1. Introduction

Fresh oxy and deoxy hemocyanin of *Helix pomatia* do not show an electron paramagnetic resonance (EPR) signal. The same result has been found for other hemocyanins [1, 2]. Aged hemocyanin of *Jasus lalandii* [3] and *H. pomatia* has been reported to show an EPR signal [4]. EPR signals were found for *H₂O₂* [1] and NO-treated [5] hemocyanin. In this communication we report the effect of NO and NO⁻ on hemocyanin of *H. pomatia*. In the case of NO-treated hemocyanin we find dipolar coupling between 2 Cu²⁺ ions in the active site.

2. Materials and methods

Hemocyanin of *H. pomatia* was isolated according to Konings et al. [6]. Protein and copper concentrations were determined as previously described [6]. All experiments were carried out in 0.1 M sodium acetate buffer at pH 5.7. The initial protein concentration was 46 mg/ml (1.84 mM Cu). The EPR was measured on a Varian E-4 spectrometer equipped with an E-257 variable temperature accessory. Anaerobic EPR experiments were carried out in an EPR tube with 2 side arms as described by Palmer [7]. Deoxygenation at room temp was done by several alternating evacuations and equilibrations with high purity nitrogen gas; during the procedure the solution was shaken. Under these conditions the volume of the sample usually decreases by a factor of about 1.5. In the NO⁻ treatment, a protein solution in one side arm of the EPR tube was mixed with a sodium nitrite-containing buffer solution in the other side arm. In the NO treatment, a protein solution was incubated with nitric oxide gas at less than 1 atm. After 3 min excess NO was pumped out and after transferring the solution to the quartz side arm, the cell was filled with nitrogen. Thereafter the EPR tube was placed in crushed ice. Incubation with NO for longer than 15 min resulted in partially denatured protein samples. Reagent grade chemicals were used. Dialysis procedures were carried out at 4°C. EPR intensities were calculated according to Vännigard [8], using the integral of the low field hyperfine component, compared to a standard solution of 25 vol% glycerol–water, 1 mM in CuSO₄·5H₂O. Unless noted otherwise, a microwave power setting of 10 mW was used.

3. Results

3.1. Monomer signal in sodium nitrite and nitrogen monoxide treated hemocyanin

EPR measurements were carried out with the following samples:

a) 0.4 ml Hemocyanin solution after 24 hr incubation with 0.1 ml buffer solution which was 18.65 mM in sodium nitrite; incubation was under air at 20°C.

b) As a, but incubated for 3 hr under nitrogen.

c) 0.2 ml Hemocyanin solution together with 0.2 ml buffer solution which was 37.3 mM in ascorbic acid (at pH 5.7) and with 0.1 ml buffer solution which was 18.65 mM in sodium nitrite; incubation was for 3 hr under nitrogen at 20°C.
Fig. 1. EPR spectrum of hemocyanin plus ascorbic acid solution in 0.1 M sodium acetate buffer, pH 5.7, after 3 hr incubation under nitrogen with sodium nitrite solution as described under sect. 2 and 3.1.c. Protein concentration was 24 mg/ml, copper concentration 0.96 mM. Temp 90°K. Microwave power 10 mW. Frequency 9.07 GHz. Modulation amplitude 100 gauss. $g_\parallel = 2.30; g_\perp = 2.03; A_\parallel = 125$ gauss = 0.0134 cm$^{-1}$.

3.2. Dipolar Cu$^{2+}$ signal in nitrogen monoxide treated hemocyanin

0.5 ml Hemocyanin solution was incubated with NO for 3 min. Protein concentration as determined after the experiment was 65 mg/ml. The yellowish-green solution gave the EPR spectrum which is shown in fig. 2. The spectrum shows a monomer signal which corresponds to about 20% of the copper in the protein. The monomer signal is superimposed on a broad signal of about 1400 gauss width. The broad signal corresponds to at least 50% of the copper in the protein. In fig. 2 we have manually subtracted the monomer signal by comparison with the pure monomer signal of fig. 1. Spectrum a in fig. 3 shows the spectrum of NO-treated hemocyanin around 1500 gauss where a half-field signal ($\Delta m_s = 2$) with 7 hyperfine lines is expected in the case of dipole-coupled Cu$^{2+}$ ions [9]. For comparison, the spectrum of our Cu$^{2+}$ standard solution is given (fig. 3b), which shows no half-field signal. An NO-saturated 0.1 M acetate buffer showed no signal under the conditions where the NO-hemocyanin showed the broad signal of fig. 2. After one day dialysis against 0.1 M sodium phosphate buffer which was 0.025 M in EDTA and after dialysing back to 0.1 M acetate buffer pH 5.7 we still saw some broadening in the EPR spectrum. We also saw a weak half-field signal and observed about the same amount of monomer as before EDTA dialysis. Copper determination after EDTA dialysis showed a decrease of the copper content of the protein of about 20%. EPR signals (around $g = 2$) before and after EDTA dialysis did not show saturation between 2 and 50 mW microwave power at 96°K. EPR signals did not
Fig. 2. EPR spectrum of hemocyanin in 0.1 M sodium acetate, pH 5.7, after 3 min incubation with NO as described under sect. 2 and 3.2. Protein concentration was 65 mg/ml, copper concentration 2.6 mM. Temp 96°K. Microwave power 10 mW. Frequency 9.07 GHz. Modulation amplitude 10 gauss. Monomer signal: $g_\parallel = 2.30; g_\perp = 2.03; A_\parallel = 125$ gauss = 0.0134 cm$^{-1}$. The dotted (---) line shows the dipole signal obtained after subtraction of the monomer signal.

change in form and the intensity did not deviate from normal Curie behaviour between 96°K and 230°K. Experiments carried out in 0.1 M sodium phosphate buffer pH 7.0 gave the same result as described above.

4. Discussion

We interpret the results obtained under sect. 3.1. as oxidation by NO of one Cu$^+$ in the active site to Cu$^{2+}$, while the other Cu$^+$ ion remains unaltered:

$$2 \text{Cu}^+ + \text{NO} \rightarrow \text{Cu}^{2+} - \text{NO} + \text{Cu}^+$$

NO in 3.1. cases a and b can arise from the reaction:

$$3 \text{NO}_2^- \rightarrow \text{NO}_3^- + 2 \text{NO} + \text{H}_2\text{O}$$

We assume that both NO$_2^-$ and ascorbic acid inhibit the oxidation of the second copper in the active site. The mono-nitrosyl hemocyanin we got in sect. 3.1. showed to be stable against EDTA dialysis. So we believe that the copper in the protein is still in the "active" site.

The results obtained with nitrogen monoxide-treated hemocyanin show very clearly that in this case 2 dipole-coupled Cu$^{2+}$ ions occur, together with a monomer that is identical to the monomer we obtained after nitrite treatment. A theoretical treatment of dipolar coupling in Cu$^{2+}$ systems has been given by Boas et al. [9]. In our case dipolar coupling follows from the appearance of the broad signal around $g = 2$ (fig. 2) and from the presence of the half-field signal around 1500 gauss (fig. 3). The broad signal around $g = 2$ cannot be caused by NO dissolved in the buffer solution as is shown experimentally under sect. 3.2., as well as theoretically [11]. The line shape of both the broad $g = 2$ line and the half-field signal agree well with the computer-simulated curves of Boas et al. [9]. Comparison with these curves indicates that the distance between the two Cu$^{2+}$ ions is 5.0 ± 0.5 Å. Computer spectrum analysis will be done in order to make this distance more certain. As expected, the half-field signal has an intensity more than 50 times less than the signal intensity around $g = 2$. From the 7 lines expected in first order, 5 lines are well resolved. The hyperfine splitting between the lines at half-field is 62.5 gauss which is one half of the hyperfine splitting of the monomer signal around $g = 2$, as is theoretically predicted. This is evidence for the fact that the signal arises from 2 coupled Cu$^{2+}$ ions, which are in similar surroundings as the Cu$^{2+}$ of the monomer.
Fig. 3. EPR spectra around half-field (1500 gauss). (a) Hemo-
cyamin in 0.1 M sodium acetate, pH 5.7, after 3 min incu-
bation with NO as described under sect. 2 and 3.2. Protein
concentration was 65 mg/ml, copper concentration 2.6 raM.
Temp 96°K. Microwave power 160 mW. Frequency 9.07
GHz. Modulation amplitude 10 gauss. Hyperfine splitting
62.5 gauss. Scan time 62.5 gauss/min, time constant 10 sec.
(b) Cu⁺⁺ standard which is used for intensity determinations.
EPR conditions are the same as in sect. 3.a with the excep-
tion of a scan time of 125 gauss/min and a time constant of
3 sec. The solution is under air.

The fact that there is no deviation of normal
Curie behaviour of the EPR signal between 96°K
and 230°K is evidence for the absence of appreciable
exchange coupling between the Cu²⁺ ions. The ex-
change coupling constant J should be less than 30°K
(20 cm⁻¹). After EDTA dialysis the monomer sig-
nal has the same intensity, while the dipolar signal
diminishes. Although this observation is as yet un-
explained, it may be possible that, as we do not
lose much copper from the protein, the “active”
site which contains 2 NO molecules is reduced re-
versibly while dialysing against EDTA buffer solu-
tion under air. We shall study this aspect further
and we intend to do magnetic susceptibility mea-
surements in order to get a good value for the
amount of paramagnetic copper and for the value
of the exchange coupling constant in the nitrogen
monoxide treated hemocyanin.

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