# Spectroscopic Study of Ferric–Histidine Chelates and Their Catalytic Activity on the Peroxidation of Methyllinoleate in Methanol

Md. RAFIQUZZAMAN, Kyoko YOSHINAGA, Keiko KOMAGOE, and Keietsu TAMAGAKE*

*Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan.*
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**The rate of peroxidation of methyllinoleate in methanol was measured by monitoring oxygen consumption in the presence and absence of ferric chloride and L-histidine. Histidine enhanced the ferric-inducing peroxidation of methyllinoleate in a non-aqueous system, which was likewise reported in an aqueous solvent. The rate depended on the ratio of the relative concentration of iron to histidine. The ratio of 1:6 in a molar concentration gave the maximum rate. Spectroscopic observation for the first time strongly suggested the formation of two ferric–histidine chelates of the composition of 1:1 and 1:2 in methanol. The apparent formation constants of the 1:1 and 1:2 complexes, log $\beta_1$ and log $\beta_2$, were calculated to be 4.6 and 8.3, respectively. Correlation of the peroxidation rates to the spectroscopically observed species led to the conclusion that the 1:2 chelate was responsible for the maximum peroxidation rate of methyllinoleate under the conditions of this investigation.**

**Keywords** lipid peroxidation; ferric–histidine complex; UV absorption spectrum; methyllinoleate

Lipid peroxidation is known to produce food spoilage, membrane damage and the degradation of subcellular particles.[1-4] It is also associated with many pathological events and disease states.[5-7] Transition metals, especially iron and its chelates, catalyze lipid peroxidation.[8-14] The involvement of iron–histidine in lipid peroxidation has received much attention. Ferrous ion in the presence of histidine promotes lipid peroxidation.[14-16] Erickson *et al.* demonstrated that histidine can either enhance or inhibit ferrous or ferric and ADP-catalyzed lipid peroxidation to a significant degree.[17-18] They believed a mixed ligand complex (iron, ADP and histidine) was formed in the system.

Hearon *et al.*[19] observed a 1:2 ferrous to histidine complex potentiometrically, but failed to demonstrate the formation of a ferric–histidine complex. Isak *et al.*,[20] by the same method, found 1:1 and 1:2 ferrous histidine complexes in weakly acidic media. Moreover, they observed that the maximum rate of oxidation of indigocarmine was due to $Fe^{2+}(His)_2$ in the presence of $H_2O_2$. Yakubov *et al.*[21] mentioned the existence of two 1:1, two 1:2 and one 2:2 ferric to D,L-histidine complexes in acidic solutions on the basis of their electrochemical studies. To our knowledge, the composition and structural aspects of a ferric–histidine complex using absorption spectroscopy have not previously appeared in literature. The relative peroxidation activity of different ferric–histidine complexes still remain unclear. Therefore, simultaneous investigation of the peroxidation and the catalytic ferric–histidine chelate identification was necessary. However, weak binding between histidine and ferric ion might be the reason there has been no previous spectroscopic report on any ferric–histidine chelate. In methanol, we were able to follow the ferric–histidine species spectroscopically. Such spectroscopic observations are important for investigating catalytic species and correlating their oxidative activity. Micelle[10,13] and liposome[12,22,23] peroxidation studies have an advantage as they resemble a biological system, but those systems may be too complex for studying the basic mechanism of peroxidation compared to methanol, which provides a homogeneous and simple environment.

In the present study, we have aimed at correlating the peroxidation activity with the catalytic species of ferric–histidine in methanol solution.

## Materials and Methods

**Materials** Methyllinoleate was from Tokyo Chemical Industry, Co., Ltd. L-Histidine and ferric chloride were from Wako Pure Chemical Industries, Ltd. Methanol was from Ishizu Seiyaku, Ltd. Deionized and distilled water was prepared by this faculty.

**Lipid Peroxidation Assay** Peroxidation of methyllinoleate was measured by monitoring oxygen consumption with a UC-P(G) type oxygen electrode of ABLE Co., Ltd. Stock solutions of methyllinoleate (1 M), histidine (1 mM) and ferric or ferrous chloride (10 mM) were prepared in methanol. The required volume of each was added to the reaction vessel to give the final concentration as written in the legend of the figures. The reaction was started by injecting ferric chloride with a microsyringe into the mixture of mythyllinoleate and histidine. The total volume of the reaction mixture was 3.5 ml. Each time, fresh solutions were prepared and used for the experiment.

**Spectral Studies** All spectra were taken with a Hewlett Packard Model 8450A diode array spectrophotometer over a wavelength range of 200—800 nm. Obtained data were analyzed using an NEC PC 9801RA Computer.

## Results and Discussion

Figure 1 shows the oxygen consumption by methyllinoleate in different systems. The addition of 0.05 mM ferric chloride to 0.4 M methyllinoleate in methanol increased oxygen consumption at the rate of 3% $min^{-1}$ (curve B). A large increase in the rate was observed when ferric chloride was added to a solution which already contained 0.3 mM of histidine together with methyllinoleate (curve C). The enhancement reached more than ten times, giving a rate of 34% $min^{-1}$. The addition of histidine alone to the linoleate solution showed no enhancement at all (curve A). These observations strongly indicate the formation of a ferric–histidine complex, which is more active than ferric chloride in the catalytic peroxidation of methyllinoleate in methanol. To our surprise, however, the addition of a higher quantity of ferric chloride to a solution with the same histidine and linoleate content lowered the

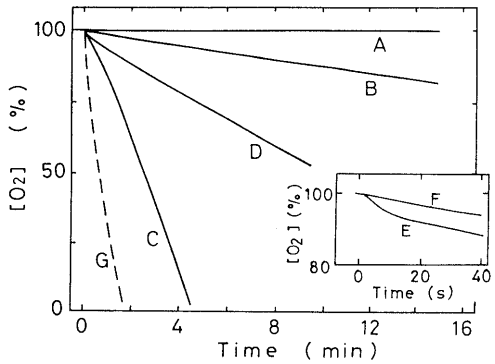


Fig. 1. The Effect of Ferric Chloride and Histidine on Oxygen Consumption by Methyllinoleate in Methanol

Oxygen consumption was measured with an oxygen electrode under the various concentration conditions of ferric chloride and histidine as follows. (A), 0.3 mM histidine and 0 mM $FeCl_3$; (B), 0 mM histidine and 0.05 mM $FeCl_3$; (C), 0.3 mM histidine and 0.05 mM $FeCl_3$; (D), 0.3 mM histidine and 0.3 mM $FeCl_3$. The reaction was started by the injection of ferric chloride, except in (A), where instead of ferric chloride an equivalent volume of solvent was injected. In each case, the concentration of methyllinoleate was 0.4 M and the total volume of the reaction mixture was 3.5 ml. The abscissa indicates the time after the injection of ferric chloride solution. Inset figure shows the effect of ferrous chloride compared to ferric chloride. The experiment was done with 0.3 mM histidine, 0.2 M methyllinoleate and (E), 0.1 mM $FeCl_2$; (F), 0.1 mM $FeCl_3$.

(G), 0.2 M partially oxidized methyllinoleate (purchased methyllinoleate was kept at room temperature to accumulate more LOOH by autoxidation), 0.3 mM histidine and 0.05 mM $FeCl_3$.

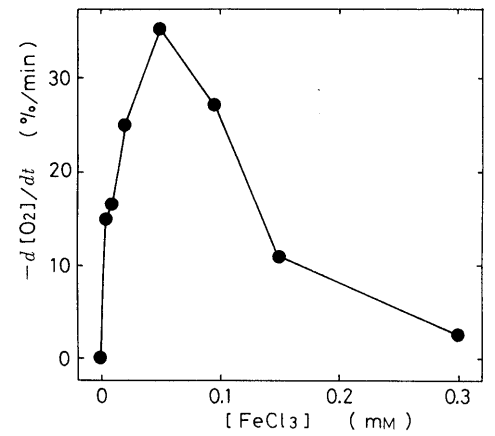


Fig. 2. Peroxidation Rate of Methyllinoleate as a Function of Concentration of Ferric Chloride in the Presence of Histidine in Methanol

The peroxidation rates were calculated from the initial decay of oxygen consumption curves measured with 0.4 M methyllinoleate, 0.3 mM histidine and various concentrations of ferric chloride. For further details of the assay see the legend in Fig. 1.

peroxidation rate significantly (curve D). The inset figure and curve G are discussed elsewhere in this paper.

Figure 2 shows the change in peroxidation rate as a function of ferric chloride concentration in the linoleate solution where the histidine concentration was kept at 0.3 mM. The maximum oxygen consumption rate, 34% $min^{-1}$, was observed at $[FeCl_3]=0.05$ mM, which indicates a 1 : 6 ferric to histidine molar ratio. This rate decreased to 3% $min^{-1}$ at $[FeCl_3]=0.3$ mM, or a 1 : 1 ferric to histidine molar ratio. The change in peroxidation rate as a function of histidine concentration (Fig. 3), under a constant ferric chloride concentration (0.02 mM), was also examined. As shown in Fig. 3, the rising peroxidation rate curve is more or less quadratic, rather than linear, below 0.05 mM of histidine, with an initial lag phase. Both Figs.

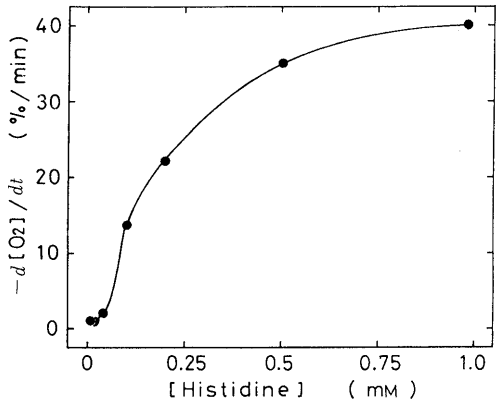


Fig. 3. Peroxidation Rate of Methyllinoleate as a Function of Concentration of Histidine in the Presence of Ferric Chloride in Methanol

The peroxidation rates were calculated from the initial decay of oxygen consumption curves measured with 0.4 M methyllinoleate, 0.02 mM ferric chloride and various concentrations of histidine. For further details of the assay, see the legend in Fig. 1.

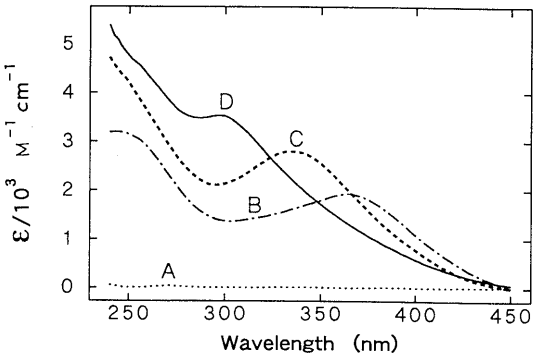


Fig. 4. Absorption Spectra of Histidine, $FeCl_3$ and Their Mixture Solution

(A), 0.1 mM histidine; (B), 0.05 mM $FeCl_3$; (C), mixture of 0.15 mM $FeCl_3$ and 0.15 mM histidine; (D), as (C) but with 0.05 mM $FeCl_3$ and 0.3 mM histidine.

2 and 3 suggest that there exists more than one species of chelates, and that the more highly coordinated iron is more active in the peroxidation.

The UV absorption spectra of the solutions of ferric chloride and histidine mixture were also changed greatly, depending on the molar ratio of ferric chloride to histidine, as shown in Fig. 4. The absorption band at 367 nm (curve B) of ferric chloride shifted to 330 nm (curve C) in the mixed solution of 0.15 mM $FeCl_3$ and 0.15 mM histidine, indicating the formation of a ferric–histidine complex in methanol since histidine alone has no absorption above 210 nm (curve A). All spectra observed in the solution, where $[\text{histidine}]/[FeCl_3]<1$, were virtually reproduced by the computational superposition of the two spectra, curve B and curve C, provided that $[FeCl_3]>0.05$ mM. An increase in histidine concentration to $[\text{histidine}]/[FeCl_3]=6$, where the maximum peroxidation rate was observed, caused a further shift of the peak to 300 nm (curve D). All other spectra observed in the solution, where $[\text{histidine}]/[FeCl_3]>2$, were well explained by the combination of the two spectra, curve C and curve D.

As described above, the formation of two types of ferric–histidine chelates in methanol were confirmed spectroscopically. The 330 nm species (curve C) should be

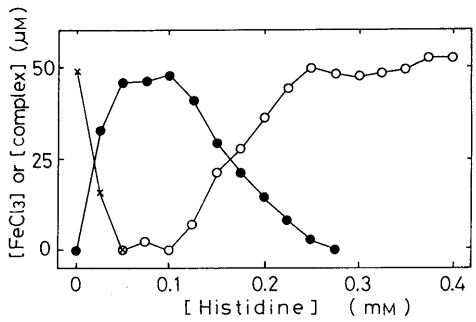


Fig. 5. Component Analysis of Ferric–Histidine Complexes as a Function of Concentration of Histidine in Methanol

The analysis was done by the spectroscopic curve fitting of the spectra observed with 50 $\mu$M ferric chloride and 0—0.4 mM histidine. Spectra B, C and D in Fig. 4 were assumed to be those of the pure $FeCl_3$, $Fe^{3+}$His and $Fe^{3+}(His)_2$, respectively. ×, $FeCl_3$; ○, 1:2 ferric–histidine complex; ●, 1:1 ferric–histidine complex.

a 1:1 ferric–histidine complex since it was the first appearance in a low histidine concentration. The assignment of the 300 nm species (curve D) to a 1:2 ferric–histidine complex would be also reasonable, since it appeared at a higher [histidine]/[$FeCl_3$] molar ratio (6:1). Another similarly high [histidine]/[$FeCl_2$] molar ratio (4:1) has also been reported for the 1:2 ferrous–histidine complex.[19]

Figure 5 shows the result of the component analysis of the spectra observed in the solutions with various [histidine]/[$FeCl_3$] by computer simulation, assuming that spectra B, C and D in Fig. 4 are those of the pure components, $FeCl_3$, $Fe^{3+}$His and $Fe^{3+}(His)_2$, respectively. The profiles obtained are apparently what we could expect theoretically for a 1:1 complex and 1:2 complex. That, in turn, would support our assignments of the two different ferric–histidine components. The apparent formation constants of the 1:1 and 1:2 complexes, $\log\beta_1$ and $\log\beta_2$, were calculated to be 4.6 and 8.3, respectively, based on the analysis in Fig. 5. Spectroscopic study of the iron–histidine chelates in an aqueous solution has not yet appeared in literature. So far, only potentiometric[19,20] and electrochemical[21] studies have been used to detect iron–histidine complexes in an aqueous system. The species reported are $Fe^{2+}$His, $Fe^{2+}(His)_2$ and the ferric-D,L-histidines such as $Fe^{3+}$His, $Fe^{3+}(His)_2$ and $Fe_2{}^{3+}(His)_2$.

The maximum peroxidation rate (Fig. 2) took place under the same condition as that of the appearance of the spectrum of the 1:2 complex. Therefore, we concluded that the 1:2 complex was responsible for the maximum peroxidation of methyllinoleate. Now we can explain Fig. 2 as follows. An increasing peroxidation rate up to the rate maximum was due to the increase in concentration of a 1:2 ferric–histidine complex. At the molar ratio of 1:6 ([$Fe^{3+}$] = 0.05 mM, [His] = 0.3 mM) the complete formation of a 1:2 complex was achieved, and hence the peroxidation rate was maximum. A further increase in $FeCl_3$, after the rate maximum, caused the conversion of the 1:2 complex to 1:1. So the rate was decreasing. The lag phase in the rate of oxygen consumption as observed in Fig. 3 can be explained due to the lower activity of the 1:1 compared to the 1:2 ferric–histidine complex.

Unfortunately, structural information on the assigned complexes is not available experimentally. However, we would like to propose a hexacoordinated form for $Fe^{3+}(His)_2$ based on both the modeling work and the consideration of other metal histidine chelates.[24] Even $Cu^{2+}$, which is usually known to form a square planar complex, has been reported to form a 1:2 octahedral complex where both histidine molecules act as a terdentate ligand.[25]

In previous studies of the catalytic peroxidation of lipids with an iron–histidine complex, ferrous species[14-18] received more attention than ferric species.[17-18] In our methanol system, ferrous–histidine showed a much faster oxygen consumption rate (inset of Fig. 1, curve E), however it was only temporary. Within 10 seconds, after the addition of ferrous chloride, the slope of the decaying process (curve E) became exactly the same as that of ferric–histidine (curve F) system. This could be explained by the fast equilibrium between the ferric and ferrous state through Fenton type reactions (Eq. 1 and 2) in the peroxidation processes,

$$Fe^{2+}(His)_2 + LOOH \xrightarrow{k_2} Fe^{3+}(His)_2 + OH^- + LO\cdot \quad (1)$$

$$Fe^{3+}(His)_2 + LOOH \xrightarrow{k_3} Fe^{2+}(His)_2 + H^+ + LOO\cdot \quad (2)$$

Here, LOOH is a linoleate hydroperoxide, a product of the autoxidation of methyllinoleate. Hence, [$Fe^{3+}(His)_2$]/[$Fe^{2+}(His)_2$] = $k_2/k_3$ holds at the stationary state. If $k_2 \gg k_3$, as in the case of the Fenton reaction,[26] where $k_2/k_3 = 10^3$—$10^4$, virtually all species will rapidly become ferric-histidine, even though one starts as ferrous–histidine. Thus, the activity of $Fe^{3+}(His)_2$ determines the overall rate of peroxidation since Eq. 2 is the rate determining step. Partially oxidized methyllinoleate, which contained more LOOH, showed a faster oxygen consumption rate, as shown by curve G in Fig. 1. This would support the above mechanism. Ferric and its histidine chelates act in methanol similarly to their activity in aqueous system toward the peroxidation of a lipid, such as methyllinoleate. The methanol system provided not only a homogeneous and simple environment for peroxidation study, but it also facilitated the spectroscopic observation of ferric and histidine complexes which are not observable in water.

In the present study, ferric–histidine complexes with compositions of 1:1 and 1:2 were found to form. The 1:2 complex was more active than the 1:1, moreover, the 1:2 complex was found to be responsible for the maximum peroxidation rate. But why the 1:2 ferric–histidine complex produced the maximum peroxidation activity remains unclear. To find the answer, it is important to study both the structural and electrochemical properties[27] of ferric-histidine complexes. Such studies will lead to a better understanding of the role of ferric–histidine chelates in peroxidation processes *in vitro* and *in vivo*.

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27) Isak *et al.*[20] reported the value for the 1:2 complex as 0.256 V, but unfortunately no value appeared for 1:1 complex in the same literature. We also tried to measure the oxidation potentials for those complexes in methanol solution, but it was not successful because the background current was too high.