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# Mechanism of Oxidation of Oxymyoglobin by Copper Ions: Comparison of Sperm Whale, Horse, and Pig Myoglobins

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**Abstract**—The influence of $Cu^{2+}$ concentration, pH, and ionic strength of the solution as well as redox-inactive zinc ions on the rate of oxidation of sperm whale, horse, and pig oxymyoglobins (oxy-Mb) by copper ions has been studied. These myoglobins have homologous spatial structures and equal redox potentials but differ in the number of histidines located on the surface of the proteins. It was shown that oxy-Mb can be oxidized in the presence of $Cu^{2+}$ through two distinct pathways depending on which histidine binds the reagent and how stable the complex is. A slow pH-dependent catalytic process is observed in the presence of equimolar $Cu^{2+}$ concentration for sperm whale and horse oxymyoglobins. The curves of pH dependence in both cases are sigmoid with $pK_{eff}$ corresponding to the ionization. The process is caused by the strong binding of $Cu^{2+}$ to His113 and His116, an analogous His residue being absent in pig Mb. In contrast, rapid oxidation of 10-15% of pig oxy-Mb is observed under the same conditions (fast phase), which is not accompanied by catalysis because the reduced copper is apparently not reoxidized. The complexing of $Cu^{2+}$ with His97 situated near the heme is probably responsible for the fast phase of the reaction. The affinity of His97 for $Cu^{2+}$ must be significantly lower than those of the "catalytic" His residues since the fast phase does not contribute markedly to the rate of sperm whale and horse oxy-Mb oxidation. Increasing copper concentration does not produce a proportional growth in the oxidation rate of sperm whale and horse oxy-Mbs. Which $Cu^{2+}$ binding sites of Mb make main contributions to the His reaction rate at different $Cu^{2+}$/Mb ratios from 0.25 to 10 is discussed.

*Key words*: myoglobin, copper, redox reactions

The mechanism of oxidation of myoglobin (Mb) and hemoglobin (Hb) by divalent cupric compounds is of interest because of the presence of similar substances in real biological structures. These redox systems resemble mitochondrial cytochrome oxidase, which contains simultaneously both heme and copper active centers.

Metal compounds with high redox potentials, such as bipyridyl and phenanthroline $Cu^{2+}$ complexes ($E_0$ = 480 and 590 mV, respectively), react with these heme proteins by the simple outer-sphere mechanism where electron transfer proceeds through overlapping π-orbitals of the heme and the metal complex [1]. In this case, the reaction rate is proportional to the difference of standard redox potentials and electronic self-exchange rates of the protein and the reagent. The reaction rate usually does not depend on pH or ionic strength of the solution. An essentially different mechanism takes place in reactions of Mb and Hb with copper compounds with sufficiently low redox potentials ($E_0$ ~ 100-150 mV) unable to oxidize the protein by the simple outer-sphere mechanism. In this case, the metal compound is first complexed to some specific protein site(s) with substitution of one or several ligands of a metal ion by protein ligands and subsequent intramolecular electron transfer (site-specific outer-sphere mechanism). As a result, the reaction rate becomes dependent also on the stability of the metal–protein complex and factors of the medium influencing its formation (pH, ionic strength) [2, 3]. For example, oxidation of oxy-Mb by $Cu^{2+}$ proceeds according to the scheme:

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

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

*Abbreviations*: Mb) myoglobin; oxy-Mb) oxymyoglobin; deoxy-Mb) deoxymyoglobin; Hb) hemoglobin; oxy-Hb) oxyhemoglobin.

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

It has been shown that the immediate electron transfer occurs in the copper complex of ligand-free deoxy-Mb (stage 3). Dissociation of the ligand (stage 2) is rate limiting for the process [2].

Transformation of oxy-Mb and oxy-Hb into oxidized met-forms, which is catalyzed by small amounts of ions or complexes of divalent copper, is of special interest in biology [4, 5]. The reaction also proceeds through formation of the specific reagent–protein complex(es) according to Eqs. (1)-(3). Reduced copper is then reoxidized by oxygen to $Cu^{2+}$, providing the closure of the catalytic cycle (catalysis is not observed under anaerobic conditions). Protons of the medium participate in the electron transfer from bound $Cu^+$ to $O_2$ [6]:

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

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

The detailed physical mechanism of oxidation of oxy-Mb by copper compounds is still not clear. One reason is that sperm whale Mb, which contains the maximal number of histidines, has been the sole object of examination. Out of twelve histidines of sperm whale Mb, five are inside the protein and inaccessible to the solvent. The seven histidines located on the surface of the protein at various distances from the heme can be protonated and bind to copper with different affinities.

From equilibrium dialysis data [7], up to six $Cu^{2+}$ ions can bind to sperm whale met-Mb at various pH values. Three binding sites have apparently the highest affinity for $Cu^{2+}$ as they are saturated already at small $[Cu^{2+}]/[Mb]$ molar ratios, from 1 up to 4 (the binding constants correspond to $10^5$-$10^6$ $M^{-1}$). The high affinity of these sites to copper can be explained by the formation of chelated $Cu^{2+}$ complexes with the participation of the protein ligands. With 10-fold molar excess of $Cu^{2+}$, all six sites are saturated and denaturation of the protein is observed. The affinity of Mb for copper is pH-dependent because protons compete with $Cu^{2+}$ for binding. Redox-inactive $Zn^{2+}$ ions evidently bind to sperm whale Mb at the same sites as $Cu^{2+}$ ions do. However, the affinity of $Zn^{2+}$ for Mb is clearly lower [7]. Even in 20-fold excess of $Zn^{2+}$, when all six sites must be occupied by zinc, the latter is easily displaced from three sites on addition of 1-4 equivalents of copper. Only one $Zn^{2+}$ remains bound to Mb in the presence of 10-fold excess of copper, i.e., competes with copper for this site. 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 [1, 2]. It has been shown [8] that only one His site in sperm whale oxy-Mb is saturated at $[Zn^{2+}]/[Mb]$ ratios from 5 to 1 (the binding constant is $4.4 \cdot 10^5$ $M^{-1}$ at pH 6).

High-resolution NMR data on complexes of Cu(II)NTA with sperm whale carboxy-Mb (at pH 4.7) and met-Mb (at pH 5.4) are in accord with the results of equilibrium dialysis of Mb and can be used to locate the sites of metal binding [2]. The C2H and C4H resonances of five histidines are found to be especially sensitive to small additives of the copper complex. The resonances of His113, His116, and His48 are widened most, and the resonances of His12 and His119 to a lesser degree, suggesting that the first three residues bind copper most strongly.

X-Ray data have been obtained by the difference Fourier method for sperm whale met-Mb crystals maintained in the presence of 3-4-fold molar excess $Zn(CH_3COO)_2$ and 80-fold excess $CuCl_2$ at pH 6 [9]. It was concluded that there is only one binding site in both cases: $Cu^{2+}$ is complexed with Mb at His12(A10), and $Zn^{2+}$ near His119(GH1), which is 0.89 nm from His12(A10). The nearby functional groups of Lys16(A14) and Asp122(GH4) must additionally coordinate the $Cu^{2+}$ and $Zn^{2+}$, thus explaining the high affinity of these sites for the metal ion as well as competition between them for binding. The X-ray diffraction data coincide with the above data for zinc, but contradict the NMR and equilibrium dialysis results for copper. Note that the interpretation of the X-ray diffraction data on $Cu^{2+}$ location in Mb is possibly erroneous because all six binding sites should be saturated by copper at the $Cu^{2+}$ concentration used.

Thus, the exact number of copper binding sites in Mb depending on $Cu^{2+}$ concentration is not clear, as well as their location in the Mb structure. Besides, it is not known how effective different sites are in the electron transfer from the heme and whether there is a correlation between the stability of the $Cu^{2+}$–protein complex and the contribution of this site to the overall oxidation rate.

In the present work, kinetics of oxidation of sperm whale, horse, and pig oxymyoglobins by $Cu^{2+}$ ions has been studied. These myoglobins have homologous spatial structures and identical redox potentials but differ in the number of His residues located on their surfaces. In comparison to sperm whale Mb, horse Mb lacks His12(A10), which is replaced by Gln, and in pig Mb three histidine residues, His12(A10), His113(G14), and His116(G17) are replaced by Gln [10, 11]. The influence of pH, ionic strength, and $Cu^{2+}$ and $Zn^{2+}$ concentration on the rate of oxidation of the three oxymyoglobins is investigated.

## MATERIALS AND METHODS

Sperm whale myoglobin (fraction IV) was isolated from skeletal muscles and purified as described earlier [12]. Tris (Serva, Germany), $CuCl_2$, $ZnCl_2$, maleic anhydride (chemical grade), and KCl (analytical grade) were used without additional purification.

Oxy-Mb was obtained under aerobic conditions by reduction of met-Mb with sodium dithionite and removing the latter by gel filtration through a Sephadex G-25 column. A complex of oxy-Mb with $Zn^{2+}$ was obtained by mixing the protein solution with concentrated $ZnCl_2$ solution (10 μl) [8]. All experiments with zinc complexes of myoglobin ( $[Zn^{2+}]/[Mb]$ ratios were 2, 5, 10, and 20) were carried out in Tris-malate buffer (pH 6.0-7.5) in which, unlike phosphate buffer, insoluble salts of zinc are not formed.

The rate of oxymyoglobin oxidation was studied spectrophotometrically by the changes in absorption at 581 or 543 nm using a Specord UV-VIS spectrophotometer (Germany) with a thermostatted cuvette holder. All experiments were carried out at 20°C. The initial concentration of oxymyoglobin was $2.25 \cdot 10^{-5}$ M. For the required $[Cu^{2+}]/[MbO_2]$ ratios, 10 μl of a concentrated $CuCl_2$ solution was added to the reaction mixture using a Hamilton microsyringe.

Kinetic curves were monitored over the time interval during which the reaction amplitude varied by 15-20%, usually including its initial linear part. The reaction rate was characterized by the initial rate ($v_0$). The $MbO_2$ concentration was determined spectrophotometrically using extinction coefficients ($M^{-1} \cdot cm^{-1}$) of 13,600 at 543 nm and 14,200 at 581 nm.

## RESULTS

The kinetic curves of sperm whale and pig $MbO_2$ oxidation in the presence of one $Cu^{2+}$ equivalent (pH 7.5) are shown in Fig. 1. The kinetics of horse $MbO_2$ oxidation under the same conditions coincides with the curve of sperm whale $MbO_2$ (Fig. 1, curve *1*). The common character of the copper-induced oxidation of sperm whale and horse oxymyoglobin differs greatly from that of pig oxy-Mb. The first two proteins are completely oxidized to met-Mb, and a slow process is observed in both cases. In contrast, a fast (less than 1 min) oxidation of 10-15% of the pig oxy-Mb is observed (fast phase), but its further transformation to met-Mb does not occur (Fig. 1, curve *2*). That is, for sperm whale and horse $MbO_2$ there is essentially no fast phase of oxidation, while with pig $MbO_2$ there is no slow phase.

Figure 2 shows the effect of pH in the pH interval 5-8 on the rate of sperm whale and horse oxy-Mb oxidation in the presence of one $Cu^{2+}$ equivalent at low ionic strength ($I$ = 0.01). Both pH dependences have pronounced sigmoid shape, indicating that the reaction rate is influenced by ionization of a group with $pK_{eff}$ corresponding to the ionization of His. The $pK_{eff}$ values for the two proteins differ by 0.3-0.4 pH unit, being 6.3-6.4 for sperm whale $MbO_2$ and 6.7-6.8 for horse $MbO_2$. In the presence of 5-fold molar excess of $Cu^{2+}$, the shape of the pH dependence changes (Fig. 3), pointing to the participation of some other process dependent on pH in a different way.

Increasing ionic strength in the interval 0-0.1 has practically no influence on the rate of sperm whale and horse $MbO_2$ oxidation in the presence of $Cu^{2+}$ (pH 7.5),

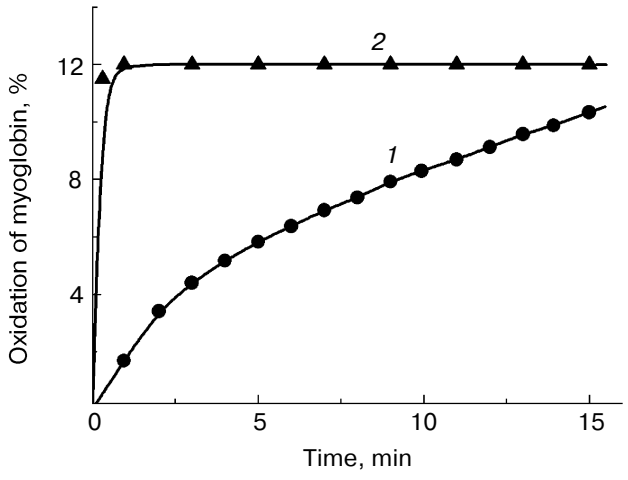


**Fig. 1.** Kinetics of oxidation of sperm whale oxy-Mb (*1*) and pig oxy-Mb (*2*) in the presence of one equivalent of $CuCl_2$ (0.01 M Tris-malate buffer, pH 7.5, 20°C).

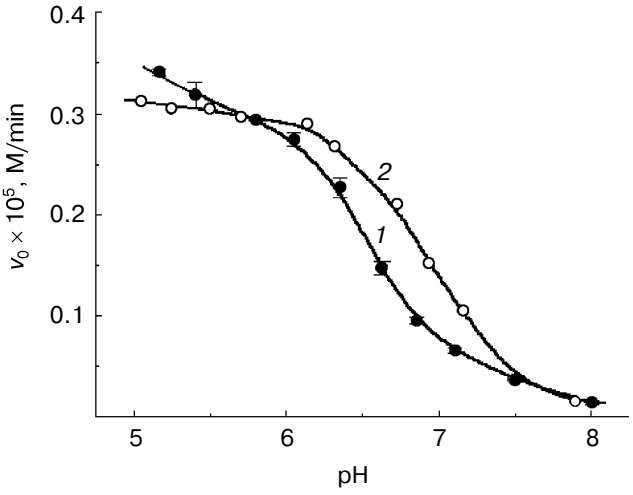


**Fig. 2.** The pH dependence of the rate of sperm whale $MbO_2$ (*1*) and horse $MbO_2$ (*2*) oxidation in the presence of one equivalent $CuCl_2$ (0.01 M Tris-malate buffer, 20°C).

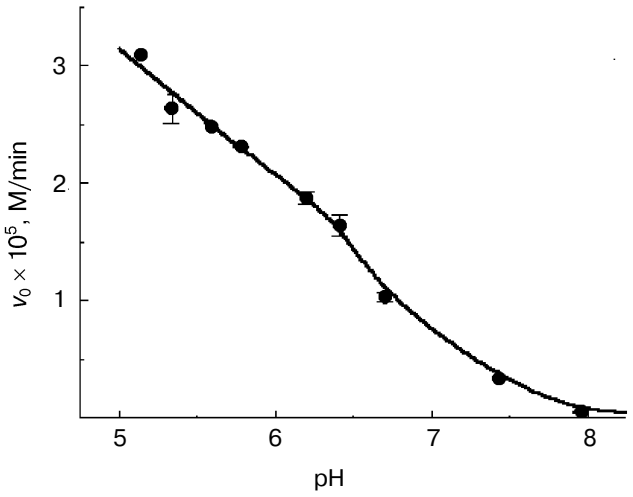


**Fig. 3.** The pH dependence of the rate of sperm whale $MbO_2$ oxidation with fivefold molar excess of $CuCl_2$ (0.01 M Tris-malate buffer, 20°C).

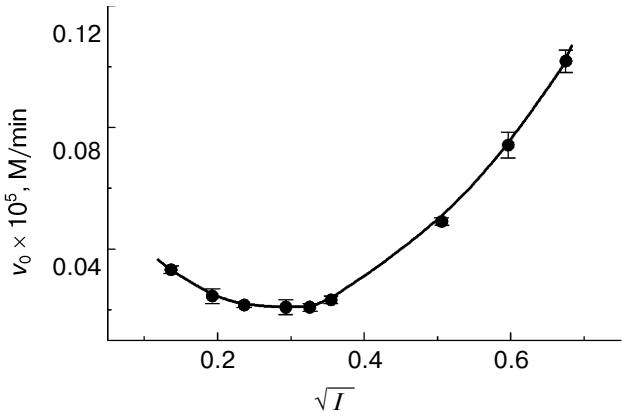


**Fig. 4.** Ionic strength dependence of the rate of sperm whale $MbO_2$ oxidation in the presence of one equivalent $CuCl_2$ in 0.01 M Tris-malate buffer, pH 7.5, 20°C.

though both reagents, the protein and the copper ions, are charged (Fig. 4). This implies that the complexing of copper to oxy-Mb (stage 1) does not limit the overall rate of the process. As ionic strength is further increased to $I = 0.4$, the reaction rate increases 4-5-fold, which can be explained by changes in the redox potentials of Mb and $Cu^{2+}$ [13].

The dependences of initial oxy-Mb oxidation rate on copper concentration at pH 6 and 7.5 are identical for sperm whale and horse oxy-Mbs (Fig. 5a). They are complex in that there is no additivity in the increasing of the reaction rate with $Cu^{2+}$ concentration. Two parts can be clearly seen on the concentration dependence curve. The first one, at $[Cu^{2+}]/[MbO_2]$ ratios from 0 to about 5, where the oxidation rate initially grows with increasing copper concentration and then reaches a plateau, and the second, at 8-fold and more excess of copper, where a sharp increase of the rate is observed. In the first part of the concentration curve, up to $[Cu^{2+}]/[MbO_2]$ ratio 3 : 1, the oxidation rate grows linearly with $Cu^{2+}$ concentration at pH 6, while at pH 7.5 the dependence noticeably deviates from linearity (Fig. 5b). The addition of $Zn^{2+}$ at 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 1).

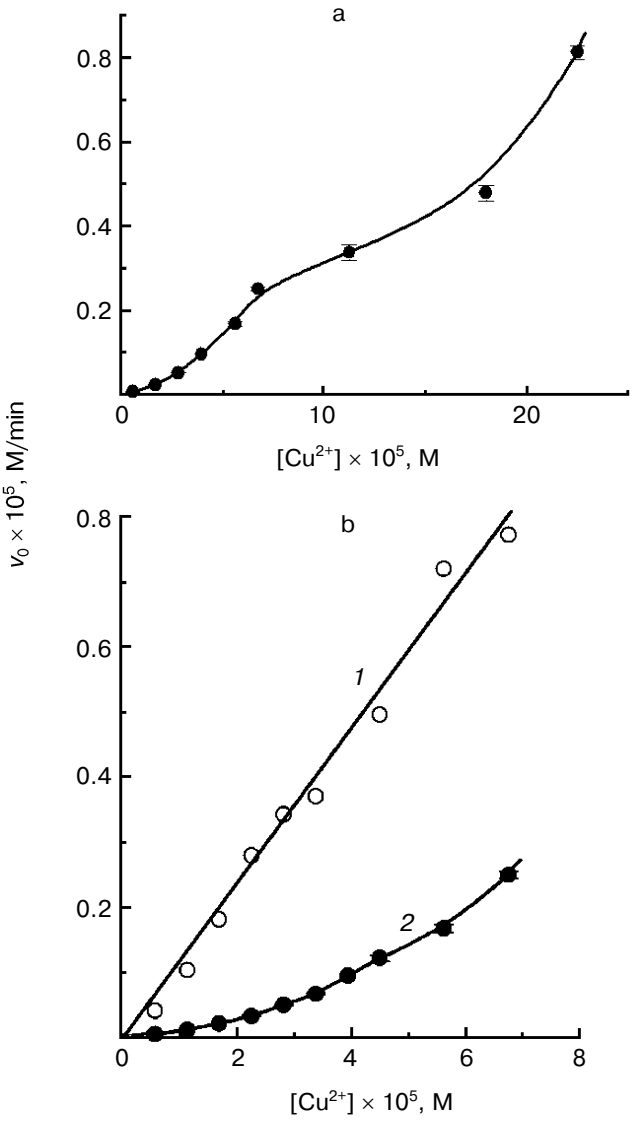


**Fig. 5.** a) Dependence of sperm whale $MbO_2$ oxidation rate on $Cu^{2+}$ concentration (0.01 M Tris-malate buffer, pH 7.5, 20°C). b) $Cu^{2+}$ concentration dependence for sperm whale $MbO_2$ in the initial part of the curve, for $[Cu^{2+}]/[MbO_2]$ ratios from 0 to 3, at pH 6 (*1*) and at pH 7.5 (*2*) (0.01 M Tris-malate buffer, 20°C).

**Table 1.** Influence of zinc ions on the rate of oxymyoglobin oxidation ($v_0 \times 10^5$, M/min) at various $[Cu^{2+}]/[MbO_2]$ ratios (0.01 M Tris-malate buffer, 20°C)

| $[Zn^{2+}]/[MbO_2]$ | Sperm whale Mb | | | | | | Horse Mb | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH 6.3 | | | pH 7.5 | | | pH 6.3 | | |
| | 1.25 : 1 | 5 : 1 | 10 : 1 | 3 : 1 | 8 : 1 | 10 : 1 | 1.25 : 1 | 5 : 1 | 10 : 1 |
| No zinc | 0.35 ± 0.02 | 1.84 ± 0.14 | 3.93 ± 0.04 | 0.26 ± 0.01 | 0.44 ± 0.02 | 0.85 ± 0.06 | 0.39 ± 0.02 | 2.2 ± 0.17 | 5.25 ± 0.15 |
| 2 : 1 | — | — | — | 0.21 ± 0.03 | 0.54 ± 0.05 | — | — | — | — |
| 5 : 1 | 0.37 ± 0.01 | 1.77 ± 0.08 | 3.83 ± 0.10 | — | — | — | 0.47 ± 0.04 | 1.98 ± 0.12 | 4.61 ± 0.13 |
| 10 : 1 | — | — | — | — | 0.46 ± 0.02 | 0.79 ± 0.05 | — | — | — |
| 20 : 1 | — | — | — | 0.25 ± 0.02 | 0.52 ± 0.04 | 0.81 ± 0.03 | — | — | — |

**Table 2.** Values of ionization p*K* of protonated His residues in myoglobins and their distances (*r*) to the Fe atom of the heme

| Source of Mb | His12(A10) | | His48(CD6) | | His81(EF4) | | His97(FG3) | | His113(G14) | | His116(G17) | | His119(GH1) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 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 | 5.4-5.8 | 26.8 | 5.5 | 16.5 | 6.7 | 23.4 | 5.6 | 6.6 | 5.6 | 19.4 | 6.5 | 24.0 | 6.1 | 22.5 |
| Horse | — | — | 5.5 | 18.0 | 6.6-6.7 | 23.0 | — | 6.7 | 5.4-5.5 | 19.9 | 6.6-6.7 | 24.1 | — | 23.3 |
| Pig | — | — | (6.8) | 17.1 | (6.2-6.4) | 23.6 | (5.8) | 6.2 | — | — | — | — | (5.54) | 22.6 |

* The p*K* values are taken from Cocco et al. [15] and Bashford et al. [16]. In brackets: p*K* values from Carver and Bradbury [11].
** The distances from Fe atom of the heme to nitrogen ND1 of histidine were calculated with the MOLMOL 2.5.1 program using Mb coordinates from the PDB database.

## DISCUSSION

The results suggest that oxy-Mb oxidation in the presence of copper can proceed by different pathways depending on which histidine is complexed with the reagent and what the stability of the complex is. The slow process taking place in sperm whale and horse oxy-Mbs is obviously explained by strong binding of $Cu^{2+}$ to His12, His113, and His116, residues that are absent in pig Mb. It is mainly due to the last two residues, which are identical for sperm whale and horse Mbs (His12 in horse Mb is replaced by Gln). Due to this, conditions arise for fast reoxidation of reduced copper bound to the protein, resulting in oxidation of all of the oxy-Mb by even small $Cu^{2+}$ concentrations (catalysis). This process should prevail at low concentrations of $Cu^{2+}$ when these binding sites of Mb are saturated. The catalytic process is slow because it is probably limited by the rate of $Cu^{+}$ reoxidation and dissociation of bound $Cu^{2+}$ from myoglobin (stages 3-5). In contrast, the fast oxidation of 10-15% of pig oxy-Mb (the fast phase) is not accompanied by the catalysis since in this case reoxidation of the reduced copper does not occur. The $Cu^{+}$ ions perhaps easily dissociate into the solution, where they are known to be slowly oxidized by oxygen with the participation of protons [14]. Indeed, some further slow oxidation of pig oxy-Mb after the fast phase can be seen only at pH 5.

The amino acid residues contacting the heme group are strictly conserved in the three studied myoglobins. Besides His, amino acids in 19 more positions are replaced in horse and pig Mbs in comparison to sperm whale Mb [10, 11]. However, all these replacements are identical or homologous (except for Thr87 in pig Mb instead of Lys87 in sperm whale and horse Mbs), which makes it impossible to explain the special kinetic behavior of pig oxy-Mb by these replacements. The analysis of primary and spatial Mb structures shows that the nearest environment of His residues is very similar in all three myoglobins, so that complexes of equal affinity for $Cu^{2+}$ should be formed at the $Cu^{2+}$ binding sites.

Of four histidines, His48(CD6), His81(EF4), His97(FG3), and His119(GH1), that are common for studied myoglobins, $Cu^{2+}$ binding to His48 and His97 closest to the heme (Table 2) are most probably responsible for the fast reaction phase. The greatest contribution should be from His97, which is located only 0.62 nm from the heme. The affinity of His97 to $Cu^{2+}$ is apparently much less than His48 and the "catalytic" His residues as no appreciable contribution of the fast phase to the kinetics of the sperm whale and horse oxy-Mb reactions is observed even though His97 is present. The His48, His81, and His119 residues of pig Mb not participating in the catalysis should compete with His97 for copper binding, in particular, His48 with the highest affinity based on the NMR data. The fact that only ~10-15% of this protein is oxidized at equimolar $Cu^{2+}$ concentration supports this conclusion. Lower affinity of His97 for copper can be explained by its H-bonding to the propionate $COO^{-}$-group of the heme and a poor accessibility to the solvent [10, 11].

The fast reaction phase was observed earlier when studying oxidation of oxyhemoglobins in the presence of copper ions [4, 17]. Only the β-chains of Hb are oxidized, and these are close to Mb in their structure and redox properties. At the same copper concentration (0.5 $Cu^{2+}$ per heme), distinctions in kinetics of horse and human oxy-Hb oxidation were revealed: 50 and 8% of the protein, respectively, was oxidized during the fast phase (3 min). Only one $Cu^{2+}$ binding site was found in horse Hb by equilibrium dialysis, which was shown to be situated near Cys93 on the proximal side of the heme. This is in good agreement with the binding of $Cu^{2+}$ to His97(FG4) of Hb β-subunits, similar to His97(FG3) of myoglobins. At the same time, two copper binding sites were found in human Hb in which β-chains, apart from His97(FG4), contain His116(G18) resembling His116(G17) of myoglobin. As His116 binds $Cu^{2+}$ with higher affinity than His97, the amount of the protein oxidized in the fast phase should decrease, as observed experimentally. Both these residues are absent in the Hb α-chains, which are resistant to oxidation by copper.

Histidine 97 in Mb and Hb plays an important role in stabilization of the heme position. Therefore, its complexing with copper should result in a changed local conformation and stability of the protein, thus increasing availability of intrinsic histidines, especially distal His64(E7), to copper. In turn, this must result in protein denaturation. In our experiments, no denaturation of sperm whale and horse oxy-Mbs at the $Cu^{2+}$ concentrations used was observed, but the denaturation of pig oxy-Mb did take place at $[Cu^{2+}]/[Mb]$ ratio more than 1. Earlier, changes in the absorption of met-Mb in the Soret region were noted at more than twofold excess of copper [18], pointing to changes in the heme cavity, and denaturation of oxy-Hb was noted as well [4, 17].

Thus, the His113 and His116 residues that are common for sperm whale and horse Mbs are apparently responsible for the catalysis. These histidines are close to each other in the Mb structure (the smallest distance between them is 0.62 nm). Another pair of histidines, His12 and His119, present in sperm whale Mb (the distance between them is 0.89 nm), either does not participate in the catalysis at all or its efficiency is much less than of the His113-His116 pair since the kinetics of the oxidation of the two proteins practically coincide. Besides, the addition of zinc ions into the reaction mixture at $[Zn^{2+}]/[Mb]$ ratios from 1 to 20 when only one site in the Mb structure near His119 is saturated does not appreciably influence the reaction rate (Table 1). Also against the participation of His119 in the oxy-Mb oxidation catalyzed by $Cu^{2+}$ is the lack of catalysis in pig Mb where this residue is present. That is, His119 located in

the same Mb region as the catalytic His113 and His116 residues does not contribute significantly to the Mb oxidation even if it binds $Cu^{2+}$. The same is true for the His81 residue. All discussed residues, His81, His113, His116, and His119, are located far from the heme (Table 2).

The sigmoid pH-dependence curves for sperm whale and horse Mbs with transition p$K$ 6.4-6.7 that are registered at equimolar concentration of copper (Fig. 2) point to the effect of ionization a His residue on the catalysis rate. From the fact that the p$K$ values found for sperm whale and horse Mbs differ by ~0.3 pH unit, it is possible to conclude that the rate of oxy-Mb oxidation catalyzed by $Cu^{2+}$ is influenced by ionization of His116. This His is protonated with p$K$ 6.5 in sperm whale Mb and with p$K$ 6.7 in horse Mb (Table 2). Upon complexing of $Cu^{2+}$ with His113, which has the greatest affinity for copper according to the NMR data, protonation of His116 located nearby should increase the probability of electron tunneling from the heme to the given Mb site because the positive electrostatic potential there becomes higher. Besides, it is very probable that the His116 ionization enhances reoxidation of $Cu^{+}$ bound to neighboring His113, which proceeds with participation of $O_2$ and protons, providing an optimal arrangement of the reagents just like in enzymes. We recently found a similar effect in the reaction of sperm whale oxy-Mb oxidation catalyzed by ferrocyanide, where His119 ionization at the binding site of the anion $[Fe(CN)_6]^{4-}$ strongly increased the reaction rate [19].

As shown, the sigmoid shape of the pH-dependence curves becomes less pronounced at higher $Cu^{2+}$ concentration, and p$K_{eff}$ is moved to more acidic pH value (Fig. 3). It should be noted that increase of the rate at pH < 6 is generally inherent in redox reactions of heme proteins with different low molecular weight reagents and also in autooxidation of oxy-Mb and oxy-Hb [1-5, 20]. The pH dependence of oxy-Mb oxidation by copper can be due to the influence of proton concentration on different stages of the process. While $Cu^{2+}$ binding to His residues of oxy-Mb (stage 1) must be worsened at acidic pH because of competition of copper ions with protons, the rates of intramolecular electron transfer and reoxidation of reduced copper (stages 3 and 4) should be increased [2, 4, 5]. Since the reaction rate increases considerably at pH < 7, complexing of $Cu^{2+}$ with oxy-Mb obviously does not limit the process. The character of the ionic strength dependence is also in favor of this (Fig. 4). In the course of catalytic oxidation of oxy-Mb in the presence of copper (stages 3), as well as during autooxidation of oxy-Mb, the primary reaction product must be superoxide anion. The reason for general acceleration of reaction rates at acidic pH can be because reduction of $O_2$ is thermodynamically much more favorable in the presence of protons since the $E_o$ of the $O_2 + H^+/HO_2$ system is −37 mV, whereas for the $O_2/O_2^-$ pair it is −330 mV [21].

The data do not completely explain the complicated shape of the $Cu^{2+}$ concentration dependence (Fig. 5, a and b), so additional investigations are necessary. However, well-justified assumptions can be made about which $Cu^{2+}$ binding sites give the main contributions to the reaction rate on the first part of the curve where the $[Cu^{2+}]/[Mb]$ ratio varies from 0 up to approximately 5. According to the equilibrium dialysis data, at least three copper ions should be bound to Mb under these conditions. As His113, His116, and His48 have the greatest affinity from the NMR data, these very residues are probably saturated first. The nonlinear character of the concentration dependence curve in this interval of $Cu^{2+}$ concentrations at pH 7.5 (Fig. 5b) suggests that at least two copper complexes take part in the catalysis with different efficiencies. Evidently, these are the copper ions bound to His113 and His116. At the same time, at pH 6 only the one bound to His113 participates in the reaction. Protonation of His116 at pH < 7 and Coulomb repulsion of the charges interferes with binding of the second $Cu^{2+}$ to His116, so that only one metal ion can then be complexed in the His113 and His116 region. It is in agreement with the nearness of His113 and His116 to each other and with the influence of His116 ionization on the catalysis. The fact that proton resonances of both histidines are widened in NMR spectrum at pH 5.6 [2] can be explained by an influence of paramagnetic $Cu^{2+}$ on both residues. The low rates of oxy-Mb oxidation at pH 7.5 with any excess of copper are apparently related to the lack of both sufficient proton concentration and the assistance from the side of protonated His116, which is necessary for the reoxidation of the copper.

As to the "non-catalytic" His48 residue, it possibly also contributes to the reaction rate in the first part of the concentration dependence, especially for higher than equimolar copper concentrations. The pH dependence of this particular process is apparently different than for that catalyzed by His116 ionization. The different shape of the pH dependence at the ratio $[Cu^{2+}]/[Mb] = 5$ (Fig. 3) compared to the curve at equimolar $Cu^{2+}$ concentration (Fig. 2) is in favor of this interpretation.

A sharp increase in the oxy-Mb oxidation rate occurs in the second part of the concentration curve at the ratio $[Cu^{2+}]/[Mb] > 8$ where, as already mentioned, up to six copper ions can be bound to Mb. The rate increase can be due to both the involvement of additional $Cu^{2+}$ binding centers and structural changes in the protein, with both these factors acting interdependently. Because His113, His116, and His48 having higher affinity for $Cu^{2+}$ and should be completely saturated at high concentrations of copper, the additional contribution from $Cu^{2+}$ bound to "non-catalytic" residues His81, His119, and, in particular, to His97 located close to the heme is probable. It is possible that in this part of the concentration dependence even the distal His64(E7) can be involved in complexing with copper.

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