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Kyoto University
Electron injection from mitochondrial transcription factor A to DNA associated with thymine dimer photo repair

Fumitaka Hashiyaa, Shinji Itoe, Hiroshi Sugiyamaab,

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a Department of Chemistry, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo, Kyoto 606-8502, Japan
b Institute for Integrated Cell-Material Sciences, Institute for Advanced Study, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
c Medical Research Support Center, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

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ABSTRACT

Electron transfer through π-stacked arrays of double-stranded DNA contributes to the redox chemistry of bases, including guanine oxidation and thymine–thymine dimer repair by photolyase. 5-Bromouracil is an attractive photoreactive thymine analogue that can be used to investigate electron transfer in DNA, and is a useful probe for protein–DNA interaction analysis. In the present study using BrU we found that UV irradiation facilitated electron injection from mitochondrial transcription factor A into DNA. We also observed that this electron injection could lead to repair of a thymine–thymine dimer.

1. Introduction

A charge transfer through π-stacked arrays of double-stranded DNA contribute to critical redox chemistry for DNA. For example, a positive charge transfer promotes oxidative damage to guanine in DNA, which possibly relates to the presence of mutation hot spots in the genome.1–4 It has also been reported that an electron transfer from FADH² through tryptophan residues is crucial for DNA repair involving thymine–thymine dimer photo repair by photolyase.5,6 5-Bromouracil (BrU) is an attractive photoreactive thymine analogue that can be used to investigate electron transfer in dsDNA: because electron transfer within DNA generates a reactive 5-yl-uracil radical from BrU, such an electron transfer can be detected by the consequent reaction product. Replacement of thymine in DNA with BrU results in retention of almost all the functional properties of DNA, even in living cells. Hence, BrU has also been used to evaluate protein–DNA interactions.7–15 Using BrU we previously demonstrated electron transfer from the archaeal DNA-binding protein sso7d to DNA and showed that this electron transfer repaired thymine–thymine dimers in a similar fashion to photolyase.16,17

Mitochondrial transcription factor A (TFAM) is a DNA-binding protein comprising a tandem high-mobility group (HMG) box domain and a short C-terminal tail; it plays a critical role during transcription, replication, and maintenance of the mitochondrial genome. The mammalian mitochondrial genome contains three promoters: light-strand promoter (LSP), heavy-strand promoter 1 (HSP1), and heavy-strand promoter 2 (HSP2), which drive mitochondrial DNA (mtDNA) transcription. Because truncated RNA transcripts from LSP initiate mtDNA replication, TFAM is also an essential factor for replication; thus, knockout of TFAM is embryonic lethal18,19 and knockdown leads to a decreased copy number of mtDNA.20 TFAM also contributes to form nucleoids, which resemble the nucleosome in eukaryotes. Ngo and coworkers reported the crystal structures of TFAM binding to LSP, HSP1, and non-sequence-specific DNA in which DNA was constrained into a U-shape to allow compaction and stabilization of mtDNA.21,22 Approximately 1000 molecules of TFAM are present per mtDNA molecule, which is enough to cover the entire length of mtDNA given that TFAM occupies about 25 bp.23 In the present study, we focused on tyrosine and tryptophan residues located in the DNA-binding site of human TFAM that were sufficiently close together to cause long-range electron transfer for thymine residues which can be replaced with BrU, and demonstrated electron injection from TFAM into DNA using a BrU probe. TFAM bound to DNA not only through the strongly positioning sequence in LSP or HSP1, but also in a non-sequence-specific manner, and injected electrons into the DNA. We also demonstrated that the electron injection facilitated photo repair of thymine–thymine dimers.
2. Material and methods

2.1. Preparation of recombinant TFAM

The human TFAM cDNA was prepared by PrimeScript RT reagent Kit (TaKaRa). PCR was carried out with primer 1 and 2 (Supplementary Table 1) to connect NdeI and XhoI sites using KOD plus Neo (Toyobo). The product which encodes residues 43–246, corresponding to full length TFAM after cleavage of the N-terminal mitochondrial leader sequence was inserted into pET28a expression vector. The sequence confirmed TFAM expression plasmid was transformed into BL21(DE3). Single colony was incubated with 500ml of auto-inducer medium, LB medium containing 2g/l lactose, 400mg/l glucose and 150mg/l MgSO4, containing 50μg/l kanamycin at 37°C for 15h. The cells were harvested and resuspended with resuspend buffer (20mM Tris-HCl, 100mM NaCl, 20mM imidazole, 1mM benzamidine, 1mM 2-mercaptoethanol, pH 7.5). The resuspended solution was incubated with 10 units/ml benzonase nuclease (SIGMA-ALDRICH) and 1mg/ml lysozyme (Nacalai) at 4°C for 1h and subsequently sonicated for 5min (10 sec on and 20 sec off) on ice. After centrifugation, the supernatant was purified by affinity chromatography using HisTrap FF (GE healthcare) on AKTA pure 25 protein purification system (GE healthcare) with elution buffer (20mM Tris-HCl, 500 mM NaCl, 40–400 mM imidazole, 1 mM benzamidine, 1 mM 2-mercaptoethanol, pH 7.5). The fractions containing His-tagged TFAM were subsequently purified by gel filtration chromatography using Superdex 200 Inclusion 10/300 GL (GE healthcare) with gel filtration buffer (20mM Tris-HCl, 300mM NaCl, 1 mM 2-mercaptoethanol, pH 7.5). The fractions containing His-tagged TFAM were concentrated by Amicon Ultra 4 (Merck) which cutoff molecular weight bellow 10kDa and stored at 4°C or −80°C for long term. The concentration of recombinant TFAM was measured by Qubit 4 Fluorometer (ThermoFisher).

2.2. Preparation of DNA fragments

FAM labelled LSP top 1–4 and HSP top 1–4 DNA fragments were purchased from Japan Bio Services. The complementary strands for the fragments, LSP bottom or HSP bottom, were purchased from SIGMA-ALDRICH. Thymine dimer containing DNA fragment, LSP top T< T, was purchased from Tsukuba Oligo Service. Each top strand and its complementary bottom strand were mixed in irradiation buffer (10mM sodium cacodylate, 10mM NaCl, pH 7.0) and incubated at 37°C for 15h and subsequently sonicated for 5 min. After the incubation the temperature was gradually decreased to form double stranded LSP or HSP. Non-sequence specific DNA was prepared by conventional PCR amplification with 601 core sequence as a template, primer 3 and TexasRed labeled primer 4 except that dBrUTP was used instead of dTTP. The sequences of each DNA oligo are shown in Supplementary Table 1. 601 core sequence is as follows:

5’dTGGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTGTAGCAAGC TCTAGCACCCTAAACGCCACGTACCGCGTGTCACCCCGCCTTTATACC
1 μM LSP or HSP DNA fragment was dissolved in irradiation buffer in the absence or in the presence of 1.4 μM TFAM and incubated for 30 min at room temperature. The volume of each samples was 40 μl. Photoirradiation was performed under aerobic condition using a 3UV™ Transilluminator (UVP) for 302 nm irradiation or a HM-3 hyper monochromatic light source (JASCO) for 280, 300 and 310 nm monochromatic irradiation. After the irradiation, 5 μl of 10x uracil DNA glycosylase (UDG) buffer, 5 U of UDG (NEW ENGLAND BioLabs) and 5 μl of Milli Q water were added for each samples and incubated at 37 °C for 60 min for locating uracil residues or 5 μl of 10x pyrimidine dimer glycosylase (PDG) buffer, 20 U of PDG (NEW ENGLAND BioLabs), 0.5 μl of 100x BSA and 4.5 μl of Milli Q water were added and incubated at 37 °C for 180 min for locating thymine dimer. Finally, all reaction mixtures were dried up using vacuum, dissolved in 40 μl of Loading Dye (prepared from 300 μl of EDAT, 200 μl of Milli Q water and 10 μl of formamide colored with New fuchsin) and heated at 95°C for 5 min. Mini slab gel filled with denaturing polyacrylamide gel (8 M urea, 20% polyacrylamide, 1x TBE buffer, pH 8.3) was used to detect DNA cleavages. Each LSP or HSP fragments treated with piperidine and heat which produced cleavage on 5-halopyrimidine site, in this case BrU, were applied as ladder in the PAGE assay.

2.3. Photoirradiation and denaturing polyacrylamide gel electrophoresis (PAGE) assay for LSP or HSP

100 nM non-sequence specific DNA fragment was dissolved in irradiation buffer in the absence or in the presence of 600 nM TFAM and incubated for 30 min at room temperature. The volume of each samples was 15 μl. Photoirradiation was performed under aerobic condition using a 3UV™ Transilluminator (UVP) for 302 nm irradiation. After the irradiation, 3 μl of 10x UDG buffer, 5 U of UDG and 12 μl of Milli Q water were added for each samples and incubated at 37 °C for 60 min. Finally, all reaction mixtures were dried up using vacuum, dissolved in 15 μl of Loading Dye and heated at 95 °C for 5 min. DNA sequence ladder was prepared from Texas Red labeled primer 4 using Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit. Photo irradiated samples dissolved in Loading Dye were analyzed with sequence ladder by SQ5500E (HITACHI) slab gel sequencer filled with denaturing polyacrylamide gel (6 M urea, 6% polyacrylamide, 1x TBE buffer, pH 8.3) to detect DNA cleavages.

2.4. Photoirradiation and slab gel sequencing assay for non-sequence specific DNA

1.4 μM TFAM was dissolved in irradiation buffer in the absence or in the presence of 1 μM LSP4 and incubated for 30 min at room temperature. Photoirradiation was performed under aerobic condition using a 3UV™ Transilluminator (UVP). After drying up using vacuum, all samples were dissolved in SDS sample buffer (30 mM Tris-HCl, 1% SDS, 2.5% sucrose, 50 mM 2-mercaptoethanol, pH 6.8) and heated at 95 °C for 5 min. SDS-PAGE was carried out to separate each products and In-Gel Tryptic Digestion Kit (ThermoFisher) was used to obtain peptide fragments. Tryptic digestion samples were analyzed using TripleTOF5600+ (SCIEX).

3. Results

3.1. Electron injection into LSP or HSP

A TFAM–DNA complex comprising recombinant human TFAM and a 28-bp DNA fragment derived from LSP was used for the photoirradiation experiment. Four LSP variants (LSP1–4) were prepared by replacing thymines 21 or 22, which are close to the HMG box A in TFAM, with BrU. According to the crystal structure of the TFAM–LSP complex (PDB ID: 3TMM), the distances between thymines 21 and 22 and the proximate aromatic amino acid, Tyr57, are 5.87 and 8.36 Å, respectively. This is close enough to cause long-range electron transfer (Fig. 1A). The binding affinity for an LSP1 that did not contain BrU was evaluated by electrophoretic mobility shift assay (EMSA). The decrease in the amount of free LSP reached a plateau at 1.0 eq TFAM (Fig. 1B). The TFAM-binding affinity of an LSP2 in which both thymines 21 and 22 were replaced with BrU was almost identical to that of LSP1, suggesting that BrU replacement did not affect the binding affinity (Fig. S1).
As described previously, photoirradiation at 302 nm and subsequent treatment with heat and uracil–DNA glycosylase (UDG) selectively produced DNA cleavage at uracil sites generated from BrU by electron transfer (24). Incidentally, these DNA cleavages were not produced in the absence of UDG (Fig. S2). Denaturing polyacrylamide gel electrophoresis (PAGE) assay demonstrated that photoirradiation of an LSP1 fragment that did not contain BrU did not produce cleavage in either the absence or presence of TFAM (Fig. 1C). In contrast, photoirradiation of LSP2, LSP3, and LSP4 in the presence of TFAM led to dramatic DNA cleavage at the locations of the uracil residues generated by electron transfer, whereas little DNA cleavage was observed in the absence of TFAM (Fig. 1D, E, and F). Photoirradiation also produced an upper band shift in the presence of TFAM that resulted from a TFAM–DNA crosslink. According to the intensity analysis of all bands resulting from crosslinked, full-length, and cleaved products, LSP4, which possesses a BrU at position 22, showed a higher efficiency of DNA cleavage than LSP3, which possesses a BrU at position 21. This suggested that a BrU at position 22 more effectively generated uracil radicals.

Further analysis of the TFAM–LSP4 crosslinked product was carried out by SDS–PAGE. On a fluorescence image, DNA band shifts were observed with increasing photoirradiation time, which is consistent with the results of denaturing PAGE (Fig. 2A). Photoirradiation in the presence of LSP4 led to the appearance of two protein bands in the Coomassie Brilliant Blue-stained image (Fig. 2B). The retardation of a lower band matched the shifted band in the fluorescence image. LS/MS/MS analysis revealed that both an upper and a lower band contained peptide fragments derived from TFAM; therefore, the lower band contained a TFAM–LSP4 crosslinked product and the upper band possibly contained covalently crosslinked TFAM proteins.

Similar photoirradiation experiments were carried out using another TFAM–DNA complex comprised of TFAM and a 22-bp DNA fragment derived from HSP1. Four variants of HSP were prepared by replacing thymines 19 or 20, which are close to the HMG box B in TFAM. According to the crystal structure of the TFAM–HSP1 complex (PDB ID: 4NOD), the distances between thymines 19 and 20 and the proximate aromatic amino acid, Tyr162, are 6.91 and 8.08 Å, respectively (Fig. 3A). The binding affinity of HSP differed slightly from that of LSP. The decrease in the amount of free HSP reached a plateau at 0.8 eq of TFAM whereas that of LSP reached a plateau at 1.0 eq (Fig. 3B) and the BrU replacement did not affect the TFAM-binding affinity for HSP (Fig. S3). The photoirradiation for these TFAM–HSP complexes gave similar results to those obtained with the TFAM–LSP complex (Fig. 3C, D, E, and F). It is noteworthy that HSP4, which possesses a BrU at position 20, showed higher photoreactivity than HSP3, which possesses a BrU at position 21. This suggested that a 3′ BrU was more photoreactive than a 5′ BrU in photoirradiation of both LSP and HSP.
even though the 3’ BrU is more distant from the proximate tyrosine than the 5’ BrU. Previously, we examined the photoreactivity of BrU-substituted DNA and reported that uracil radicals were effectively generated in certain sequences: 5’/3’-G/C[A]n=1, 2, 3 BrUBrU-3’/5’. We termed this a “hot-spot sequence”. In BrU-substituted DNA, photoirradiation of the hot-spot sequence facilitates electron transfer from G and the electron is trapped by BrUBrU to form anion radical [BrUBrU]•−. Subsequent release of a Br− generates the uracil-5-yl radical24,25. We
performed photoirradiation of the self-complementary hot-spot sequence 5′-CGA\textsubscript{BrU}BrUBrUCG-3′ and 5′-GCBrUBrUAAGC-3′. HPLC analysis revealed that an uracil residue was effectively generated from a 3′ Br\textsubscript{U} rather than 5′ in both hot-spot sequences\textsuperscript{24}. These results suggest that a 3′ Br\textsubscript{U} is more photoreactive than 5′ in [Br\textsubscript{U}Br\textsubscript{U}]•− and releases Br− effectively to generate uracil radical. Consistent with the results, a 3′ Br\textsubscript{U} in both LSP and HSP effectively generated uracil radical in the present study.

3.2. Electron injection into nonspecific DNA

In addition to the strongly positioning sequences in LSP and HSP1, it has been reported that TFAM interacts nonspecifically with DNA to compact it. To evaluate the electron transfer to a non-sequence-specific DNA, a 147-bp DNA fragment in which all thymine residues were substituted with \textsuperscript{32}P was prepared and used to photoirradiation. Further, this electron transfer facilitated photo repair of thymine–thymine dimers. Photolyase catalyzes electron transfer from FADH\textsuperscript{+} to DNA through its tryptophan residues to repair thymine–thymine dimers, a common UV-induced DNA lesion\textsuperscript{5,6}. In addition, facilitation by photoirradiation of electron transfer from an aromatic amino acid residue, especially tryptophan or tyrosine, to DNA repairs the thymine–thymine dimer\textsuperscript{16,17,26,27}. Because we had demonstrated electron transfer from TFAM, we evaluated the photo repair of thymine–thymine dimers by electron transfer. A DNA fragment LSP-T < > T in which thymines 21 and 22 formed a thymine–cyclobutane dimer, was prepared and used to generate a thymine–cyclobutanetamine dimer by electron transfer. A DNA fragment LSP-T < > T, in which thymines 21 and 22 formed a thymine–cyclobutane dimer, was prepared and used to generate a complex with DNA. Consistent with previous results, photoirradiation of the non-sequence-specific DNA produced cleavage at hot-spot sequences and positions at positions 1–4 in both the absence and presence of TFAM (Fig. 4B). Besides such background cleavage, TFAM enhanced the photosensitivity of the DNA and produced new DNA cleavage mainly at Br\textsubscript{U}-substituted sites. These results suggest that TFAM can bind to DNA in a non-sequence-specific manner and cause electron transfer.

3.3. Thymine-thymine dimer photo-restoration

Photolyase catalyzes electron transfer from FADH\textsuperscript{+} to DNA through its tryptophan residues to repair thymine–thymine dimers, a common UV-induced DNA lesion\textsuperscript{5,6}. In addition, facilitation by photoirradiation of electron transfer from an aromatic amino acid residue, especially tryptophan or tyrosine, to DNA repairs the thymine–thymine dimer\textsuperscript{16,17,26,27}. Because we had demonstrated electron transfer from TFAM, we evaluated the photo repair of thymine–thymine dimers by electron transfer. A DNA fragment LSP-T < > T, in which thymines 21 and 22 formed a thymine–cyclobutane dimer, was prepared and used to generate a complex with DNA. Consistent with previous results, photoirradiation of the non-sequence-specific DNA produced cleavage at hot-spot sequences and positions at positions 1–4 in both the absence and presence of TFAM (Fig. 4B). Besides such background cleavage, TFAM enhanced the photosensitivity of the DNA and produced new DNA cleavage mainly at Br\textsubscript{U}-substituted sites. These results suggest that TFAM can bind to DNA in a non-sequence-specific manner and cause electron transfer.

4. Discussion

A prokaryotic DNA packing protein, HU, which like TFAM constrains DNA into a U-shape, preferentially interacts with damaged DNA, for example at a base-pair mismatch or base flip out, and recruits damage-repair enzymes\textsuperscript{28}. TFAM also preferentially interacts with cisplatin-damaged DNA and oxidized DNA\textsuperscript{29}. In the present study, we detected no binding preference of TFAM for intact DNA over damaged DNA containing thymine–thymine dimers. However, there is sufficient TFAM in mitochondria to cover the entire mtDNA and allow damaged base pairs to interact with TFAM. TFAM could interact not only with strongly positioning sequences on LSP and HSP, but also with a non-sequence-specific DNA, causing electron transfer into DNA during photoirradiation. Further, this electron transfer facilitated photo repair of thymine–thymine dimers.

mtDNA is physiologically vulnerable because of the mitochondrial respiratory chain that generates reactive oxygen species (ROS), and may therefore accumulate more oxidative DNA damage than nuclear DNA. It is known that oxidative DNA damage in mitochondria is repaired by base excision repair (BER).\textsuperscript{30} However, the possibility of repair of thymine–thymine dimers in mtDNA by BER has been discounted by radioisotope-labeled thymidine incorporation experiments, although the accumulation of thymine–thymine dimers depletes mtDNA.\textsuperscript{31} TFAM contributes to mtDNA replication because the truncated DNA transcripts from LSP become a primer to initiate mtDNA replication. Further, this electron transfer facilitated photo repair of thymine–thymine dimers by electron transfer and thereby maintains mtDNA.

5. Conclusion

Using \textsuperscript{32}P as a probe, we demonstrated electron transfer from TFAM to DNA that was facilitated by photoirradiation. TFAM effectively interacted with not only strongly positioning sequences in LSP and HSP1 but with a non-sequence-specific DNA fragment to cause electron transfer. The binding affinity of TFAM for intact DNA and damaged DNA containing thymine dimers was identical and the electron transfer from TFAM enhanced thymine dimer photo repair. The contribution of TFAM to mtDNA maintenance remains elusive and the present results may provide an important insight into mtDNA stabilization by TFAM.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2018.11.044.

References


