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Production, Purification, and Characterization of ^{15}N -Labeled DNA Repair Proteins as Internal Standards for Mass Spectrometric Measurements

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Abstract

Oxidatively induced DNA damage is caused in living organisms by a variety of damaging agents, resulting in the formation of a multiplicity of lesions, which are mutagenic and cytotoxic. Unless repaired by DNA repair mechanisms before DNA replication, DNA lesions can lead to genomic instability, which is one of the hallmarks of cancer. Oxidatively induced DNA damage is mainly repaired by base excision repair pathway with the involvement of a plethora of proteins. Cancer tissues develop greater DNA repair capacity than normal tissues by overexpressing DNA repair proteins. Increased DNA repair in tumors that removes DNA lesions generated by therapeutic agents before they became toxic is a major mechanism in the development of therapy resistance. Evidence suggests that DNA repair capacity may be a predictive biomarker of patient response. Thus, knowledge of DNA–protein expressions in disease-free and cancerous tissues may help predict and guide development of treatments and yield the best therapeutic response. Our laboratory has developed methodologies that use mass spectrometry with isotope dilution for the measurement of expression of DNA repair proteins in human tissues and cultured cells. For this purpose, full-length ^{15}N -labeled analogs of a number of human DNA repair proteins have been produced and purified to be used as internal standards for positive identification and accurate quantification. This chapter describes in detail the protocols of this work. The use of ^{15}N -labeled proteins as internal standards for the measurement of several DNA repair proteins in vivo is also presented.

1. INTRODUCTION

Exogenous and endogenous sources such as free radicals and ionizing radiation generate oxidatively induced DNA damage by a variety of mechanisms, resulting in the formation of modified bases and sugars, DNA–protein cross-links, strand breaks, base-free sites, and tandem lesions such as 8,5'-cyclopurine-2'-deoxyribonucleosides and clustered damaged sites (reviewed in Dizdaroglu & Jaruga, 2012; Evans, Dizdaroglu, & Cooke, 2004). This type of DNA damage is thought to play an important role in disease processes such as carcinogenesis and aging (reviewed in Dizdaroglu, 2015; Friedberg et al., 2006). Elaborate DNA repair pathways exist in mammalian cells with approximately 150 different proteins involved (reviewed in Friedberg et al., 2006; Wood, Mitchell, & Lindahl, 2005). DNA repair deficiencies cause accumulation of DNA damage and mutations, leading to genomic instability, which is a major factor in carcinogenesis (Beckman & Loeb, 2005; Helleday, Petermann, Lundin, Hodgson, & Sharma, 2008; Hoeijmakers, 2001; Kelley, 2012; Liu, Yin, & Pu, 2007; Loeb, 2011; Madhusudan & Middleton, 2005). Oxidatively induced DNA damage is mainly repaired by base excision repair (BER) and also by nucleotide excision repair (NER), albeit to a lesser extent (Friedberg et al., 2006). In the first step of BER, DNA glycosylases hydrolyze the *N*-glycosidic bond releasing the damaged base and generating an abasic site, followed by the action of a series of other BER enzymes (Gros, Saparbaev, & Laval, 2002; Hegde, Hazra, & Mitra, 2008). Formamidopyrimidine DNA glycosylase (Fpg, also called MutM) is one of the main DNA glycosylases in *Escherichia coli*, which specifically excises 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 8-hydroxyguanine (8-OH-Gua) from DNA containing multiple lesions (Boiteux, Gajewski, Laval, & Dizdaroglu, 1992; Dizdaroglu, 2005). In eukaryotes, 8-oxoguanine-DNA glycosylase (OGG1) including human OGG1 (hOGG1), which is a functional homolog of Fpg, exhibits a strong specificity for FapyGua and 8-OH-Gua, but does not act on FapyAde (Audebert, Radicella, & Dizdaroglu, 2000; Dherin, Radicella, Dizdaroglu, & Boiteux, 1999; Hu et al., 2005; Sidorenko, Grollman, Jaruga, Dizdaroglu, & Zharkov, 2009).

Mammalian NEIL1 protein, which is a *E. coli* Nei-like DNA glycosylase with an additional β,δ -elimination activity, has been discovered in eukaryotes (Bandaru, Sunkara, Wallace, & Bond, 2002; Hazra et al., 2002). This enzyme is unique in that it specifically removes FapyAde and FapyGua from DNA with multiple lesions, without exhibiting any significant activity for 8-OH-Gua (Bandaru et al., 2002; Chan et al., 2009; Doublet, Bandaru, Bond, & Wallace, 2004; Hazra et al., 2002; Hu et al., 2005; Jaruga, Birincioglu, Rosenquist, & Dizdaroglu, 2004; Liu et al., 2010; Muftuoglu et al., 2009; Rosenquist et al., 2003; Roy et al., 2007). The involvement of NEIL1 in NER has also been suggested on the basis of accumulation of 8,5'-cyclopurine-2'-deoxyribonucleosides in *neil1*^{-/-} mice (Jaruga, Xiao, Vartanian, Lloyd, & Dizdaroglu, 2010). Homologs of *E. coli* Nth have also been found in yeast and mammals (Aspinwall et al., 1997; Roldán-Arjona, Anselmino, & Lindahl, 1996). Mammalian NTH1 mainly acted on pyrimidine-derived lesions, exhibiting a narrower substrate specificity than *E. coli* Nth (Karahalil, Roldan-Arjona, & Dizdaroglu, 1998; Roldán-Arjona et al., 1996); however, evidence has also been provided for purine-derived FapyAde to be the physiological substrate of NTH1 (Chan et al., 2009; Hu et al., 2005).

Other enzymes such as apurinic/apyrimidinic endonuclease 1 (APE1) and DNA polymerase β (Pol β) also play vital roles in BER. Abasic sites, which are left behind following removal of modified DNA bases by DNA glycosylases, are processed by APE1 (Dempfle, Herman, & Chen, 1991). APE1 provides over 95% of the total endonuclease function with some additional critical functions (Abbotts & Madhusudan, 2010; Barnes et al., 2009; Dempfle & Harrison, 1994; Dempfle et al., 1991; Fishel, Vascotto, & Kelley, 2013; Gros, Ishchenko, Ide, Elder, & Saparbaev, 2004; Tell, Quadrifoglio, Tiribelli, & Kelley, 2009; Wilson & Barsky, 2001; Xanthoudakis & Curran, 1992). The action of APE1 generates a one-nucleotide gap with a 3'-OH and a 5'-terminal 2'-deoxyribose phosphate (dRP) moiety. Pol β , which is found in all vertebrate species (Beard & Wilson, 2006, 2014; Braithwaite & Ito, 1993), binds to this gap and performs DNA synthesis with its DNA polymerase activity, filling in the gap. It then removes the blocking dRP moiety, paving the way for other enzymes such as DNA ligases to close the resulting gap to complete the repair of DNA (Beard & Wilson, 2000, 2006; Matsumoto & Kim, 1995; Srivastava et al., 1998).

Defects in BER enzymes such as those discussed above are associated with neurological disorders and cancer (Sampath, McCullough, & Lloyd, 2012; Wallace, Murphy, & Sweasy, 2012; Wilson, Kim, Berquist, & Sigurdson, 2011). Polymorphic variants of these enzymes have been found in human populations in connection to various cancer incidences (reviewed in Dizdaroğlu, 2015). For example, *neil1*^{-/-} mice developed obesity and symptoms of metabolic syndrome (Vartanian et al., 2006). Male and female *neil1*^{-/-} and *nth1*^{-/-} mice developed pulmonary and hepatocellular tumors to an extent up to $\approx$ 15%. A dramatic increase up to 75% in cancer incidence has been observed in *neil1*^{-/-} *nth1*^{-/-} male and female mice (Chan et al., 2009). In mice with both deleted alleles of *ape1*, early embryonic lethality has been observed; similarly, APE1 heterozygous mice exhibited increased oxidative stress, spontaneous mutagenesis and cancer incidences, and reduced survival of pups and embryos (Cabelof, 2012; Hakem, 2008; Huamani et al., 2004; Meira et al., 2001; Xanthoudakis, Smeyne, Wallace, & Curran, 1996). Thus, accumulated evidence unequivocally points to the crucial role of BER enzymes in the maintenance of genetic stability and disease prevention.

It is now well known that overexpression of DNA repair proteins that may increase the DNA repair capacity is common in cancer (Helleday et al., 2008; Kelley, 2012; Madhusudan & Middleton, 2005). Increased and effective DNA repair capacity in tumors that removes DNA lesions generated by ionizing radiation and/or chemotherapy before they became toxic is a major mechanism to develop therapy resistance. Evidence suggests that DNA repair capacity might be a predictive biomarker of patient response (Kelley, 2012; Perry, Sultana, & Madhusudan, 2012). In this respect, BER proteins have emerged as biomarkers for assessment of the effectiveness and outcome of therapy (Chan & Bristow, 2010; Curtin, 2013; Fishel et al., 2013; Helleday, 2008; Helleday et al., 2008; Illuzzi & Wilson, 2012; Kelley, 2012; Madhusudan & Middleton, 2005; Moeller, Arap, & Pasqualini, 2010; Perry et al., 2012). Targeting MTH1 protein, which is not a BER protein, has been also suggested to be beneficial in cancer therapy (Gad et al., 2014; Huber et al., 2014). This enzyme dephosphorylates modified 2'-deoxynucleoside triphosphates in the nucleotide pool so that they cannot be incorporated into DNA during DNA replication, preventing cell death and mutations (Maki & Sekiguchi, 1992; Mo, Maki, & Sekiguchi, 1992; Sakai et al., 2002;

Sakumi et al., 1993; Sekiguchi & Tsuzuki, 2002; Tsuzuki et al., 2001). Its overexpression has been observed in many cancers (Gad et al., 2014; Huber et al., 2014; Kennedy, Cueto, Belinsky, Lechner, & Pryor, 1998; Okamoto et al., 1996; Speina et al., 2005).

The determination of the overexpression or underexpression of DNA repair proteins in normal and cancer tissues may help predict and guide development of treatments, potentially yielding the greatest therapeutic response (Kelley, 2012). In general, expression levels of DNA repair proteins have been estimated by Western blot methods. With these methods, proteins are separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and identified by the use of an antibody specific to a target protein, meaning that they completely depend on a reliable and specific antibody resource. Antibodies may potentially exhibit some off-target binding, leading to false results. No positive identification and no absolute quantification are provided because of the lack of mass spectrometric evidence and suitable internal standards. Quantification is based on the estimation of stained area only. The molecular mass of a target protein is only estimated by the use of a molecular mass ladder of proteins. If DNA repair proteins are to be used as reliable biomarkers in cancer, their expression levels must be accurately measured in tissues, providing positive identification of a target protein and its absolute quantification. Mass spectrometry is the most suitable technique of choice for the measurement of proteins. The application of this technique would be essential for positive identification and accurate quantification of DNA repair proteins in human tissues using proper internal standards. Our laboratory has recently developed methodologies and stable isotope-labeled standards for the measurements of DNA repair proteins using liquid chromatography–tandem mass spectrometry (LC-MS/MS) with isotope dilution (Coskun et al., 2015; Dizdaroglu, Reddy, & Jaruga, 2011; Kirkali et al., 2013; Reddy, Jaruga, Nelson, Lowenthal, & Dizdaroglu, 2011; Reddy et al., 2013). Stable isotope-labeled analogs of DNA repair proteins, which are to be used as internal standards, were produced, purified, and characterized. This chapter describes the procedures used for this purpose and the application of the developed internal standards for the measurement of DNA repair proteins.

2. MATERIALS AND METHODS

2.1 Materials

Restriction endonucleases, DNA ligase, and calf intestinal alkaline phosphatase were obtained from New England Biolabs (MA). Isopropyl β -D-thiogalactoside (IPTG), acrylamide, bisacrylamide, and Tris(2-carboxyethyl)phosphine hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). Diethylaminoethyl (DEAE) cellulose (DE52) was from Whatman Laboratories. Shodex carboxymethyl cellulose HPLC preparative column (CM 2025) was from Phenomenex. YM10 membrane filters were from EMD Millipore Corporation (Bedford, MA). Ni-Sepharose column HisTrap HP and MonoQ anion-exchange column were obtained from GE Healthcare, Bio-Science (Uppsala, Sweden). BugBuster protein extraction reagent was from Millipore (Billerica, MA). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN). Benzonase nuclease was from Novagen (Denmark). ^{15}N - NH_4Cl was obtained from Sigma Chemical Company (St. Louis, MO).

2.2 *E. coli* Strains and Plasmids

The pET11a expression vector, for native protein production of *EcFpg* based on the IPTG induction, was obtained from Novagen, Madison, WI. The relevant strains for cloning and expression are *E. coli* Novablue (K12) and BL21(DE3), respectively. The human *ogg1* gene in the expression vector pET15b for His-tagged production of the protein was kindly provided by Dr. Dmitry Zharkov through Prof. Arthur Grollman's laboratory. The human *neil1* gene in the expression vector pET28 for His-tagged production of the protein was kindly provided by Dr. Stephen Lloyd (Oregon Health and Science University, Portland, Oregon). The human *ape1* gene was provided by Dr. David M. Wilson III of the National Institute on Aging, National Institutes of Health, Baltimore, Maryland, USA.

2.3 Preparation of Minimal Medium

The composition of the medium was: 6 g NaH₂PO₄, 3 g K₂HPO₄, 0.5 g NaCl, and 1 g ¹⁵N-NH₄Cl, 5 g glucose, 246 mg MgSO₄·7H₂O per liter. Ampicillin or kanamycin was added to a final concentration of 50 µg/mL or 25 µg/mL, respectively.

2.4 DNA Procedures

E. coli Novablue (K12) harboring a recombinant plasmid was grown at 37 °C overnight in 10 mL Luria-Bertani medium (Miller, 1972), containing the appropriate antibiotic. Minipreparations of plasmid DNA were purified using a Qiagen Kit. Digestion of DNA with restriction enzymes was performed according to the manufacturer's recommendation. DNA fragments were separated by electrophoresis on SeaKem GTG agarose or NuSieve GTG agarose. DNA was purified using a Qiagen Kit. Ligation of DNA fragments was performed as described (Sambrook, Fritsch, & Maniatis, 1989). Competent cells of the *E. coli* strains used here were prepared by the Hanahan method (Hanahan, 1983; Sambrook et al., 1989).

3. PRODUCTION AND PURIFICATION OF *E. COLI* ¹⁵N-Fpg PROTEIN

3.1 Cloning of *fpg* Into pET11a Vector

The coding sequence for *E. coli fpg* gene from the plasmid pFpg was provided by Dr. Timothy R. O'Connor of the Beckman Research Institute of the City of Hope, Duarte, CA (O'Connor, Boiteux, & Laval, 1989). It was amplified by PCR with Pfu polymerase using a 5'-end primer (5'-GGAATTC CAT ATG CCT GAA TTA CCC GAA G-3') and a 3'-end primer (5'-CCG CTC GAG TTA CTT CTG GCA CTG CCG AC-3'). The 5'-end primer sequence contained the *NdeI* restriction recognition sequence CATATG wherein ATG served as the initiation codon for protein expression. The 3'-end primer contained the TAA translation stop codon. The amplified product was digested with *NdeI* to produce the gene with 5'-*NdeI* protruding end and 3'-blunt end. The DNA fragment was purified from a 1% agarose gel, and cloned into the *NdeI* and *Bam*HI (first digested with *Bam*HI, filled in with DNA polymerase to make this blunt end, then digested with *NdeI*) sites of the pET11a expression vector. A recombinant plasmid pET11a/Fpg was isolated from *E. coli* Nova Blue cells. *E. coli* BL21 (DE3) was transformed with the recombinant plasmid to induce protein expression with IPTG.

3.2 Production of ^{15}N -Fpg

E. coli BL21 (DE3) harboring pET1 1a/Fpg recombinant plasmid was grown at 37 °C for 20 h on LB agar plate containing 100 µg/mL ampicillin. A colony was carefully (without touching into the LB medium) transferred to 10 mL minimal medium containing 1 mg ^{15}N - NH_4Cl /mL and 50 µg ampicillin/ mL. Cells were grown for 1 h at 37 °C at 250 rpm in a 50 mL tube. This inoculum was transferred to 140 mL minimal medium, in a 500 mL baffled flask, containing ^{15}N - NH_4Cl and ampicillin as above. This culture was grown at 37 °C for 16 h. Next, 3×50 mL of this seed culture was transferred to 3×450 mL of minimal medium containing ^{15}N - NH_4Cl and ampicillin in 3×2 L baffled flasks. IPTG was added to a final concentration of 30 µmol/L to induce ^{15}N -Fpg production at 37° C for 1 h. Next, the culture was shifted 42° C for 5 h to continue ^{15}N -Fpg production. Cells were harvested at $6000 \times g$ for 20 min and washed with 25 mmol/L Tris-HCl buffer, pH 7.5. The wet weight of cells obtained in this procedure was $\approx 3\text{g}$.

3.3 Purification of ^{15}N -Fpg

Cell pellet was suspended in 40 ml of 50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L β -mercaptoethanol, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 50 mmol/L NaCl (lysis buffer 1), containing one tablet of protease inhibitors. Cell suspension was passed through a French press at 7.1×10^4 kPa twice. The cell-free extract was centrifuged at $10,000 \times g$ for 20 min. The supernatant was mixed with 5 g of DE52 anion-exchange resin equilibrated with the lysis buffer 1 in a 500-mL bottle at 80 rpm for 1 h at 4 °C and then poured into a column. The flow through containing nearly all the ^{15}N -Fpg and fewer cellular proteins was collected. The resin was washed with 10 mL of the lysis buffer 1 and added to the flow through. The ^{15}N -Fpg enriched pool (50 mL) was dialyzed overnight against 1 L of 20 mmol/L potassium phosphate buffer, pH 7.4. The dialyzed pool was centrifuged at $100,000 \times g$ for 1 h to remove any particulate material. The supernatant was chromatographed on a HPLC-Shodex carboxymethyl cellulose column (2 cm $\times$ 20 cm) equilibrated with 20 mmol/L potassium phosphate buffer, pH 7.4. The column was washed with 100 mL of 20 mmol/L potassium phosphate buffer, pH 7.4 until the A_{280} stabilized. Then ^{15}N -Fpg was eluted with a 0–0.5 mol/L potassium chloride gradient (250 mL each) in 20 mmol/L potassium phosphate buffer, pH 7.4. Pure ^{15}N -Fpg was eluted as a sharp peak at ≈ 0.3 mol/L KCl. Fractions containing ^{15}N -Fpg were pooled, concentrated on YM3 membrane filter, and dialyzed overnight against 500 mL of 50% glycerol, 20 mmol/L Tris-HCl, 1 mmol/L EDTA, and 10 mmol/L β -mercaptoethanol, pH 7.5. Protein concentration was determined by the Lowry method using bovine serum albumin (BSA) as standard (Lowry, Rosebrough, Farr, & Randall, 1951). Total yield of the protein was 1 mg. The SDS-PAGE and Coomassie staining of ^{15}N -Fpg (lane 5) and protein molecular mass standards (lane 6) are shown in Fig. 1, indicating high purity of the labeled protein (Reddy et al., 2011).

4. PRODUCTION AND PURIFICATION OF ^{15}N -hOGG1

4.1 Production of ^{15}N -hOGG1

E. coli BL21(DE3) harboring pET15b/hOGG1 recombinant plasmid was grown at 37 °C for 20 h on LB agar plate containing 100 µg/mL ampicillin. A colony was carefully (without

touching into the LB medium) transferred to 100 mL minimal medium containing 1 mg ^{15}N - NH_4Cl /mL and 50 μg ampicillin/mL. Cells were grown overnight (16 h) at 37 °C at 250 rpm in a 250-mL baffled flask. This inoculum was transferred to 1000 mL minimal medium, in a 2000-mL baffled flask, containing ^{15}N - NH_4Cl and ampicillin as above. This culture was grown at 37 °C for 1 h and briefly cooled in ice water to room temperature. Next, IPTG was added to a final concentration of 100 $\mu\text{mol/L}$ to induce ^{15}N -hOGG1 production at 24 °C overnight. Cells were harvested at $6000 \times g$ for 20 min and washed with 25 mmol/L Tris-HCl buffer, pH 7.5. The wet weight of cells obtained in this procedure was $\approx 2\text{g}$.

4.2 Purification of ^{15}N -hOGG1

The cell pellet was suspended in 20 mL of 50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L β -mercaptoethanol, 300 mmol/L NaCl, and 10 mmol/L imidazole (lysis buffer 2) containing one tablet of protease inhibitors. Cell suspension was passed through a French press at 7.1×10^4 kPa. The cell-free extract was centrifuged at $40000 \times g$ for 1 h. Meanwhile, 2 mL of nickel-agarose slurry (1 mL resin) was washed with the lysis buffer 2 in a 30 mL Bio-Rad polypropylene column. The supernatant was added to the resin and mixed on a rocker for 2 h at 4 °C. The flow through was collected, and the column was washed successively with three 10-mL aliquots of the lysis buffer 2. Next, the resin was washed three times with 3-mL aliquots of the lysis buffer 2 containing an additional 20 mmol/L imidazole (total 30 mmol/L). Next, the ^{15}N -hOGG1 was eluted with five 5-mL aliquots of the lysis buffer containing 100 mmol/L imidazole. The first 5 mL contained about 50% of ^{15}N -hOGG1 ($\approx 95\%$ pure) with minor contaminants as judged by SDS-PAGE. The subsequent four elutions contained the remaining ^{15}N -hOGG1 with higher purity ($\approx 98\%$). All the five elutions containing ^{15}N -hOGG1 were pooled, concentrated on YM3 membrane filter, and dialyzed overnight against 500 mL of 50% glycerol, 20 mmol/L Tris-HCl, 1 mmol/L EDTA, and 10 mmol/L β -mercaptoethanol, pH 7.5. Mass measurements and activity analysis of ^{15}N -hOGG1 were carried out. Protein concentration was determined by the Lowry method using BSA as standard (Lowry et al., 1951). Total yield of the protein was 5.3 mg. Figure 2 shows the SDS-PAGE and Coomassie staining of ^{15}N -hOGG1 (lane 5) and protein molecular mass standards (lane 6), indicating high purity of the labeled protein (Reddy et al., 2011).

5. PRODUCTION AND PURIFICATION OF ^{15}N -hNEIL1

5.1 Production of ^{15}N -hNEIL1

E. coli BL21 (DE3) harboring pET28/human *neil1* recombinant plasmid was grown at 37 °C for 20 h on LB agar plate containing 100 μg ampicillin and 40 μg chloramphenicol/mL. A colony was carefully (without touching into the LB medium) transferred to 10 mL minimal medium containing 1 mg ^{15}N - NH_4Cl /mL, 50 μg ampicillin, and 20 μg chloramphenicol/mL. Cells were grown for 16 h at 37 °C at 250 rpm in a 50 mL tube. This inoculum was transferred to 90 mL minimal medium, in a 250 mL flask, containing ^{15}N - NH_4Cl , ampicillin, and chloramphenicol as above. This culture was grown at 37 °C for 9 h. Next, this seed culture was transferred to 900 mL of minimal medium containing ^{15}N - NH_4Cl , ampicillin, and chloramphenicol in a 2 L flask. This culture was grown at 37 °C for 4 h. Cells ($A_{600} = 0.45$) were briefly cooled in ice and ^{15}N -hNEIL1 production was induced with 100 μmol IPTG/mL at 25 °C for 16 h. Cells were harvested at $6000 \times g$ for 20 min and

washed with 25 mmol/L Tris-HCl buffer (pH 7.5). The wet weight of cells obtained in this procedure was 2.8 g/L culture.

5.2 Purification of ^{15}N -hNEIL1

Cell pellet was suspended at a rate of 1 g/10 mL of 50 mmol/L Na_2HPO_4 - NaH_2PO_4 buffer (pH 8.0), 10 mmol/L β -mercaptoethanol, 10 mmol/L imidazole, and 300 mmol/L NaCl (lysis buffer). One tablet of protease inhibitors was added to the cell suspension. Cell suspension was passed through a French press at 7.1×10^4 kPa twice. The cell-free extract was centrifuged at $36000 \times g$ for 30 min. Meanwhile, 1 mL of nickel-agarose slurry (0.5 mL resin) was washed with the lysis buffer in a 30-mL Bio-Rad polypropylene column. The supernatant was added to the resin and mixed on a rocker for 2 h at 4 °C. The flow through was collected, and the column was washed successively with three 10-mL aliquots of the lysis buffer. Next, the resin was washed successively two times with 10 mL aliquots of the lysis buffer containing an additional 30 mmol/L imidazole (total 40 mmol/L). Next, the resin was washed successively three times with 5-mL aliquots of the lysis buffer containing an additional 70 mmol/L imidazole (total 80 mmol/L). The first imidazole₈₀ wash did elute about 30% of ^{15}N -hNEIL1 with a number of contaminating proteins as judged by SDS-PAGE. The second and third imidazole₈₀ washes eluted about 30% of nearly homogeneous ^{15}N -hNEIL1. Next, ^{15}N -hNEIL1 was eluted successively with three 5 mL aliquots of the lysis buffer containing 150 mmol/L imidazole. The first 5 mL contained about 30% of ^{15}N -hNEIL1. The subsequent two elutions contained the remaining ^{15}N -hNEIL1. The second and third imidazole₈₀ elutions and all the imidazole₁₅₀ elutions were pooled and dialyzed overnight against 1 L of 50 mmol/L Na_2HPO_4 - NaH_2PO_4 buffer (pH 8.0) and 300 mmol/L NaCl. Slight precipitate of ^{15}N -hNEIL1 appeared after dialysis even in the presence of 300 mmol/L NaCl. The precipitate was centrifuged off from the dialyzed pool, and the clear supernatant was concentrated on NMLW5 membrane in an Amicon cell. The above purification procedure developed for cells from 1 L culture was proportionately scaled up to 5 L as and when needed. Protein concentration was determined by the Lowry method using BSA as the standard (Lowry et al., 1951). The final yield of ^{15}N -hNEIL1 was ≈ 2 mg/L culture. The SDS-PAGE and Coomassie staining of ^{15}N -hNEIL1 (lane 5) and protein molecular mass standards (lane 6) are presented in Fig. 3. A high purity of the labeled protein can be seen with a minor contaminant at ≈ 17 kDa (Reddy et al., 2013).

6. PRODUCTION AND PURIFICATION OF ^{15}N -hAPE1

6.1 Production of ^{15}N -hAPE1

E. coli BL21 (DE3) harboring pET11/hAPE1 recombinant plasmid was grown at 37 °C for 20 h on LB agar plate containing 100 μg ampicillin/mL. A colony was carefully (without touching into the LB medium) transferred to 10 mL minimal medium containing 50 μg ampicillin/mL. Cells were grown for 3 h at 37 °C at 250 rpm in a 50 mL tube. This inoculum was transferred to 200 mL minimal medium with 50 μg ampicillin/mL in a 500 mL flask. This culture was grown at 37 °C for 14 h. Next, each 50 mL of this seed culture was transferred to 4×1000 mL of minimal medium containing ^{15}N - NH_4Cl and ampicillin in 4×2 L flasks. This culture was grown at 37 °C for 6 h and then at 20 °C for 90 min. Cell density at this stage was $A_{600} = 0.5$. The production of ^{15}N -hAPE1 was induced with 100

$\mu\text{mol IPTG/mL}$ at $20\text{ }^{\circ}\text{C}$ for 15 h. Cells were harvested at $6000 \times g$ for 20 min and washed with $25\text{ mmol/L Tris-HCl}$ buffer ($\text{pH } 7.5$). The wet weight of cells obtained in this procedure was 2.25 g/L culture for a total of 9 g wet weight of cells.

6.2 Purification of ^{15}N -hAPE1

Nine grams of cells from 4-L culture were suspended in 90 mL of lysis buffer A: $50\text{ mmol/L HEPES-KOH}$ buffer, $\text{pH } 7.5$, containing 5% glycerol, 50 mmol/L KCl , and 1 mmol/L DTT . Cells were broken by passing through a French press at $7.1 \times 10^4\text{ kPa}$. The cell-free extract was centrifuged at $70000 \times g$ for 1 h. The supernatant was mixed with 5 g of DE52 anion-exchange resin equilibrated with the buffer A. The supernatant/resin slurry in a 250-ml bottle was mixed at 178 rpm for 1 h and then poured into a column. The flow through containing nearly all the ^{15}N -hAE1 and other cellular proteins was collected. The resin was washed with 10 mL of the buffer A and added to the flow through. The ^{15}N -hAPE1 enriched pool (100 mL) was chromatographed on a HPLC-Shodex carboxymethyl cellulose column ($2.0\text{ cm} \times 25\text{ cm}$) equilibrated with buffer A. The column was washed with 100 mL of buffer A until the A_{280} stabilized. Then ^{15}N -hAPE1 was eluted with a potassium chloride gradient generated from buffer A and buffer A containing 0.65 mol/L KCl (250 mL each). Pure ^{15}N -hAPE1 was eluted as a sharp peak at $\approx 0.3\text{ mol/L KCl}$. Fractions containing ^{15}N -hAPE1 were pooled, concentrated to $\approx 10\text{ mg/mL}$ on a YM10 membrane filter. The yield of ^{15}N -hAPE1 from 4 L culture of minimal medium was 60 mg. Aliquots of the protein were stored at $-70\text{ }^{\circ}\text{C}$. Figure 4, lane 6, shows the SDS-PAGE analysis and Coomassie staining of ^{15}N -hAPE1 and protein molecular mass standards (lane 7), indicating high purity of the labeled protein (Kirkali et al., 2013).

7. PRODUCTION AND PURIFICATION OF ^{15}N -hMTH1

^{15}N -hMTH1 was produced according to the procedures used for the production of hMTH1 (Svensson et al., 2011). ^{15}N -hMTH1 was expressed from pET28a-hMTH1 in *E. coli* BL21(DE3) grown in a minimal medium (6 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g NaCl, 5 g glucose, 120.4 mg MgSO_4 per liter) containing 1 g $^{15}\text{N-NH}_4\text{Cl}$. The production was induced by addition of $200\text{ }\mu\text{mol/L}$ isopropyl β -D-1-thiogalactopyranoside. Cells were harvested by centrifugation after 16 h at $20\text{ }^{\circ}\text{C}$, and the obtained pellet was dissolved in BugBuster protein extraction reagent supplemented with complete protease inhibitor cocktail and benzonase nuclease. The resulting suspension was centrifuged, and the cleared lysate was subjected to chromatography on Ni-Sepharose column HisTrap HP. Bound proteins were eluted using a linear gradient of imidazole ($25\text{--}500\text{ mmol/L}$) and analyzed by SDS-PAGE. Fractions containing ^{15}N -hMTH1 were after dialysis loaded onto a MonoQ anion-exchange column and eluted with a linear gradient of NaCl. Eluted fractions were analyzed using SDS-PAGE with Coomassie staining. Fractions containing ^{15}N -hMTH1 protein were pooled and stored in 20 mmol/L HEPES , 225 mmol/L NaCl , 10% glycerol, and 1 mmol/L Tris (2-carboxyethyl)-phosphine hydrochloride at $-80\text{ }^{\circ}\text{C}$. The purity of the isolated ^{15}N -hMTH1 was checked using SDS-PAGE with Coomassie staining. Figure 5 illustrates the SDS-PAGE analysis of ^{15}N -hMTH1 along with hMTH1 and protein standards, indicating high purity of both proteins (Coskun et al., 2015).

8. MASS SPECTROMETRIC ANALYSIS OF ^{15}N -LABELED PROTEINS

Mass spectrometry was used to measure the molecular masses and to check the isotopic purities of ^{15}N -labeled DNA repair proteins that were produced as described above. MALDI-ToF mass spectrometry was applied to measure the molecular masses of *E. coli* ^{15}N -Fpg and ^{15}N -hOGG1 (Reddy et al., 2011). The protonated average molecular mass (MH^+) of *E. coli* ^{15}N -Fpg amounts to 30641 Da, as calculated using the NIST *Mass and Fragment Calculator*. The measurement yielded a value of 30442 Da. It is well known that the N-terminal Met is removed from proteins by peptide deformylase and methionineaminopeptidase as an essential process, when the penultimate residue is small and uncharged as Pro next to Met (Gigliione, Boularot, & Meinnel, 2004). Without the N-terminal Met, the calculated value of the MH^+ of *E. coli* ^{15}N -Fpg amounted to 30509 Da. The measured value (30442 Da) is 99.8% of this calculated value. Using the same approach, the MH^+ of His-tagged ^{15}N -hOGG1 was found to be 41834 Da. The His-tag had a sequence of MGSSHHHHHHSSGLVPRGSHEL. The calculated MH^+ of this protein amounts to 41688 Da, which is 99.7% of the measured value. The measured MH^+ of His (KLAAALEHHHHHH)-tagged ^{15}N -hNEIL1 amounted to 45833 Da (unpublished results). The calculated MH^+ of this protein was 45785 Da, which is 99.9% of the measured value. Considering that the MALDI-ToF mass spectrometry provides a mass accuracy of 0.1%, these values represent more than 99% ^{15}N -labeling of *E. coli* Fpg, hOGG1, and hNEIL1 proteins.

An Orbitrap mass spectrometer coupled to an HPLC system, which has a mass accuracy of 0.001%, was used to measure the molecular mass of ^{15}N -hAPE1 (Kirkali et al., 2013). The calculated MH^+ of ^{15}N -hAPE1 with the loss of Met amounts to 35858 Da. The measured MH^+ was 35854 Da, which differs by 4 Da only from the calculated value and thus represents a 99.99% labeling. Within the mass accuracy of the Orbitrap mass spectrometer, this result showed that a complete ^{15}N -labeling of hAPE1 was achieved.

The molecular mass of ^{15}N -hMTH1 was measured using a high-resolution QToF mass spectrometer coupled to an HPLC system (Coskun et al., 2015). Without the N-terminal Met, the calculated MH^+ of ^{15}N -hMTH1 amounts to 20229.8 Da. The measured MH^+ of ^{15}N -hMTH1 was 20216.2 Da, which is 99.93% of the calculated value. Taken together, the measurement of the average molecular masses of the produced full-length ^{15}N -labeled proteins confirmed their identity and an almost complete ^{15}N -labeling, meaning that these labeled proteins can be used as excellent internal standards for the measurement of DNA repair proteins by LC-MS/MS or any other MS technique.

9. APPLICATIONS TO THE MEASUREMENT OF DNA REPAIR PROTEINS

9.1 Development of Methodologies

Following production of ^{15}N -labeled DNA repair proteins, we developed methodologies using these proteins as internal standards to positively identify and accurately quantify corresponding DNA repair proteins in human cultured cells and tissues with the use of LC-MS/MS. For this purpose, ^{15}N -labeled proteins and their unlabeled analogs were hydrolyzed with trypsin and then analyzed by LC-MS/MS. A number of tryptic peptides of each protein

were identified on the basis of their full-scan mass spectra and product ion spectra. Identified tryptic peptides yielded protein scores of more than 100 with the use of the “Mascot” search engine (<http://www.matrixscience.com>), according to which protein scores greater than 70 are considered significant ($p < 0.05$) for positive identification. Moreover, using the SwissProt database (<http://prospector.ucsf.edu/prospector/cgi-bin/mssearch.cgi>) and the taxonomy *Homo sapiens* (20,200 sequences), several combinations of only four out of many identified tryptic peptides provided a 100% match with studied proteins. This means that the simultaneous measurement of just four tryptic peptides would be sufficient to positively identify and quantify a target protein. As an example of full-scan mass spectra, Fig. 6 illustrates the mass spectrum of one of the tryptic peptides of hAPE1 and its ^{15}N -labeled analog (Kirkali et al., 2013). In general, mass spectra of such peptides contain an MH^+ ion of lower abundance (ion at m/z 1131 in Fig. 6) and a doubly protonated (charged) molecular ion $[(\text{M}+2\text{H})^{2+}]$ as the most abundant ion (m/z 566.1 in Fig. 6) (Kinter & Sherman, 2000). The mass spectrum of the ^{15}N -labeled peptide exhibited a shift of 14 Da in the mass of MH^+ of the unlabeled peptide, consistent with the fourteen ^{15}N atoms in the molecule. The shift in the mass of $(\text{M}+2\text{H})^{2+}$ was also in agreement with the mass difference of 14 Da. To obtain product ion spectra, optimum collision energies using $(\text{M}+2\text{H})^{2+}$ ions as the precursor ions were measured (Coskun et al., 2015; Reddy et al., 2013). These were in agreement with previously determined collision energies of peptides with similar masses (Kinter & Sherman, 2000). Product ion spectra of identified tryptic peptides were recorded using the determined collision energy for each peptide. As examples, product ion spectra of the peptides in Fig. 6 are illustrated in Fig. 7 (Kirkali et al., 2013). Such spectra are generally dominated by so-called γ -series ions with b -series ions being of lesser intensity (Kinter & Sherman, 2000). In Fig. 7A, typical γ -ions from the γ_1 -ion to the γ_9 -ion, with the most intense ion being the γ_8 -ion at m/z 904, are seen with only a few b -ions. The ^{15}N -labeled analog gave an essentially identical spectrum with mass shifts according to the ^{15}N -content of the fragments (Fig. 7B). The observed masses of all γ -series ions and b -series ions in product ion spectra of analyzed tryptic peptides perfectly matched the corresponding theoretical masses calculated using NIST *Mass and Fragment Calculator* (www.nist.gov/mml/bmd/bioanalytical/massfragcalc.cfm).

Selected reaction monitoring (SRM) was used to analyze trypsin hydro-lysates of proteins and their ^{15}N -labeled analogs to establish the sensitive and accurate measurement of proteins in vivo. Mass transitions from $(\text{M}+2\text{H})^{2+}$ to the most intense γ -ion in product ion spectra were used. Ion-current profiles of the mass transitions of tryptic peptides exhibited excellent peak shapes and a baseline separation between peptides. As expected, each tryptic peptide and its respective ^{15}N -labeled analog coeluted at the same retention time (Coskun et al., 2015; Dizdaroglu et al., 2011; Kirkali et al., 2013; Reddy et al., 2013). To further ascertain the identity of a target peptide, multiple typical transitions were simultaneously monitored. All typical transitions of tryptic peptides lined up at their appropriate retention times, confirming their identity. The analytical sensitivity of the LC-MS/MS instrument was measured. Depending on the peptide, the on-column limit of detection varied from 10 to 20 fmol with a signal-to-noise ratio of at least 3, whereas the limit of quantification was approximately 50–100 fmol of target peptide with a signal-to-noise ratio of 10.

9.2 Measurement of DNA Repair Proteins In Vivo

hAPE1 has been positively identified and quantified in nuclear and cytoplasmic extracts of three human cell lines and in mouse liver (Kirkali et al., 2013), and in human cancerous and disease-free breast tissues (unpublished data). Different expression levels of hAPE1 were observed depending of the cell line and tissue types. As an example, Fig. 8 illustrates ion-current profiles of mass transitions of five tryptic peptides of hAPE1 and ^{15}N -hAPE1 obtained using the tryptic digest of a nuclear extract of human mammary gland epithelial adenocarcinoma (MCF-7) cells. The signals of five peptides of hAPE1 and ^{15}N -hAPE1, which was added to protein extracts as an internal standard, lined up at the corresponding retention times, unequivocally identifying hAPE1 in this human cell line. The expression levels of hAPE1 in cell lines and tissues were calculated by using the measured signals of mass transitions of each tryptic peptide and their ^{15}N -labeled analogs, the amount of the internal standard, and the protein amount in each protein extract. In addition, we demonstrated the detection of APE1 variants with single amino acid replacements, i.e., Gln51His and Gly241Arg, which occur in several cancers (Pieretti, Khattar, & Smith, 2001), and in the general population (Hadi, Coleman, Fidelis, Mohrenweiser, & Wilson, 2000; Mohrenweiser, Xi, Vazquez-Matias, & Jones, 2002).

hMTH1 has been unequivocally identified and quantified in human malignant and disease-free breast tissues and in four cultured human cell lines (Coskun et al., 2015). Extreme expression of MTH1 in malignant breast tumors was observed, suggesting that cancer cells are addicted to MTH1 for their survival. In agreement with this finding, cultured MCF-7 cells exhibited a much greater expression level of hMTH1 than their normal counterpart mammary gland epithelial (MCF-10A) cells.

10. CONCLUSIONS

In this chapter, we described the production and purification of full-length ^{15}N -labeled DNA proteins to be used as internal standards for the measurement of DNA repair proteins in human tissues in vivo by LC-MS/MS or by other mass spectrometric techniques. These internal standards are absolutely essential for positive identification and accurate quantification of target proteins. The use of these labeled proteins has been demonstrated for the measurement of several DNA repair proteins in human tissues and cultured cells. Such measurements will be of fundamental importance for the determination of DNA repair capacity, the use of DNA repair proteins as biomarkers, and the development of DNA repair inhibitors in cancer, and eventually in other diseases as well.

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Certain commercial equipment or materials are identified in this chapter in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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Figure 1.

SDS (13%)-PAGE analysis of the purification steps of *E. coli*¹⁵N-Fpg: lane 1, 21 µg protein of the extract of BL21(DE3) cells harboring pET11a control plasmid; lane 2, 31 µg protein of the extract of BL21(DE3) cells harboring Fpg/pET11a plasmid induced with IPTG; lane 3, 26 µg of 10000 × *g* supernatant; lane 4, 8.6 µg flow through from DEAE cellulose column, 29 µg; lane 5, 8.5 µg ¹⁵N-Fpg eluted from CM cellulose column, 26 µg; and lane 6, molecular mass markers. *Data from Reddy et al. (2011).*

Figure 2.

SDS (10%)-PAGE analysis of the purification steps of ¹⁵N-hOGG1: lane 1, 20 µg protein of the extract of BL21(DE3) cells harboring pET11a control plasmid; lane 2, 21 µg protein of the extract of BL21(DE3) cells harboring hOGG1/pET15b plasmid induced with IPTG; lane 3, 17 µg of 48000 × *g* supernatant; lane 4, 15.5 µg from Ni-agarose flow through; lane 5, 8.3 µg ¹⁵N-hOGG1 eluted from Ni-agarose column; and lane 6, molecular mass markers. *Data from Reddy et al. (2011).*

Figure 3.

SDS (10%)-PAGE analysis of the purification steps of ¹⁵N-hNEIL1: lane 1, uninduced cell extract, 24 µg; lane 2, induced cell extract, 28 µg; lane 3, 20000 × g supernatant, 22 µg; lane 4, flow through from nickel resin, 17 µg; lane 5, pure ¹⁵N-NEIL1 eluted from nickel resin, 15 µg; and lane 6, molecular mass markers. *Data from Reddy et al. (2013).*

Figure 4.

SDS (11%)-PAGE analysis of the purification steps of ^{15}N -hAPE1: lane 1, uninduced cell extract, 24 μg ; lane 2, induced cell extract, 28 μg ; lane 3, 70000 $\times$ g supernatant, 24 μg ; lane 4, flow through from DEAE cellulose column, 17 μg ; lane 5, flow through from CM cellulose column, 16 μg ; lane 6, pure ^{15}N -APE1 eluted from CM cellulose column, 21 μg ; and lane 7, molecular mass markers. Data from *Kirkali et al. (2013)*.

Figure 5. SDS-PAGE analysis of purified hMTH1 and ^{15}N -hMTH1. Lane 1, hMTH1; lane 2, ^{15}N -hMTH1; and lane 3, molecular mass markers. *Data from Coskun et al. (2015).*

Figure 6. Full-scan mass spectra of the tryptic peptides GAVAEDGDEL R (A) and ^{15}N -GAVAEDGDEL R (B). Data from Kirkali et al. (2013).

Figure 7.

Product ion spectra of GAVAEDGDEL R (A) and ^{15}N -GAVAEDGDEL R (B). The $(\text{M}+2\text{H})^{2+}$ ions m/z 566.3 (A) and m/z 573.3 (B) were used as the precursor ions. The insert shows the fragmentation pathways leading to the b - and y -ions. Data from Kirkali et al. (2013).

Figure 8.

Ion-current profiles of mass transitions of five tryptic peptides of hAPE1 and ^{15}N -hAPE1 obtained using the tryptic digest of a nuclear extract of MCF-7 cells. The extract was spiked with an aliquot of ^{15}N -hAPE1 prior to separation by HPLC. Data from *Kirkali et al. (2013)*.