Photo-induced DNA cleavage by zinc-substituted myoglobin with a redesigned active center

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1. Materials and Methods

1.1 Materials

All reagents were of analytical grade commercially available and were used without further purification. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was purchased from dojindo (Shanghai, China). Zinc protoporphyrin (ZnPP), 2,2,6,6-Tetramethylpiperidine (TEMP) and Plasmid DNA (pUC19) were purchased from Thermo Fisher Scientific Inc (USA). Water was purified using a Direct-Q 3UV apparatus (18.2 MΩ cm) to prepare buffers.

1.2 Preparation and reconstitution of apoMbs with ZnPP

Wild-type (WT) sperm whale Mb was expressed in BL21 (DE3) cells using the Mb gene of pMbt7-7 and purified using the procedure described previously [1]. H64A Mb gene was constructed using the QuikChange Site Directed Mutagenesis Kit (Stratagene). The mutation was confirmed by DNA sequencing assay. F43H Mb, H64A Mb, F43H/H64A Mb and L29E Mb mutants were expressed and purified as described in previous studies [2, 3]. Apo-forms of Mbs (apo-Mb, apo-L29E Mb, apo-F43H Mb, apo-H64A Mb and apo-F43H/H64A Mb) were prepared by removing the heme using acidic butanone method as previously described [4]. A 2-fold molar excess of ZnPP in DMSO was added dropwise to potassium phosphate buffer (100 mM, pH 7.0) containing apoMb within 30 minutes. After the final addition of ZnPP, the solution was stirred at 4 °C overnight to ensure an equilibrium was reached. The protein solution was centrifuged to remove the insoluble ZnPP at 4 °C, followed by concentration to ~6 mL. The bright magenta color protein was then passed down a DEAE column pre-equilibrated in potassium phosphate buffer (100 mM, pH 7.0) to remove any excess ZnPP. The protein solution was dialyzed in chelexed potassium phosphate buffer (100 mM, pH 7.0) overnight to remove free metal ions. Stock of ZnPP-Mbs was stored at -20°C for used. Incorporation of ZnPP into apoMbs was confirmed by UV-Vis. The extinction coefficient of ZnPP-Mbs was determined using hemochromagen method as previously reported (ε551 = 12.3 mM⁻¹cm⁻¹ for
ZnPP-pyridine complex) [5]. Protein concentration was determined with an extinction coefficient of $\varepsilon_{409} = 157$ mM$^{-1}$·cm$^{-1}$ for WT Mb, $\varepsilon_{406} = 155$ mM$^{-1}$·cm$^{-1}$ for F43H Mb, $\varepsilon_{408} = 155$ mM$^{-1}$·cm$^{-1}$ for H64A Mb, $\varepsilon_{407} = 155$ mM$^{-1}$·cm$^{-1}$ for F43H/H64A Mb, $\varepsilon_{413} = 135$ mM$^{-1}$·cm$^{-1}$ for L29E Mb, $\varepsilon_{428} = 142$ mM$^{-1}$·cm$^{-1}$ for ZnPP-WT Mb, $\varepsilon_{428} = 133$ mM$^{-1}$·cm$^{-1}$ for ZnPP-F43H Mb, $\varepsilon_{429} = 135$ mM$^{-1}$·cm$^{-1}$ for ZnPP-H64A Mb, $\varepsilon_{427} = 133$ mM$^{-1}$·cm$^{-1}$ for ZnPP-F43H/H64A Mb, $\varepsilon_{428} = 136$ mM$^{-1}$·cm$^{-1}$ for ZnPP-L29E Mb, respectively, as calculated using the standard hemochromogen method [6].

1.3 Mass spectrometry

H64A Mb mutant was newly constructed in this study, and the protein mass spectrum measurement was carried out on G2-XS QToF mass spectrometer (Waters). The desalted protein solution (~20 μM) was mixed with 1% formic acid, which was transferred into the mass spectrometer chamber for measurement under positive mode. The multiple m/z peaks were transformed to the protein molecular weight by using software MaxEnt1.

1.4 Photo-induced DNA cleavage studies

DNA photo-cleavage activities of reconstituted ZnPP-Mbs (ZnPP-WT Mb, ZnPP-F43H Mb, ZnPP-H64A Mb and ZnPP-F43H/H64A Mb) were measured using plasmid DNA relaxation assay. The supercoiled pUC19 DNA (0.01 μg/μL) was treated with corresponding ZnPP-Mbs (10 μM), and the sample was photo-irradiated for various time intervals by using a LED light resource (420~440 nm, 3W) in potassium phosphate buffer (100 mM, pH 7.0) at 37 °C. The reaction products were stored at -20 °C with an addition of loading buffer. The samples were loaded to a 0.8% agarose gel and electrophoresed in at 75 V for 2 hrs in TAE buffer (40 mM Tris base, 2 mM EDTA and 20 mM acetic acid). After electrophoresis, the gel was stained with an ethidium bromide solution (0.2 μg/mL) for 30 min, then photographed and analyzed on the Tanon-1600 gel imaging system. Inhibition studies of DNA photocleavage were performed by addition of various scavengers or quencher, including histidine, dimethyl sulfoxide (DMSO), mannitol, superoxide dismutase.
(SOD), ascorbic acid, and NaN₃. The following procedure was similar to the DNA cleavage activity study. Control experiments with free ZnPp and hemin were performed under the same conditions. The bands in gel electrophoresis were quantified by Tanon 1600/1600R Gel Imaging System (Tanon Science & Technology Co., Ltd., China).

1.5 Spin-trapping studies

Electron paramagnetic resonance (EPR) spin trapping was used to detect hydroxyl radical (·OH) and singlet oxygen (¹O₂) formed by photo-irradiation of ZnPp-Mbs (ZnPp-WT Mb, ZnPp-F43H Mb, ZnPp-H64A Mb and ZnPp-F43H/H64A Mb). The trapping agents, DMPO and TEMP, were used for the detection of ·OH and ¹O₂, respectively. For the detection of ·OH, the sample containing 0.5 mM reconstituted protein and 100 mM DMPO was photo-irradiated for 10 min at 37 °C in potassium phosphate buffer (100 mM, pH 7.0), then was transferred into four EPR capillary tubes with total volume of 40 μL. The spectra were recorded on Bruker A300 spectrometer (X-band) at room temperature, with frequency of 9.43 GHz, center field 3510 G and sweep width 110 G, microwave power 0.595 mW and modulation amplitude 3.0 G. Under the same conditions, the detection assay of ¹O₂ generation by ZnPp-Mbs was performed by the addition of trapping agent TEMP.
**Figure S1.** MS spectra of H64A Mb: calculated molecular weight for the apo-protein, 17265 Da, and the observed, 17264.5±0.5 Da.
Figure S2. UV-vis spectra of Mbs (black solid line) and ZnPP-Mbs (red solid line) in potassium phosphate buffer (100 mM, pH 7.0). (A) WT Mb and ZnPP-WT Mb; (B) F43H Mb and ZnPP-F43H Mb; (C) H64A Mb and ZnPP-H64A Mb; and (D) F43H/H64A Mb and ZnPP-F43H/H64A Mb.
Figure S3. Agarose gel electrophoresis assay of photo-induced pUC19 DNA (0.01 μg/μL) cleavage by WT Mb (A), F43H Mb (B), F43H/H64A Mb (C), H64A Mb (D), ZnPP (E) and hemin (F) (10 μM) after irradiation at 420~440 nm at 37 °C, respectively. Lanes 2-6, sample exposure time was 15, 30, 45, 60, 90 min; lane 1, DNA alone stocked under irradiation, and lane 7, DNA incubated with protein in dark for 90 min. SC and NC represent supercoiled circular DNA and nicked circular DNA, respectively.
Figure S4. (A) UV-vis spectra of L29E Mb (black solid line) and ZnPP-L29E Mb (red solid line) in 100 mM KH₂PO₄ buffer (pH 7.0). (B) Agarose gel electrophoresis assay of photo-induced pUC19 DNA (0.01 μg/μL) cleavage by ZnPP-L29E Mb after irradiation at 420~440 nm at 37 °C for 15, 30, 45, 60, 90 min (lanes 2-6), lane 1, DNA alone stocked under irradiation, and lane 7, DNA incubated with protein in dark for 90 min. SC and NC represent supercoiled circular DNA and nicked circular DNA, respectively.
Fig. S5 (A) Agarose gel electrophoresis of pUC19 DNA after incubation with ZnPP-F43H Mb or ZnPP-F43H/H64A Mb (10 µM) under photo-irradiation at 37 °C for 45 min in the presence of \(^1\text{O}_2\) scavenger, NaN\(_3\) (1 mM). UV-Vis spectra of ZnPP-F43H Mb (B) and ZnPP-F43H/H64A Mb (C) (5 µM) in the absence (black line) and presence (red line) of NaN\(_3\) (1 mM) in potassium phosphate buffer (100 mM, pH 7.0).
Figure S6. EPR spectra of TEMP adducts of singlet oxygen generated by Mbs or ZnPP-Mbs. Spectra were recorded after irradiation at 420~440 nm for 30 min at 37 °C in the presence of 0.5 mM protein and 5 mM TEMP. (A) WT Mb and ZnPP-WT Mb; (B) F43H Mb and ZnPP-F43H Mb; (C) H64A Mb and ZnPP-H64A Mb; (D) F43H/H64A Mb and ZnPP-F43H/H64A Mb.
References:


