Bioorganic & Medicinal Chemistry 24 (2016) 4254–4262

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Simple and accurate single base resolution analysis of 5-hydroxymethylcytosine by catalytic oxidative bisulfite sequencing using micelle incarcerated oxidants

Seketsu Fukuzawa [a,b,*], Saori Takahashi [c], Kazuo Tachibana [a], Shoji Tajima [c], Isao Suetake [b,c,*]

[a] Department of Chemistry, School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[b] CREST, Japan Agency for Medical Research and Development (AMED), 1-7-1 Otemachi, Chiyoda-ku, Tokyo 100-0004, Japan
[c] Laboratory of Epigenetics, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 13 June 2016
Revised 7 July 2016
Accepted 9 July 2016
Available online 11 July 2016

Keywords:
5-Hydroxymethylcytosine
Bisulfite sequencing
Oxidation
DNA demethylation
Single base resolution

ABSTRACT

Oxidation of 5-methylcytosine (5mC) is catalyzed by ten-eleven translocation (TET) enzymes to produce 5-hydroxymethylcytosine (5hmC) and following oxidative products. The oxidized nucleotides were shown to be the intermediates for DNA demethylation, as the nucleotides are removed by base excision repair system initiated by thymine DNA glycosylase. A simple and accurate method to determine initial oxidation product 5hmC at single base resolution in genomic DNA is necessary to understand demethylation mechanism. Recently, we have developed a new catalytic oxidation reaction using micelle-incarcerated oxidants to oxidize 5hmC to form 5-formylcytosine (5fC), and subsequent bisulfite sequencing can determine the positions of 5hmC in DNA. In the present study, we described the optimization of the catalytic oxidative bisulfite sequencing (coBS-seq), and its application to the analysis of 5hmC in genomic DNA at single base resolution in a quantitative manner. As the oxidation step showed quite low damage on genomic DNA, the method allows us to down scale the sample to be analyzed.

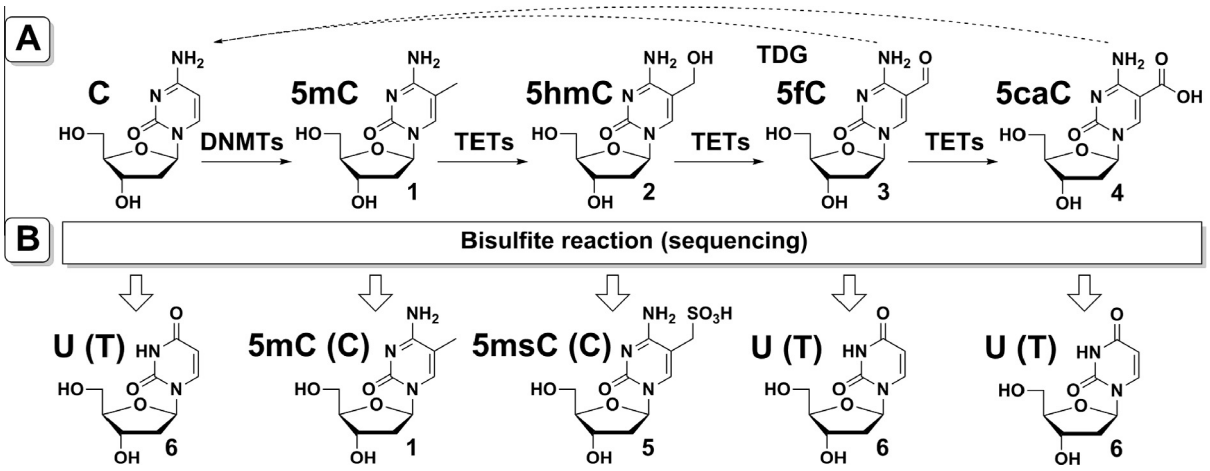
1. Introduction

Among four bases in genomic DNA, cytosine (C) is often methylated at C5 position when it is in the CpG sequence. 5-Methylcytosine (5mC, **1**), one of the epigenetic factors, plays important roles in gene suppression.[1] Hydroxylated form of 5mC, 5-hydroxymethylcytosine (5hmC, **2**) was found in some bacterial and animal viruses[2], and recently, found to be enriched in brain.[3,4] It was also found that 5mC is oxidized to 5hmC by oxygenase, ten-eleven translocation (TET) enzymes,[5] and further oxidized to 5-formylcytosine[6,7] (5fC, **3**), and 5-carboxylcytosine[7] (5caC, **4**). 5fC and 5caC are proposed to be excised by thymine DNA glycosylase[8] (TDG) to initiate base excision repair leading to demethylation (Scheme 1).[9,10] 5hmC seems to be not only an intermediate of demethylation process but its existence by itself is proposed to contribute to cell reprogramming and self-renewal of embryonic stem cells.[11] 5hmC is most abundant of the three oxidized methylated cytosine modifications, and highly exists in specific cell types such as pluripotent stem cells and neurons. As the methylation of cytosine and its demethylation play quite important roles in biological phenomena through regulation of gene expression, decoding of their patterns must be a cue for understanding epigenetic regulation.

Bisulfite sequencing is the standard method to determine the position of 5mC in genomic DNA at single base resolution.[12–14] However, this technique alone cannot distinguish between 5mC and 5hmC. Because bisulfite treatment converts 5hmC to cytosine 5-methylenesulfonate (5msC, **5**),[15,16] being read as cytosine by DNA polymerase (Scheme 1) just like 5mC. Several methods to identify 5hmC at single base resolution have been reported. Two of them, TET-assisted bisulfite[17,18] (TAB) and oxidative bisulfite[19] (oxBS) sequencings (seqs) are the major 5hmC identification methods. In both methods, oxidation of cytosine analogs is the key step, either by enzymatically (TAB) or chemically (oxBS) with TET or a metal oxide, respectively. However, these methods have their limitations. TAB-seq requires highly active TET1 enzyme, of which recombinant currently can only be expressed in insect cells and must carefully be purified.[20] As for oxBS-seq, it is difficult to optimize the conditions to avoid extensive DNA degradation during chemical oxidation reaction.[20] Recently, we have reported a mild catalytic oxidation of 5hmC in DNA using sodium dodecyl sulfate (SDS) micelle-incarcerated oxidants of, bis(acetoxy)

* Corresponding authors. Tel.: +81 3 5841 4358; fax: +81 3 5841 4357 (S.F.); tel.: +81 6 6879 8628; fax: +81 6 6879 8629 (I.S.).
E-mail addresses: seketsuf@chem.s.u-tokyo.ac.jp (S. Fukuzawa), suetake@protein.osaka-u.ac.jp (I. Suetake).

http://dx.doi.org/10.1016/j.bmc.2016.07.016
0968-0896/



Scheme 1. Methylation and demethylation intermediates of cytosine and their bisulfite modifications. (A) Cytosine (C) is methylated by DNA methyltransferase (DNMT), and 5mC is oxidized to 5hmC, 5fC, 5caC by TET enzymes. 5fC and 5caC are excised by TDG. (B) On bisulfite reaction, C, 5fC, and 5caC are converted to uracil (U, **6**), while 5mC is not. 5hmC is converted to 5msC. After PCR amplification, each base is read as the base indicated with parenthesis; U to thymine (T), and 5mC or 5msC to C.

iodobenzene (BAIB), and 2-hydroxy-2-azaadamantane[21–23] (AZADOL). An oxoammonium salt of AZADOL is the active species which specifically reacts with hydroxyls (Fig. 1).[24] By incarcerating BAIB in SDS micelles, BAIB and DNA were segregated (Fig. 1). Under the conditions, 5hmC was selectively oxidized to 5fC avoiding degradation of DNA.[24] As subsequent bisulfite reaction translates 5fC to uracil (U, **6**), which distinguishes between 5mC and 5hmC (Scheme 1), the position of 5hmC in DNA can be determined.[24] In the present study, we report the optimization of the reaction in detail, and application to the analysis of 5hmC in genomic DNA. The catalytic oxidative bisulfite sequencing (coBS-seq) allows us a quantitative and reproducible detection of 5hmC in genomic DNA as a specific cytosine.

2. Results

2.1. Catalytic oxidation of ssDNA in the absence of SDS micelles

Catalytic oxidation using 2-azaadamantane *N*-oxyl (AZADO) family requires co-oxidant, such as BAIB, which is a single electron oxidant. Initially we oxidized 10 bp single-stranded (ss) DNA containing 5hmC using AZADO and BAIB in the absence of SDS. The reaction proceeded in a pH dependent manner, which was optimal at 0.75 M sodium phosphate, pH 6.8, but was not completed even with high concentration of 118 mM (38 mg/mL) BAIB and 1.2 mM (0.19 mg/mL) AZADO (Table S1 and Fig. S1). Reaction time more than 30 min or increasing temperature caused DNA degradation (Fig. S2), which was due mainly to the oxidative damage by BAIB (Fig. S3). BAIB, which is a single electron oxidant, oxidized not only AZADO but also substrate DNA. Under the condition, double-stranded (ds) DNA was also degraded (data not shown).

2.2. Catalytic oxidation of dsDNA with micelle-incarcerated oxidant

To prevent DNA degradation caused by BAIB oxidation, BAIB, which is highly hydrophobic, was incarcerated into SDS micelles to segregate BAIB from DNA that resides in hydrophilic circumstances in the reaction mixture (Fig. 1).[24] We hypothesized that this segregation may protect DNA from degradation during the oxidation. Since BAIB was too hydrophobic to dissolve in aqueous media, its stock suspension (310 mM) used in above experiments was prepared in acetonitrile. To improve its solubility in an aqueous solution, SDS was used. The maximal stock solution was

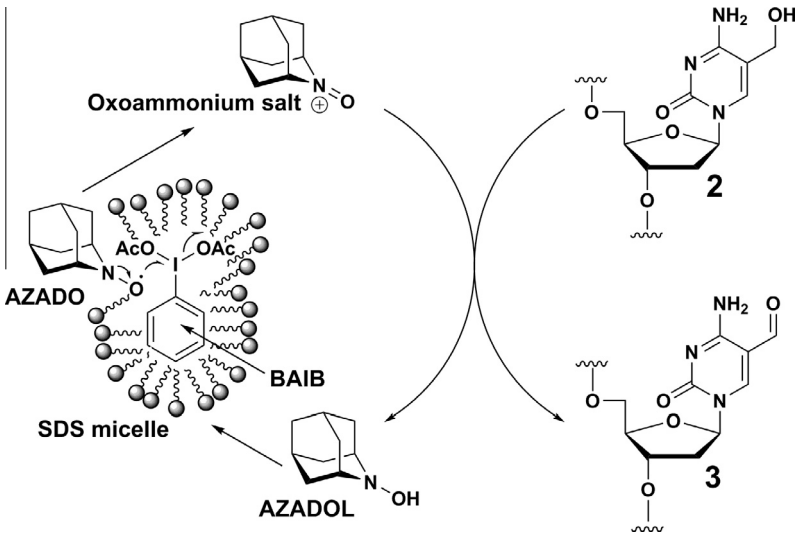


Figure 1. Catalytic oxidation reaction of 5hmC with AZADOL and BAIB. SDS micelle segregates BAIB from DNA.