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Fenton-like reaction of the iron(II)-histidine complex generates hydroxyl radicals: implications for oxidative stress and Alzheimer's disease

Can Wang,[a] Zheng Wang,[a] Binglin Zeng,[a] Meiqing Zheng,[b] Nao Xiao[a] and Zhongwei Zhao*[a,c]

[a] School of Pharmaceutical Sciences, Capital Medical University, Beijing 100069, China;

[b] Core Facility Center, Capital Medical University, Beijing 100069, China

[c] College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China

* Correspondence author. Email: zzhao@ccmu.edu.cn (Z. Zhao)

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Materials and Methods

Materials

The ferrous ammonium sulfate, ferrous sulfate, sodium phosphate dibasic, L-Histidine, 3′-(*p*-aminophenyl) fluorescein (APF), 5,5-Dimethyl-1-pyrroline N-oxide (DMPO), dimethyl sulfoxide (DMSO) and 3-(N-morpholino) propane sulfonic acid (MOPS) were all analytical reagent grade (AR grade) and purchased from Sigma-Aldrich. The H_2O_2 (30%, AR grade) was purchased from Beijing Modern Oriental Fine Chemistry Co. Ltd. and its concentration was determined spectrophotometrically by using the extinction coefficient at 240 nm ($\varepsilon_{240\,nm} = 43.6\,M^{-1}\,cm^{-1}$).[1] All solutions were prepared with Millipore deionized water.

UV-vis Spectrophotometry

UV-vis spectrophotometric measurements were carried out by using a Shimadzu UV-2600 Spectrophotometer with 1 cm light path. The iron(II) ions can chelate with one or two histidine ligands to form the iron(II)-histidine and iron(II)-(histidine)$_2$ complexes with the logarithms of the stability constants of 5.85 and 4.30 respectively.[2] The 1 mM iron(II)-histidine complex solutions were prepared by dissolving the ferrous salts in N_2 saturated solutions containing histidine at pH7.0, and these solutions were continuously flushed with N_2 to prevent iron(II) oxidation. The molar ratio of histidine:iron(II) was kept at 5:1 to fully bind all iron(II) ions by histidine. At this molar ratio, the concentrations of iron(II) and histidine, and pH7.0, about 99.6% iron(II) ions were complexed by histidine, and about 85% and 15% of the complexes were in the form of iron(II)-histidine and iron(II)-(histidine)$_2$, respectively.[2] This 1 mM iron(II)-histidine complex solution was then 1:1 mixed with N_2 saturated 5 mM phosphate buffer (pH7.0) to obtain the working solution of

0.5 mM iron(II)-histidine complex (in 2.5 mM phosphate, pH7.0). The cuvette was filled up with the N_2 saturated solution and covered with stopper to minimize the effect of oxygen from the air. The Fenton-like reaction was initiated by adding 6.9 mM H_2O_2 (17 μL) to the 0.5 mM iron(II)-histidine complex solution (1.7 mL) prepared as the above. The iron(II)-histidine oxidation was observed as the absorbance increase in the near UV to visible region.

EPR Spin-trapping Spectroscopy

EPR experiments were performed by using a JEOL JFA300 EPR spectrometer at room temperature. The following parameters were adopted: center field, 3360 G; sweep width, 75 G; modulation width, 3.5 G; modulation amplitude, 200; modulation frequency, 100 KHz; microwave power, 1 mW. The iron(II)-histidine complex solutions were prepared in the same way as described in the UV-vis spectrophotometry section. The commercial DMPO was purified with charcoal to eliminate the background noise. The Fenton-like reactions were initiated by adding 6.9 mM H_2O_2 to the 0.5 mM iron(II)-histidine complex solution containing 40 mM DMPO (in 2.5 mM phosphate at pH7.0). EPR spin-trapping spectra were recorded 2 min after initiating the reaction and measurements were repeated at least three times until reproducible spectra were obtained.

Fluorescence Spectroscopy

Fluorescence measurements were performed by using a Hitachi F-2500 fluorescence spectrometer. The fluorescence emissions were measured with the fluorescence probe APF having the excitation and emission wavelengths at 490 nm and 520 nm respectively.[3, 4] The APF molecule itself is not fluorescent, but its

reaction with •OH yields a highly fluorescent product.[4] APF has been reported as a •OH-specific probe because its fluorescence emissions for $O_2^{\bullet-}$ and H_2O_2 compared with that for hydroxyl radical are less than 0.7% and 4%, respectively.[3] The iron(II)-histidine complex solutions were prepared in the same way as described in the UV-vis spectrophotometry section. The Fenton-like reaction was initiated by adding 6.9 mM H_2O_2 to the N_2 saturated solution containing 0.5 mM iron(II)-histidine complex and 2.5 μM APF (in 2.5 mM phosphate at pH7.0). At least three reproducible measurements were performed and data obtained were averaged with standard deviations.

NMR Spectroscopy

All ^{1}H-NMR spectra were recorded by using a 500 MHz Bruker DRX-500 NMR Spectrometer at room temperature. The iron(II)-histidine complex solutions were prepared in the same way as described in the UV-vis spectrophotometry section, and all solutions were saturated with N_2. The Fenton-like reaction was initiated by adding 6.9 mM H_2O_2 to 0.5 mM iron(II)-histidine complex solution containing 50 mM DMSO (2.5 mM phosphate, pH7.0, total volume 0.5 mL), followed by adding 0.055 mL of deuterium oxide (D_2O) to lock the field frequency.[5] The ^{1}H-NMR spectra were recorded 2 min after initiating the reactions.

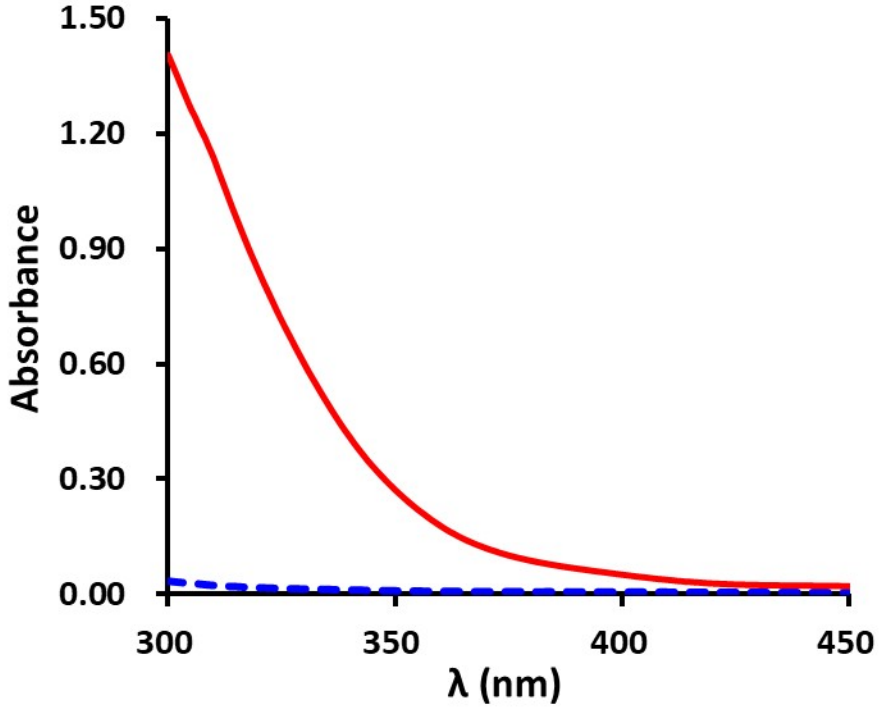

Fig. S1 Oxidation of the iron(II)-histidine complex by H_2O_2 observed by using the UV-vis spectrophotometry. The absorption spectra were recorded from the iron(II)-histidine complex (0.5 mM) in the N_2 saturated buffer (2.5 mM phosphate, pH7.0) (dashed line) and the iron(III)-histidine complex obtained by adding 6.9 mM H_2O_2 to the above iron(II)-histidine complex solution (solid line).

More Analysis on the EPR Spectra

When the H_2O_2 was added to the solution containing the iron(II)-histidine complex and DMPO, the observed EPR spectrum (Fig. 1, upper spectrum) exhibited the characteristic of DMPO-OH spin adduct with hyperfine splitting constants of a_N = 15.02 G and a_H^β = 14.85 G and g factor of 2.0060 in good agreement with those reported in literature.[6, 7]

Furthermore, in the presence of DMSO methyl radical ($CH_3^\bullet$) should be produced from the reaction of hydroxyl radical ($^\bullet$OH) with DMSO. Indeed, a spectrum with 6 lines of EPR signals can be noticed in the presence of DMSO (Fig. 1. lower spectrum), which can be assigned to DMPO- $CH_3^\bullet$ adduct reported in the literature (see Fig. S6 in the reference).[8]

However, it is not clear why the EPR signal intensity we detected was much smaller than that observed in the above study,[8] although both studies were all dealing with Fenton-like reactions of transition metal complexes. This difference in EPR signal intensity might be due to the presence of a strong reducing agent, ascorbic acid, in the reaction system reported in literature,[8] which may deserve further investigation.

Fenton-like Reaction of Iron(II)-citrate Complex

For the Fenton-like reaction of iron(II)-citrate complex, we have demonstrated convincing NMR spectroscopy evidence that $^\bullet$OH is generated from this reaction at neutral pH.[9] Later, two other studies on this Fenton-like reaction were also reported,[10, 11] one followed the other, from which the authors questioned $^\bullet$OH generation from the reaction (see the text). The earlier study was carried out on either predominantly the iron(II)-carbonate complex or the mixture of iron(II)-carbonate and iron(II)-citrate complexes,[10] due to the greater stability constants for iron(II)-

carbonate complex than that for iron(II)-citrate complex (see the text). As a result, when bicarbonate buffer was used and the iron(II)-carbonate complex (not the iron(II)-citrate complex) was the dominant iron(II) species, $CO_3^{\bullet-}$ was observed.[10] However, when phosphate buffer was used, citrate radical and iron(IV) were proposed to be formed, and it was not conclusive whether $^{\bullet}OH$ was generated from the reaction.[10]

The later study[11] was intended to confirm the conclusion from the earlier one, [10] and it was carried out by using DNA as a marker to identify the oxidizing species generated from the Fenton-like reaction of iron(II)-citrate complex in bicarbonate and phosphate buffers respectively.[11] When bicarbonate buffer was used, $CO_3^{\bullet-}$ was observed. However, we noticed that only 1.1 equivalents of citrate (55 μM) was used to bind the iron(II) (50 μM), [11] which was not sufficient because at least a 5:1 ratio of citrate to iron(II) is needed to fully bind iron(II) ions.[12] Furthermore, by considering the high carbonate concentration used (25 mM) and the stronger binding of iron(II) with carbonate than with citrate (see the text), it was very likely that the iron(II)-carbonate complex, rather than the iron(II)-citrate complex, would be the predominant iron(II) complex in the reaction system. Therefore, as expected, $CO_3^{\bullet-}$ was observed in the reaction.[11] On the other hand, when phosphate buffer was used and the iron(II)-citrate was the predominant iron(II) complex due to the stronger binding of iron(II) with citrate than that with phosphate (see the text), $^{\bullet}OH$ was generated.[11] This result fully agrees with our previous finding that $^{\bullet}OH$ is generated from the Fenton-like reaction the iron(II)-citrate complex at neutral pH.[9]

Reason for Using Phosphate Not Bicarbonate Buffer

One may ask why we carried out this study in phosphate not bicarbonate buffer? Phosphate cannot compete with histidine for binding iron(II) because of its much

smaller stability constant with iron(II) (log$K$ is 2.23)[13] than that of histidine (see above). Otherwise, if we used bicarbonate buffer, there would be both iron(II)-carbonate and iron(II)-histidine complexes in the reaction system, resulting in complicated chemistry like those reported (see below).[10, 11] In addition, H_2O_2 can react with bicarbonate to form HCO_4^- that is a stronger oxidant than H_2O_2,[14] leading to more complications. Thus, phosphate buffer gives us a pure (clean) reaction system for studying the Fenton-like reaction of only the iron(II)-histidine complex.

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