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# Direct determination of the redox status of cysteine residues in proteins *in vivo*

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ABSTRACT

The redox states of proteins in cells are key factors in many cellular processes. To determine the redox status of cysteinyl thiol groups in proteins *in vivo*, we developed a new maleimide reagent, a photocleavable maleimide-conjugated single stranded DNA (DNA-PCMal). The DNA moiety of DNA-PCMal is easily removed by UV-irradiation, allowing DNA-PCMal to be used in Western blotting applications. Thereby the state of thiol groups in intracellular proteins can be directly evaluated. This new maleimide compound can provide information concerning redox proteins *in vivo*, which is important for our understanding of redox networks in the cell.



## 1. Introduction

The redox status of cellular proteins is strongly affected by the conditions of the cell. The thiols of cysteine residues in proteins are subjected to redox-mediated modifications due to their high reactivity. The disulfide bond formed by two vicinal cysteines is a well-known redox-mediated modification of cysteines. Other modifications of the thiol group of cysteine, such as glutathionylation, S-nitrosylation, hyperoxidation, and palmitoylation, have also been reported [1,2]. These reversible, or occasionally irreversible, cysteine modifications regulate enzymatic activity or protein function. Therefore, these modifications have a great impact on various physiological phenomena *in vivo*.

In this study, we intended to develop a useful method to determine thiol redox status *in vivo*. For this purpose, we studied glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a model of thiol modification. This enzyme is a key enzyme of glycolysis with many moonlighting functions in mammalian cells [3,4]. GAPDH has three cysteines. Cys152, which is located at the active site, is known to be highly sensitive to oxidative modifications. When GAPDH is exposed to oxidative stress caused by oxidants, Cys152 undergoes oxidative modifications, such as glutathionylation, S-nitrosylation, and hyperoxidation, as well as intramolecular disulfide bond formation with Cys156 [5–7]. Multiple modifications sometimes occur concurrently. When HEK-293T cells were exposed to $H_2O_2$, sulfonation at Cys152 and disulfide bond formation between Cys152 and Cys156 were simultaneously observed. These oxidations resulted in the inactivation of the enzymatic activity and induced an interaction with p54nrb [7]. In contrast, $H_2O_2$ exposure induced reversible S-thiolation in the GAPDH found in yeast cells, which results in the induction of apoptosis [8]. In this case, the reduction of the S-thiolation product is mainly catalyzed by glutaredoxin, grx5 [9].

Maleimide reagents are generally used to assess the redox status of the thiols in a protein of interest. Because the maleimide group specifically reacts with the reduced form of a thiol, the number of bound maleimides varies depending on the redox situations of the thiols on the protein. This difference of the number of maleimide compounds incorporated is easily detected as a change in mobility on SDS–PAGE. When proteins labeled with maleimide compounds are separated by SDS–PAGE, the mobility of the modified protein depends on its molecular weight, which is dependent upon the number of bound maleimide compounds. So far, the

*Abbreviations:* NHS, *N*-hydroxysuccinimide; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; PEG-Mal, methoxypolyethylene glycol-maleimide; PEO, biotin-$PEG_{11}$-maleimide; ssDNA, single-stranded DNA; Trxβ, fusion protein of the cytosolic thioredoxin *h1* from *Arabidopsis thaliana* and the β subunit of the $F_1$-ATPase from *Thermosynechococcus elongatus* BP-1; NTR, NADPH-thioredoxin reductase from *A. thaliana*; roGFP, redox sensitive GFP derivative; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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maleimides 4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid (AMS) [10] and polyethylene glycol-maleimide (PEG-Mal) [11] have generally been used to distinguish the redox states of proteins. However, determination of the number of reduced thiols is sometimes very difficult owing to the chemical properties of these modifiers. To overcome the difficulties of these maleimide compounds, we have developed the single-stranded DNA (ssDNA)-based maleimide compound (DNA-Mal) [12]. When a protein is labeled with DNA-Mal and analyzed on SDS–PAGE, DNA-Mal shifts its mobility by an identical 9.3 kDa per molecule of DNA-Mal. This substantial and accurate mobility shift allowed us to directly determine the number of bound DNA-Mal molecules, and thus the number of reduced thiols regardless of the molecular size of the protein of interest.

However, DNA-Mal also has a disadvantage, just like PEG-Mal. These large chemical modifiers often affect the efficiency of protein transfer from the gel to the membrane during Western blotting analysis. We suspect that the lower efficiencies observed are caused by the larger electrophoretic molecular weight of the labeled proteins. In addition, the transfer efficiency must be affected by the number of maleimide compounds bound to the protein. Accordingly, DNA-Mal and PEG-Mal are not applicable to Western blotting methods or for the quantification of the proteins by staining. To overcome this disadvantage, we have introduced a photocleavable group between the ssDNA and the maleimide moiety and designated these molecules as DNA-PCMal. When a DNA-PCMal-labeled protein is separated by SDS–PAGE and subsequently irradiated with UV on the polyacrylamide gel, the DNA moiety is removed. Consequently, the labeled protein can be electrophoretically transferred to the membrane, generally with the same efficiency as non-labeled proteins. Thus, DNA-PCMal can be used as a ruler to titrate reduced thiols, even under Western blotting conditions.

## 2. Materials and methods

### 2.1. Materials

5′-Aminohexyl PC ssDNA was purchased from Tsukuba Oligo Service (Ibaraki, Japan). $SM(PEG)_2$ and maleimide-$PEG_{11}$-biotin (PEO) were purchased from Thermo Fisher Scientific (Massachusetts, USA). Methoxypolyethylene glycol maleimide (SIGMA–ALDRICH, Missouri, USA) with an average molecular weight of 5000 Da was used as PEG-Mal in this study. Antibodies against human GAPDH and penta·His were purchased from GeneTex (Los Angeles, USA) and Qiagen (Venlo, Netherlands), respectively. $CF_1$ γ-subunit antibody was prepared in a previous study [13]. All other chemicals were of the highest grade commercially available.

### 2.2. Synthesis of DNA-PCMal

The nucleotide sequence of 5′-aminohexyl PC ssDNA was designed to be the same as that of DNA-Mal (5′-TAC-CTCTCCCTAACTACACAACCT-3′). The introduction of the maleimide moiety into 5′-aminohexyl PC ssDNA and the subsequent purification were performed as previously described [12], with slight modifications. After the anion exchange chromatography, the purified DNA-PCMal was desalted by ultrafiltration and then lyophilized. All procedures were performed under dark or dim light conditions. The concentrations of DNA-PCMal were determined by measuring the absorbance at 260 nm ($\varepsilon_{260nm}$ = 244.3 $mM^{-1}$ $cm^{-1}$).

### 2.3. Proteins

The expression and purification of Trxβ, NTR [12], and roGFP [14] were performed as described. A gene encoding *Arabidopsis thaliana* methionine synthase (MetE) (AT5G17920) with his-tag appended to its N-terminus was cloned into the expression vector pET23a. *Escherichia coli* strain BL21 (DE3) harboring the resulting plasmid was cultured at 37 °C in 2 × YT medium supplemented with 50 μg/mL ampicillin and 1 mM $ZnCl_2$ until $A_{600}$ reached 0.7. Expression was induced by the addition of IPTG to a final concentration of 1 mM, and then incubated at 21 °C for 16 h. MetE protein was purified using a nickel-nitrilotriacetic acid Superflow (Qiagen) followed by a Superdex 200 (GE Healthcare, UK).

### 2.4. Two-dimensional gel electrophoresis

DNA-PCMal-labeled protein was separated using 8% BisTris/MOPS-PAGE gel, and the gel was then soaked in 360 mM BisTris–HCl (pH 6.8) for 10 min to exchange the buffer. The lanes of the gel containing proteins were excised, placed on top of 12% BisTris gel and then fixed. A second electrophoresis was performed using MES running buffer (Life Technologies, USA).

### 2.5. SDS–PAGE and Western blotting analysis

Proteins were subjected to neutral pH gel systems (BisTris/MES or BisTris/MOPS) to analyze their molecular weights. After SDS–PAGE, the proteins were transferred to a PVDF membrane at 100 mA constant current for 60 min using a semi-dry blotter and Towbin buffer. Following procedures were performed according to standard methods, and chemi-luminescence signals on the membrane were detected using ECL prime (GE Healthcare) and a LAS-3000mini (Fuji Film, Tokyo).

### 2.6. Preparation of HeLa cell proteins and labeling

HeLa cells at 60–70% confluence were incubated in the presence or absence of redox reagents, and the cells in the dishes were then washed three times with PBS. Cold 10% TCA was directly added to fix the redox state in the HeLa cells. At this point, cell lysate was extracted from the cell and the denatured proteins were precipitated by centrifugation. The proteins obtained were immediately dissolved in the extraction buffer (Ex. Buffer) [50 mM Tris–HCl (pH 6.8), 1 mM EDTA, 0.5 M NaCl, 2% SDS, and the protease inhibitor cocktail Complete (Roche, Manheim, Germany)] containing 2.5 mM maleimide compounds, and the free thiols were labeled for 90 min at 37 °C. Following UV-irradiation of the electrophoresed gel for 15 min, proteins were electrophoretically transferred to the PVDF membrane as described above. The samples were kept in the dark during the labeling and SDS–PAGE procedures to avoid undesired cleavage of DNA-PCMal.

## 3. Results and discussion

### 3.1. Constraint of DNA-Mal as a thiol-labeling modifier

Compared with unlabeled and AMS-labeled GAPDH, we only observed a substantially weak signal from DNA- and PEG-Mal-labeled GAPDH in HeLa cells (Fig. 1A) when proteins were visualized by Western blot analysis. Similar result was obtained from the spinach $CF_1$ γ-subunit (Fig. 1B). These weak band intensities are attributable to the low efficiency of the protein transfer process because the protein bands visualized in the PEG-Mal and DNA-Mal lanes were much weaker than those in other lanes when the total proteins on the membrane were directly stained with CBB. These results clearly indicate that these large maleimide compounds prevent the transfer from gel to membrane, rather than preventing the antigen–antibody interaction. If this is the case, removal of the large molecule used for protein labeling from the protein after

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