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The Main Role of Inner Histidines in the Molecular Mechanism of Myoglobin Oxidation Catalyzed by Copper Compounds

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In the presence of Cu^{2+} and $Cu(Gly)_2$, the oxidation of two native MbO_2's (Mb = myoglobin), from the sperm whale and horse, and also two chemically modified sperm whale MbO_2's alkylated at solvent-accessible histidines by sodium bromoacetate (CM-MbO_2) and by iodoacetamide (CA-MbO_2) have been studied at different pH's, ionic strengths, and concentrations of the copper reagent. The influence of competitive redox-inactive zinc ions on the reaction rate is investigated as well. Localization of $Cu(Gly)_2$ in sperm whale met-Mb and CM-met-Mb has been examined using the high-resolution NMR method. The obtained data suggest that binding of copper compounds to the surface histidines (all of them are 1.8−2.7 nm apart from the heme) has only a minor, no more than 35%, contribution to the overall reaction rate, in particular under a large excess of the reagent (more than 8−10-fold). The noticeable contribution of His113(116), His48, and His81, which have the greatest affinity to copper according to NMR data, is revealed only at small concentrations of copper, less than a 5-fold excess relative to the protein. The main contribution to the reaction rate must be from the binding of copper to the inner histidines, His97 (0.62 nm from the heme), and possibly to the distal His64. Both are inaccessible to the modification by alkylating reagents and have much lower affinity to copper than all surface histidines, because they are hydrogen-bonded, the former with the carboxyl group of the heme propionate and the second with the liganded O_2.

The maintenance of the reduced state of the heme complex in HbO_2 and MbO_2 is very important for their functioning, as oxidized met forms of these respiratory proteins are not capable of binding O_2 (Hb = hemoglobin, Mb = myoglobin). At the same time, both can be spontaneously oxidized under aerobic conditions (autoxidation),[1,2] the process being largely enhanced by various metal salts and complexes.[3,4] While metals are essential for the normal function of many biological structures, they became toxic when present in excess. The mechanism of Mb oxidation by copper compounds[5−7] is of special interest, as this protein is present in high concentration in muscles and the redox reaction plays a very important role in the damage of the myocardium during ischemia and subsequent reperfusion.[8,9] Oxidized met-Hb and met-Mb can be again reduced by low-specific cell diaphorases and also by special NADH-dependent enzymes, met-Hb-reductase of erythrocytes, and muscle met-Mb-reductase (NADH-Cyt b_5-reductases).[10]

Metal compounds with high redox potentials, such as bipyridyl and phenantroline Cu^{2+} complexes (E_0 is 480 and 590 mV, respectively), can oxidize heme proteins by direct interaction with the heme group through overlapping π orbitals of the heme and the metal complex (simple outer-sphere electron transfer).[3,11] However, the Cu^{2+} compounds with medium redox potentials ($E_0 \leq 100-150$ mV) not capable of such reaction with HbO_2 and MbO_2 will initially form some specific complex with protein with subsequent intramolecular electron transfer in the complex (site-specific outer-sphere electron transfer mechanism). In the former case, the reaction rate is basically proportional to the difference in redox potentials between the protein and the reagent, as well as to their electronic self-exchange rates, but in the latter, it becomes dependent also on the stability of the protein−reagent complex and different factors influencing its formation.[3,4,7,12]

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Catalytic transformation of MbO_2 (and HbO_2) into the met form in the presence of small amounts of Cu^{2+} or its complexes, which is of special interest for biology, also proceeds via a site-specific electron transfer mechanism according to the following scheme:[3]

$$MbO_2 + Cu(II)L_n \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} MbO_2 \cdot Cu(II)L_{n-m} + mL \quad (1)$$

$$MbO_2 \cdot Cu(II)L_{n-m} \underset{k_2}{\overset{k_{-2}}{\rightleftarrows}} Mb(2) \cdot Cu(II)L_{n-m} + O_2 \quad (2)$$

$$Mb(2) \cdot Cu(II)L_{n-m} \xrightarrow{k_3} Mb(3) \cdot Cu(I)L_{n-m} \quad (3)$$

$$Mb(3) \cdot Cu(I)L_{n-m} + O_2 + H^+ \xrightarrow{k_4} Mb(3) \cdot Cu(II)L_{n-m} + HO_2 \quad (4)$$

$$Mb(3) \cdot Cu(II)L_{n-m} + mL \underset{k_5}{\overset{k_{-5}}{\rightleftarrows}} Mb(3)H_2O + Cu(II)L_n \quad (5)$$

In Cu^{2+} binding (reaction 1), histidines are shown to participate.[5−7] Immediate electron transfer is proven to proceed in the copper complex of ligand-free deoxy-Mb (reaction 3), and dissociation of the bound ligand (reaction 2) is the rate-limiting stage.[3] Reduced Cu^+ is reoxidized then to Cu^{2+} by dissolved oxygen with the participation of protons (reaction 4), providing a closing of the catalytic cycle and regeneration of the catalyst (reaction 5). Catalysis is not observed under anaerobic conditions.[5,6] Active oxygen forms (O^{2-}, HO_2, et al.), produced in reaction 4, must disproportionate rapidly to H_2O_2, converting then into O_2 and H_2O by met-Mb, which have very high peroxidase activity.[1]

In spite of the fact that the oxidation of respiratory proteins catalyzed by copper has long been studied, its molecular mechanism is still not clear. The obtained data are insufficient and contradictory. According to most investigators, the saturation observed in the reagent concentration curves, the linear correlation between the rate constant (k_{obs}), and the stability constant (K) of the copper complex, as well as the inhibiting effect of redox-inactive Zn^{2+} ions competitive with Cu^{2+} for binding to the protein, are in favor of initial formation of the complex between copper compounds and myoglobin (site-specific outer-sphere electron transfer mechanism).[3,7,12] However, the amount and exact location of different Cu^{2+}−His complexes and their contributions to the overall reaction rate are not clear. For example, out of 12 histidines of sperm whale Mb, six residues located on the protein surface are capable of binding copper. According to NMR data,[3] three of them, His113, 116, and 48, have the greatest affinity to copper, but if they are replaced by Ala, Asp, and Ala, respectively, the rate of MbO_2 oxidation by $Cu(Gly)_2$ is shown to decrease insignificantly (only by 15−35%).[12]

Note that some authors did not observe any inhibiting effects of redox-inactive competitive Zn^{2+} and Ni^{2+} ions on the HbO_2 and MbO_2 oxidation by copper compounds, concluding that specific Cu^{2+} binding to protein is not necessary, and electron transfer can proceed through a simple outer-sphere mechanism (at least at the excess of the reagent) or possibly both variants might take place under different conditions.[11]

In the present report, we compare the oxidation rates in the presence of Cu^{2+} and $Cu(Gly)_2$ of two native oxymyoglobins (MbO_2), sperm whale and horse, differing in the amount of surface histidines, with those of two chemically modified sperm whale MbO_2 derivatives, which were alkylated at all accessible histidines by sodium bromoacetate (CM-MbO_2) and iodoacetamide (CA-MbO_2). For all proteins, the effect of reagent concentration, pH, ionic strength, and concentration of the redox-inactive zinc ions on the reaction rate is investigated. Through the use of the high-resolution NMR method, localization of $Cu(Gly)_2$ in native sperm whale and horse met-Mb and in CM-met-Mb is examined.

Materials and Methods

Sperm whale myoglobin in the met form (fraction IV) is isolated from skeletal muscles and purified as described previously.[13] Horse myoglobin (Sigma, U. S. A.) has been gel-filtered through a Sephadex G-25 column equilibrated in a 0.1 M phosphate buffer, pH 7.5.

Sperm whale met-Mb carboxymethylated at all accessible histidines (CM-Mb) has been obtained by incubation of the protein with 0.2 M bromoacetate in a 0.1 M phosphate buffer, pH 7, within 6−7 days at room temperature.[14] Under these conditions, all accessible histidines have been shown to modify. After separation of the excess reagent by intensive dialysis against a 0.01 M phosphate buffer, pH 6, the reaction mixture was additionally fractionated on the Sephadex CM-25. CM-Mb is eluted by a 0.01 M phosphate buffer, pH 6, as a single fraction, while intact Mb goes out only in a 0.1 M phosphate buffer, pH 6.5.[15]

Sperm whale met-Mb carboxyamidated at all accessible histidines (CA-Mb) is obtained by chemical modification of met-Mb in the presence of 0.2 M iodoacetamide under the same conditions.[16] After 5−7 days, the excess reagent was separated by gel-filtration through a Sephadex G-25, equilibrated in a 0.01 M phosphate buffer, pH 6. The reaction mixture was then fractionated by ion-exchange chromatography on a CM-Sephadexe-25 in a stepwise gradient, 0.01−0.1 M, with a phosphate buffer, pH 6.5.[15] The main fraction of CA-Mb, which was eluted with a 0.05 M phosphate buffer and used in our experiments, makes 60% of the total protein.

Oxy forms of myoglobin have been prepared by a reduction of met-Mb with sodium dithionite and separating the latter by gel-filtration through a Sephadex G-25 column in 10 mM KCl, pH 7.5, under aerobic conditions. A complex of oxy-Mb with Zn^{2+} is obtained by mixing of the protein with a concentrated $ZnCl_2$ solution ($[Zn^{2+}]/[Mb]$ ratios are 1.25, 5, and 10).

Tris (Serva, Germany), $CuCl_2$, $ZnCl_2$, maleinic anhydride (all of chem. grade), glycine (Reanal, Hungary), and KCl (analyt. grade) are used without additional purification.

The MbO_2 concentration has been determined spectrophotometrically, using extinction coefficients (M^{-1} cm^{-1}) equal to 13 600 at 543 nm and 14 200 at 581 nm. For modified CM-MbO_2 and CA-MbO_2, the same coefficients are used, as the modification does not affect absorption of the heme.[15,16]

The kinetics of MbO_2 oxidation have been studied spectrophotometrically at 581 (or 543) nm, using a biradial Specord UV−vis spectrophotometer (Germany) at 20 °C. The kinetic

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curves were monitored over the time interval, in which the reaction amplitude varies by 15−20%, covering usually its initial linear part. The reaction rate was characterized by the initial rate (V_0). The concentration of oxymyoglobin was 2.25×10^{-5} M. For the necessary variation of the $[Cu^{2+}]/[MbO_2]$ ratio from 0.2 up to 50, the concentrated $CuCl_2$ or $Cu(Gly)_2$ solution (10 μL) was added to the reaction mixture with a Hamilton microsyringe. The $Cu(Gly)_2$ complex was prepared by mixing the $CuCl_2$ solution with glycine. All experiments were carried out in a Tris-maleate buffer (1:1), in which, unlike with the phosphate buffer, insoluble copper and zinc salts are not formed.

Isoelectrofocusing of sperm whale and horse met-Mb, CM-Mb, and CA-Mb was conducted in 5% polyacrylamide gel with pH 4−9 ampholines, according to Fesenko et al.[17] After separation, a carved protein band was eluted by 2.5 mL of bidistilled water, the pH of eluate being determined within ±0.05 pH accuracy. For native sperm whale and horse met-Mb, pI values of 8.3 and 7.4 were obtained, respectively. CM-Mb yielded a single wide band with a pI of 5.2 and CA-Mb, a band with a pI of 8.2.

The high-resolution NMR spectra were recorded at room temperature, first on a Bruker WM-400 spectrometer and then on a Bruker AV-600. Spectra were accumulated (256 scans for each experiment) in 16 000 data points. Samples were prepared by the incubation of myoglobin in a 10 mM acetate buffer (in ^2H_2O), pH 5.2−5.5, or a 10 mM Tris-HCl buffer (in ^2H_2O), pH 7.1−7.5, within 12 h at 4 °C. To 500 μL of met-Mb solution with a concentration of 6 mg/mL was added 5 μL of a $Cu(Gly)_2$ solution up to $[Cu(Gly)_2]/[Mb]$ ratio equal to 0.3 (at pH 7.1−7.5 and 5.5) and 0.08 (at pH 5.2). At a $[Cu(Gly)_2]/[Mb]$ ratio of 0.3 (pH 5.2), nonspecific broadening of all NMR spectra was observed because of the protein aggregation.

Results

Characterization of Chemically Modified Myoglobins. Six of 12 histidines of sperm whale myoglobin, His12, 48, 81, 113, 116, and 119, all located near the surface, are fully accessible to the solvent and can easily react with H^+ ions, bromoacetate, and iodoacetamide, while the inner residues, His24, 36, 64 (distal), 82, 93 (proximal), and 97, are poorly accessible or inaccessible and cannot be modified (Figure 1). Chemical modification of myoglobins by these chemical reagents was studied in detail by Gurd at al.[14−16] Using tryptic or chymotryptic hydrolysis of carboxymethylated CM-Mb and carboxyamidated CA-Mb followed by amino acid analysis of the peptides, they showed that His12, 113, 116, and 81 form mainly bisubstituted derivatives, while His48 and His119 form monoalkylated compounds and inner residues do not modify. As Gurd at al. analyzed the whole reaction mixture (after separation of the excess reagent), we additionally fractionated it by ion-exchange chromatography[15] and used main fractions of CM- and CA-Mb in the experiments to be sure that all surface residues are carboxyalkylated (see Materials and Methods).

The absorption, ORD, and CD spectra in the UV, Soret, and visible regions as well as the thermal and pH stability of CM- and CA-myoglobins do not differ from those of intact Mb, thus pointing out that the conformation of the heme cavity and the whole protein is maintained.[18] As a consequence, the modification also does not influence O_2 binding or the redox properties of myoglobin, in particular the rate of MbO_2 autoxidation.[15,19,20] The main difference of CM-Mb and CA-Mb from the intact protein consists in the overall molecule charge. This change is insignificant for carboxyamidated Mb (pI 8.2 for CA-Mb and 8.3 for intact Mb), while carboxymethylated CM-Mb is much more negative (pI 5.2) due to introduced COO^- groups.

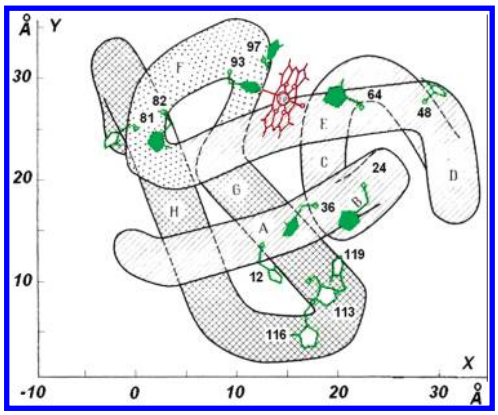

Figure 1. Spatial structure of sperm whale myoglobin in the *xy* projection and the location of buried (solid green) and surface (open green) histidines in it. The heme is shown in red.

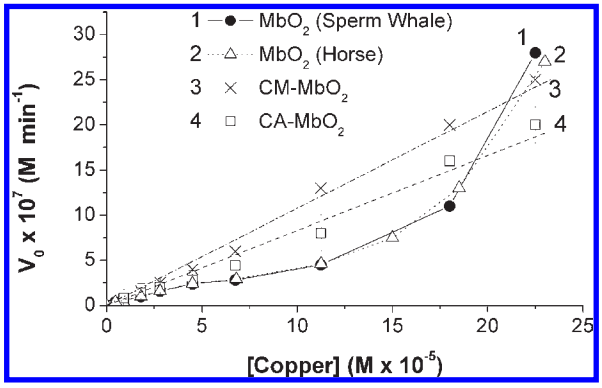

Figure 2. The Cu^{2+} concentration dependence of the oxidation rate of sperm whale and horse MbO_2 (curves 1 and 2), CM-MbO_2 (curve 3), and CA-MbO_2 (curve 4) for $[Cu^{2+}]/[Mb]$ ratios from 0.2 to 10. The protein concentration is 2.25×10^{-5} M, 0.01 M Tris-maleate buffer (1:1), pH 7.5, 20 °C.

Oxidation of Native and Modified Myoglobins in the Presence of Copper Compounds. The influence of Cu^{2+} concentration on the initial oxidation rate of sperm whale MbO_2, horse MbO_2, CM-MbO_2, and CA-MbO_2 at pH 7.5 is shown in Figure 2 (curves 1−4). The concentration dependences are identical for sperm whale and horse myoglobins (curves 1 and 2). Both are complex in shape, clearly demonstrating two parts on the concentration curve. The first one is observed at $[Cu^{2+}]/[MbO_2]$ ratios from 0.2 to about 5, where the oxidation rate initially grows with increasing copper concentration and then reaches a plateau. The second part, at the 8-fold and

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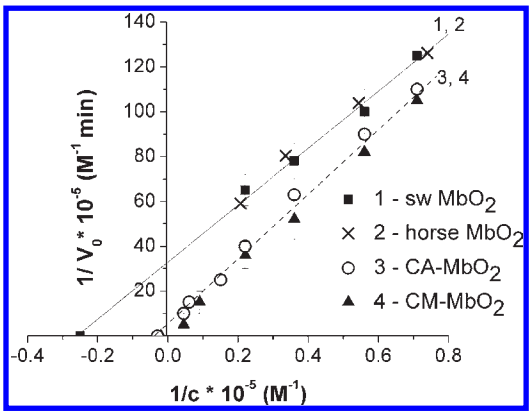

Figure 3. The Cu^{2+} concentration dependence in reverse coordinates, $1/V_0$ versus $1/c^{Cu}$, of the oxidation rate of native sperm whale and horse MbO_2 at low copper concentrations, up to a 3-fold molar excess of Cu^{2+} (curves 1 and 2) and of CM-MbO_2 and CA-MbO_2 at [Cu^{2+}]/[Mb] ratios from 0.2 to 10 (Curves 3 and 4).

more excess of copper, shows a sharp increase of the rate. The oxidation rate of both modified CM-MbO_2 and CA-MbO_2 changes with increasing Cu^{2+} concentration in a similar way, linearly increasing at all intervals of copper concentrations measured (Figure 2, curves 3 and 4), but differently than that of the native myoglobins (curves 1 and 2). The reaction rates of CM-MbO_2 and CA-MbO_2 are close in value, the difference being within the experimental error.

Figure 3 presents the obtained concentration dependences in the reverse coordinates, $1/V_0$ versus $1/c^{Cu}$. For the native sperm whale and horse myoglobins, straight lines manage to appear only for low copper concentrations, up to a 3-fold molar excess (curves 1 and 2), the Cu^{2+} binding constant being $0.28 \times 10^5\ M^{-1}$ (K_m is about 4×10^{-5} M and V_{max} 3.1×10^{-7} M/min). For both CM-MbO_2 and CA-MbO_2, close straight lines are seen (curves 3 and 4), giving a Cu^{2+} binding constant of about $0.35 \times 10^4\ M^{-1}$ ($K_m = 2.9 \times 10^{-4}$ M and $V_{max} = 0.2 \times 10^{-5}$ M/min) in the whole [Cu^{2+}] interval studied.

The concentration dependences for the reactions of the native and modified oxymyoglobins with a glycine complex of copper, $Cu(Gly)_2$, are similar in shape to those inherent for the reactions with Cu^{2+}, though the oxidation rates are much lower (Table 1).

Figure 4 shows the effect of pH on the rate of oxidation of oxymyoglobins studied in the presence of one molar equivalent of Cu^{2+} (ionic strength $I = 0.01$). The pH-dependence curves for sperm whale and horse proteins have a pronounced sigmoidal shape with a pK_{eff} of about 6.7, indicating that the reaction rate is influenced by ionization of some histidines (Figure 4, curves 1 and 2). As the transition interval takes only 2 pH units, at least two (or more) His residues must participate (it takes 3.5−4 pH units for the single group). A quite different shape of the pH-dependence curve is observed for CM-MbO_2 and CA-MbO_2 under the same conditions (Figure 4, curves 3 and 4). The oxidation rates of the modified MbO_2 constantly increase with decreasing pH identically in both cases, not reaching a plateau. The pH dependencies represent in this case an intermediate shape between the sigmoid and monotonic curves with a pK_{eff} < 6. At pH < 6.5, the rates of CM-MbO_2 and CA-MbO_2 oxidation are about ~1.5 times higher than that of intact sperm whale and horse MbO_2 (Figure 4, curves 1 and 2). In the presence of a 10-fold molar excess of Cu^{2+} (Figure 5, curves 1−4), all of the pH dependences are practically identical and similar in shape to those observed for CM-MbO_2 and CA-MbO_2 in the presence of one Cu^{2+} equivalent, though the reaction rates are almost 20 times higher.

Increasing the ionic strength in the 0−0.1 range does not practically influence the rate of sperm whale and horse MbO_2 oxidation in the presence of Cu^{2+} (pH 7.5), though both reagents, the protein and the copper ions, are charged (not shown). As the ionic strength is further increased, up to $I = 0.4$, the reaction rate grows 4−5 times, which can be explained by alterations in Mb and Cu^{2+} redox potentials.[21]

The addition of Zn^{2+} in various [Zn^{2+}]/[MbO_2] ratios to the reaction mixture at different Cu^{2+} concentrations has no appreciable effect on the rate of MbO_2 oxidation (Table 2).

Analysis of Copper Binding to Myoglobin by NMR Method. The addition of $Cu(Gly)_2$ to sperm whale met-Mb (at pH 7.1) results in a broadening of the $C^{\delta}H$ and $C^{\varepsilon}H$ resonances of all surface histidines in the NMR spectrum (Figure 6a−c). According to the assignment of Cocco et al.,[22] the resonances of four histidines are most strongly broadened, His113 ($C^{\delta}H$, 7.6; $C^{\varepsilon}H$, 6.6), His116 ($C^{\delta}H$, 7.7; $C^{\varepsilon}H$, 7.25), His48 ($C^{\delta}H$, 7.1; $C^{\varepsilon}H$, 7.85), and His81 ($C^{\delta}H$, 7.0; $C^{\varepsilon}H$, 7.9), which may indicate preferable binding of copper to these residues. As the distance between His113 and His116 is only 0.62 nm, the broadening resonances of both histidines are possibly caused by binding copper only to one of them. Indeed, only His116 resonances are broadened at pH 5.2 . The resonances of His12 ($C^{\delta}H$, 6.9; $C^{\varepsilon}H$, 7.85), His119 ($C^{\delta}H$, 6.7; $C^{\varepsilon}H$, 7.8), and of inner His24 ($C^{\delta}H$, 6.2; $C^{\varepsilon}H$, 7.65) that is H-bonded to His119 are broadened at pH 7.1 to much lesser extent. Taking into account a small amount of the copper complex, some broadening of His12, 119, and 24 resonances might be due to the high affinity binding of paramagnetic copper to His113(116), 48, and 81.

In the NMR spectrum of horse met-Mb (Figure 7a−c), the resonances of three histidines, His113 ($C^{\delta}H$, 7.65; $C^{\varepsilon}H$, 6.67), His116 ($C^{\delta}H$, 7.22; $C^{\varepsilon}H$, 7.8), and His48 ($C^{\delta}H$, 6.9; $C^{\varepsilon}H$, 7.88), are most strongly broadened at pH 7.5 in the presence of $Cu(Gly)_2$. The His resonance is also strongly broadened at 6.93 ppm as well as a much lesser one at about 7.15 ppm, for which an assignment is missed.[22] They must obviously belong to His81 and His119, because they are broadened in sperm whale met-Mb under these conditions (His 12 in horse met-Mb is replaced by Gln).

At pH 5.5, the primary broadening of the $C^{\delta}H$ and $C^{\varepsilon}H$ resonances of the same histidines is observed for sperm whale and horse met-Mb, as it is at pH 7.1−7.5 (not shown). At more acidic pH 5.2, however, the signals of His48 ($C^{\delta}H$, 7.14; $C^{\varepsilon}H$, 8.02), His12 ($C^{\delta}H$, 7.35; $C^{\varepsilon}H$,

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Table 1. Initial Oxidation Rates of the Native and Chemically Modified Oxymyoglobins in the Presence of Different Copper Reagents ($V_0 \times 10^7$, M min^{-1}), 0.01 M Tris-maleate buffer (1:1), pH 7.5, 20 °C

reagent	$[CuCl_2]$/[myoglobin]			$[Cu(Gly)_2]$/[myoglobin]		
sample	1.25	5	10	1.25	5	10
MbO_2 (sperm whale)	1.5 ± 0.5	4.2 ± 0.5	30 ± 3.0	0.5 ± 0.2	1.4 ± 0.5	4.8 ± 0.5
MbO_2 (horse)	1.6 ± 0.5	4.5 ± 0.5	28 ± 3	0.3 ± 0.2	1.3 ± 0.5	5 ± 0.5
CM-MbO_2 (sperm whale)	2.3 ± 0.5	10.2 ± 0.5	25 ± 2.5	0.4 ± 0.2	1.7 ± 0.5	4.6 ± 0.5
CA-MbO_2 (sperm whale)	2.6 ± 0.5	8.6 ± 0.5	20 ± 2.0	0.5 ± 0.2	1.6 ± 0.5	4.5 ± 0.5

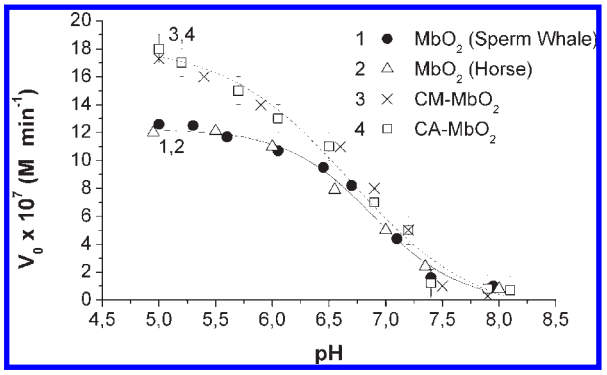

Figure 4. The pH dependence of oxidation rates of sperm whale and horse MbO_2 (curves 1 and 2) and CM- and CA-MbO_2 (curves 3 and 4) in the presence of one molar equivalent of $CuCl_2$. Protein concentration is 2.25×10^{-5} M, 0.01 M Tris-maleate buffer (1:1), 20 °C.

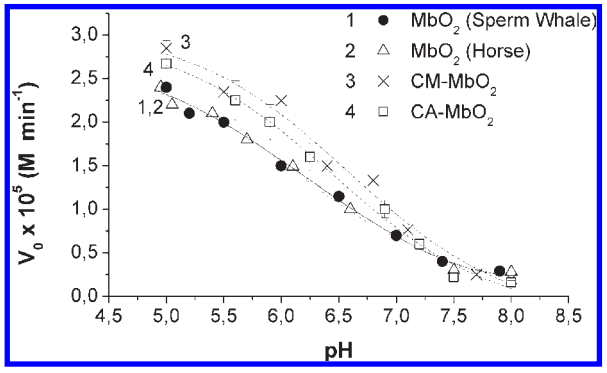

Figure 5. The pH dependence of oxidation rates of sperm whale and horse MbO_2 (curves 1 and 2), CM-MbO_2 (curve 3), and CA-MbO_2 (curve 4) in the presence of a 10-fold molar excess of $CuCl_2$. The protein concentration is 2.25×10^{-5} M, 0.01 M Tris-maleate buffer (1:1), 20 °C.

8.55), and His81 ($C^{\delta}H$, 7.17; $C^{\varepsilon}H$, 8.6) in sperm whale met-Mb are broadened only a bit less than the most strongly broadened resonances of His116 ($C^{\delta}H$, 8.52; $C^{\varepsilon}H$, 7.47; Figure 8a−c). But the resonances of His113 ($C^{\delta}H$, 7.8; $C^{\varepsilon}H$, 7.0), which are most strongly broadened at pH 7.1, are broadened very modestly, and the signals of His119 ($C^{\delta}H$, 6.83; $C^{\varepsilon}H$, 8.65) do not change. On the contrary, broadening of the inner His36 ($C^{\delta}H$, 8.32; $C^{\varepsilon}H$, 8.57) and His82 ($C^{\delta}H$, 6.75; $C^{\varepsilon}H$, 8.3) resonances is clearly seen, which obviously indicates conformational changes in the protein at pH 5.2.

Thus, His113(116), His48, and His81, common for sperm whale and horse myoglobins, can be considered the most preferable sites for the binding of copper at pH 5.5−7.5. Our results slightly differ from the high-resolution NMR data obtained earlier by complexing CuNTA with sperm whale MbCO (pH 4.7) and met-Mb (pH 5.4), according to which His113, 116, and 48 have the greatest affinity to copper.[3]

With the addition $Cu(Gly)_2$ to carboxymethylated CM-met-Mb, no broadening of His resonances in the NMR spectrum is observed (Figure 9a−c). The spectrum was registered at pH 5.2, as the assignment of His resonances in sperm whale CM-met-Mb had been done only at this pH value.[23] The lack of the broadening confirms that the copper reagent does not bind to modified histidines. It specifies also that all broadened His resonances in the NMR spectra of native myoglobins are the result of only paramagnetic copper ions bound to the protein, but not those in the solution, as their concentration is small.

Discussion

Copper binding to sperm whale met-Mb was studied earlier using two different techniques, equilibrium dialysis and almost complete precipitation of the protein by the metal ions, which gave similar results.[24] At small $[Cu^{2+}]$/[Mb] molar ratios, from 1 up to 4, nearly all copper was bound to myoglobin (initial pH 7−7,1), which corresponds to a high affinity of 3−4 His residues to Cu^{2+}. Their high affinity can be explained by the formation of chelated Cu^{2+} complexes with the protein groups situated nearby. With the 10-fold molar excess of copper, six Cu^{2+} ions bind to Mb, and at higher ratios, up to 40, maximally 7.2 copper ions were bound, implying that the affinity of remaining 3−4 sites must be about 1−2 orders lower. Note that carboxyalkylated Mb was shown to be able to bind some Cu^{2+}.[16]

Redox-inactive Zn^{2+} ions bind to sperm whale Mb at the same sites as Cu^{2+} ions do, but their affinity to Zn^{2+} is much smaller.[24] Even in a 20-fold excess of Zn^{2+} when six sites are occupied by zinc, the last is easily displaced from three sites already upon the addition of 1−4 equivalents of copper. One Zn^{2+}, however, remains bound to Mb even in the presence of a 10-fold excess of copper, that is, competes for this site with Cu^{2+} (the binding constant was shown to be 4.4×10^5 M^{-1} at pH 6[7]). The different affinity of Cu^{2+} and Zn^{2+} for the same binding sites in Mb can be explained by different structures of complexes formed, square for copper and tetrahedral for zinc.[3,11]

Our NMR data allow the assignment of 3−4 sites with high affinity to Cu^{2+} as His113(116), His48, His81, and possibly His119, common for sperm whale and horse myoglobins. Their Cu^{2+} binding constant is evaluated to be about 0.28×10^5 M^{-1} (Figure 3). At $[Cu^{2+}]/[MbO_2]$ ratios from 0.2 to about 5, all of these histidines must be completely saturated with Cu^{2+}, but the oxidation rates for both native MbO_2's are very low (Figure 2, curves 1 and 2). Moreover, no saturation of the MbO_2 oxidation rate is seen on the concentration

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Table 2. Effect of Zinc Ions on the MbO_2 Oxidation Rate ($V_0 \times 10^6$, M min^{-1}) at Various $[Cu^{2+}]/[MbO_2]$ Ratios, 0.01 M Tris-Maleate Buffer (1:1), pH 6.5, 20 °C

	sperm whale myoglobin			horse myoglobin		
	$[Cu^{2+}]/[MbO_2]$			$[Cu^{2+}]/[MbO_2]$		
$[Zn^{2+}]/[MbO_2]$	1.25:1	5:1	10:1	1.25:1	5:1	10: 1
	0.1 ± 0.05	0.61 ± 0.1	3.0 ± 0.3	0.13 ± 0.05	0.65 ± 0.1	2.8 ± 0.3
5:1	0.12 ± 0.05	0.59 ± 0.1	2.8 ± 0.3	0.16 ± 0.05	0.66 ± 0.1	2.5 ± 0.3
10:1	0.15 ± 0.05	0.65 ± 0.1	3.3 ± 0.3	0.12 ± 0.05	0.74 ± 0.1	2.6 ± 0.3
20:1	0.11 ± 0.05	0.72 ± 0.1	2.6 ± 0.3	0.14 ± 0.05	0.62 ± 0.1	2.7 ± 0.3

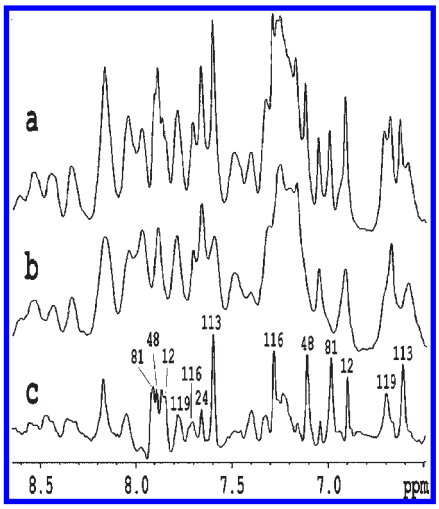

Figure 6. Region of the 1D 400 MHz ^{1}H NMR spectra of sperm whale met-Mb at pH 7.1 (a), the same with the addition of $Cu(Gly)_2$ up to 0.1 mM concentration (b), and the differential ^{1}H NMR spectrum (c). The myoglobin concentration is 0.3 mM, 0.01 M Tris-DCl buffer, pH 7.1, 298 K.

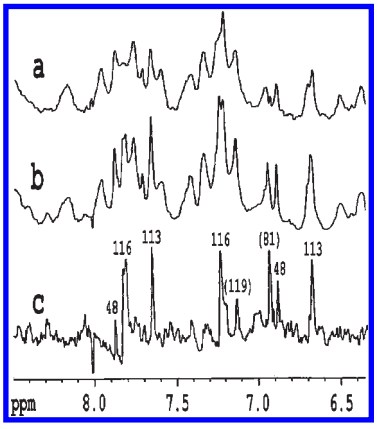

Figure 7. Region of the 1D 400 MHz ^{1}H NMR spectra of horse met-Mb at pH 7.5 (a), the same with the addition of $Cu(Gly)_2$ up to 0.1 mM concentration (b), and the differential ^{1}H NMR spectrum (c). The myoglobin concentration is 0.3 mM, 0.01 M Tris-DCl buffer, pH 7.5, 298 K.

curves at a $[Cu^{2+}]/[Mb]$ ratio > 8, where all six surface histidines must have bound Cu^{2+}.[24]

The obtained results suggest that copper complexes with all surface histidines, which are far from the heme (Table 3), introduce only a minor (if any) contribution to the overall reaction rate. This conclusion is supported by the fact that the oxidation of CM-MbO_2 and CA-MbO_2 in the presence of Cu^{2+} proceeds practically as effectively or even more effectively as that of intact MbO_2 (Figure 2, curves 3 and 4), though the surface histidines in the modified myoglobins are

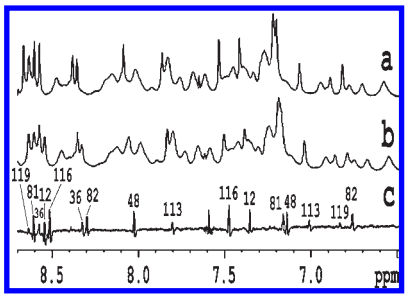

Figure 8. Region of the 1D 600 MHz 1H-NMR spectra of sperm whale met-Mb at pH 5.2 (a), the same with addition of $Cu(Gly)_2$ up to 0.03 mM concentration (b), and the differential 1H-NMR spectrum (c). Myoglobin concentration is 0.3 mM, 0.01 M Tris-DCl buffer, pH 5.2, 298 K.

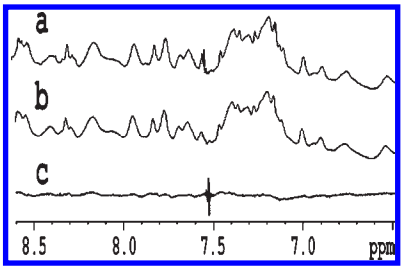

Figure 9. Region of the 1D 600 MHz ^{1}H NMR spectra of carboxymethylated sperm whale CM-met-Mb at pH 5.2 (a), the same with the addition of $Cu(Gly)_2$ up to 0.03 mM concentration (b), and the differential ^{1}H NMR spectrum (c). The myoglobin concentration is 0.3 mM, 0.01 M Tris-DCl buffer, pH 5.2, 298 K.

not able to bind copper (Figure 9). The absence of inhibition by Zn^{2+} ions under these conditions, when they can compete with Cu^{2+} for binding to surface histidines, is also in favor of it (Table 2).

Horse Mb lacks His12 that is changed to Gln, while amino acid replacements in 16 more positions of horse Mb are homologous. The ionization pK values of histidines in horse and sperm whale myoglobins are very close to each other (Table 3) because of the large similarity in their nearest environments. Amino acid residues contacting the heme group are strictly invariant in all mammal myoglobins. Similar concentration dependences for sperm whale and horse myoglobins (Figure 2, curves 1 and 2) imply that His12 does not participate in the reaction at all. Neither cysteine nor cystine residues are present in these proteins.

A sharp increase in the MbO_2 oxidation rate occurs in the second part of the concentration curve, at $[Cu^{2+}]/[Mb]$ ratios > 8 (Figure 2, curves 1 and 2), and can be due to involvement in the reaction of the Cu^{2+} complexes formed by inner histidines, which are poorly accessible to the solvent and do not alkylate by the reagents. Some structural changes in the protein could also proceed at high Cu^{2+}concentrations, as evidenced by cooperative shape of the curves 1 and 2 (both processes could take place simultaneously). At last, direct

Table 3. Values of Ionization pK of Protonated Histidines in Sperm Whale and Horse Myoglobins and Their Distances (*r*) to Fe Atom of the Heme

source of Mb	liganded form	His12(A10)		His48(CD6)		His81(EF4)		His97(FG3)		His113(G14)		His116(G17)		His119(GH1)		His36(C1)	
		p*K*	r[c], Å	p*K*	r, Å	p*K*	r, Å	p*K*	r, Å	p*K*	r, Å	p*K*	r, Å	p*K*	r, Å	p*K*	r, Å
sperm whale	met-Mb[a]	6.4	26.8	6.7	16.5	6.7	23.4		6.6	5.0	19.4	6.6	24.0	6.2	22.5	8.2	15.6
	MbCN[b]	6.4		6.55		6.35		5.55		5.6		6.7		5.6		8.1	
	MbCO[b]	6.25		6.4		6.2		5.85		5.6		6.75		5.6		6.75	
horse	met-Mb[a]			6.8	18.0	6.7	23.0		6.7	5.4	19.9	6.65	24.1	6.4	23.3	7.9	15.8
	MbCO[b]			6.6		6.4		5.9		5.8		6.8		5.53		6.07	

[a] The p*K* values are taken from Cocco et al.[22] and. [b] From Carver et al.[23] [c] The distances between the Fe atom and nitrogen ND1 of histidine are calculated with the MOLMOL 2.5.1 program using atomic coordinates of myoglobins from the PDB database.

interaction of Cu^{2+} ions with the heme (simple outer-sphere electron transfer) is possible.

From the inner histidines, four residues, His24, 64 (distal), 82, and 93 (proximal), are buried and can titrate only at pH < 5 during the acid denaturation. Two others, His36 (15.6 nm from the heme) and 97 (0.62 nm apart from the heme), are partly accessible (Figure 1). While His36 ionizes only at pH ≥ 8, His97, capable of being titrated in the pH 5−8 range (Table 3), can be considered as the residue, Cu^{2+} binding to which being most probably responsible for the fast oxidation of CM-MbO_2 and CA-MbO_2. His97 is located near proximal His93, and its affinity to copper must be about one order lower than that of surface histidines, which can be explained by H-bonding to the COO^- group of the heme propionate A. In favor of His97 participation is also the fact that it is present in all animal myoglobins and the β chains of hemoglobin, which all are oxidized by copper, but absent in the Hb α chains resistant to the oxidation.[6,7] In the α chains, Arg92 (instead of His97) forms the H bond with the heme propionate A, while distal His58 occupies the same position as His64.

The greatest contribution of His 97 in the reaction rate should be, for native sperm whale and horse MbO_2, at least, up to a $[Cu^{2+}]/[MbO_2]$ ratio equal to 8−10 (Figure 2, curves 1 and 2). It follows also from very similar pH dependences for all myoglobins under these conditions (Figure 5). At smaller Cu^{2+} concentrations, the surface His48, 113(116), and 81 can successfully compete with His97 for copper, reducing the overall reaction rate, which is really observed. As sited above, seven Cu^{2+} ions principally can bind to native Mb,[24] and the modified proteins are able to bind some copper,[16] meaning the involvement of inner histidines in the binding.

Both His97 and Arg45 play very important roles in stabilization of the heme position, forming H bonds with the heme propionates A and D, respectively. Besides, His97 makes an additional contribution in this stabilization, as its imidazole ring is almost parallel to the heme at the 0.36 nm distance below pyrrole D. Complexing His97 with Cu^{2+} should result in a changed local conformation and stability of the heme cavity, increasing in turn the availability of other intrinsic histidines, first of all, that of distal His64, to copper. A cooperative increase of the reaction rate of both native myoglobins at an 8−10-fold and more molar excess of Cu^{2+} (Figure 2, curves 1 and 2) probably implies that both His97 and His64 participate now in Cu^{2+} binding and electron transfer from the heme. However, no cooperativity is seen for the modified myoglobins (Figure 2, curves 3 and 4).

Unfortunately, direct evidence of copper complexing with inner His97 (or His64) isn't apparent using the NMR technique, as proton signals of His97 are not visible in the spectra of high-spin met and low-spin CN derivatives of CM- and CA-Mb [ref 23, this work]. In addition, they did not register in the NMR spectra of diamagnetic MbCO with and without copper.[3] Site-directed mutagenesis also cannot be useful, as any replacement of His97 (or His64) must lead to remarkable changes in the Mb autoxidation rate. It was shown that the latter increases 2−20 times upon any replacement in positions 45 and 64.[2]

The shape of the pH dependences for all myoglobins studied with a 10-fold excess of Cu^{2+} (Figure 5), as well as for the CM-MbO_2 and CA-MbO_2 reactions with one molar equivalent of Cu^{2+} (Figure 4, curves 3 and 4), suggests the effect of distal His64(E7) ionization. Such a shape is typical for the pH-dependent MbO_2 autoxidation,[1,2] though the rate of the Cu^{2+}-catalyzed reaction is much higher. The distortion of the H bond between distal His64(E7) and liganded O_2 is known to play a major role in the mechanism of autoxidation.[1,2] The absence of this H bond in soybean leghemoglobin (LbO_2) enhances it by hundreds of times.

The rate increase at pH < 6 is generally inherent in the reactions of different heme proteins with low molecular weight redox reagents, and also in the MbO_2 and HbO_2 autoxidation and their copper-catalyzed oxidation.[1−7] As the superoxide anion must be the primary product of the reactions (see stage 4), O_2 reduction is thermodynamically much more favorable in the presence of protons (E_o of the $O_2 + H^+/HO_2$ system is −37 mV, whereas for the O_2/O_2^- pair, it is −330 mV).[25] That is why the sigmoid pH-dependence curves for sperm whale and horse MbO_2 with transition p$K \sim 6.7$ registered at an equimolar concentration of copper (Figure 4, curves 1 and 2) are quite unusual and point to the inhibiting effect of some surface His ionization on the oxidation rate. Most probably, these are His116 (p*K* 6.6), His81 (p*K* 6.7), and His48 (p*K* 6.7−6.8) (Table 3). At pH > p*K*, these surface histidines having the greatest affinity for copper are able to successfully compete with His97 for copper, thus inhibiting the catalysis. At pH < p*K*, the inhibiting effect diminishes due to the competition of protons with Cu^{2+} for binding. As the reaction rate considerably grows at pH < 7, Cu^{2+} complexing to MbO_2 does not obviously limit the process.

Contributions of copper complexes with His48, 113, and 116 to the overall reaction rate can be evaluated from the data on the oxidation of mutant sperm whale oxymyoglobins, in which they are substituted, by $Cu(Gly)_2$.[12] The greatest effect on the reaction rate (37%) is via the replacement of His48 nearest to the heme by Ala, while the replacement of His116 by Asp reduces it only by 16%, and the replacement of His113 by Ala has practically no influence. A similar

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situation was observed earlier for human hemoglobin, in which the His2 of the β chain, having the greatest affinity for copper, does not participate in the redox transformation.[6]

Most data in the literature[3,4,7,12] are now in favor of a site-specific mechanism of the copper-catalyzed oxidation of myoglobins, as (i) saturation on the concentration curves is always observed at a 30−100-fold excess of copper reagents, (ii) the reaction rate is dependent on the stability of the copper−ligand complex, and (iii) the inhibiting effect takes place at large but not at low $Zn^{2+}$and Ni^{2+} concentrations (240−275-fold molar excess), supporting our idea about the involvement of inner histidines. The copper-catalyzed oxidation of soybean LbO_2 (redox potential is 270 mV) by copper reagents is very convincing evidence for the site-specific mechanism of the reaction.[26] It is shown that the oxidation of this protein, in which only two histidines, proximal and distal, are present, involves binding the reagent with distal His61 oriented to the solvent. The situation is reminiscent of that with the His97 position in myoglobins.[23]

Against the simple outer-sphere electron transfer are our findings that the oxidation rates of CM- and CA-MbO_2, different in the overall molecule charge, by copper do not practically differ. Also, the rate of sperm whale and horse MbO_2 oxidation does not depend on ionic strength in the 0−0.1 interval, though both reagents, the protein and copper compound, are charged. Against it is also the fact that α chains of hemoglobin (E_o is +110 mV) are not oxidized by copper, though the potential difference allows it, while β chains similar to myoglobin are oxidized.[5,6] Note that, contrary to cytochrome c with partially exposed heme, simple outer-sphere electron transfer is less probable for myoglobin, as its heme is buried and poorly accessible to the solvent.[27] As copper compounds have a high affinity for imidazole,[5−7] the reaction proceeding through the transient copper complex with His97 or (and) His 64 located near the heme seems to be much more favorable.

The present work is in concept of actual modern investigations of the binding steheometry of the copper and other metal ions to proteins, the properties of binding sites, and the mechanisms of production of active O_2 forms that damage cells.[28−30]

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