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Short communication

Redox Activity of the Mini-αA-crystallin–Cu(II) Complex and Its Biological Relevance

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Cu(II) has been known to be a redox-active metal whose complexes with proteins/peptides have been linked to oxidative stress in many diseases. Oxidative stress and Cu(II) are also known to induce the expression of the small heat shock proteins α-crystallins (both αA- and αB- crystallins). Recent studies have demonstrated that αA-crystallin binds Cu(II) and suppresses Cu(II)-mediated generation of reactive oxygen species as well as the oxidation of ascorbic acid. In this work, the redox activity of the complex formed between Cu(II) and mini-αA-crystallin (a Cu(II)-binding peptide consisting of residues 71–88 of αA-crystallin) was studied. Voltammetric measurements of Cu(II) bound by the mini-αA-crystallin demonstrated that the Cu(II) redox cycle is modulated by mini-αA-crystallin and dependent upon critically on pH, which were supported by direct kinetic assays that reported the production of H_2O_2 from ascorbic acid and oxygen. Our findings also indicated that mini-αA-crystallin possesses the properties of promoting the controlled H_2O_2 production and inhibiting the formation of hydroxide free radical.

Keywords: α-crystallin; Cu(II); oxidative stress; electrochemistry

1. INTRODUCTION

Oxidative stress resulted from the reactive oxygen species (ROS) has been definitely recognized as a pathogenetic factor that induces the formation of protein inclusions as well as selective degeneration and further leads to excitotoxicity and mitochondrial dysfunction. Although copper acquisition is a fundamental process in almost all living organisms (e.g. O_2 transport, DNA synthesis and electron transport), free copper ion exhibits high cytotoxicity with its ability to initiate redox cycling, which in turn generates reactive oxygen species (ROS). For example, increased accumulation

of Cu(II) is manifested in several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, prion disease, Wilson's disease and Menkes disease [1-3]. αA-crystallin, a member of small heat shock proteins (sHSP), is a major structural protein in the ocular lenses of all vertebrates [4]. It has been suggested that treatment of lens epithelial cells with Cu(II) leads to selective up-regulation of the sHSP, including both αA- and αB-crystallin [5]. Several studies also demonstrated that lens proteins tightly bind Cu(II) and suppress Cu(II)-mediated generation of ROS as well as the oxidation of ascorbic acid (AA) [6-9]. Typically, Raju et al. identified and characterized the Cu(II)-binding site in αA-crystallin and found that the mini-αA-crystallin (a peptide consisting of residues 71–88 of αA-crystallin) prevented copper-induced oxidation of AA by the formation of the 1:1 mini-αA-crystallin–Cu(II) complex [6]. Although there has been several studies of the interaction of mini-αA-crystallin and Cu(II), the role of α-crystallins in oxidative stress and in Cu(II)-mediated processes is not clearly understood. Consequently, it has been difficult to rationalize cell-based and in vivo findings about the possible functions of the Cu(II) complexes of αA-crystallin [4]. In this work, we accurately measured the redox potentials of the mini-αA-crystallin–Cu(II) complex by employing electrochemical techniques and reported its redox activity (cycling). The implication of our findings to the physiological functions of αA-crystallin was also discussed.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Fmoc-protected amino acids, Wang resin, benzazimidol, diisopropylcarbodiimide and piperidine were obtained from GL Biochem Ltd. (Shanghai China). Other reagents were all of AR grade. They were obtained from Aldrich Chemicals or Beijing Chemical Reagent Co. (Beijing, China). Mini-αA-crystallin peptide with a sequence of DFVIFLDVKHFSPEDLTVK) was prepared by solid phase Fmoc chemistry. The coupling reaction was 0.5 h. The Fmoc group was deprotected with 20% piperidine. The crude product was purified by Waters 2545 preparative reversed phase high performance liquid chromatography (HPLC). Mobile phase A and B were water and acetonitrile, respectively. 0.1% Trifluoroacetic acid was included in the two mobile phases.

2.2 Voltammetric measurements

Cyclic voltammograms of mini-αA-crystallin–Cu(II) and free Cu(II) were collected on a CHI 660E electrochemical electrochemical workstation (CHI Instruments, Shanghai, China). A glassy carbon disk electrode (3 mm in diameter) and a platinum wire were used as the working and counter electrodes, respectively. The reference electrode was Ag/AgCl and the electrolyte solution was a 10 mM phosphase buffer (pH 7.4) containing 50 mM Na_2SO_4. The scan rate was 5 mV/s. Prior to each experiment, the glassy carbon electrode was polished with diamond pastes of 1 and 0.3-μm in

diameter. Voltammetric experiment in the absence of O_2 was carried out in a glove box. Before assays, the glove box was purged with high-purity N_2 to keep the O_2 concentration below 0.05 ppm.

2.3 Kinetic measurement

The AA oxidation was followed by Uv-vis spectrophotometry (Cary 50, Varian Inc.). The kinetic experiments were conducted at 25 °C in phosphate buffer (pH, 7.4). At this pH, AA exists predominately in the ascorbate monoanion form and throughout this work we use AA interchangeably to represent the ascorbate monoanion. The initial oxidation rates of AA were measured from the decrease of absorbance at 265 nm, where AA shows a maximum absorption ($\varepsilon = 1.5 \times 10^4\ M^{-1}cm^{-1}$). Within the initial 120 s, the absorbance decreases linearly with time. The absorbance value was plotted by excluding that contributed by αA-crystallin at 265 nm. To minimize the free Cu(II) concentration, the [mini-αA-crystallin]/[Cu(II)] ratio was at 10/1. At such a ratio, even if the binding constant between mini-αA-crystallin and Cu(II) was in the submicromolar, copper would still exist predominantly in the form of a complex. We should emphasize that any uncomplexed Cu(II) can catalyze rapidly the oxidation of AA and reduce H_2O_2 to OH• via the Harber–Weiss-like mechanism [10]. Therefore, keeping the free Cu(II) concentration at the minimum by using a high [mini-αA-crystallin]/[Cu(II)] ratio is critical for avoiding such a complication.

2.4 Detection of H_2O_2

The concentrations of produced H_2O_2 were determined with H_2O_2 detection kit. The reaction solution were injected through a six-port rotary valve into a flowing stream of phosphate buffer (10 mM, pH 7.4) delivered by a Harvard syring pump at a flow rate of 6 mL/h. The concentration of generated H_2O_2 was determined by comparing the measured current to that of a calibration curve constructed with H_2O_2 standard solutions. Before each analysis, the sample mixtures were diluted by 5 times.

3. RESULTS AND DISCUSSION

3.1 Redox behavior of mini-αA-crystallin–Cu(II)

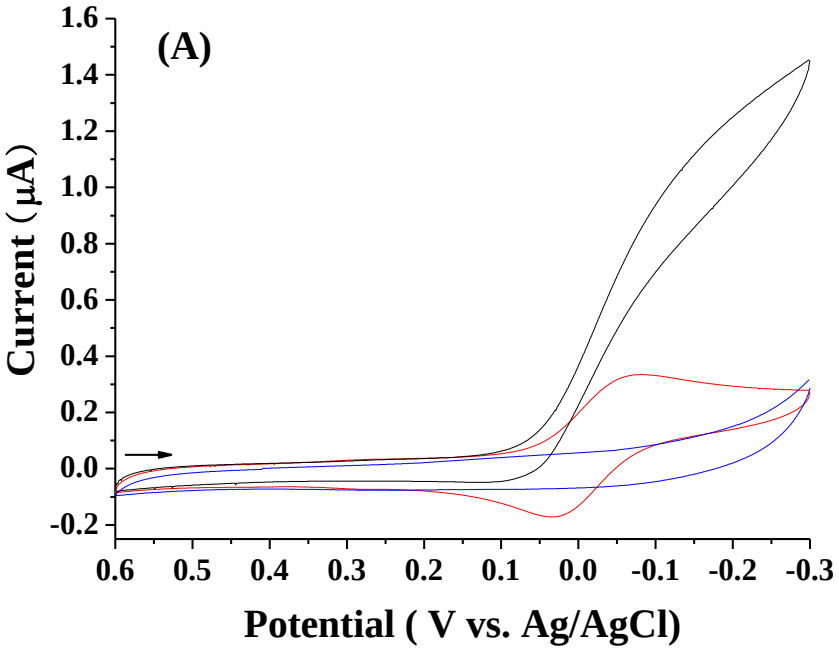
Mini-αA-crystallin is known to form a 1:1 mini-αA-crystallin–Cu(II) complex. Fig. 1A depicts the cyclic voltammograms (CVs) of mini-αA-crystallin–Cu(II) in the absence and presence of oxygen. Mini-αA-crystallin is redox inactive (blue curve), in contrast, a mixture of mini-αA-crystallin and one equivalent copper exhibits a pair of quasi-reversible oxidation (E_{pa} = 0.031 V) and reduction (E_{pc} = –0.081 V) peaks (red curve). The shape of waves is drastically different from those of free copper (see the red curve in Fig. 1B), indicating the Cu(II) is ligated by mini-αA-crystallin. From the peak potentials the redox potential of the complex is calculated to be –0.025 V (vs. Ag/AgCl). Usually, the

toxicity of redox active metal originates from its involvement in redox cycling, producing reactive oxygen species. For example, free Cu(II) will be reduced by biological reductants, and the oxygen present regenerates the Cu(II) from the reduced Cu(I) and itself is reduced to either H_2O_2 or hydroxide free radical. The catalytic cycling continues until oxygen or biological reductants are depleted. To find out if such self-enforced redox cycling is possible, we conducted CV of the complex in the presence of O_2. By the technique, the Cu(II) complex can be directly reduced to Cu(I) in the complex in-situ and the catalytic regeneration of Cu(II) by oxygen can be detected directly. As shown in the black curve of Fig. 1A, its oxidation peak vanishes and reduction current is increased by a few times, a typical catalytic mechanism of electrochemical reduction followed by chemical oxidation. The mechanism is mini-αA-crystallin–Cu(II) + e → mini-αA-crystallin–Cu(I), mini-αA-crystallin–Cu(I) + O_2 → mini-αA-crystallin–Cu(II) + H_2O_2. Overall, the results indicate that mini-αA-crystallin–Cu(II) is capable of catalytic redox cycling.

Furthermore, based on the shifts in the reduction potentials of the complex with respect to that of free Cu(II), we estimated that the binding affinities of mini-αA-crystallin towards Cu(II) and Cu(I) are comparable. The redox potential of Cu(II) complexed with mini-αA-crystallin can be depicted by the following equation [11]:

$$\Delta E^0 = E^0_{OP-Cu(II)/OP-Cu(I)} - E^0_{Cu(II)/Cu(I)} = \frac{RT}{F}\ln(\frac{K_A^{'}}{K_A}) \qquad (6)$$

where $E^0_{OP-Cu(II)/OP-Cu(I)}$ is the standard redox potential of mini-αA-crystallin–Cu(II)/mini-αA-crystallin–Cu(I) (–0.025 V) and $E^0_{Cu(II)/Cu(I)}$ is that of Cu(II)/Cu(I) (–0.040 V). T, F, and R are temperature (298 K), the Faraday's constant, and the gas constant, respectively. K_A' is the binding affinity of mini-αA-crystallin with Cu(I) and K_A is the one of the peptide with Cu(II). From the above equation, the K_A'/K_A ratio was determined to be 1.8.



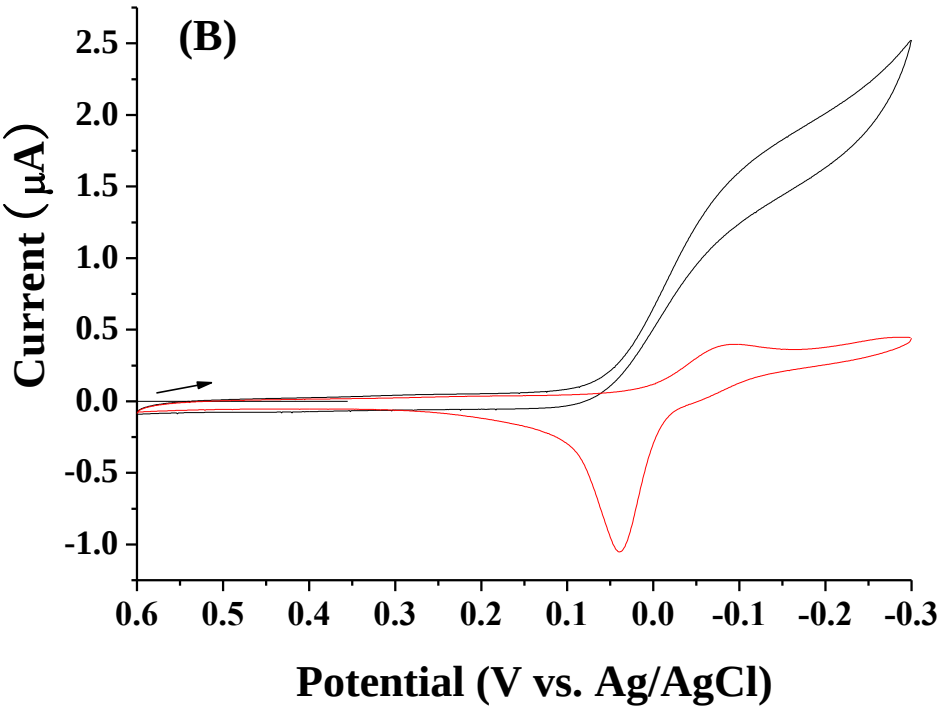


Figure 1. (A) Cyclic voltammograms (CVs) of mini-αA-crystallin (blue curve) in N_2-saturated solution and mini-αA-crystallin–Cu(II) in N_2-saturated (red curve) and O_2-purged solutions (black curve). (B) CVs of free Cu(II) in the absence (red curve) and presence (black curve) of O_2. The concentrations of mini-αA-crystallin and Cu(II) in all cases were 100 μM. The arrow indicates the initial scan direction.

3.2 Catalytic oxidation of AA by mini-αA-crystallin–Cu(II)

Above results demonstrated that the mini-αA-crystallin–Cu(II) is capable of redox cycling by electrochemical reduction. To further explore the biological relevance of the finding, we used a very common and abundant biological reductant, AA, to verify if the redox cycling happens or not. The effectiveness of the redox cycling can be characterized by the kinetics of AA catalytic oxidation by O_2. Fig. 2A shows the absorbance of AA vs. time curves in the absence and presence of free Cu(II) and mini-αA-crystallin–Cu(II) in air saturated solution. The AA consumption rates are drastically different. In Cu(II)-free solution, the absorbance remains little changed over 20 min, which is indicative of slow AA auto-oxidation. As expected, in the presence of 5 μM free Cu(II), the AA consumption rate is drastically increased. The AA consumption rate in the presence of mini-αA-crystallin–Cu(II) is lower than that in the absence of any Cu(II) species but higher than that in the presence of free Cu(II).

The catalytic reaction not only exhausts AA and O_2, what is worse, it generates H_2O_2 and in some cases H_2O_2 further turns into hydroxide free radical. Fig. 2B shows the concentration of H_2O_2 produced after 20 min of reaction. Clearly, H_2O_2 concentration produced in the presence of different

Cu(II) species echoes the corresponding AA consumption rate. The general mechanism of Cu(II) redox cycling can be described by the following reaction equations:

2 mini-αA-crystallin–Cu(II) (free Cu(II)) + AA + H_2O

= 2 mini-αA-crystallin–Cu(I) (free Cu(I)) + DA + $2H^+$ (1)

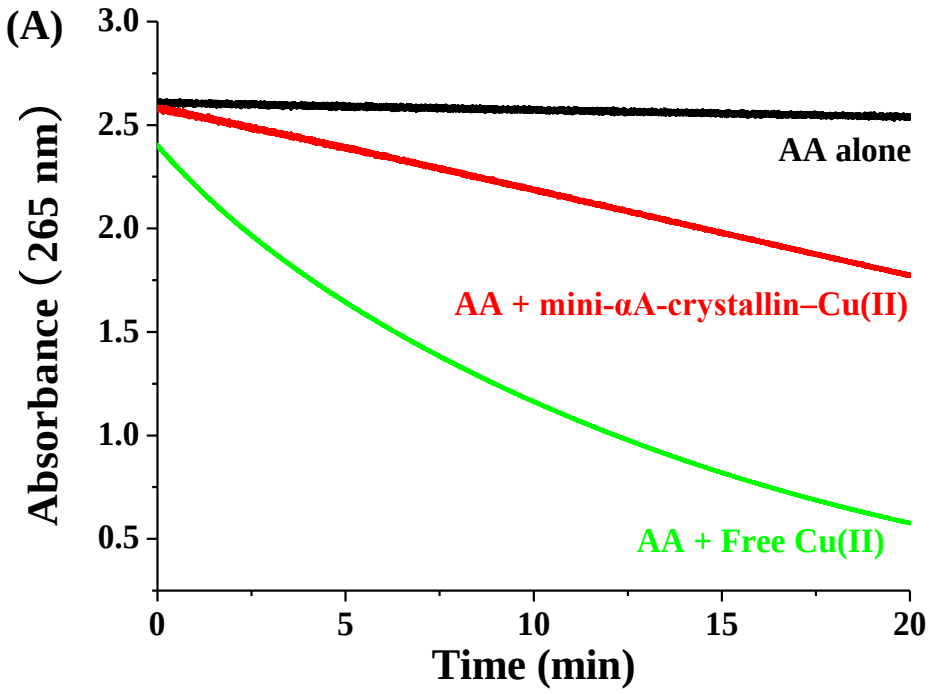
2 mini-αA-crystallin–Cu(I) (free Cu(I)) + O_2 + $2H^+$

= 2 mini-αA-crystallin–Cu(II) (free Cu(II)) + H_2O_2 (2)

where DA is dehydroascorbate, the oxidaion production of AA. The happening of the redox cycling depends on the relative redox potentials of the redox couples. Table 1 lists the redox potentials of a few redox couples of interest. For free Cu(II) water is the coordination ligand [11]. The potential of Cu(I)/Cu(II) is higher than that of AA/DA [12], so reaction 1 is thermodynamically favored and the reaction occurs spontaneously. In addition, the potential of O_2/H_2O_2 is higher than that of Cu(I)/Cu(II) [13], so reaction 2 also occurs spontaneously and regenerates Cu(II), completing the cycle. The regenerated Cu(II) reacts again with AA, starting a new cycle. When Cu(II) is ligated by mini-αA-crystallin, forming the complex of mini-αA-crystallin–Cu(II), the reactions 1 and 2 become slower.

Table 1. Redox potential of Cu(II) species and select biological redox couples

System	E (V vs. Ag/AgCl)	References
O_2/H_2O_2	0.099	[13]
Mini-αA-crystallin–Cu(II)	–0.025	This work
Free Cu(II)	–0.040	[11]
AA	–0.145	[12]



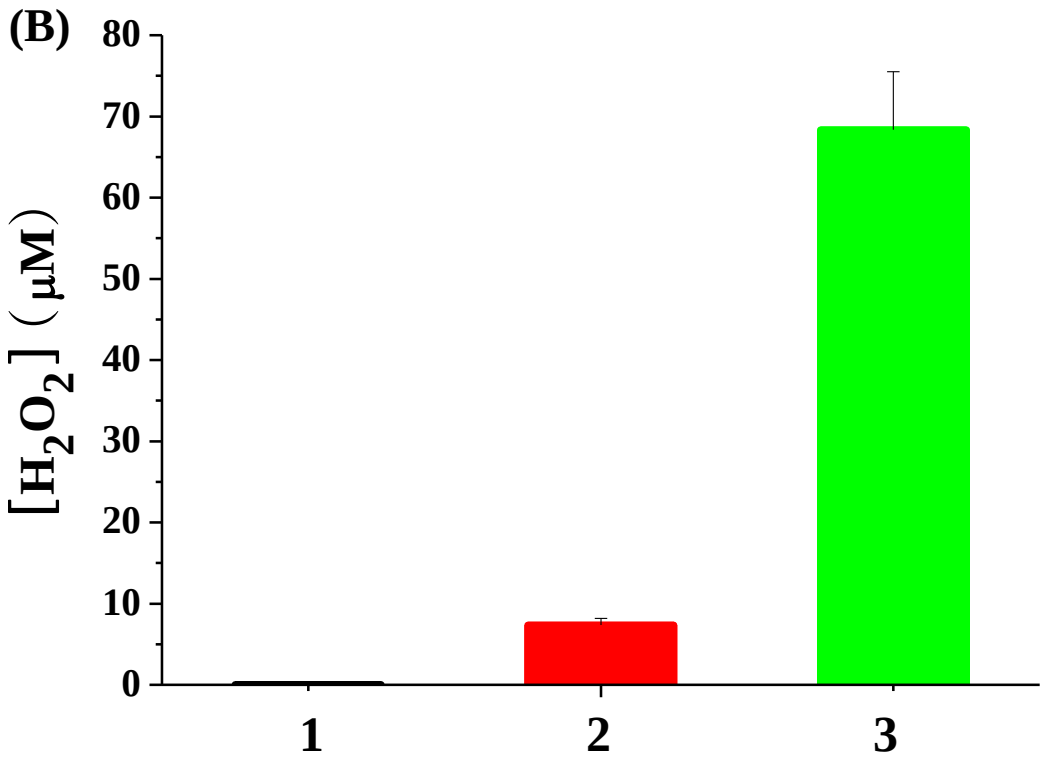


Figure 2. (A) Change of AA (200 μM) absorbance as a function of reaction time in the absence and presence of different Cu(II) species. (B) Catalytic H_2O_2 generation from AA by mini-αA-crystallin–Cu(II) (red bar) and free Cu(II) (green bar). Mini-αA-crystallin (100 μM) was mixed with Cu(II) (5 μM) for 10 min to form the mini-αA-crystallin–Cu(II) complex. For panel B, the Cu(II) species was incubated separately with AA (200 μM) for 20 min. Control incubation was performed in the absence of any Cu(II) species (black bar).

3.3 Redox behavior of mini-αA-crystallin–Cu(II) at different pH

It is reported that the amide nitrogens become protonated at pH below the physiological value (~6.5 or lower) [14]. To figure out whether the binding of αA-crystallin or its peptidic variants to Cu(II) is dependant upon pH, we investigated the effect of pH on the CV responses of mini-αA-crystallin–Cu(II). We therefore examined the voltammetric responses of mini-αA-crystallin–Cu(II) at different pH values. As shown in Fig. 3, there is no difference in the CVs of mini-αA-crystallin–Cu(II) between pH 7.4 and 6.8. However, at pH below 6.0, the CV is similar to that of free Cu(II) in the absence of O_2 (cf. blue curve in Fig. 3B and red curve in Fig. 1B). The redox peaks associated with mini-αA-crystallin–Cu(II) were not observed. These results suggest that Cu(II) released from mini-αA-crystallin–Cu(II), that is, low pH disfavored the mini-αA-crystallin–Cu binding.

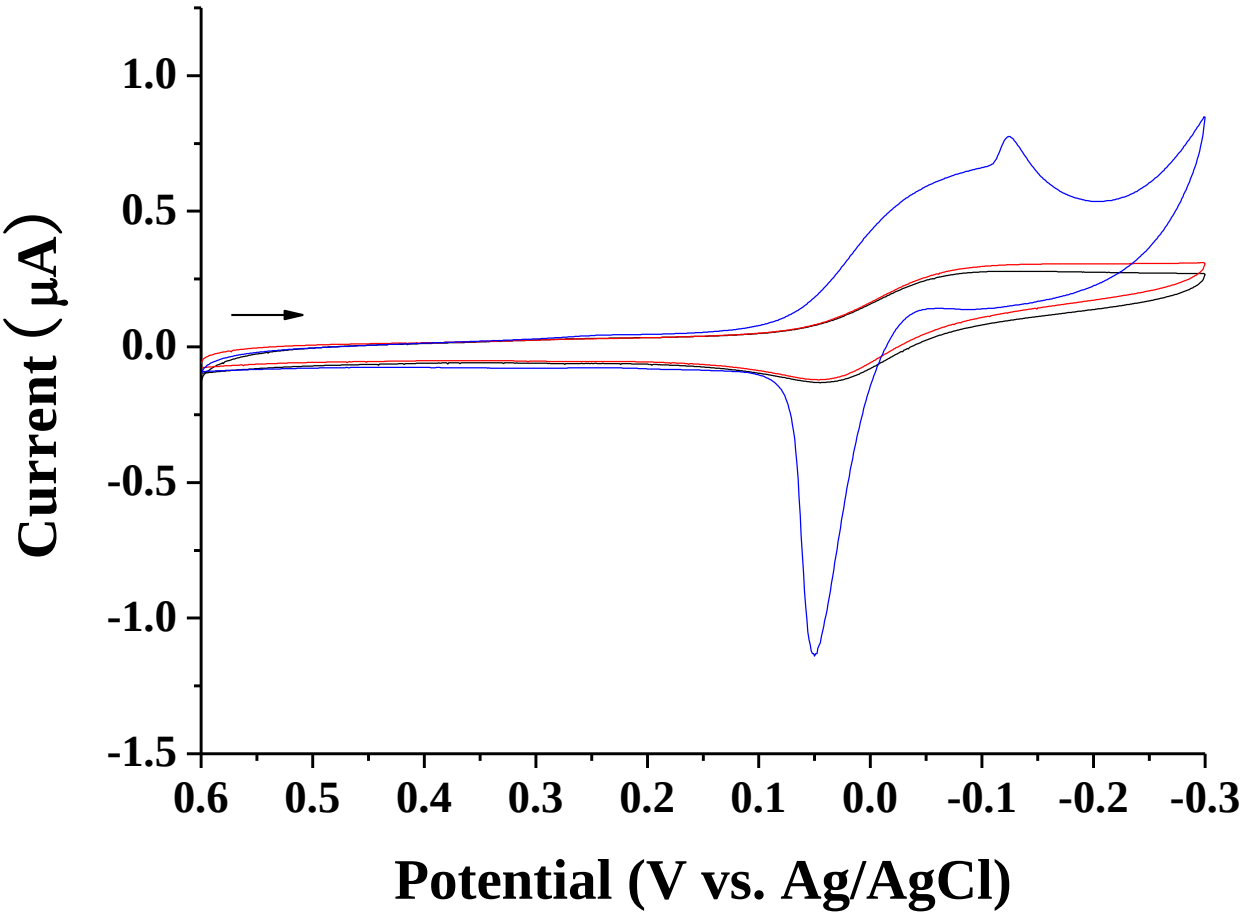


Figure 3. CVs of mini-αA-crystallin–Cu(II) at different pH values: 7.4 (black), 6.8 (red) and 6.0 (blue). The other experimental conditions were the same as those in Fig. 1.

3.4 Effect of reaction time and solution pH on the production of H_2O_2

Furthermore, we tested the production of H_2O_2 as a function of incubation time at different pH. Fig. 4 displays the time-dependence of the H_2O_2 concentration at different pH. The amount of H_2O_2 generated by mini-αA-crystallin–Cu(II) at pH 7.4 (black) or 6.8 (red) increases gradually with the incubation time. At pH 6.0 (green), the amount of H_2O_2 generated by mini-αA-crystallin–Cu(II) increased and then greatly decreased, which is similar to that in the case of free Cu(II) (blue). In this process, the increase is due to the production of H_2O_2 via reactions (1) and (2) by free Cu(II). The decrease can be attributed to the decomposition of H_2O_2 by free Cu(II) via the Harber-Weiss reaction (reactions 3-5) [10]. Thus, with the production of H_2O_2, reactions (3)–(5) occur, which in turn causes the reduction of H_2O_2 and the formation of more reactive and pernicious hydroxyl radicals.

$$Cu(II) + H_2O_2 \rightarrow Cu(I)\bullet O_2H + H^+ \quad (3)$$

$$Cu(I)\bullet O_2H + H_2O_2 \rightarrow Cu(I) + O_2 + OH\bullet + H_2O \quad (4)$$

$$H^+ + Cu(I) + H_2O_2 \rightarrow Cu(II) + OH\bullet + H_2O \quad (5)$$

Overall, mini-αA-crystallin–Cu(II) facilitated the redox cycling of Cu(II) and the accumulation of H_2O_2 in the presence of O_2. The catalytic cycling ended until the exhaustion of AA or O_2. However,

as can be seen from Fig. 4, at the beginning of the redox cycle, the amount of H_2O_2 generated by mini-αA-crystallin–Cu(II) is much lower than that in the case of free Cu(II). Notice that in free Cu(II) solution, the sharp increase in H_2O_2 concentration is followed by a relatively rapid decay. Such a decay can produce the much more pernicious hydroxyl radicals by the Harber-Weiss reaction between H_2O_2 and free Cu(II). Contrary to the free Cu(II) solution, the concentration of H_2O_2 generated in the mini-αA-crystallin–Cu(II) solution is controlled and increases only gradually with time. Although the gradual production of H_2O_2 may also trigger oxidative stress, it should be mentioned that in vivo H_2O_2 is readily scavenged by enzymes such as catalase and glutathione peroxidase, as well as antioxidants such as AA and uric acid.

Therefore, a buildup of H_2O_2 given rise by the mini-αA-crystallin–Cu(II) complex is impossible. It has been estimated that concentrations of up to 250 μM H_2O_2 could be produced within brain neuropil every minute [15]. Notice that the H_2O_2 concentration depicted in Fig. 2B is much lower than the known endogenous H_2O_2 concentration in the central nervous system. Thus, the capacity of endogenous H_2O_2 homeostasis in vivo should be sufficient to weaken the negative effect caused by the produced H_2O_2. Additionally, H_2O_2 is less toxic than the hydroxyl radicals and will not readily inflict oxidative damage.

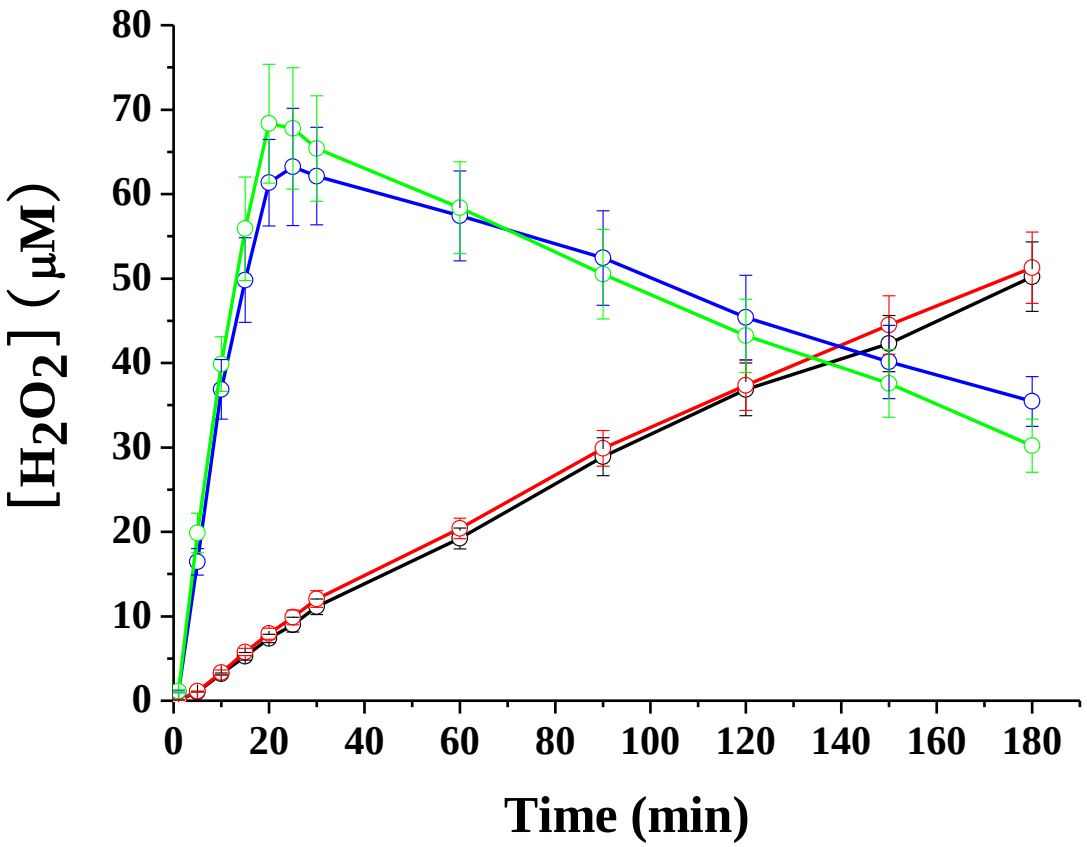


Figure 4. Variations of [H_2O_2] generated by free Cu(II) (blue) at pH 7.4 and by mini-αA-crystallin–Cu(II) at pH 7.4 (black), 6.8 (red) and 6.0 (green). The concentrations of Cu(II) and mini-αA-crystallin are the same as those in Fig. 2.

4. CONCLUSIONS

In summary, we investigated the redox activity of the mini-αA-crystallin–Cu(II) complex. The results confirmed that mini-αA-crystallin–Cu(II) was reduced to mini-αA-crystallin–Cu(I) by electrochemical reduction or biological species of AA. Furthermore, the resulting mini-αA-crystallin–Cu(I) complex was oxidized to mini-αA-crystallin–Cu(II) by O_2. The Cu(II) redox cycle is modulated by mini-αA-crystallin and dependent upon critically on pH. Based on the shifts in the reduction potential of mini-αA-crystallin–Cu(II) with respect to that of free Cu(II), we found that the binding affinities of mini-αA-crystallin towards Cu(II) and Cu(I) are comparable. Moreover, our findings also indicated that mini-αA-crystallin possesses the properties of promoting the controlled H_2O_2 production and inhibiting the formation of hydroxide free radical by binding to Cu(II).

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