxidized hemocyanin with NO gas under almost anaerobic conditions is indeed to prevent double oxidation of the active site, as suggested by Schoot Uiterkamp (1972).

Hydroxylamine reduces not only the singly oxidized active sites, but also the doubly oxidized sites. The oxygen binding capacity of such regenerated samples is almost completely restored.[4]

The doubly oxidized active sites are not stable at room temperature (Figure 4 and Table II) and partly degenerate into singly oxidized ones. Which compound takes part in the reduction is yet unknown. However, our experiments indicate that it seems unlikely that the reductant is a functional amino acid residue at the active site.

As was pointed out before, it is very difficult to estimate standard redox potentials in our case. Our experiments indicate that the first copper ion in the active site is more easily oxidized than the second one. This does not necessarily indicate an inequivalence of the two coppers. While it is possible that the copper ions in the active site have different accessibilities, such differences may be caused by oxidation of the first copper ion. Another possibility is that the standard redox potential of the second copper ion is higher than that of the first one. Again this difference may originate from the oxidation of the first copper ion. The electrostatic interaction between the two copper ions in the active site at a distance of about 6 Å (Schoot Uiterkamp et al., 1974) can be such that the standard potential of the second oxidation step is appreciably higher than that of the first step. Anyhow, the analysis of dipolar coupled EPR spectra, obtained after oxidation of the second copper ion in the active site, shows that both copper ions are magnetically equivalent (Schoot Uiterkamp et al., 1974).

The accelerated formation of *Helix pomatia* "methemocyanin" by azide and fluoride has been described by Witters and Lontie (1975). A reduction of these "methemocyanin" samples with hydrogen sulphide was completed in a short time (De Ley et al., 1975). Indirect evidence was presented that the "methemocyanin" samples, obtained in this manner, contained a pair of exchange-coupled cupric ions in the active site (Dr. R. Witters, Dr. M. De Ley, and Dr. R. Lontie, Vth Hemocyanin Workshop at Malta, 1976). In contrast to this, our EPR spectra and those of Schoot Uiterkamp et al. (1974) indicate that, in doubly oxidized hemocyanin, a dipolar coupled cupric pair with negligible exchange coupling exists in the active site. In addition we were able to reduce the singly oxidized protein with sodium sulfide at pH 7.0, as judged from the EPR spectrum, but these reduced samples did not bind oxygen (H. Hoving, unpublished results). A detailed comparative study of "methemocyanin" and singly and doubly oxidized hemocyanin samples will therefore be necessary to discover the differences between these three oxidation states of hemocyanin.

Oxygen binding properties of hybrid molecules of apohemocyanin and native hemocyanin have indicated that the interactions between native subunits are essential for cooperative oxygen binding (Van Driel, 1973). However, these experiments give no indication which stage of oxygen binding triggers a conformational change. Dr. R. Lontie has suggested experiments with hybrid molecules of "methemocyanin" and native hemocyanin to get more information about the cooperativity of hemocyanin (Vth Hemocyanin Workshop at Malta, 1976). Such hybrid experiments with singly and doubly oxidized hemocyanin and native hemocyanin will now allow us to study the oxygen binding mechanism in more detail. For example, the hypothesis could be tested that oxygen binding occurs in two steps involving an intermediate singly oxidized state.

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[4] One of the reviewers brought to our attention the Ph.D. thesis of Dr. C. Simo (University of Oregon Medical School, 1966). In this thesis the NO reaction with *Cancer magister* hemocyanin was studied in detail, including superhyperfine structure in the EPR signal. It seems interesting to note that, in case of *Cancer magister* hemocyanin, it was possible to reduce the singly oxidized protein with ascorbic acid, whereas doubly oxidized *Helix pomatia* hemocyanin could only be reduced to its singly oxidized state with this reducing agent.

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Complete Amino Acid Sequence of Azotoflavin, a Flavodoxin from *Azotobacter vinelandii*†

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ABSTRACT: Amino acid sequence studies which have led to the sequence determination of the *Azotobacter vinelandii* flavodoxin (azotoflavin) are presented in this report. The total sequence of the flavoprotein is of interest since it is the first group II flavodoxin whose sequence has been determined completely. The amino acid sequence of azotoflavin, a flavin mononucleotide (FMN) redox protein, is: H_2N-Ala-Lys-Ile-Gly-Leu-Phe-Phe-Gly-Ser-Asn-Thr-Gly-Lys-Thr-Arg-Lys-Val-Ala-Lys-Ser-Ile-Lys-Lys-Arg-Phe-Asp-Asp-Glu-Thr-Met-Ser-Asp-Ala-Leu-Asn-Val-Asn-Arg-Val-Ser-Ala-Glu-Asp-Phe-Ala-Gln-Tyr-Gln-Phe-Leu-Ile-Leu-Gly-Thr-Pro-Thr-Leu-Gly-Glu-Gly-Glu-Leu-Pro-Gly-Leu-Ser-Ser-Asp-Cys-Glu-Asn-Glu-Ser-Trp-Glu-Glu-Phe-Leu-Pro-Lys-Ile-Glu-Gly-Leu-Asp-Phe-Ser-Gly-Lys-Thr-Val-Ala-Leu-Phe-Gly-Leu-Gly-Asp-Gln-Val-Gly-Tyr-Pro-Glu-Asp-Tyr-Leu-Asp-Ala-Leu-Gly-Glu-Leu-Tyr-Ser-Phe-Phe-Lys-Asp-Arg-Gly-Ala-Lys-Ile-Val-Gly-Ser-Trp-Ser-Thr-Asp-Gly-Tyr-Glu-Phe-Glu-Ser-Ser-Glu-Ala-Val-Val-Asp-Gly-Lys-Phe-Val-Gly-Leu-Ala-Leu-Asp-Leu-Asp-Asn-Gln-Ser-Gly-Lys-Thr-Asp-Glu-Arg-Val-Ala-Ala-Trp-Leu-Ala-Gln-Ile-Ala-Pro-Glu-Phe-Gly-Leu-Ser-Leu-COOH. This single polypeptide chain protein consists of 179 amino acids and contains a single cysteine residue at position 69. Dimerization of two azotoflavin molecules by disulfide bond formation results in the inactivation of azotoflavin as an electron carrier. Azotoflavin differs from previously studied flavodoxins in that the two residues flanking the planar FMN ring are a glutamic acid and a tyrosine residue. Sequence and conformational comparisons of the various group I and group II flavodoxins suggest that the major difference between these two types is a lengthening of the COOH-terminal region of the group II flavodoxin, which further suggests that all of the flavodoxins have arisen from the same ancestral precursor.

Azotoflavin is a naturally occurring FMN[1] redox protein which was isolated from the obligate aerobe *Azotobacter vinelandii.* Since this flavoprotein was first reported as an electron carrier in *Azotobacter vinelandii* (Shethna et al., 1965, 1966), it has been known by the names "Shethna flavoprotein" (Edmondson and Tollin, 1971b), "*Azotobacter* free-radical flavoprotein" (Hinkson and Bulen, 1967), and "Azotoflavin" (Benemann et al., 1969). Earlier work by Hinkson and Bulen (1967), Benemann et al. (1969), and also Cusanovich and Edmondson (1971) had shown that the Shethna flavoprotein would not replace ferredoxin in the photosynthetic reduction of $NADP^+$ by spinach chloroplasts or in the phosphoroclastic assay of extracts from *Clostridia.* This flavoprotein could, however, replace ferredoxin in assays with *Azotobacter* nitrogenase (Benemann et al., 1969), and more recent studies by van Lin and Bothe (1972) have demonstrated that at high concentrations and under anaerobic conditions it could also replace ferredoxin in NADP reduction by illuminated spinach chloroplasts or by molecular hydrogen and hydrogenase from *Clostridium pasteurianum.* Thus, the Shethna flavoprotein was classified as a flavodoxin. Azotoflavin (*Azotobacter* flavodoxin) differs from other flavodoxins

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[1] Abbreviations used are: FMN, flavin mononucleotide; NADP, nicotinamide adenine dinucleotide phosphate.