^{1}H NMR Red–Ox Titration of Copper(II)–Histidine and –Oligopeptide Complexes with L(+)-Ascorbic Acid

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Abstract

Cu(II) complexes with histidine and analogues and their interaction with the vitamin C red–ox system were studied by means of ^{1}H NMR spectroscopy. It could be shown that the paramagnetic line broadening as well as the red–ox activity observed by NMR at pH 7 depend on the pK_R value of the imidazole residue. A reaction scheme is proposed which describes the NMR red–ox titration of the histidine–copper complexes. The exceptional role of Gly–Gly–His as a model for the protein binding site of copper ions and as an anti-tumor agent in correlation with ascorbic acid is interpreted in terms of its binding capability and red–ox activity as seen by the NMR method.

Introduction

Copper is known as an essential trace element in living tissue; *e.g.* it is responsible for the activity of some enzymes participating in electron and dioxygen transport and metabolism [1, 2]. The biological efficacy of copper is based, presumably, on its red–ox properties and interconnected for that reason with the red–ox system of vitamin C [3]. Thus, it has been found that ascorbate is cytotoxic against tumor cells in the presence of cupric ions [4], a fact which could be explained by the production of ȮH radicals due to a Fenton-type reaction between cuprous complexes and H_2O_2. Ascorbate may reduce the oxidized metal, hence recycling it for further catalytical action [5].

Because this reduction of ligated copper is univalent, ascorbate is oxidized to its radical form [6]. Indeed, an increase in concentration of this radical could be observed in human blood samples in the presence of certain copper proteins or low molecular weight complexes of copper with histidine or histidine-containing oligopeptides [7].

To study the red–ox properties of these complexes and their interference with ascorbate the technique of red–ox titration by means of proton magnetic resonance (^{1}H NMR) spectroscopy has been applied.

Materials and Methods

L-Histidine (His), L-1-methyl-histidine (m^{1}His), and L-3-methyl-histidine (m^{3}His) were purchased from Sigma, Munich, glycyl–histidine (Gly–His) and glycyl–glycyl–histidine (Gly–Gly–His) from Serva, Heidelberg, $CuSO_4 \cdot 5(H_2O)$ and sodium-L(+)-ascorbate (ASC) from Merck, Darmstadt. D_2O was obtained from Sharp and Dohme, Munich.

The ^{1}H NMR spectra were measured at 30 °C with a Varian HA-100 CW spectrometer operating at 100 MHz and interfaced to a Telefunken TR 86 computer. An addition of 5 vol% tert. butanol to the samples served for the field-frequency lock. The line widths at half height (Δ_{obs}) as calculated by a Lorentzian fit of the resonance signals contain an error of less than 10%.

The pH values (pH*: pH-meter reading without correction for the isotopic effect) were adjusted by the addition of small amounts of concentrated NaOD or DCl to the D_2O solutions and measured directly within the 5 mm sample tube by a combination electrode made by Ingold, Frankfurt. Pure argon was bubbled through the samples for at least 5 min to eliminate dissolved oxygen.

Results

It is well known that Cu(II) ions cause a paramagnetic line broadening in the NMR spectra of the ligands in copper complexes. This broadening is proportional to the concentration ratio between metal ion and ligands and depends on the strength and geometry of the complexes. It also depends on the type of relaxation mechanism and contributions arising from chemical exchange [8].

It is useful to characterize the effect by a line broadening factor LBF defined as

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TABLE I. Imidazole pK Values of the Copper Ligands Studied as Estimated from the pH dependence of the H-4 Resonance (pK_R^*) and NMR Sensitivity $s = k_a \cdot \Delta_2/\Delta_1$ of the Line Broadening Effect on H-4 Due to the Binding of Cu(II) Ions at pH* 7. The parameters k_b/k_c and m were obtained by fitting the experimental data (Fig. 3) according to eqn. 12. The potential difference ΔE (defined by eqn. 6) is calculated from the mean value of m according to eqn. 10 with $K_d = 1.5 \times 10^{-9}$ (pH 7; 25 °C).

Ligand	pK_R^*	s [10^3] (pH* 7)	k_b/k_c	m [10^{-3}]	ΔE [V]
m^3His	5.7	0.4 ± 0.1	>1000	–	–
His	6.0	0.8 ± 0.1	>1000	–	–
m^1His	6.4	22 ± 1	56.2 ± 3.6	12 ± 10	0.21
Gly–His	6.8	17 ± 1	672 ± 58	3 ± 7	0.19
Gly–Gly–His	6.9	3.3 ± 0.2	4.6 ± 0.3	54 ± 20	0.23

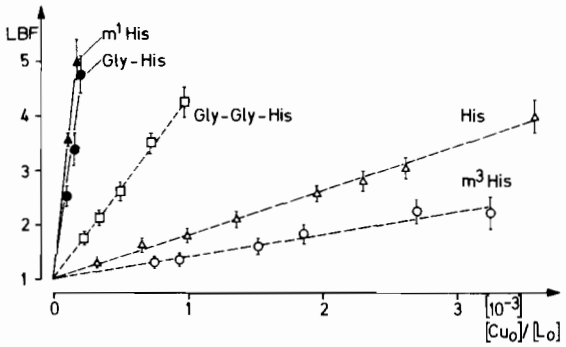


Fig. 1. The dependence of the line broadening factor LBF (measured at the imidazole H-4 of the ligands) on the ratio of copper and ligand concentrations at pH* 7. The copper concentrations used were 0.1 mM (m^3His, His, and Gly–Gly–His) and 0.01 mM (m^1His and Gly–His).

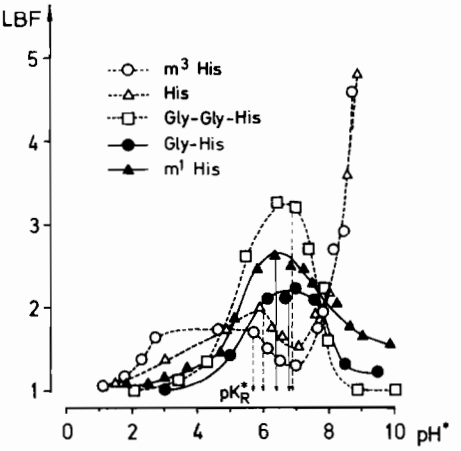


Fig. 2. pH dependence of the line broadening factor LBF. The Cu(II) concentrations were the same as in Fig. 1. The ligand concentration was 150 mM. The pK_R^* values of the imidazole residue of the ligands are indicated by arrows.

$$\text{LBF} = \frac{\text{line width in the presence of Cu(II)}}{\text{line width without Cu(II)}} \qquad (1)$$

The H-4 proton of the histidine imidazole ring is common to all ligands used in this study. Therefore, the ^{1}H NMR signal of this proton has been used to compare the paramagnetic line broadening of the different complexes.

As is shown in Fig. 1, the line broadening increases with increasing Cu(II) concentration. There are, however, large differences in the response of the line broadening of the different complexes to the amount of the paramagnetic agent added. The concentration required to produce a certain effect increases in the following order: m^1His < Gly–His < Gly–Gly–His < His < m^3His.

The copper interaction competes with the protonation of the amino acids. Therefore, the broadening effect should depend on the pH. This is especially true around the pK_R value of the imidazole ring. This pK_R value which is listed in Table I for all ligands used was obtained from the pH-dependence of the chemical shift of the H-4 NMR signal.

The pH dependence of the line broadening effect on H-4 is shown in Fig. 2. It can be seen that LBF reaches a maximum near the pK_R values. In the case of His and m^3His the line broadening decreases towards higher pH values and increases again above pH 7. The pH range of maximum LBF is broader in the case of m^3His compared with the other ligands. At pH 7, the absolute LBF values exhibit a minimum for His and m^3His, whereas they are still close to their relative maximum for the other ligands.

The paramagnetic line broadening observed in the NMR spectra of the copper ligands may be used to follow the reduction of paramagnetic Cu(II) to diamagnetic Cu(I). Thus, the NMR technique appears to be well suited for monitoring red–ox transitions.

As shown in Fig. 3, the observed relative line width of H-4 (normalized to a certain concentration of Cu(II)) decreases with the addition of ascorbate as a reductant. The sigmoid titration curve obtained depends on the red–ox potentials and other molec-

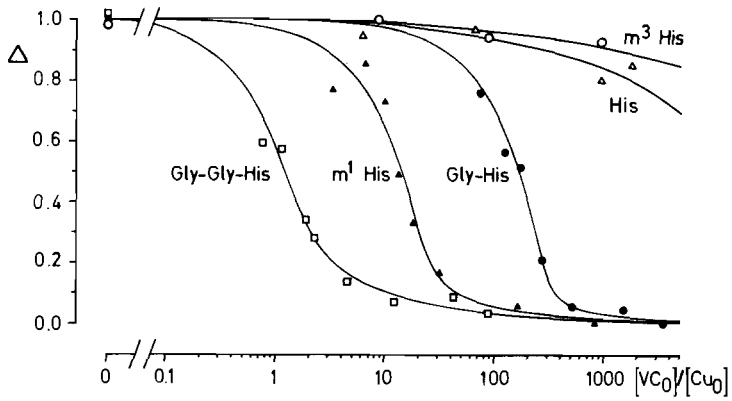


Fig. 3. Observed relative line width Δ (for definition see eqn. 8) of the imidazole H-4 NMR signal as a function of the total vitamin C concentration at pH* 7. The absolute concentrations were: 150 mM of the ligands and 0.13 mM (Gly–Gly–His), 0.015 mM (m^1His), 0.02 mM (Gly–His), and 0.1 mM (His, m^3His) of copper. Eqn. 12 has been used to fit the experimental data.

ular properties of the reductant and the ligands used. This has been proved *e.g.* with histidine. A 50% reduction of the relative line width can be obtained in this case with reduced glutathione (GSH) at a concentration ratio of $[GSH]/[Cu_o] \approx 7$ or with dithiothreitol at a corresponding ratio of about 0.5. In contrast, nicotine adenine dinucleotide (NADH) seems to be as ineffective as shown for ASC in Fig. 3.

It is also seen in this figure that the concentration of ASC required for a 50% reduction of the relative line width increases by a factor of about 10 from Gly–Gly–His to m^1His and Gly–His, whereas the complex with m^3His is virtually uninfluenced even at high concentrations of ASC.

Theory

Paramagnetic line broadening requires an exchange of ligands between the free and complexed state, which must be fast on the NMR time scale. If the exchange time is too long, the respective complexes do not contribute to the line broadening of the ligands. As has been pointed out *e.g.* for Gly–Gly–His [9], there might be situations where the major complex species in solution is in slow exchange with the ligand, so that the effects observed in the NMR spectra will be mainly dependent on the properties of a minor complex species, which is less tightly bound and for which, thus, the condition of fast exchange is met.

The concentration ratio of this 'NMR active' part (denoted by Cu(II)*L) to the total Cu(II) concentration is represented by

$$k_a = \frac{[Cu(II)^*L]}{[Cu(II)_o]} \tag{2}$$

The line width observed in the NMR spectrum of the ligand L is given by

$$\Delta_{obs} = \frac{[L]}{[L_o]}\Delta_1 + \frac{[Cu(II)^*L]}{[L_o]}\Delta_2 \tag{3}$$

Δ_1 expresses the line width in the absence of Cu(II) and Δ_2 stands for the asymptotic (theoretical) line width of the complex. The line broadening factor of eqn. 1 is, then, given by

$$LBF = \frac{\Delta_{obs}}{\Delta_1} = \frac{[L]}{[L_o]} + \frac{[Cu(II)^*L]}{[L_o]}\frac{\Delta_2}{\Delta_1} \tag{4}$$

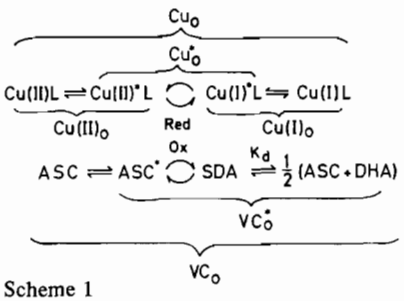
With eqn. 2 and $[L] \approx [L_o]$ (*i.e.* $[L_o] \gg [Cu(II)_o]$) one obtains

$$LBF = 1 + \frac{k_a\Delta_2}{\Delta_1}\frac{[Cu(II)_o]}{[L_o]} \tag{5}$$

whereby the ratio $s = k_a \cdot \Delta_2/\Delta_1$ may be interpreted as a NMR sensitivity factor which describes the linear relationships shown in Fig. 1. It also reflects the geometry and strength of binding of the complexes investigated, which, however, will not be considered in detail in this study.

For the red–ox reaction, in a similar way to above, it has to be assumed that only part of the complexes coexisting in solution are capable of exchanging electrons with the reductant. This phenomenon is well known *e.g.* for ligated porphyrins, where only the free porphyrin can take part in the red–ox reaction [10]. Here, similar principles seem to apply, and the red–ox equilibrium of the NMR active Cu(II)*L/Cu(I)*L system may be formulated according to Scheme 1.

It is shown in the scheme that ASC might only in part (indicated by ASC*) be capable of reducing the ligated Cu(II) due to a concomitant interaction with the ligands. It is also considered that the ascorbyl radicals (SDA) formed by the univalent oxidation



Scheme 1

of ASC* disproportionate to ASC and dehydroascorbic acid (DHA) as described by the constant K_d. The concentration of free copper ions is neglected due to their strong binding to amino acids.

The potential difference ΔE between the red–ox potentials at pH 7 of the corresponding red–ox couples Cu(II)*L/Cu(I)*L and ASC*/SDA is given by the Nernst equation applied to both systems, which at T = 25 °C results in

$$\Delta E = E_o'(\text{ASC*/SDA}) - E_o'(\text{Cu(II)*L/Cu(I)*L}) = 0.06 \log \frac{[\text{Cu(II)*L}]\,[\text{ASC*}]}{[\text{Cu(I)*L}]\,[\text{SDA}]} \quad (6)$$

The concentrations of the species involved are given by

$$[\text{Cu}_o^*] = [\text{Cu(II)*L}] + [\text{Cu(I)*L}]$$

$$[\text{VC}_o^*] = [\text{ASC*}] + [\text{DHA}] + [\text{SDA}] \approx \approx [\text{ASC*}] + [\text{DHA}] \quad (7)$$

$$[\text{SDA}] = (K_d[\text{ASC*}]\,[\text{DHA}])^{1/2}$$

$$[\text{DHA}] = [\text{Cu(I)*L}]/2$$

If the line width of the ligand observed in the absence of Cu(II) and ASC is denoted by Δ_1, the line width obtained after the addition of a given amount of Cu(II) by Δ_{obs} (defined by eqn. 3), and the line width obtained in the presence of the same amount of Cu(II) and a certain amount of ASC by Δ_{obs}^{ASC}, then the ratio of the differences is proportional to $[\text{Cu(II)*L}]/[\text{Cu}_o^*]$ according to

$$\Delta = \frac{\Delta_{obs}^{ASC} - \Delta_1}{\Delta_{obs} - \Delta_1} = \frac{[\text{Cu(II)*L}]}{[\text{Cu}_o^*]} \quad (8)$$

With eqns. 6, 7 and 8 one obtains

$$\frac{[\text{VC}_o^*]}{[\text{Cu}_o^*]} = 0.5 \left[(1-\Delta) + m\frac{(1-\Delta)^3}{\Delta^2}\right] \quad (9)$$

with

$$m = K_d \cdot 10^{\Delta E/0.03} \quad (10)$$

Using

$$k_b = \frac{[\text{Cu}_o^*]}{[\text{Cu}_o]} \text{ and } k_c = \frac{[\text{VC}_o^*]}{[\text{VC}_o]} \quad (11)$$

for correlating the red–ox active to the total concentrations of copper and vitamin C eqn. 9 can be written as

$$\frac{[\text{VC}_o]}{[\text{Cu}_o]} = 0.5\frac{k_b}{k_c}\left[(1-\Delta) + m\frac{(1-\Delta)^3}{\Delta^2}\right] \quad (12)$$

The fit of the experimental data according to this formula (Fig. 3) results in values of the parameters m and k_b/k_c which are tabulated in Table I.

Discussion

The red–ox titration curve obtained for the reduction of Cu(II) complexes by ASC should depend primarily on the difference of the red–ox potentials involved. Equilibria other than the red–ox reaction (occurring prior or posterior to the reduction) as well as a characteristic NMR sensitivity have also to be considered if NMR spectroscopy is used to monitor the red–ox reaction.

The NMR line width of a ligand signal depends on the concentration ratio of Cu(II) to the ligand, on the complex geometry and strength of binding (expressed by the complex line width Δ_2), and on the fraction k_a of 'NMR visible' complexes as well. As shown by the linear correlations in Fig. 1, an NMR sensitivity factor can be defined for each complex. This factor, which is independent of the actual concentrations used, combines the ratio between the line width of the complex (Δ_2) and of the free state (Δ_1) as well as the fraction k_a.

The NMR sensitivity depends on the pH. It exhibits a maximum around the pK_R value of the imidazole residue and decreases at higher pH values (Fig. 2). Thus, the fast exchange, necessary for the complexes to be observed by NMR around pH 7, seems to depend mainly on this pK_R value.

The reducibility of Cu(II) complexes requires an access of the reductant to the copper ions, *i.e.* an exchange of a copper binding site similar to that postulated for the paramagnetic effect on NMR signals. Therefore, a concentration fraction k_b similar to that included in the NMR sensitivity (k_a) is tentatively used to calculate the concentration of the red–ox active copper complexes. Furthermore, the inactivation of ASC by complexation is considered by the constant $1/k_c$. The parameters k_b/k_c and m can be obtained by a fit of the experimental

data according to the reaction scheme given above.

The parameter m includes the SDA disproportionation constant K_d and the differences ΔE between the potential of the ASC*/SDA system and that of the copper complex. Using +0.32 V for the ASC*/SDA potential and $K_d = 1.5 \times 10^{-9}$ [11] the values of ΔE given in Table I are obtained. According to these values the potential of free Cu(II)/Cu(I) (E_o = +0.16 V) seems to be diminished by about 70 mV (Gly–Gly–His) to 30 mV (Gly–His) due to complexation. This seems to be reasonable in comparison with values known for similar complexes [1].

The concentration ratio k_c might be correlated to the association constant K_c of the interaction between vitamin C (likewise in its reduced or oxidized form) and the ligand L by $(1 - k_c)/k_c = K_c \cdot [L]$. With [L] = 0.15 M and assuming $k_b = 1$, the values of k_b/k_c from Table I correspond to K_c = 24 M^{-1} (Gly–Gly–His), 370 M^{-1} (m^1His), 4500 M^{-1} (Gly–His), and over 7000 M^{-1} (His and m^3His). If it is taken into account that k_b might be less than 1, the values of K_c should be even higher.

The increase of k_b/k_c in the sequence

$$\text{Gly–Gly–His} < m^1\text{His} < \text{Gly–His} < \text{His} \leqslant m^3\text{His}$$

corresponds to the decrease of pK_R with the exception of Gly–His, for which the ratio k_b/k_c seems to be too high. Because the NMR sensitivity is similar to that of m^1His, it might be concluded that the values of k_b do not differ too much either. Hence, the binding of Gly–His to ASC seems to be stronger than expected, which might be caused by the influence of an additional binding site.

The NMR sensitivity increases with increasing pK_R within the series m^3His, His, and m^1His. The sensitivity of Gly–Gly–His is less than expected. This may be caused by a relatively low exchange rate, *i.e.* tight binding of Cu(II) or a competition of binding sites other than the imidazole ring of histidine. This would also be an explanation for the sensitivity of Gly–His, which is less than that found with m^1His. Moreover, the decrease of the sensitivity from Gly–His to Gly–Gly–His might be seen as a consequence of a change in the stoichiometry of the complexes, because Gly–Gly–His prefers 1:1 instead of 2:1 complexes with Cu(II), in contrast to the other ligands [12, 13].

It might be concluded from the results obtained under the conditions of NMR and red–ox activities mentioned above, that the Gly–Gly–His ligand binds on one side a Cu(II) ion strong enough to avoid its direct (presumably toxic) attack on biomolecules. On the other side, this ligand binds ASC less than other ligands, and the Gly–Gly–His–Cu(II)-complex is of an adequate flexibility and exchange rate at least at one site (presumably at the histidine residue) to allow a relatively free access of ASC to Cu(II) for reduction.

Gly–Gly–His simulates the copper binding site of serum albumine [13] and may also represent a good model for the active site of copper enzymes [14]. This ligand might be exceptional not only in the transport and storage of copper ions but also due to its facilitated oxidation of ASC to SDA. This radical is highly effective in the radical biochemistry of living cells especially in its nascent form and is known to be directly or indirectly indicative for the biological activity of radicals *e.g.* during the protection of biomembranes against oxidative stress or in the patho-biochemistry of cancer [3]. The easy reducibility of Cu(II) in its complex with Gly–Gly–His as seen by the NMR red–ox titration is, therefore, in good agreement with the observation of an expressed anti-tumor activity of this complex in correlation with ASC [4].

Acknowledgements

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