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DNA cleavage with oxymyoglobin and cysteine-introduced metmyoglobin†

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Double strand DNA was cleaved oxidatively by incubation with oxygenated myoglobin, and Lys96Cys sperm whale myoglobin in its stable ferric form functioned as an artificial nuclease under air by formation of an oxygenated species, owing to electron transfer from the SH group of the introduced cysteine to the heme.

For many enzymes, metal centres function as active sites in biological redox reactions, and much research has been performed 15 on the development of artificial metal centres for applications in molecular biology, biotechnology, and chemotherapy.[1-6] For example, artificial metal complexes and metal-binding peptides have been reported for DNA scission, repair, and signal transduction.[2,3,7-15] Bleomycin has been shown to cleave DNA, 20 where the low-spin ferric-hydroperoxo species, BLM-Fe^{III}-OOH, was detected before DNA cleavage, following production of the ferryl-oxo species and hydrogen-atom abstraction from the C-4' of a deoxyribose sugar moiety.[7,16,17] For metalloproteins, heme proteins such as horse myoglobin (Mb) and bovine cytochrome *c* 25 have been shown to cleave DNA in the presence of hydrogen peroxide.[18,19]

Mb stores molecular oxygen in muscles. Most of the Mb proteins consist of eight α-helices, forming a globular structure (Figure 1).[20,21] A histidine is coordinated to its heme iron, to which 30 molecular oxygen binds at the opposite position of the coordinated histidine. We have shown that the heme of cysteine-introduced sperm whale metMb is reduced by electron transfer from the introduced cysteine to the heme during incubation, and carbon monoxide binds to the heme, although most of the mammalian Mb 35 do not possess a cysteine residue.[22] In this study, we show that double strand DNA is cleaved oxidatively by incubation with oxygenated Mb (oxyMb). We also show that cysteine-introduced sperm whale metMb converts to oxyMb by incubation under air, and thus it exhibits DNA cleavage activity without addition of a 40 strong reducing agent.

DNA cleavage by sperm whale oxyMb was investigated under agarose gel electrophoresis. Nicked circular (~26 %) and linear

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(~34 %) forms of plasmid DNA were observed after incubation of 45 supercoiled pUC19 DNA with wild-type (WT) oxyMb for 5 min, whereas no cleavage of DNA was detected after incubation with WT metMb for 20 min (Figure 2). Similar results for DNA cleavage were obtained for WT horse Mb (ESI†, Figure S1).
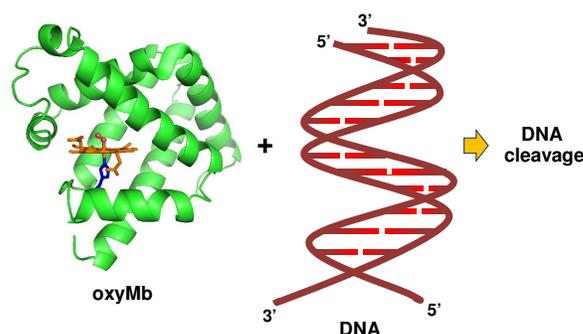

Fig. 1. Schematic representation of sperm whale oxyMb (PDB: 1A6M) and double strand DNA. The heme (orange) and coordinated histidine (blue) of Mb are shown as stick models. The coordinated dioxygen of Mb is shown 65 as a sphere and stick model in red. Mb may interact with DNA at its minor groove.
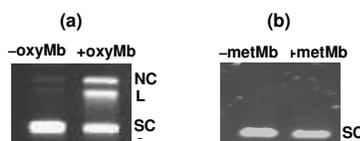

Fig. 2. Agarose gel electrophoresis of pUC19 DNA after incubation with WT sperm whale (a) oxyMb and (b) metMb. Incubation was performed under air at 37 °C for 5 and 20 min for oxyMb and metMb, respectively. Incubation of pUC19 DNA (0.02 μg/μl) with Mb (8 μM) was performed in 10 mM potassium phosphate buffer, pH 7.2. SC, NC, and L represent supercoiled, nicked circular, and linear forms of DNA, respectively.

The double strand DNA cleavage activity of oxyMb did not change significantly in the presence of low NaCl concentration (50 mM), whereas it was inhibited in the presence of high NaCl concentration (500 mM) (Figure 3, lanes 3 and 4). These results show that weak electrostatic interaction exists between oxyMb and 85 DNA. The DNA cleavage activity decreased significantly in the presence of a minor groove binder, 4',6-diamidino-2-phenylindole (DAPI), but not in the presence of a major groove binder, methyl green (Figure 3, lanes 5 and 6), indicating that sperm whale oxyMb interacted with the minor groove of DNA. Interaction of proteins

with the minor groove of DNA has been reported for several proteins, including the TATA-binding protein, male sex determining factor SRY, lymphoid enhancer-binding factor 1, and the integration host factor.[23] DNA cleavage was also performed by incubation of pUC19 DNA with WT sperm whale oxyMb for 20 min in the presence of radical scavengers, such as singlet oxygen scavengers (histidine and sodium azide), a superoxide scavenger (ascorbic acid), and hydroxyl radical scavengers (potassium iodide and mannitol) (Figure 3, lanes 7–11). Histidine, sodium azide, potassium iodide, and mannitol did not inhibit DNA cleavage activity of WT oxyMb. Furthermore, no inhibition was observed in the DNA cleavage activity by an addition of catalase (Figure 3, lane 13). These results indicated that singlet oxygen and hydroxyl radical were not responsible for the DNA cleavage.[24] Although ascorbic acid inhibited DNA cleavage (Figure 3, lane 9), it has been reported that superoxide anion does not react directly with DNA.[25] In addition, no inhibition of DNA cleavage was observed by an addition of superoxide dismutase (SOD) or both SOD and catalase (Figure 3, lanes 12 and 14). These results indicate that superoxide was also not involved in the DNA cleavage reaction.
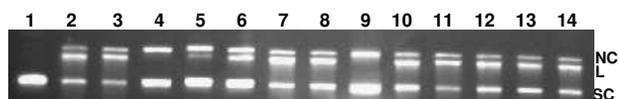
Fig. 3. Agarose gel electrophoresis of pUC19 DNA after incubation with WT sperm whale oxyMb at 37 °C for 20 min in the presence of various reagents: Lane 1, DNA control; lanes 2–14, with oxyMb; lane 3, with 50 mM NaCl; lane 4, with 500 mM NaCl; lane 5, with 50 μM DAPI; lane 6, with 50 μM methyl green; lane 7, with 25 mM histidine; lane 8, with 25 mM sodium azide; lane 9, with 50 μM ascorbic acid; lane 10, with 50 mM potassium iodide; lane 11, 50 mM mannitol; lane 12, with SOD (15 units); lane 13, with catalase (15 units); lane 14, with SOD (15 units) and catalase (15 units). SC, NC, and L represent supercoiled, nicked circular, and linear forms of DNA, respectively. Incubation conditions were the same as those in Figure 2.

Ligation assay was performed using T4 DNA ligase to determine whether the plasmid DNA was cleaved with WT sperm whale oxyMb oxidatively or hydrolytically (ESI[†], Figure S2). The nicked circular and linear DNA products obtained by incubation with oxyMb were isolated and treated with T4 ligase, but cells did not grow after transformation. These results showed that the cleavage of DNA by oxyMb was oxidative.

Horse Mb has been shown to cleave DNA by forming the active ferryl-oxo species.[18] Ascorbic acid inhibited the DNA cleavage with oxyMb (Figure 3, lane 9). Ascorbic acid has been reported to react with the ferryl-oxo species of the heme,[26] and thus the active ferryl-oxo species may have been produced from oxyMb in the presence of DNA. A structural change in oxyMb may be induced by DNA, and the bound dioxygen may react with a nearby amino acid, producing an active ferric-hydroperoxo or/and ferryl-oxo species and an amino acid radical. It is known that the ferric-hydroperoxo (Fe^{III}-OOH) species easily converts to an active ferryl-oxo species ($Fe^{IV}=O$) by receiving an electron from the protein moiety in Mb.[7] In fact, it has been proposed that the ferric-hydroperoxo and ferryl-oxo species of bleomycin are responsible for the hydrogen atom abstraction from the sugar moiety on the DNA strand.[16] In the present study, the produced ferryl-oxo species may have attacked the DNA, initiating cleavage of the double strand. Production of a predominant amount of the linear form of DNA along with its nicked circular form by incubation with

oxyMb indicated effective double-strand DNA cleavage, as in the case of using bleomycin.[7,27] Therefore, we suggest a similar DNA cleavage mechanism for oxygenated Mb and bleomycin. Interestingly, ascorbic acid inhibited the amount of double-strand DNA cleavage (Figure 3, lane 9). Ascorbic acid may function as a reducing agent and promote the formation of the ferryl-oxo species from oxyMb with decrease in production of amino acid radicals. However, detailed study is necessary to elucidate the mechanism of DNA cleavage with oxyMb.

The heme of cysteine-introduced mutant Mb has been reported to be reduced under CO atmosphere by electron transfer from the cysteine residue to the heme.[22] Therefore, we envisaged that the intramolecular electron transfer in K96C sperm whale metMb, in which Lys96 is positioned close to the heme (< 10 Å) (ESI[†], Figure S3), may induce formation of the oxygenated species and initiate DNA cleavage. By incubation of K96C metMb at relatively high protein concentration (460 μM) under air, new peaks were generated at 543 and 581 nm in its absorption spectrum, whereas the intensity of the 504-nm absorption band decreased (Figure 4). The 543- and 581-nm bands are characteristic for oxyMb, whereas the 504-nm band is characteristic for metMb.[28] However, at relatively low protein concentration (8 μM), changes in the absorption spectrum of K96C metMb were not significant by incubation under air at 37ºC for 6 h (ESI[†], Figure S4). In addition, the spectral changes in the 500−600 nm region were not detectable for WT Mb even by incubation at high protein conditions (ESI[†], Figure S5). These results show that the heme of K96C metMb was reduced by incubation at high protein concentrations, and subsequently molecular oxygen bound to the reduced heme, although the electron transfer from the cysteine residue to the heme by incubation under air was less effective than that under CO atmosphere.[22] In addition to the observation of the absorption bands related to the oxygenated complex of K96C metMb in the absorption spectrum after the incubation, a dimer band was detected in the SDS-PAGE analysis in the absence of 2-mercaptoethanol (ESI[†], Figure S6). The dimer band disappeared with an addition of 2-mercaptoethanol in the SDS-PAGE analysis (ESI[†], Figure S6). These results show that K96C metMb dimerized under air through a disulphide bond, presumably due to electron transfer from the SH group of the introduced K96C cysteine to the oxidized heme forming a cysteinyl radical, which dimerized.
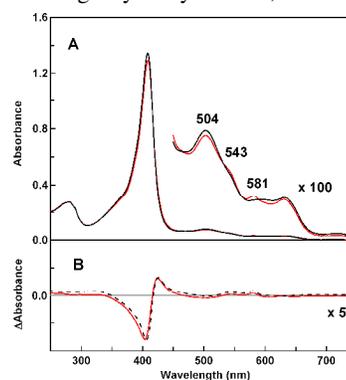
Fig. 4. Absorption spectra of K96C sperm whale metMb before (black) and after (red) incubation at high protein concentration (460 μM) under air at 37 ºC for 6 h. A normalized difference spectrum between K96C oxyMb and metMb is also depicted by the black broken line. The absorption spectra of Mb (8 μM) was measured in 10 mM potassium phosphate buffer, pH 7.2.

The amount of nicked circular and linear forms of DNA observed in the agarose gel electrophoresis gradually increased by longer incubation of supercoiled pUC19 DNA with K96C sperm whale metMb (0–120 min) (Figure 5), whereas only the initial supercoiled form of DNA was detected after incubation with WT metMb (Figure 2b). Cleavage of pUC19 DNA decreased significantly in the presence of DAPI (ESI†, Figure S7), indicating that interaction of K96C Mb with the minor groove of DNA was important, similar to the interaction of WT oxyMb with DNA. DNA cleavage by K96C metMb also showed effective double-strand DNA cleavage and was inhibited with ascorbic acid but not with histidine, potassium azide, potassium iodide, or mannitol, as in the case for oxyMb, supporting the hypothesis that the oxygenated complex of K96C Mb was involved in the DNA cleavage (ESI†, Figure S7). The cells did not grow after transformation of the isolated nicked circular and linear DNA products obtained by incubation with K96C metMb and subsequent treatment with T4 ligase (ESI†, Figure S2), showing that the DNA cleavage by K96C metMb was also oxidative. However, the K96C metMb dimer with an intermolecular disulfide bond cleaved pUC19 DNA more effectively than WT oxyMb (ESI†, Figure S8), indicating the importance of the cysteine residue (K96C) but a different mechanism for DNA cleavage with the K96C metMb dimer compared to WT oxyMb.

DNA cleavage was not observed by incubation of pUC19 DNA with V66C or K102C metMb, where the distance between the heme and introduced cysteine was longer for these mutants than that of K96C metMb (ESI†, Figure S9). These results show that the position of the cysteine introduced in the protein is important for the DNA cleavage activity. It has been shown that dimerization of mutant Mb under CO atmosphere occurs one or two orders faster in K96C Mb than in K102C and V66C Mb, which is related to the effectiveness of electron transfer from the cysteine to the oxidized heme.[22] These results support the hypothesis that oxyMb produced by electron transfer initiated DNA cleavage.
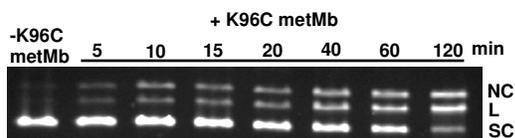

Fig. 5. Agarose gel electrophoresis of pUC19 DNA after incubation with K96C sperm whale metMb. Incubation was performed under air at 37 °C for 0–120 min. SC, NC, and L represent supercoiled, nicked circular, and linear forms of DNA, respectively. Incubation conditions were the same as those in Figure 2.

In summary, oxyMb initiated oxidative cleavage of double strand DNA. By incubation of K96C metMb under air, oxygenated and intermolecular disulfide-linked Mb dimer was formed. These results imply electron transfer from the introduced cysteine to the oxidized heme in K96C metMb. K96C metMb also exhibited DNA cleavage activity, acting as a DNA nuclease. Most metal complexes and metalloproteins rely on an addition of strong reducing agents to bring about a redox active species necessary for the nuclease activity. In the present study, the double strand DNA cleavage occurred in the absence of a strong reducing agent, and thus electron transfer from a cysteine to a heme may be a useful tool for development of artificial DNA nucleases.

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