Autoxidation of Ascorbic Acid Catalyzed by the Copper(II) Bound to L-Histidine Oligopeptides, (His)_iGly and Acetyl-(His)_iGly (*i*=9, 19, 29). Relationship between Catalytic Activity and Coordination Mode

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Spectroscopic and kinetic studies on the autoxidation of ascorbic acid catalyzed by copper complexes of histidine oligopeptides, (His)_iGly (*i*=4, 9, 19, 29), and their acetyl derivatives, Ac-(His)_iGly (*i*=9, 19) have been carried out at pH 4.4 and 25°C under dioxygen. The reaction was monitored at 260 nm using a stopped-flow spectrophotometric technique. The reaction fitted the “Michaelis–Menten” mechanism, and ascorbate was oxidized by the “Ping-Pong” mechanism. The Cu(II) complexed with the oligopeptide (*i*≥9) enhanced the reaction approximately two-fold relative to the aqueous Cu(II). The catalytic activity depends on the molecular weight which is related to the number of histidyl residues and on the coordination mode of the copper-binding site. Results of circular dichroism (CD) experiments revealed the existence of two types of Cu(II). The catalytically active Cu(II), which is accommodated in the imidazole clusters composed of at least six histidyl residues, exhibits *d–d* transition bands at 520 and 630 nm, and is easily dissociable, enhances the autoxidation; Ac-(His)₉Gly is likely to accommodate approximately three active Cu(II) ions. The Cu(II), which is complexed tightly with the terminal H₂N–X–Y–His– moiety, where X and Y denote amino acids, inhibits the autoxidation, and exhibits absorption bands at 480 and 550 nm.

Key words autoxidation; ascorbic acid; copper ion; histidine peptide; circular dichroism; active site

A variety of metalloenzymes, which contain copper ions in the active center, function in electron transfer, *e.g.*, plastocyanin, storage and transport of dioxygen, *e.g.*, hemocyanin, disproportionation of superoxides, *e.g.*, superoxide dismutase, oxygenation, *e.g.*, tyrosinase, and oxidation, *e.g.*, ascorbate oxidase.²⁾ These functions involve primarily electron transfer, and the copper ions, which are located in histidine-rich clusters coordinating with the imidazole nitrogens, comprise the active site.^{3–5)} Recently, X-ray crystallographic studies of metalloenzymes have provided much information on the coordination environment around the active centers.

The copper ion in the oxidase is turned over between the Cu(II) and Cu(I) state during the reaction. The Cu(II) is reduced to Cu(I) by the substrate. The Cu(I) in low molecular-weight complexes, as well as in the oxidase, is able to associate with dioxygen to form an adduct, L–Cu(I)–O₂,⁶⁾ which can either dissociate back to Cu(I)–L and O₂ or disproportionate to the Cu(II) species and superoxide ions; where L denotes a ligand. The oxygen adduct can also react with another Cu(I)–L to form a peroxide, L–Cu(I)–O₂–Cu(I)–L, which subsequently undergoes redox cleavage to form the Cu(II) species and hydrogen peroxide (H₂O₂). In the presence of an appropriate electron donor (reductant) namely ascorbic acid or cysteine, the L–Cu(I)–O₂ is likely to cleave into the Cu(II) species and H₂O₂.⁷⁾ The reductant is likely to donate two electrons to the receptor. Thus, copper complexes are capable of catalyzing autoxidation of ascorbic acid and thiol compounds of biological relevance.^{8–11)}

Addition of metal chelating agents modifies the activities of the catalyst. Generally, metal chelating agents such as EDTA inhibit the autoxidation. In contrast, poly-L-histidine (PLH) which could coordinate with Cu(II) is shown to enhance considerably the autoxidation of ascorbic acid.^{12–14)} We have been studying the importance of histidyl residues

and imidazole clusters in constructing the metal-binding sites.^{15–17)} Results from equilibrium dialysis experiments revealed that PLH complexes contain at least two types of Cu(II), which were classified as either catalytically active or inactive/inhibitory ions.¹⁵⁾ The active Cu(II) readily dissociated from the complexes on addition of NaClO₄ and CH₃COONa.

The PLH synthesized by polymerization methods is composed of a population of histidine oligomers with various molecular weights. Therefore, the interrelation among coordination modes of Cu(II), the molecular weight, and the activity of the catalyst is not well understood. In order to clarify the coordination mode and structure of histidine peptides with Cu(II), we intended to synthesize chemically homogeneous oligopeptides, (His)_iGly (*i*=9, 19, 29), which possess two types of metal-binding sites, one each at the amino terminus and in the imidazole-rich cluster, and their *N*-acetylated derivatives Ac-(His)_iGly (*i*=9, 19), which lack the amino terminal binding site. Results from spectroscopic and kinetic experiments reveal that the Cu(II) with *d–d* transition bands at 520 and 630 nm is catalytically active and that with the bands at 480 and 550 nm is inhibitory.

Experimental

Materials Ultrosyn resin, 9-fluorenylmethoxycarbonylglycine pentafluorophenyl ester (Fmoc-Gly-OPFP) and *N*_α-(9-fluorenylmethoxycarbonyl)-*N*_{im}-*tert*-butoxycarbonyl-L-histidine pentafluorophenyl ester (Fmoc-His(Boc)-OPFP) purchased from Pharmacia Co. (Sweden) were used as received. Histidine, glycine, 2-*tert*-butoxycarbonyloxyimino-2-phenylacetoneitrile (Boc-ON), trifluoroacetic acid (TFA), *N,N*-dimethylformamide (DMF), and 20% piperidine in DMF were obtained from Kokusan Chemical Co. (Japan). *N*-hydroxybenzotriazole (HOBt), diethyl phosphorocyanidate (DEPC), benzyl alcohol, *p*-toluenesulfonic acid (*p*-TsOH), anisole and acetic anhydride were purchased from Wako Pure Chemical Co. (Japan). 4-Methoxybenzenesulfonyl chloride (MBS-Cl) was purchased from Aldrich Chemical Co. (U.S.A.). HisGly was a product of BACHEM Feinchemikalien

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AG (Switzerland).

Solid-Phase Synthesis of Histidine Oligopeptides The histidine oligopeptides, (His)_iGly (*i*=9, 19, 29), and their acetyl derivatives, Ac-(His)_iGly (*i*=9, 19), were synthesized by a solid-phase technique on an semi-automatic peptide synthesizer LKB BIOLYNX 4175 (Bromma, Sweden) using Ultrosyn resin. Firstly, Fmoc-His(Boc)-OPFP was coupled to the glycine attached on Ultrosyn resin by HOBT to yield Fmoc-His(Boc)-Gly-resin. Next, the Fmoc at the amino terminus of Fmoc-His(Boc)-Gly-resin was removed with a 20% piperidine in DMF to yield His(Boc)-Gly-resin, which was subsequently coupled to Fmoc-His(Boc)-OPFP affording the Fmoc-His(Boc)-His(Boc)-Gly-resin. The same procedure was repeated until the appropriate Fmoc-[His(Boc)]_{*i*}-Gly-resin was synthesized. Finally, the Fmoc group at the amino terminus of the Fmoc-[His(Boc)]_{*i*}-Gly-resin was removed by a 20% piperidine in DMF to yield [His(Boc)]_{*i*}-Gly-resin. Cleavage and deprotection of the peptide from the resin were achieved by treatment with 95% TFA for 1 h at room temperature. The *N*-acetyl derivatives of [His(Boc)]_{*i*}-Gly were prepared by treatment with 10% acetic anhydride in DMF. The synthesized histidine oligopeptide mixtures were separated and purified by high performance liquid chromatography on the TSKgel SP-2SW column (Tosoh Co., Japan) according to the method described previously.¹⁸⁾

Liquid-Phase Synthesis of Histidine Oligopeptides (His)₄Gly was synthesized by a conventional liquid-phase method. Starting materials were Boc-His(MBS)-OH and Gly-OBzl *p*-TsOH, and DEPC was used as a coupling agent. Firstly, Boc-His(MBS)-Gly-OBzl was prepared and subsequently treated with 50% TFA for 50 min affording NH₂-His(MBS)-Gly-OBzl. This was then coupled with Boc-His(MBS)-OH to yield Boc-[His(MBS)]₂-Gly-OBzl. Finally, Boc-[His(MBS)]₄-Gly-OBzl prepared by stepwise coupling was treated with hydrogen fluoride (HF) in the presence of anisole to afford (His)₄Gly. Stepwise procedures are summarized in Chart 1. The peptide was purified according to the method described previously.¹⁸⁾

Procedure for the Measurement of Ascorbate Autoxidation Routinely, the reactions were carried out at 25 °C and pH 4.4 under dioxygen. Solutions of $8.0 \times 10^{-5} \text{ mol dm}^{-3}$ ascorbate and $4.0 \times 10^{-6} \text{ mol dm}^{-3}$ Cu(II) complexes, which were prepared freshly before kinetic runs in 0.02 mol dm⁻³ acetate buffer, were mixed with a UNION MX-7 rapid-mixing apparatus to start the reaction, and subsequent changes in absorbance at 260 nm were monitored on a UNION SM-401 spectrophotometer. Before and during the measurement, the solutions were purged with water-saturated O₂ at 100 ml/min. The activity, v_{obsd} , was obtained from the initial rate of ascorbate consumption. The dead-time of the mixing apparatus was assessed within 1 s.

Circular Dichroism (CD) Measurement Measurements of CD were carried out at room temperature and pH 4.4 in 0.02 mol dm⁻³ acetate buffer, and the concentration of Cu(II) was fixed at $8.1 \times 10^{-5} \text{ mol dm}^{-3}$. The spectrum was recorded from 300 nm to 700 nm on a JASCO J-40 spectropolarimeter. Cell length used here was 5 cm.

Analysis of Kinetic Data The oxidation of ascorbate obeys the "Michaelis-Menten" mechanism and the rate, v_{obsd} , is expressed by Eq. 1.¹⁵⁾

$$v_{\text{obsd}} = v_{\text{max}} / \{1 + (K_m / [\text{AscH}]_0)\} \quad (1)$$

where [AscH]₀ stands for the total concentration of ascorbate. The terms, v_{max} and K_m , are the maximum rate and the "Michaelis-Menten" constant given by Eqs. 2 and 3, respectively;

$$v_{\text{max}} = k_2[\text{Cu}]_0 \quad (2)$$

$$K_m = (k_{-1} + k_2) / k_1 \quad (3)$$

where [Cu]₀ represents the total concentration of copper.

The kinetic parameters, v_{max} and K_m , were calculated from the v_{obsd} -[AscH]₀ plot by a nonlinear least-squares method programmed by one of authors (K. H.).

Results and Discussion

Catalytic Activities Both (His)_iGly and Ac-(His)_iGly (*i*=9, 19) accelerated the copper-catalyzed autoxidation of ascorbic acid. The rate-pH plots showed bell-shaped curves with a maximum around pH 4.4. Subsequently, spectroscopic and kinetic experiments were done at pH 4.4. The systems without Cu(II) lack the catalytic activity.

(1) **Coppers Associated with (His)_iGly:** Plots of the relative rate, v/v_0 , against the concentrations of (His)_iGly (*i*=1, 4,

Chart 1. Solution Synthesis of (His)₄Gly

Fig. 1. Plot of the Autoxidation Rate, v/v_0 , against $[(\text{His})_i\text{Gly}]/[\text{Cu(II)}]_0$ at pH 4.4

(×—×): HisGly; (□—□): (His)₄Gly; (△—△): (His)₉Gly; (●—●): (His)₁₉Gly; (○—○): (His)₂₉Gly. [Ascorbate] = $8.0 \times 10^{-5} \text{ mol dm}^{-3}$, [Cu(II)] = $4.0 \times 10^{-6} \text{ mol dm}^{-3}$, at 25 °C under O₂. Data are the average of at least three experiments.

9, 19, 29) are shown in Fig. 1, where v_0 denotes the rate in the absence of peptides, [Cu(II)]₀ is fixed at $4.0 \times 10^{-6} \text{ mol dm}^{-3}$, and [(His)_iGly] is varied. The rate depended not only on the concentration but also the molecular weight of the peptides. The Cu(II) complexed with HisGly did not affect the autoxidation; the rate was approximately the same as that of aqueous Cu(II). In the pH range from 4 to 5, HisGly forms CuL or CuL₂ species with a histamine-like coordination structure, where L represents the peptide.¹⁹⁾ This suggests that Cu(II) with histamine-like coordination structures neither accelerates nor inhibits the autoxidation. Under optimum conditions, the Cu(II) complexed with (His)₄Gly enhanced the reaction approximately 1.2-fold, but the Cu(II) with (His)₉Gly increased the rate 2.5-fold. Thus, the molecular weight of the peptide, *i.e.*, numbers of serial histidyl residues, contributes to the enhancement of the catalytic activity. When the v/v_0 values exhibited maxima, the peptide would bind enough Cu(II) ions to work efficiently. In terms of maximum v/v_0 , the various (His)_iGly complexes ranked in increasing order as follows;

Fig. 2. Plot of the Autoxidation Rate, v/v_0 , against $[\text{Peptide}]/[\text{Cu(II)}]_0$ at pH 4.4; Effect of *N*-Acetylation

(▲—▲): $(\text{His})_9\text{Gly}$; (●—●): $(\text{His})_{19}\text{Gly}$; (△—△): $\text{Ac}-(\text{His})_9\text{Gly}$; (○—○): $\text{Ac}-(\text{His})_{19}\text{Gly}$. Conditions are the same as in Fig. 1. Data are the average of at least three experiments.

$(\text{His})_4\text{Gly}$ (2) $< (\text{His})_9\text{Gly}$ (3) $< (\text{His})_{19}\text{Gly}$ (4) $< (\text{His})_{29}\text{Gly}$ (6)

where in parentheses are shown approximate numbers of histidyl residues per one molar Cu(II) , (number of $\text{His-residue}/[\text{Cu(II)}]$). The greater the molecular weight, the more copper atoms each peptide can accommodate and the more effective the complex becomes.

The v/v_0 decreased as the $[\text{L}]/[\text{Cu(II)}]_0$ increased over 0.3–0.4, and finally became less than unity. This suggests formation of inactive/inhibitory Cu(II) complexes. The $(\text{His})_i\text{Gly}$ is considered to have $(i+1)$ donor sets from one terminal amino nitrogen and i imidazole nitrogens. $(\text{His})_i\text{Gly}$ ($i \geq 9$) primarily coordinates with multi-equivalent Cu(II) forming at least two types of complexes with different coordination modes. Those Cu(II) ions would have opposite functions: one accelerates the autoxidation and the other inhibits it.

(2) Coppers Associated with $\text{Ac}-(\text{His})_i\text{Gly}$: $\text{Ac}-(\text{His})_i\text{Gly}$ lacks the binding site at the amino terminus. Their complexes were catalytically more active than the corresponding $(\text{His})_i\text{Gly}$ complexes. In Fig. 2 is shown the v/v_0 -plots for $\text{Ac}-(\text{His})_i\text{Gly}$ ($i=9, 19$), along with those for $(\text{His})_i\text{Gly}$. The v/v_0 for the $\text{Ac}-(\text{His})_i\text{Gly}$ system reached a maximum around $[\text{L}]/[\text{Cu(II)}]_0=0.5$. As the $[\text{L}]/[\text{Cu(II)}]_0$ increased, the activity resulted in a rapid drop probably because the active Cu(II) sites on the peptide would be dispersed. However, the $\text{Ac}-(\text{His})_i\text{Gly}$, lacking the Cu(II) site at the amino terminus, did not make the v/v_0 drop below unity. Thus, blocking of the terminal binding site certainly induced enhancement of the reaction system. The v/v_0 for $\text{Ac}-(\text{His})_i\text{Gly}$ complexes was arranged as follows;

$\text{Ac}-(\text{His})_9\text{Gly}$ (2) $< \text{Ac}-(\text{His})_{19}\text{Gly}$ (3)

where in parentheses are shown (number of $\text{His-residue}/[\text{Cu(II)}]$). The $(\text{His})_i\text{Gly}$ could accommodate more than one molar Cu(II) , as compared with the corresponding *N*-acetylated peptides. This is probably due to the Cu(II) bound at the amino terminus. Enhancement of the activities

Fig. 3. CD Spectra of the Cu(II) Coordinated with $(\text{His})_{19}\text{Gly}$ at pH 4.4

$[(\text{His})_{19}\text{Gly}]/[\text{Cu(II)}]=0.12$ (1); 0.25 (2); 0.37 (3); 0.62 (4); 1.9 (5). $[\text{Cu(II)}]=8.1 \times 10^{-3} \text{ mol dm}^{-3}$ at room temperature.

by *N*-acetylation is as follows:

$\text{Ac}-(\text{His})_9\text{Gly}/(\text{His})_9\text{Gly}=1.4$ and $\text{Ac}-(\text{His})_{19}\text{Gly}/(\text{His})_{19}\text{Gly}=1.2$

Though the increase in the autoxidation rate is related to the increase in the total number of His-residues , the enhancement by *N*-acetylation is not. In other words, the contribution of the inhibitory Cu(II) to the reaction is diminished with the increase in total His-numbers .

Coordination Modes and Structures of the Cu(II) Complexes Circular dichroism has been shown to provide useful information about environments around metal sites.²⁰⁾ Coordination of a transition metal ion with a chiral ligand induces the CD at its $d-d$ transition band, and the λ_{max} depends on the ligand-field strength.²¹⁾ In an attempt to obtain information on the Cu(II) binding sites, measurements of the CD spectra of Cu(II) with $(\text{His})_i\text{Gly}$ ($i=9, 19, 29$) and $\text{Ac}-(\text{His})_i\text{Gly}$ ($i=9, 19$) were made.

(1) Cu(II) Complexes of $(\text{His})_i\text{Gly}$: The CD spectra of the Cu(II) with $(\text{His})_{19}\text{Gly}$ at various $[\text{L}]/[\text{Cu(II)}]_0$ ratios from 0.06 to 1.9 are shown in Fig. 3. The spectra at $[\text{L}]/[\text{Cu(II)}]_0 < 0.4$ exhibited two $d-d$ transition bands; one positive at 520 nm and the other negative at 630 nm, and its intensity increased proportionally to $[\text{L}]$, reaching a maximum at $[\text{L}]/[\text{Cu(II)}]_0=0.37$ ($\Delta\epsilon$; $0.97 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}/\text{Cu(II)}$ at 520 nm, $-0.21 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}/\text{Cu(II)}$ at 630 nm). As the $[\text{L}]/[\text{Cu(II)}]_0$ increased over 0.4, however, the spectrum underwent blue and hypochromic shifts. At $[\text{L}]/[\text{Cu(II)}]_0=1.9$, the values for λ_{max} were 480 nm ($\Delta\epsilon$; $0.20 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$) and 550 nm ($\Delta\epsilon$; $-0.25 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$). Similar results were obtained for the $(\text{His})_{29}\text{Gly}$ complex; λ_{max} ($\Delta\epsilon/\text{mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}/\text{Cu(II)}$): 520 nm (0.60), 630 nm (-0.17) at $[\text{L}]/[\text{Cu(II)}]_0=0.33$, and 480 nm (0.04), 560 nm (-0.03) at $[\text{L}]/[\text{Cu(II)}]_0=1.74$ (data not shown). On the basis of the CD spectrum, it is clear that the $(\text{His})_{19}\text{Gly}$ and $(\text{His})_{29}\text{Gly}$ complexes have the same type of coordination mode. The Cu(II) associated with $\text{Cu(II)}-(\text{His})_9\text{Gly}$ was a little different. The 520/630 nm bands were obscure at low $[\text{L}]/[\text{Cu(II)}]_0$ ratios below 0.6. But, at $[\text{L}]/[\text{Cu(II)}]_0 > 0.6$, the CD spectrum exhib-

Fig. 4. CD Spectra of Cu(II) Coordinated with Ac-(His)₁₉Gly at pH 4.4
[Ac-(His)₁₉Gly]/[Cu(II)]=0.13 (1); 0.25 (2); 0.63 (3); 1.88 (4). Conditions are the same as in Fig. 3.

ited well-separated *d-d* transition bands at 480 nm ($\Delta\epsilon$; $0.25 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$) and 550 nm ($\Delta\epsilon$; $-0.35 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$) (data not shown). (His)_{*i*}Gly (*i*=9, 19, 29) are likely to form complexes with the same coordination mode as long as the peptides are in excess.

(2) Cu(II) Complexes of Ac-(His)_{*i*}Gly: The Cu(II) in the Ac-(His)_{*i*}Gly (*i*=9, 19) complexes always showed a similar type of CD spectrum exhibiting two *d-d* bands; one positive at 520 nm and the other negative at 630 nm. It did not exhibit the 480/550 nm bands even if the peptide existed in excess in the system. The CD spectra of the Ac-(His)₁₉Gly complexes are shown in Fig. 4, where [L]/[Cu(II)]₀ is changed from 0.13 to 1.88. The intensity of the spectrum, reaching a maximum at [L]/[Cu(II)]₀=0.63, decreased without changing the λ_{max} . The spectral parameters for the Ac-(His)_{*i*}Gly complexes were as follows; λ_{max} ($\Delta\epsilon/\text{mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}/\text{Cu(II)}$): 520 nm (0.68) and 630 nm (-0.36) for Ac-(His)₉Gly complex, 520 nm (0.47) and 630 nm (-0.09) for Ac-(His)₁₉Gly complex. The $\Delta\epsilon$ for the Ac-(His)₉Gly complex is bigger than that of the Ac-(His)₁₉Gly, probably because of steric strain around the Cu(II) sites. Those *N*-acetylated peptides lacking the binding-site at the amino terminal could incorporate Cu(II) in the imidazole cluster to a limit. Thus, the 520/630 nm bands are assignable to the Cu(II) accommodated in the imidazole cluster, and the 480/550 nm bands to that at the amino terminus.

Number of Cu(II) Ions Bound to the Peptides The average number of Cu(II) ions bound to the histidine peptides ($\nu = [\text{Cu}_n\text{L}]/[\text{L}]_0$) could be determined by titration of L with Cu(II) using CD spectroscopy, where [Cu_{*n*}L] stands for the concentration of bound Cu(II). The measurements were carried out at 520 nm; [Cu(II)] was variable and [L] was fixed. The titration curves for Ac-(His)₁₉Gly and (His)₁₉Gly are shown in Figs. 5A and 5B. The maximum binding numbers for Ac-(His)₉Gly and Ac-(His)₁₉Gly were calculated as approximately 2 and 3 mol, respectively. The maximum ν for (His)₁₉Gly and for (His)₂₉Gly are difficult to determine. Since these peptides possess two types of Cu(II) ions having different λ_{max} , the titration curve appeared complicated and inter-

Fig. 5. CD Titration of Ac(His)₁₉Gly (A) and (His)₁₉Gly (B) with Cu(II) at pH 4.4

[Ac(His)₁₉Gly]= $2.6 \times 10^{-5} \text{ mol dm}^{-3}$ and [(His)₁₉Gly]= $3.0 \times 10^{-5} \text{ mol dm}^{-3}$ at room temperature.

sected on the abscissa at [Cu(II)]/[L]₀=1. The maximum ν for (His)_{*i*}Gly (*i*=19, 29) could be estimated as 3—4 mol, in which one molar Cu(II) is bound to the nitrogen at the amino terminus.

Mechanism of the Ascorbate Oxidation The autoxidation of ascorbic acid by the poly-L-histidine (degree of polymerization=*ca.* 50) complexes has been shown to obey the “Michaelis-Menten” mechanism and can be expressed briefly by Eqs. 4 and 5.¹⁵⁾

where L, Asc[−] and Asc· represent ligands, ascorbate, and its free radical, respectively. Double reciprocal plots of the rate against the concentration of the substrates give straight lines. When ascorbate was arranged as the variable substrate and dioxygen as the changing fixed substrate, the family of lines in a reciprocal plot was parallel.¹⁵⁾ This fits the “Ping-Pong” mechanism.²²⁾

Oxidation by the (His)_{*i*}Gly and Ac-(His)_{*i*}Gly complexes appeared a little complicated. In the system with low molecular-weight peptides, such as HisGly and probably (His)₄Gly, the family of lines in a reciprocal plot intersect at a point. This fits the “sequential mechanism”.^{7,22)} Spectroscopic studies have revealed that both (His)_{*i*}Gly and Ac-(His)_{*i*}Gly complexes accommodate multi-equivalent Cu(II) ions. The autoxidation by those Cu(II) complexes occurs primarily by the “Ping-Pong” mechanism. Double reciprocal plots for the systems containing (His)₁₉Gly and Ac-(His)₁₉Gly at an appropriate [L]/[Cu(II)]₀ ratio are shown in Figs. 6A and 6B. However, since the peptide is capable of accommodating different

Fig. 6. Double Reciprocal Plot of the v_{obsd} against the Concentration of Ascorbate

Partial pressure of O_2 : ($\square$ — $\square$) 10%, ($\diamond$ — $\diamond$) 20%, ($\circ$ — $\circ$) 40%, ($\triangle$ — $\triangle$) 70%, (∇ — ∇) 100%. (A) $(\text{His})_{19}\text{Gly}$, $[\text{Cu}(\text{II})]=4.0 \times 10^{-6} \text{ mol dm}^{-3}$, $[\text{L}]/[\text{Cu}(\text{II})]=0.68$ and (B) $\text{Ac}-(\text{His})_{19}\text{Gly}$, $[\text{Cu}(\text{II})]=4.0 \times 10^{-6} \text{ mol dm}^{-3}$, $[\text{L}]/[\text{Cu}(\text{II})]=0.48$. Data are the average of at least three experiments.

numbers of $\text{Cu}(\text{II})$ depending on $[\text{L}]/[\text{Cu}(\text{II})]_0$, the double reciprocal plots are not always straight lines.

The autoxidation can be expressed as a composite reactions (4-a), (4-b), ..., and (4-i).

The reaction was analyzed as a linear combination of two from Eqs. 4-a, 4-b, ..., and 4-i. By the nonlinear least-square method, the $v_{\text{obsd}}-[\text{AscH}]$ plots at various $[\text{L}]/[\text{Cu}(\text{II})]$ were resolved into two main curves and individual K_m and V_{max} values were determined. Typical examples for the system with $(\text{His})_{19}\text{Gly}$ and with $\text{Ac}-(\text{His})_{19}\text{Gly}$ are shown in Figs. 7 and 8, respectively. The plot for $(\text{His})_{19}\text{Gly}$ at $[\text{L}]/[\text{Cu}(\text{II})]_0=0.09$ was divided into a straight line and a saturation curve; the former resulted from the reaction by aque-

Fig. 7. Resolution of $v_{\text{obsd}}-[\text{AscH}]$ Plot for the $\text{Cu}(\text{II})-(\text{His})_{19}\text{Gly}$ system at pH 4.4

$[\text{Cu}(\text{II})]=4.0 \times 10^{-6} \text{ mol dm}^{-3}$, (A) $[(\text{His})_{19}\text{Gly}]/[\text{Cu}(\text{II})]=0.09$; (B) $[(\text{His})_{19}\text{Gly}]/[\text{Cu}(\text{II})]=0.35$; (C) $[(\text{His})_{19}\text{Gly}]/[\text{Cu}(\text{II})]=1.18$. Data are the average of at least three experiments.

ous $\text{Cu}(\text{II})$ and the latter resulted from the “Michaelis-Menten” mechanism. The rate- $[\text{Asc}^-]$ plot for aqueous $\text{Cu}(\text{II})$ was shown to be linear.¹⁵⁾ At $[\text{L}]/[\text{Cu}(\text{II})]_0=0.35$, the plot was expressed by a single saturation curve with $K_m=3.3 \times 10^{-5} \text{ mol dm}^{-3}$ and $v_m=3.1 \times 10^{-5} \text{ mol dm}^{-3}/\text{min}$, where both the catalytic activity and the CD intensity reached the maximum. The $(\text{His})_{19}\text{Gly}$ could accommodate multi-equivalents $\text{Cu}(\text{II})$, and the contribution of the inhibitory $\text{Cu}(\text{II})$ is neglected in the kinetic expression. As the $[\text{L}]/[\text{Cu}(\text{II})]$ ratio increased, the plot could be resolved again into two saturation curves with different parameters. The plot with a smaller v_{max} and bigger K_m is ascribable to the $\text{Cu}(\text{II})$ at the amino terminus.

The plots for $\text{Ac}-(\text{His})_{19}\text{Gly}$ were always expressed by a single saturation curve with $K_m=2.5-3.5 \times 10^{-5} \text{ mol dm}^{-3}$ and $v_m=2.6-3.3 \times 10^{-5} \text{ mol dm}^{-3}/\text{min}$, indicating that the reactions obey the “Michaelis-Menten” mechanism.

Conclusion

The catalytic activities of $\text{Cu}(\text{II})$ toward the autoxidation of ascorbic acid were enhanced more than two-fold by the complexation with histidine oligopeptide. Not only the molecular weight of the peptide but also the number of serial histidyl residues affects the enhancement. This is supported by the observation that addition of a large excess of Ac-His-NHCH_3

Fig. 8. Resolution of $v_{\text{obsd}}\text{--}[\text{AscH}]$ Plot for the Cu(II)–Ac-(His)₁₀Gly System at pH 4.4

[Cu(II)] = $4.0 \times 10^{-6} \text{ mol dm}^{-3}$, (A) [Ac-(His)₁₀Gly]/[Cu(II)] = 0.16; (B) [Ac-(His)₁₀Gly]/[Cu(II)] = 0.32; (C) [Ac-(His)₁₀Gly]/[Cu(II)] = 1.26. Data are the average of at least three experiments.

does not accelerate the autoxidation.¹⁷⁾ The Cu(II) coordinated with the amino terminal peptide fragment; *i.e.*, NH₂–X–Y–His– (X and Y denote amino acid residues), certainly inhibits the reaction. The imidazole cluster constructed with the sequence of –(His)_{*n*}– is necessary for the Cu(II) complex to have high catalytic activity. The minimum number of histidyl residues is five or six. These peptides are likely to coordinate with two Cu(II) ions or more which exhibit two CD bands at 520 and 630 nm and are readily dissociable from the complexes by the addition of NaClO₄ or CH₃COONa.¹⁵⁾

The involvement of free metal ions in *in vivo* oxidation has been questioned because most of these ions are linked to prosthetic groups or are tightly sequestered by specific binding proteins. Experiments with autoxidation by the labile Cu(II) bound to Ac-(His)₁₀Gly suggest the presence of a labile and catalytically active metal in the metalloprotein. Labile Cu(II) bound to an imidazole group, which is Chelex-removable, has been found in ceruloplasmin, and has catalytic activity inducing oxidation of low density lipoprotein.²³⁾

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References and Notes

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