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A Proof for Substitution of Endogenous Iron (II) in Lipoxygenase by Exogenous Cu^{2+}

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Abstract Soybean lipoxygenase (LOX) contains endogenous iron (II) at the active site, which is important for the enzyme activity. The activity of LOX can be accelerated by some exogenous metal ions including Cu^{2+}. However, the mechanism of the activity improvement caused by exogenous metal ions remains unclear, not only for LOX but for most other metalloenzymes. Meanwhile, the possibility that exogenous metal ions can displace endogenous iron (II) is still in discussion for a lack of a direct and quantitative proof. In this paper, a quantitative proof of replacing iron (II) inside LOX by exogenous Cu^{2+} was provided, simply using UV-Vis spectrometry with two indicators *p*-carboxylantipyrylazo and 9-(4-carboxyphenyl)-2,3,7-trihydroxyl-6-fluorine. A 0.56 μM free iron (II) was observed in the bulk solution after incubating 9.45 μM Cu^{2+} with 16.10 μM LOX at 20°C for 5 min, which is in coincidence with the decrement of Cu^{2+} in the bulk solution (0.53 μM), implying that iron (II) was replaced by Cu^{2+}.

Keywords Soybean lipoxygenase · Iron · Copper · Substitution · Trace determination · Metalloenzyme

Introduction

Lipoxygenase (LOX, oxidoreductase, EC 1.13.11.12), a metalloenzyme, plays an important role in plant physiology and food quality [1, 2]. It catalyzes the regio- and stereoselective

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incorporation of molecular oxygen into the methylene interrupted diene system of the substrate to produce a conjugated hydroperoxide product [3]. Chelation and dialysis proofs have indicated that each native LOX molecule contains one iron (II) atom, which is generally believed to be in an octahedral ligand environment [4].The switch from ferrous iron to ferric iron has been identified as the controlling step in the oxidization reaction [5–7]. On the other hand, some exogenous metal ions like Cu^{2+} can stimulate the reaction catalyzed by LOX [8]; however, the mechanism of the activity improvement caused by exogenous metal ions remains unclear. Meanwhile, the displacement of endogenous iron (II) by exogenous metal ions still lacks a direct and quantitative proof.

According to the Irving–Williams series theory and the fact that Cu^{2+} can also be trapped in octahedral crystal cave, in theory there is a chance that Cu^{2+} might occupy the octahedral crystal cave and kick out the ferrous iron. Hence, we hereby propose that Cu^{2+} trends to displace the endogenous iron (II) in LOX. To verify this hypothesis, one of the key difficulties is to prove Cu^{2+} does displace iron (II) instead of simply associating with LOX at regulatory sites, qualitatively and quantitatively. Electron paramagnetic resonance technology can analyze both endogenous iron (II) and the exogenous Cu^{2+}, but not quantitatively. So in this paper, by the first time we employed a facile two-indicators protocol by using the indicators *p*-carboxylantipyrylazo (CAP) [9] and 9-(4-carboxyphenyl)-2,3,7-trihydroxyl-6-fluorone(CTF) [10] to track the exchanging with ultraviolet-visible spectrum, in which CAP only responses to free Cu^{2+} while CTF responses to both free Fe^{2+} and Cu^{2+} at different wavelengths. This protocol may provide a simple routine method to study metal ions modification and dynamic catalytic process control for metalloenzymes.

Materials and Methods

Materials

Soybean LOX was purchased from Sigma (70,600 Umg^{-1}). CAP [9] and CTF [10] were prepared according to the literature. All other reagents used were of analytical grade. The electrical conductivity of water was 0.078 $\mu s\ cm^{-1}$ at 25°C.

Measurement of the absorbance was conducted on a TU-1901 UV-vis spectrophotometer (PGeneral, China).

Determination of Free Cu^{2+} and Fe^{2+} Concentration

Both CAP and CTF do not response to any metal ions inside native LOX. CAP and CTF were used to detect free Cu^{2+} and Fe^{2+} in solution, respectively; the absorption maxima of CAP-Cu^{2+} and CTF-Fe^{2+} complex are at a wavelength of 626 [9] and 620 nm [10], respectively. The calibration curves for Cu^{2+} and Fe^{2+} concentration were made using CAP-copper sulfate and CTF-ammonium ferrous sulfate standard solution, respectively.

Tracing the Exchange Between Cu^{2+} and Fe^{2+}

A 1.5 mL of 0.156 mM Cu^{2+}-sulfate standard solution and LOX solution (the LOX final concentration was 16.10 μM) were mixed in a 25-mL calibrated flask, incubated at 20°C for 5 min. For detection of Cu^{2+}, 4.0 mL acetic acid, and 1.0 mL of 0.2 gL^{-1} CAP were then added (in case of detecting Fe^{2+}, 5.0 mL NH_3–HAc buffer solution, 2.0 mL of 0.2%

cetyltrimethylammonium bromide solution, and 2.0 mL of 5.5×10^{-4} M CTF were added), and final volume of the mixture was made up to 25 mL with water.

The concentrations of Cu^{2+} and Fe^{2+} in the bulk solution were then obtained by measuring the absorbance of the solution at 626 and 620 nm, respectively.

The complex ratio of Cu^{2+} is defined as the percentage of the decrement of Cu^{2+} in the LOX solution after the incubation. All experiments were made in triplicate.

Results and Discussion

The Influence of the Exogenous Cu^{2+} on LOX UV Absorbance

Fe (II)-LOX has been regarded as the main form of native LOX in literatures [11, 12]. The native LOX possess a normal UV absorbance as proteins. When the LOX solution was titrated with metal ions, as shown in Fig. 1, the addition of Mn^{2+}, Mg^{2+}, Ca^{2+}, and Na^{+} had no obvious impact on the UV absorbance of the native LOX at 360nm; Ni^{2+} and Cd^{2+} slightly enhanced it, while Fe^{3+}, Fe^{2+}, Ba^{2+}, Zn^{2+}, and Cu^{2+} enhanced the absorbance obviously and concentration dependently.

Refer to the Irving-Williams stability series, among these metal ions, all ions enhanced the A_{360} can form more stable complexes than Fe (II) except Ba^{2+}. In addition, Cu (II) can form more stable complexes due to extra stabilization offered by the Jahn–Teller effect.

Fe^{3+} and Fe^{2+} also enhanced A_{360}. As it is well known, Cu^{2+} cannot oxidize Fe (II)-LOX into Fe (III)-LOX because the standard potential of Fe^{3+}/Fe^{2+} (0.77 V) is higher than that of

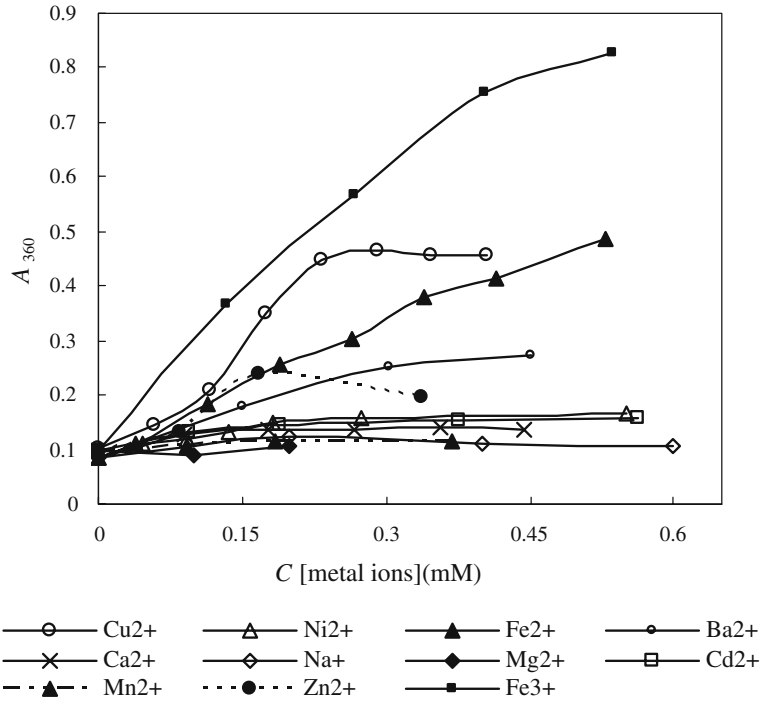

Fig. 1 Effect of exogenous metal ions on the absorbance of lipoxygenase

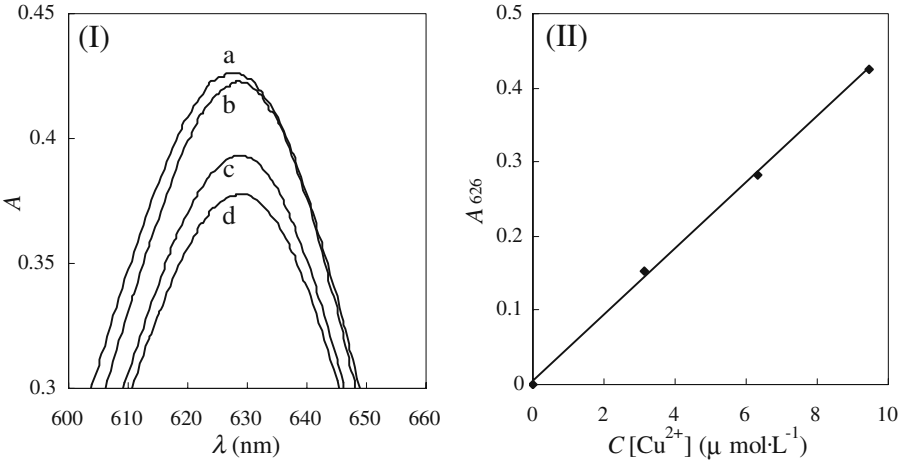

Fig. 2 (*I*) Effect of LOX concentration on the absorption of *p*-carboxylantipyrylazo-Cu^{2+}. A 9.45 μM Cu^{2+} mixed with 0, 4.83, 8.05, and 16.10 μM LOX (from *a* to *d*), incubated at 20°C for 5 min. (*II*) Calibration graph of *p*-carboxylantipyrylazo-Cu^{2+} concentration

Cu^{2+}/Cu^{+} (0.62 V). Therefore, the strengthening of UV absorbance at 360 nm after addition of exogenous Cu^{2+} could be due to some interaction between LOX and Cu^{2+}, rather than the increment of Fe (III)-LOX.

Therefore, the quantitative determination was made to test if the free Cu^{2+} concentration decreased in the bulk solution with the addition of LOX.

As shown in Fig. 2, with the increase of LOX, the free Cu^{2+} was declining in the bulk solution. Since LOX did not induce any chemical reaction with Cu^{2+}-sulfate under this condition, the only possibility for LOX shielding the absorbance of Cu^{2+}-*p*-carboxylantipyrylazo was the formation of LOX-Cu^{2+}. When the concentration of LOX was 16.10 μM, the complex ratio reached 12.3%.

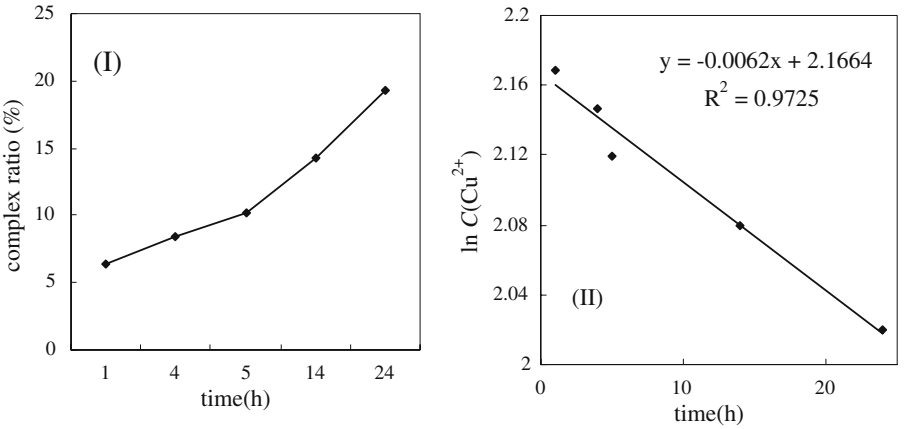

Fig. 3 (*I*) The LOX-Cu^{2+} complex ratio as a function of Cu^{2+} concentration (LOX, 4.83 μM). (*II*) The LOX-Cu^{2+} complex ratio as a function of LOX concentration (Cu^{2+}, 9.35 μM). Cu^{2+} and LOX solution were mixed and incubated at 20°C for 5 min

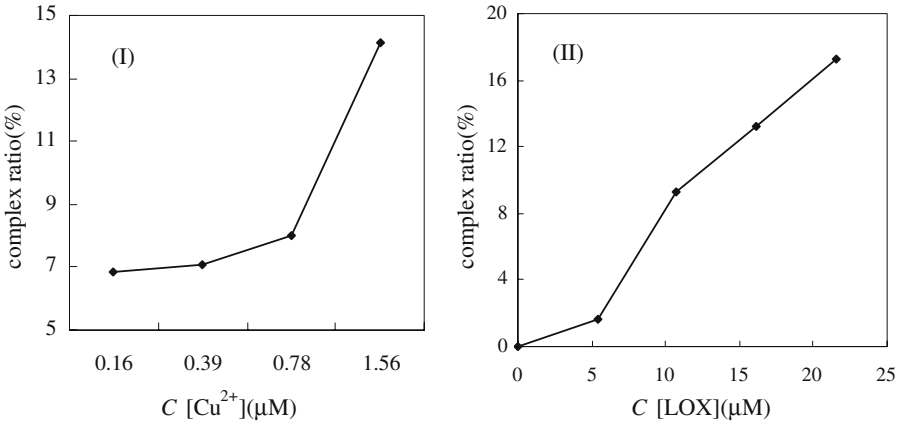

Fig. 4 (*I*) Time course of the complexation of LOX-Cu^{2+}. (*II*) Pseudo-first order kinetics of the complexation of LOX-Cu^{2+}. A 9.35 μM Cu^{2+} mixed with 4.83 μM LOX at 20°C

The Dynamics of the Formation of LOX-Cu^{2+}

The time course of the complexation and the concentration dependence were investigated to verify the dynamics of the formation of LOX-Cu^{2+}. The complex ratio increased with increasing concentrations of both Cu^{2+} and LOX (Fig. 3). As shown in Fig. 4, the complexation of Cu^{2+} and LOX followed first order reaction. The rate constant k was $1.7\times10^{-6}s^{-1}$.

The Proof of the Release of Fe^{2+} from LOX

The above experiments preliminarily proved that Cu^{2+} could form complexation with LOX, but it was still unclear whether Cu^{2+} entered the active site or simply associated with protein when mixed with native LOX solution, or if it was possible for endogenous iron to get released from the LOX. There was no absorbance at 581 nm (standing for CTF-Cu^{2+}) nor 620 nm (standing for CTF-Fe^{2+}) when CTF was mixed with LOX, so CTF does not capture Cu^{2+} or iron (II) from native LOX. After mixing CTF with LOX-Cu^{2+} solution for 5 min, the maximum absorbance of the solution shifted from 581 to 586 nm comparing with that of CTF-Cu^{2+} solution (Fig. 5I a, b); and the enhanced absorbance at 620 nm

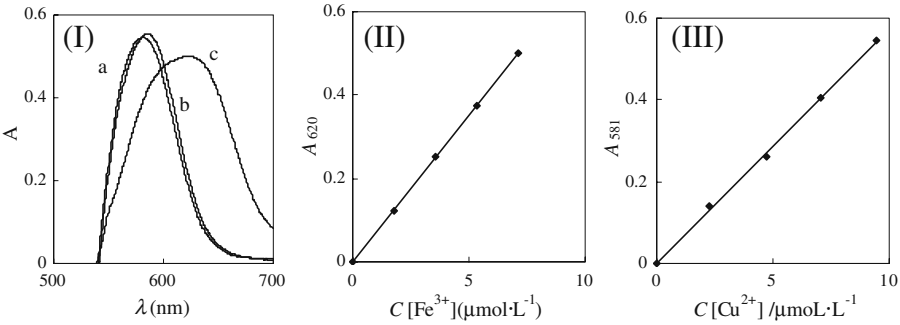

Fig. 5 (*I*) Visible spectrum of CTF-Cu^{2+}. (*a*) A 9.45 μM Cu^{2+}; (*b*) 9.45 μM Cu^{2+} with 16.10 μM LOX; (*c*) 7.14 μM Fe^{2+} at 20°. C (*II*) Calibration of 9-(4-carboxyphenyl)-2,3,7-trihydroxyl-6-fluorine-Fe^{2+} concentration. (*III*) Calibration of 9-(4-carboxyphenyl)-2,3,7-trihydroxyl-6-fluorine-Cu^{2+} concentration

suggested that Fe^{2+} appeared in the solution (Fig. 5I c). The calculated concentration of Cu^{2+} reduced by 0.53 μM and meanwhile 0.56 μM free Fe^{2+} was found in the bulk solution. Since CTF could not capture Cu^{2+} or iron (II) inside LOX, the only explanation to this coincidence is that Cu^{2+} displaced iron (II) located at the active site in native LOX. As mentioned before, LOX contains one atom of iron per molecule, therefore the detected amount of CTF-Fe^{2+} was accounted for 3.48% theoretically based on the total amount of endogenous Fe (II).

Conclusion

By means of two indicators, *p*-carboxylantipyrylazo and 9-(4-carboxyphenyl)-2,3,7-trihydroxyl-6-fluorone with ultraviolet-visible spectrometry analysis, the proof of the substitution of endogenous iron (II) trapped in LOX by exogenous Cu^{2+} was given in this paper. The addition of Cu^{2+} enhanced the UV absorbance of LOX. A 0.56 μM of Fe^{2+} was found in the bulk solution while 0.53 μM of exogenous Cu^{2+} was missing after incubating the mixture of 9.45 μM Cu^{2+} and 16.10 μM LOX for 5 min, and the substitution ratio of endogenous Fe (II) in LOX by exogenous Cu^{2+} was 3.48% under this condition.

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