could, therefore, explain why an inverse order in pyridine is observed in a strongly coordinating buffer such as tris but not in a weakly coordinating buffer such as boric acid.

Support for this interpretation is supplied by the kinetic data in boric acid as a function of pH. As the pH of the medium approaches and crosses the pK_a of boric acid (9.2), the concentration of $H_2BO_3^-$ increases substantially. This species is a better axial ligand than its conjugate acid and it can compete to some extent with pyridine for a coordination site on the iron. Since P-H_2-buf is more reactive toward oxidation than P-H_2-P, an inverse order in pyridine is observed. The magnitude of this inverse-order dependence increases with increasing concentration of the competing ligand causing the slopes of the plots in Figure 5 to increase with pH. The y intercepts of the plots remain constant throughout, however, because the outer-sphere oxidation rate of the dipyridine heme dimer ($k_2' = k_{obsd}/2[O_2] = 7 \times 10^2$ M^{-1} s^{-1}) is pH independent.[26]

All the data, therefore, point toward an outer-sphere oxidation mechanism with no evidence for the intermediacy of any oxyheme complex. These findings are consistent with the general theory that dissociative electron-transfer processes do not occur from low-valence transition metals to π-bonding ligands in low-spin complexes.[26] Instead a peripheral π transfer is the most probable route for this process in analogy with Castro's formulation for monomeric heme complexes in amine solvents.[25] Thus, a loose π complex may be formed between molecular oxygen and the metalloporphyrin followed by electron transfer from the periphery of the ring. Such loose adducts of metalloporphyrins with oxygen as well as other π systems have been previously reported.[27]

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Conclusions

(1) Excess pyridine initially converts aqueous protoheme to a dipyridine heme dimer which is stable up to 0.2 M pyridine.

(2) Above this concentration, the dimer is gradually dispersed until at 2 M pyridine, the hexacoordinate dipyridineprotoheme monomer becomes the exclusive species in solution.

(3) The oxidation rate of the dipyridine heme dimer is independent of the pyridine concentration and proceeds entirely via an outer-sphere process.

The mechanism of conversion of the dipyridine heme dimer to the hexacoordinate monomer as well as the oxidation pathway at very high pyridine concentrations is currently still under investigation and will be the subject of a separate publication.

Acknowledgments. The authors would like to thank the Research Corporation and the National Institutes of Health (HL Grant No. 24519) for financial support and Professor C. E. Castro for helpful advice.

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Reaction of Superoxide Radicals with Copper(II)-Histidine Complexes

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Abstract: A study of the catalytic effect of Cu(II)-histidine complexes upon the disproportionation of superoxide radicals shows that only one complex, $(CuHist_2H)^{3+}$, of six which are known to exist catalyzes the disproportionation in the pH range between 1 and 10. The corresponding second-order rate constant, $k_{7,9} = (3.4 \pm 0.9)10^8$ M^{-1} s^{-1}, is pH independent between 2 and 7. The kinetic results are interpreted by two alternative mechanisms. One is similar to the currently accepted mechanism for superoxide dismutase catalysis; the other is based on the assumption that a transient superoxide complex of $(CuHist_2H)^{3+}$ is formed. The latter assumption is discussed in terms of present knowledge of the structural properties of the copper complexes.

The kinetic and chemical properties of superoxide radicals ($HO_2 \rightleftharpoons O_2^- + H^+$) and their involvement in biological oxidation-reduction reactions have been reviewed in several articles in recent years.[1-5] Research on these species has intensified since the discovery of prevalent superoxide dismutases (SOD), which catalyze their disproportionation to hydrogen peroxide and oxygen.[6]

While at least four metals are known to be involved in various superoxide dismutases, the mammalian enzyme which has a copper atom at the active site, and a zinc atom which is apparently not required for activity, has been most widely studied. It has been suggested that the mechanism of catalytic dismutation of the radicals involves alternate reduction and oxidation of the copper center:[7,8]

$$\text{enzyme-Cu}^{2+} + O_2^- \rightarrow \text{enzyme-Cu}^{+} + O_2 \quad (1)$$

$$\text{enzyme-Cu}^{+} + O_2^- + 2H^+ \rightarrow \text{enzyme-Cu}^{2+} + H_2O_2 \quad (2)$$

where enzyme-Cu represents the enzyme-bound copper ion and the respective rate constants, $k_1 = 2.4 \times 10^9$ and $k_2 = 2.4 \times 10^9$ M^{-1} s^{-1},[8] are near the theoretical limit for diffusion-controlled reactions. The turnover rate is pH independent over the range 4.8-9.5 and declines slightly below pH 4.8. According to the

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authors, this decline "suggests that the enzyme actually acts upon the superoxide anion rather than HO_2".[7]

Many studies have been carried out to probe the nature of the active site of SOD. Appearance of a superhyperfine pattern with a splitting of about 14 G strongly suggests that there is hyperfine coupling of three to four nitrogens to copper, but electron spin resonance results do not indicate which amino acids furnish the nitrogens.[9] Photosensitized oxidation experiments on SOD point to the presence of three to four imidazole groups.[10] A recent X-ray crystal structure of the enzyme shows four histidine ligands to copper in a square-planar arrangement.[11]

We have been investigating copper–histidine complexes in aqueous solution as a model system for SOD. This study presents evidence that a particular state of protonation of the ligands is essential for dismutation activity of copper–histidine complexes.

Experimental Section

Pulse radiolysis experiments were carried out with the BNL Van de Graaff accelerator, whose operation is described elsewhere.[12,13] All solutions were prepared in distilled water, which was further purified by a Milli-Q reagent grade water system (Millipore Corp., Bedford, Mass.). L-Histidine (Sigma Chemical Co.) and cupric acetate (Baker Analyzed reagent) were used without further purification. Sodium formate and trisodium phosphate (Baker Analyzed reagent) were purified as described earlier.[12] Cuprous chloride (Apache Chemical Co.) was washed with N_2-saturated perchloric acid solution immediately before use to remove cupric impurities from the crystal surfaces.

The rate of decay of superoxide radicals was monitored spectrophotometrically at 250 nm over the range from pH 1.1 to 10.0. Solutions contained 0.10 M sodium formate to scavenge hydroxyl radicals, 0.4 mM histidine, and various amounts of copper acetate ($CuAc_2$) (1–100 μM). pH was adjusted with vacuum-distilled perchloric acid (G. Frederick Smith Chemical Co.) and/or with three times recrystallized trisodium phosphate.

Copper and histidine combine to form at least six different complex species over the pH range studied. We used known association constants[14-16] and a computer program reported in the literature[17] to determine the distribution of copper among various complexes under our experimental conditions. The program uses an iterative technique to calculate the equilibrium concentration of complex species from knowledge of the total concentration of copper, ligands (histidine, formate, and acetate), and pH.

Results and Discussion

In this investigation the superoxide radicals were generated by pulse radiolysis of oxygenated aqueous solutions containing formate:[18]

$$H_2O \xrightarrow{HCOONa,O_2} (HO_2 \rightleftharpoons O_2^- + H^+), H_2, H_2O_2 \qquad (I)$$

In the absence of competing reactants, superoxide radicals decay according to a pH-dependent mechanism:[19-21]

$$HO_2 \rightleftharpoons O_2^- + H^+ \qquad (3)$$

$$HO_2 + HO_2 \rightarrow H_2O_2 + O_2 \qquad (4)$$

$$HO_2 + O_2^- + H_2O \rightarrow H_2O_2 + O_2 + OH^- \qquad (5)$$

$$O_2^- + O_2^- + 2H_2O \rightarrow H_2O_2 + O_2 + 2OH^- \qquad (6)$$

Table I. Complex Species Present in Solution Containing Copper Acetate, Sodium Formate, and Histidine[a]

species	log K^*	ref
(CuHistH)	14.20	14
$(CuHist)^{2+}$	10.2	14
$[Cu(HistH)_2]^{4+}$	26.9	14
$[CuHist_2H]^{3+}$	24.0	14
$[CuHist_2]^{2+}$	18.5	14
$[CuHist_2OH]^+$	7.71	14
$[CuAc]^+$	1.67	15
$[CuAc_2]$	0.98	15
$[Cu(HCOO)]^+$	1.57	16
$[Cu(HCOO)_2]$	0.65	16
$[Cu(HCOO)_3]^-$	−0.15	16
$[Cu(HCOO)_4]^{2-}$	0.40	16

[a] K^* is the cumulative association constant for the species, e.g., $Cu^{2+} + 2Hist \rightleftharpoons (CuHist_2)^{2+}$; $K^* = (CuHist_2)^{2+}/(Cu^{2+})(Hist)^2$.

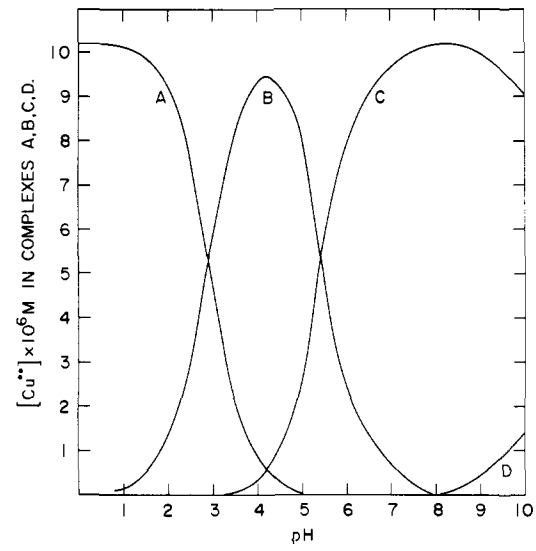

Figure 1. Distribution of Cu(II) complexes in a solution containing 0.4 mM histidine, 0.1 M formate, and 10.6 μM $Cu(CH_3COO)_2$. A = $[Cu(HistH)_2]^{4+}$; B = $[CuHist_2H]^{3+}$; C = $[CuHist_2]^{2+}$; D = $[CuHist_2OH]^+$. All other complexes do not exceed the concentrations indicated over the pH range shown: $[CuHistH]^{3+} \leq 1.0 \times 10^{-12}$ M; $[CuHist]^{2+} \leq 1 \times 10^{-10}$ M; $Cu(CH_3COO)^+ \leq 2 \times 10^{-20}$ M; $Cu(CH_3COO)_2 \leq 9 \times 10^{-26}$ M; $Cu(HCOO)^+ \leq 9 \times 10^{-17}$ M; $Cu(HCOO)_2 \leq 1 \times 10^{-18}$ M; $Cu(HCOO)_3^- \leq 2 \times 10^{-20}$ M; $Cu(HCOO)_4^{2-} \leq 5 \times 10^{-21}$ M.

The corresponding dissociation constant and second-order rate constants have recently been redetermined in our laboratory:[22]

$$K_{HO_2} = (2.05 \pm 0.39)10^{-5} \text{ M or } pK_{HO_2} = 4.69 \pm 0.08$$

$$k_4 = (8.60 \pm 0.62)10^5 \text{ M}^{-1} \text{ s}^{-1}$$

$$k_5 = (1.02 \pm 0.49)10^8 \text{ M}^{-1} \text{ s}^{-1} \text{ and } k_6 < 0.35 \text{ M}^{-1} \text{ s}^{-1}$$

Superoxide radical decay is catalyzed by the presence of small amounts of histidine-complexed copper in solution. The spontaneous dismutation reactions 4–6 return to the solution one molecule of O_2 for every two superoxide radicals. Since the superoxide originates from molecular oxygen in solution, dismutation results in a net consumption of half as much oxygen as originally was converted to superoxide. If a solution of O_2^- (formed by irradiation of a formate solution at pH 10) is mixed with a copper–histidine solution in a chamber fitted with an oxygen electrode, the measured oxygen consumption is identical with that observed when the same superoxide solution is mixed with a blank solution. This indicates that the stoichiometry for the overall reaction is the same in the presence and absence of the catalyst.

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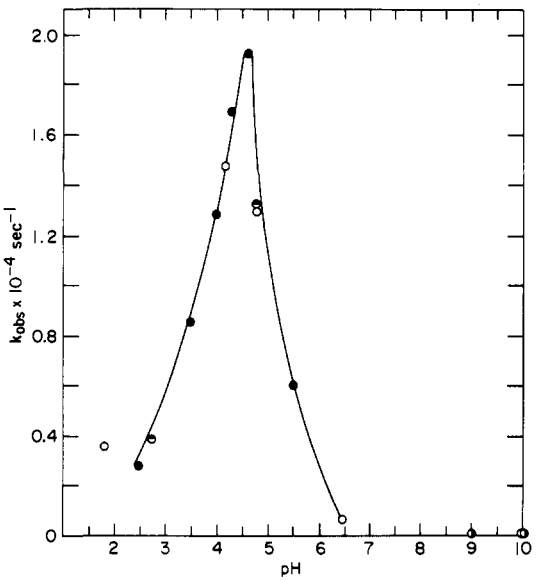

Figure 2. Pseudo-first-order rate constants for disappearance of HO_2/O_2^- at various pH values. $[Cu(CH_3COO)_2]$ = 20.8 μM, [histidine] = 0.4 mM, [formate] = 0.1 M. Data corrected for spontaneous decay of HO_2/O_2^-. Different symbols represent data collected on different days.

In determining the equilibrium distribution of copper in our solutions at a particular pH, one must consider several acetate, formate, and histidine complexes. The appropriate species and their cumulative association constants are presented in Table I.[14-16] The variation in concentration of the various histidine complexes with pH for a total copper concentration of 1.06×10^{-5} M is shown in Figure 1. Despite the potential for complications, only four of the complexes were present in concentrations above 10^{-10} M (see legend of Figure 1). The concentration of free copper is expected to be 10^{-13}% of the total present. In these experiments most of the formate and histidine (because of the large excess over copper) is present in the uncomplexed form. Appropriate blank experiments showed no interfering reactions of superoxide radicals with these substances.

Pseudo-first-order kinetics were observed for the decay of superoxide radicals (1–10 μM) between pH 2.1 and 10.0 in the presence of a total copper concentration of 1–100 μM. No transient absorption other than that due to O_2^-/HO_2 was observed in the UV and visible range under our experimental conditions. Within experimental error, the decay traces showed no residual absorption changes. The observed first-order rate constant for the copper–histidine catalyzed disproportionation of superoxide radicals varies with pH as shown in Figure 2.

Comparison of Figure 1 with Figure 2 strongly suggests that only one complex, $(CuHist_2H)^{3+}$, is responsible for the superoxide dismutase activity. Assuming that this hypothesis is correct, to compute the second-order rate constant the observed first-order rate constant is divided by the concentration of copper in the form of $(CuHist_2H)^{3+}$ at a given pH. Figure 3 shows the linear dependence of the pseudo-first-order rate constants on the concentration of this complex at pH 2.5 and 4.7. Similar plots for other pHs give the value of the second-order rate constant for $(CuHist_2H)^{3+}$-catalyzed disproportionation of superoxide radicals as $k_{7,9} = (3.4 \pm 0.9)10^8$ M^{-1} s^{-1} between pH 2 and 7. At the extreme ends of the pH range studied, the data are not as reliable because of the very low concentrations of $(CuHist_2H)^{3+}$ (e.g., 10^{-10} M at pH 10), and the concomitant large error.

A profile such as that shown in Figure 2 could arise fortuitously if O_2^- reacted specifically with complex A, while HO_2 reacted specifically with the complex C. Calculations show that such reactions would require rate constants several orders of magnitude greater than diffusion-controlled values to fit the experimental data.

Two mechanisms can be proposed for the catalytic activity of the $(CuHist_2H)^{3+}$ complex. Mechanism I (Scheme I) is similar to the currently accepted mechanism for superoxide dismutase

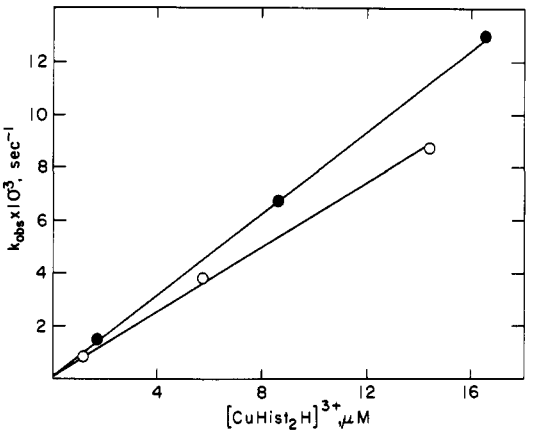

Figure 3. Pseudo-first-order rate constants for disappearance of HO_2/O_2^- as a function of $[CuHist_2H]^{3+}$ at two pH values. [Histidine] = 0.4 mM, [formate] = 0.1 M, $[Cu(CH_3COO)_2]$ = 4–50 μM for pH 2.5 (○); $[Cu(CH_3COO)_2]$ = 2–20 μM for pH 4.7 (●).

catalysis[4] (reactions 1 and 2) in which Cu(II) is reduced to Cu(I) and reoxidized to Cu(II) in a cycle as illustrated in reactions 7 and 8. Mechanism II (Scheme II) suggests the formation of an intermediate Cu(II)–superoxide radical complex in reaction 9 which can further react with another superoxide radical in a dismutation step, reaction 10. The latter reaction is analogous to reaction 5, the fast step in the spontaneous disproportionation of superoxide radicals. Although the formation of a transient complex between O_2^-/HO_2 and metal cations/metal chelates has been suggested and/or observed in the past,[23-35] no evidence exists for such a complex with copper–histidine.

Scheme I

$$(CuHist_2H)^{3+} + O_2^- \rightarrow (CuHist_2H)^{2+} + O_2 \quad (7)$$

$$(CuHist_2H)^{2+} + O_2^- \, 2H^+ \rightarrow (CuHist_2H)^{3+} + H_2O_2 \quad (8)$$

Scheme II

$$(CuHist_2H)^{3+} + O_2^- \rightarrow [(CuHist_2H)^{3+}\cdots O_2^-] \quad (9)$$

$$[(CuHist_2H)^{3+}\cdots O_2^-] + O_2^- + 2H^+ \rightarrow (CuHist_2H)^{3+} + H_2O_2 + O_2 \quad (10)$$

A similar set of equations can be written for the HO_2 species. Overall the catalytic disproportionation of superoxide radicals (HO_2/O_2^-) is described for either mechanism by

$$-d[O_2^-]/dt = 2k_{7,9}[Cu_{catalyst}][O_2^-] = k_{obsd}[O_2^-] \quad (II)$$

Expression II was derived assuming that the reverse of steps 7 and 9 proceed at a much slower rate than the sum of the forward reactions 7 and 8 and 9 and 10, respectively. An additional

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assumption, that k_8 and k_{10} are much larger than k_7 and k_9, respectively, is borne out by the observation that the base-line absorbance of $(CuHist_2H)^{3+}$ is unchanged after the reaction. With these two assumptions, both mechanisms predict the observed pseudo-first-order disappearance of superoxide radicals.

Since we could not distinguish kinetically between mechanisms I and II, we hoped to observe an absorbance peak due to the complex $[(CuHist_2H)^{3+}\cdots O_2^-]$. A shift in the absorption maximum from 245 to 270 nm of O_2^- upon attachment to metal cations had been reported earlier.[32,34,35] Unfortunately, we were unable to detect such a shift in the presence of up to 40 μM $(CuHist_2H)^{3+}$. Attempts to increase the complex concentration were prevented by the high absorbance of histidine in the region where the complex is expected to absorb. From our experience with the $[Cu^{II}\cdots O_2^-\cdots Cu^{II}]^{3+}$ complex,[36] significantly higher concentrations of $(CuHist_2H)^{3+}$ may be necessary to consume all O_2^- radicals in reaction 9 and thus prevent reaction 10 from occurring and depleting the $[CuHist_2H\cdots O_2^-]^{2+}$ concentration. We also cannot rule out the possibility that a dinuclear complex between $[(CuHist_2H)^{3+}\cdots O_2^-]$ and a second $(CuHist_2H)^{3+}$ may be formed under conditions of high concentration of the copper complex.

In principle, the dioxygen complex should be formed through the direct reaction of the cuprous complex with molecular oxygen. Because of the disproportionation of Cu(I) above pH 2, we were unable to perform experiments at the optimum pH, 4.5. We therefore attempted to form the related complex $[Cu(HistH)_2^{4+}\cdots O_2^-]$ by reacting the doubly protonated cuprous complex, $Cu(HistH)_2^{3+}$, with O_2 in a stopped-flow spectrophotometer. The $Cu(HistH)_2^{3+}$ complex was formed by dissolving excess CuCl in deoxygenated 0.1 mM histidine solution at pH 2. The small amount of Cu(I) so formed is expected to complex strongly with histidine.[24] At this pH, we do not observe absorption which can be attributed to the dioxygen complex. The absorption attributable to Cu(I) decays by reaction with O_2 at a rate $k_{11} = (4.0 \pm 0.4)10^4$ M^{-1} s^{-1} at pH 2:

$$[Cu(HistH)_2]^{3+} + O_2 \rightarrow [Cu(HistH)_2^{4+}\cdots O_2^-] \rightleftharpoons [Cu(HistH)_2]^{4+} + O_2^- \quad (11)$$

As a result of the above observations, we were unable to distinguish experimentally between mechanisms I and II. Theoretical considerations, however, favor mechanism II, as will be discussed. At present there is considerable controversy over the structure of copper–histidine complexes. A recent reference[37] summarizes the five proposed structures for $(CuHist_2)^{2+}$. At pH 4.5, the singly protonated $(CuHist_2H)^{3+}$ reaches its maximum concentration. The work of Kruck and Sarkar[14] points to the imidazole as the likely group for protonation and suggests a five-coordinated octahedral structure for $(CuHist_2H)^{3+}$ (Figure 4a). The free axial site would be occupied by a loosely bound water and easily accessible to O_2^-/HO_2. The dioxygen adduct would have a structure such as that shown in Figure 4b. That metals can bind O_2 as a ligand was shown from crystal-structure studies of Co and Fe complexes.[38,39] The active complex, $(CuHist_2H)^{3+}$, is unique in that one imidazole nitrogen is protonated, leaving just one octahedral site accessible to O_2^-/HO_2. Recent NMR and pulse radiolysis studies of the active site of bovine superoxide dismutase suggest that there is one coordination site free on the copper center and that this site becomes available through protonation of the bridging imidazole group.[40,41]

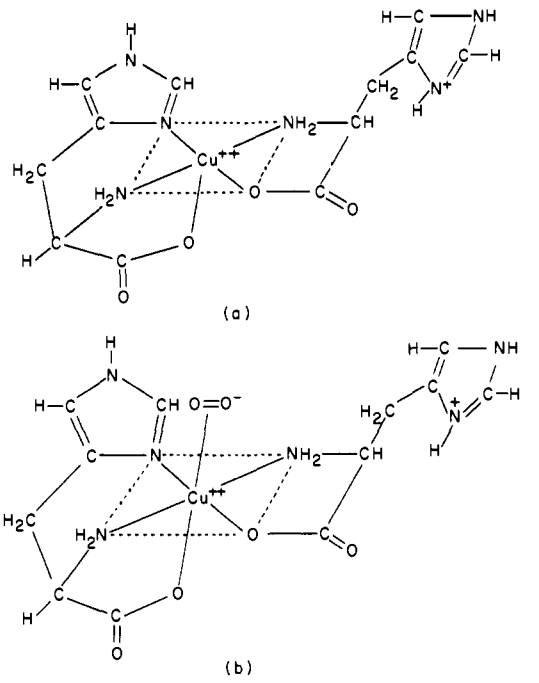

Figure 4. (a) Proposed octahedral structure for the active complex, $(CuHist_2H)^{3+}$ (see ref 14). (b) Proposed octahedral structure for the dioxygen adduct.

Although five-coordinated cuprous complexes have been proposed,[42] they appear to be limited to complexes with rigid macrocyclic ligands. In general, cuprous ion is tetrahedrally coordinated. Although to the best of our knowledge there is little hard evidence, it has been suggested unlikely that a catalytic reaction sequence would go through an intermediate that requires rearrangement of the ligands from octahedral to tetrahedral coordination.[36]

The superoxide dismutase activity of simple copper–amino acid complexes has been investigated before.[16,43-45] In one case, $(CuHist_2)^{2+}$ was found to have some dismutase activity, measured by its ability to suppress the xanthine–xanthine oxidase induced reduction of cytochrome *c*.[43] These studies have traditionally been carried out near physiological pH, where our results indicate that only a small fraction of the active complex exists. The present study illustrates that computed second-order rate constants based on the amount of metal cation added may lead to erroneous results since a mixture of complexes may be formed and not all complexes possess the same catalytic activity.

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