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Probing adenosine nucleotide-binding proteins with an affinity labeled-nucleotide probe and mass spectrometry

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Abstract

Mass spectrometry combined with chemical labeling strategies has become very important in biological analysis. Herein, we described the application of a biotin-conjugated acyl nucleotide for probing adenosine nucleotide-binding proteins. We demonstrated that the probe reacted specifically with the lysine residue at the nucleotide-binding site of two purified adenosine nucleotide-binding proteins, *Escherichia coli* RecA and *Saccharomyces cerevisiae* alcohol dehydrogenase-I (YADH-I). A single conjugate peptide with a specifically labeled lysine residue was identified, by using LC-MS/MS, from the tryptic digestion mixture of the reaction products of the nucleotide analog with RecA or YADH-I. The strategy, which involved labeling reaction, enzymatic digestion, affinity purification and LC-MS/MS analysis, was relatively simple, fast and straightforward. The method should be generally applicable for the identification of lysine residues at the nucleotide-binding site of other proteins. The biotin-conjugated acyl nucleotide probe also allowed for the enrichment and identification of nucleotide-binding proteins from complex protein mixtures; we showed that more than 50 adenosine nucleotide-binding proteins could be identified from the whole cell lysates of HeLa-S3 and WM-266-4 cells.

INTRODUCTION

Mass spectrometry (MS) has progressed extremely rapidly during the past two decades. The application of MS to the identification of chemical compounds in a mixture, including determining the structural composition of large biomolecules, becomes increasingly popular¹. When the analysis is directed towards complex biological mixtures or protein functional investigations, a few challenges, such as sample complexity and quantitation, are encountered when MS techniques are used alone. Fortunately, this can be overcome, to some extent, by combining MS with powerful separation techniques, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), in which proteins are separated based on their isoelectric points and molecular masses, or LC-based strategies, e.g., the multi-dimensional protein identification technology (MudPIT)^{2, 3}.

Aside from these technologies, chemical tagging methods that involve the modification of functional groups of amino acid residues in proteins and peptides have been described⁴. These chemical tagging or labeling reagents target specific amino acid residues or post-translational modifications (PTMs), which facilitate the enrichment of subfractions of interest via affinity purification. When stable isotope-labeled tags are employed, relative quantitation of protein expression can be readily achieved. In this context, isotope-coded affinity tag (ICAT) has become widely used⁵. Only those peptides containing certain amino acids (in this case, cysteine) can be targeted; an affinity tag, usually containing a biotin moiety, is attached to the functional group of interest via covalent linkage, which allows for reducing the sample

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complexity by affinity purification. However, these chemical tags, by measuring protein abundance, lack specificity for functional study of proteins, especially for various enzymes. To address this limitation, Cravatt and coworkers^{6,7} have developed a series of activity-based chemical tagging approaches, known as activity-based protein profiling, or ABPP, for functional proteomic studies. For instance, they reported an LC-MS strategy to identify the sites of labeling on several enzymes targeted by sulfonate ester probes⁸. In this approach, proteomes were treated with a rhodamine-tagged phenyl sulfonate ester, followed by denaturation, thiol reduction, alkylation, and trypsin digestion. The peptide mixture was then incubated with an affinity capture matrix to isolate specifically the probe-labeled peptides for the subsequent LC-MS/MS analysis.

In addition to the sulfonate ester probes, a variety of nucleotide analogs, which are usually fluorescent, photoactive or affinity-labeled, have been developed for different applications⁹⁻¹¹. Among these nucleotide analogs, ATP derivatives are the most widely used because ATP is essential for almost all living organisms and it is a substrate for numerous enzymes and ATP-binding proteins. For example, 5'-*p*-fluorosulfonylbenzoyl adenosine (FSBA), a reactive ATP analog, has been used as an activity-based probe to target nucleotide-binding proteins¹². In addition, a class of acyl nucleotide probes, as well as the methods for their synthesis and use in proteomic analysis, has been described¹³. These probes, termed tagged acyl phosphate probes (TAPPs) or more specifically nucleotide-binding protein-directed affinity probes (NBAPs), was claimed to provide enhanced simplicity and accuracy in identifying changes in the presence, amount, or activity of target proteins in a complex protein mixture, preferably nucleotide-binding proteins including protein kinases. Recently, several hundred protein kinases have been identified by using these acyl phosphate probes in an analysis of more than 100 proteomes including human, mouse, rat and dog, which has demonstrated the ability of the acyl phosphate probes to broadly profile protein kinases in native proteomes¹⁴.

In this paper, we chose two adenosine nucleotide-binding proteins, *Escherichia coli* recombinase A (RecA), an ATP/ADP-binding protein, and *Saccharomyces cerevisiae* alcohol dehydrogenase-I (YADH-I), a nicotinamide adenine dinucleotide (NAD)-binding protein, to demonstrate the utility of the affinity-labeled acyl-phosphate probe with MS in elucidating protein structure and probing nucleotide-binding sites. We also applied the probe to profile the nucleotide-binding proteins in cell lysates. The method shows the potential application of this probe in the purification, enrichment and identification of nucleotide-binding proteins from whole cell lysates.

MATERIALS and METHODS

Materials

ATP, in disodium salt form, was from MP Biochemicals (Solon, OH). D-biotin was purchased from AnaSpec Inc. (San Jose, CA), and β -alanine was from TCI America (Portland, OR). *E. coli* RecA protein was obtained from Epicentre Biotechnologies (Madison, WI). YADH-I and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO) and BioRad (Hercules, CA), respectively. These proteins were used without further purification. Streptavidin-conjugated magnetic particles and sequencing-grade modified trypsin were obtained from Roche Applied Science (Indianapolis, IN). Common reagents for synthesis were obtained from VWR. Other reagents, unless otherwise specified, were from Sigma-Aldrich.

Preparation of the Biotinylated ATP Probe

The general methods for the preparation of acyl nucleotide probes were described previously¹³. Briefly, the biotinylated ATP probe was synthesized via a two-step procedure as outlined in Scheme 1. In this regard, we first prepared 3-*N*-D-biotinylaminopropanoic acid following

previously published procedures^{15, 16}. Isobutyl chloroformate (0.32 ml, 2.47 mmol) was added to a solution containing biotin (500 mg, 2.05 mmol), DMF (40 ml), and tri-*n*-butylamine (0.64 ml, 2.69 mmol). After being incubated at room temperature for 10 min, the mixture was slowly added to a suspension of β -alanine (4.1 mmol) in DMF (40 ml) at 5°C. After stirring at 5°C for 2 hrs, the solvent was removed under reduced pressure and the crude product was dissolved in a 36-ml water and ethanol (1:1, v/v) solution at 40°C. The solution pH was adjusted to 2 with 2.0 N HCl and the mixture was kept at 0°C for 12 hrs. The desired 3-*N*-D-biotinylaminopropanoic acid was precipitated out of solution. The precipitate was filtered, washed with water, and dried under vacuum.

To make ATP soluble in organic solvent, the commercially available disodium salt of ATP was converted to the tributylammonium form by passing it through a cation exchange column packed with Spectra/Gel IE 50X8 resin (40-75 μ m), in which the H⁺ was displaced by tributylammonium ion (NHBu₃⁺). The exchange was performed by passing through the column twice at 4°C, and the collected ATP-NHBu₃⁺ fractions were lyophilized to form white solids.

The acyl adenosine-nucleotide was synthesized following the procedures described by Kreimeyer et al.¹⁷ with minor modifications. Briefly, 3-*N*-D-biotinylaminopropanoic acid (94.5 mg, 0.3 mmol) was dissolved in a 2.5-ml solvent mixture of dry CH₂Cl₂ and DMF (4:1, v/v). To the ice-cold solution were then added tri-*n*-butylamine (0.11 ml, 0.45 mmol) and ethyl chloroformate (0.03 ml, 0.3 mmol). After stirring at 0°C for 5 min, the mixture was stirred at room temperature under argon atmosphere for another 60 min. ATP-NHBu₃⁺ (0.1 mmol), dissolved in a 2.5-ml solution of CH₂Cl₂ and DMF (4:1, v/v), was then added to the above reaction mixture. The reaction was continued at room temperature and under argon atmosphere for 18 hrs, and to the reaction mixture was added toluene (5 ml), which produced white precipitates. The solid was collected by filtration and dried under vacuum. To the filtrate was added water (2 ml) and the solution was extracted with toluene. The aqueous phase was collected, lyophilized, and combined with the precipitate.

The resulting solid products were purified by HPLC with a YMC ODS-AQ column (4.8×250 mm, 120 Å in pore size, 5 μ m in particle size, Waters). The flow rate was set at 0.8 ml/min, and a 45-min linear gradient of 0-30% acetonitrile in 50 mM triethylammonium acetate (TEAA, pH 6.8) was used for the purification. A UV detector was set at 265 nm for monitoring the effluents. Appropriate HPLC fractions were collected, lyophilized, and stored at -20°C. The product was confirmed by phosphorus NMR and ESI-MS. ³¹P NMR (121 MHz, D₂O, proton decoupled): δ (in p.p.m.) -10.5 (d, 1P, J_{P-P} = 20.1 Hz), -19.0 (d, 1P, J_{P-P} = 16.4 Hz), -22.6 (d, 1P, J_{P-P} = 21.0 Hz). ESI-MS: m/z 803.0 ([M-H]⁻), 401.0 ([M-2H]²⁻).

Reaction of the Biotin-ATP Probe with Lysine

The biotin-ATP probe solution was prepared by dissolving the lyophilized compound in water and stored in a -20 °C freezer. To investigate the labeling efficiency of the biotin-ATP probe, different concentrations of lysine, ranging from 0.1 μ M to 1 mM, were incubated with the biotin-ATP probe at a concentration of 0.1 mM (reaction volume, 50 μ l).

Labeling of RecA and YADH-I with the Biotin-ATP Probe

Different concentrations of the probe, which varied from 0.1 μ M to 1 mM, were treated with 10 μ g of the protein. For the labeling reaction with protein mixtures, the amount of BSA was varied from 10 μ g to 1 mg while the amount of RecA was kept at 10 μ g.

For the labeling reaction, the biotin-ATP probe solution was added to a solution of the protein in PBS buffer (pH 7.5) and mixed by quickly flicking the tube. The labeling reaction was continued with gentle shaking at room temperature for 1 hr or at 4°C for 4 hrs. At the end of

the labeling reaction, the excess probes were removed by using Microcon YM-10 centrifugal filter (Millipore, Billerica, MA).

Cell Lysate Preparation and Labeling with the Biotin-ATP Probe

HeLa-S3 cells were purchased from National Cell Culture Center (Minneapolis, MN). The WM-266-4 cell line, derived from a metastatic site of a human malignant melanoma, was purchased from ATCC (Manassas, VA) and cultured in Eagle's Minimum Essential Medium (EMEM) supplied with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 IU/mL of penicillin, 100 µg/mL of streptomycin and 1.5 g/L of sodium bicarbonate in 5% CO₂ at 37°C. Cells were harvested and washed three times with cold PBS. Approximately 6×10⁶ cells were lysed in 500 µL lysis buffer, which contained 0.7% CHAPS, 50 mM HEPES (pH 7.4), 0.5 mM EDTA, 100 mM NaCl and a protease inhibitor cocktail, on ice for 30 min. The cell lysates were centrifuged at 16,000 g at 4°C for 30 min, and the resulting supernatants were collected. To remove free endogenous nucleotides, particularly ATP, cell lysates were subjected to gel filtration separation by using NAP-25 columns (Amersham Biosciences). Cell lysates were eluted into 1.5 ml elution buffer, which contained 50 mM HEPES (pH 7.4), 75 mM NaCl and 5% glycerol. The gel-filtered cell lysates were further concentrated by using Microcon YM-10 centrifugal filter. The proteins in cell lysates were quantified by using Quick Start™ Bradford Protein Assay (BioRAD). Salts with divalent cations were added to the concentrated cell lysate until it contained 50 mM MgCl₂, 5 mM MnCl₂, and 5 mM CaCl₂. The biotin-ATP probe was then added until its final concentration reached 100 µM. Labeling reactions were carried out with gentle shaking at room temperature for 1 hr. After reaction, cell lysates were cleaned and the buffer was exchanged to 100 mM NH₄HCO₃ solution (pH 8.5) by using Microcon centrifugal filter, followed by the addition of dithiothreitol (DTT) and iodoacetamide (IAA) to reduce and block cysteines.

Enzymatic Digestion and Affinity Purification

The labeled proteins were digested by using modified sequencing-grade trypsin at an enzyme/substrate ratio of 1:50 in 100 mM NH₄HCO₃ (pH 8.5) and at 37°C for overnight, and the digestion was stopped by adding trifluoroacetic acid (TFA) to a final concentration of 5 mM. The peptide mixture was subsequently dried in a Speedvac.

Streptavidin-immobilized magnetic beads (MBs) were used to capture the biotin-labeled peptides from RecA and YADH-I digests. Prior to the binding, the MBs were washed with Buffer A, which contained 20 mM potassium phosphate and 0.15 M NaCl (pH 7.5), for at least 3 times and resuspended in 50 µl Buffer A. The dried tryptic peptides were dissolved in 25 µl Buffer A followed by the addition of the MB suspension. Mixing was achieved by quickly flicking the tube. The mixture was then incubated at 25°C for 30 min with gentle shaking.

After incubation, MBs were magnetically immobilized, the supernatant was removed, and the beads were washed with Buffer A for three times and then with pure water for another three times. Following the washing procedures, MBs were resuspended in pure water (50 µl). The biotinylated peptides were eluted from the beads according to a modified protocol¹⁸. In this regard, the MB suspension was incubated at 70°C for 5 min and the mixture was allowed to cool to room temperature. Once beads were magnetically captured, the supernatant, which contained eluted peptides, was transferred to a clean tube. This process was repeated once and the eluates were combined, dried in a Speedvac, and stored at -20°C prior to LC-MS analysis.

Affinity columns packed with avidin-agarose resin (Sigma-Aldrich) were used to improve the capacity for capturing biotin-labeled peptides from HeLa-S3 and WM-266-4 cells. Briefly, the packed avidin-agarose resin was washed with 10 column volumes of Buffer A before the tryptic digests were loaded. After a 30-min incubation, the unbound peptides were removed by

washing the resin thoroughly with binding buffer and pure water. Following washing, labeled peptides were eluted with a mixture of CH₃CN and H₂O (1:1, v/v) at 65°C. The eluates were dried in a Speedvac and stored at -20°C prior to mass spectrometric analysis.

LC-MS/MS

LC-MS/MS analysis of the affinity-purified peptides was performed in triplicate on an LTQ linear ion-trap mass spectrometer (Thermo Electron, Waltham, MA) equipped with an electrospray ionization source. An Agilent 1100 capillary HPLC system (Agilent, Palo Alto, CA) with a Zorbax SB-C18 capillary column (5 µm in particle size, 0.5×250 mm, Agilent Technologies) was used for the separation, and the effluent from the column was coupled directly to the ionization source. Peptides were eluted from the column using a 63-min linear gradient of 2-65% CH₃CN in 0.6% aqueous solution of acetic acid, and the flow rate was 6 µl/min. The product-ion spectra were acquired in data-dependent mode, where the eight most abundant ions observed in MS scan were chosen sequentially for collisional activation to obtain MS/MS.

Data Processing

The product-ion spectra from RecA and YADH-I samples were searched against UniProt (Universal Protein Resource, <http://www.ebi.ac.uk/trembl/index.html>) using TurboSEQUEST in Bioworks 3.2 (Thermo Electron Corporation, San Jose, CA). MassAnalyzer 1.03, provided by Dr. Zhongqi Zhang at Amgen Inc., was used to further confirm the search results^{19, 20}. The product-ion spectra from the analysis of samples of HeLa-S3 and WM-266-4 cell lysates were searched against NCBI human RefSeq protein database. The sequences and the labeling sites of all the identified biotin-labeled peptides were confirmed by analyzing manually the product-ion spectra of these peptides.

RESULTS AND DISCUSSION

Reactivity of the Biotin-ATP Probe with Lysine

Many acyl phosphates are metabolic acylating reagents. A best-known example of these is acetyl coenzyme A, which is the acetyl group donor in many biological acetylation reactions²¹. In this paper, the biotin-ATP probe was used as a chemical reagent for the acylation of ε-amino group of lysine residues in proteins (The structure of the biotin-ATP probe is shown in Scheme 1). The reactivity of this probe relies mainly on the mixed carboxylic phosphoric anhydride²². In this respect, acyl phosphates are known to react efficiently with amines to form amides, and the hydrolysis of acyl phosphates is very slow compared to the rate of amide bond formation when suitable concentrations of amines are used²³.

To evaluate the reactivity of the biotin-ATP probe, different concentrations of lysine were used. ESI-MS and tandem MS (MS/MS, Figure 1) results revealed that, with high concentrations of lysine (1 mM) and long incubation time (4 hr), an acylation product of lysine was formed. In this respect, the product-ion spectrum of the [M-H]⁺ ion of the acylation product showed the formation of a fragment ion of *m/z* 145, which is attributed to the deprotonated ion of lysine (Figure 1B). At low lysine concentrations (< 10 µM), the formation of the acylation product was, however, not obvious under the otherwise identical reaction conditions.

Labeling of Adenosine Nucleotide-binding Proteins

To investigate the reaction of biotin-ATP probe with target proteins, we chose two adenosine nucleotide-binding proteins, that is, RecA and YADH-I. The former protein is a multi-functional DNA-binding protein and a critical player in both homologous recombination and post-replicative DNA repair²⁴. The X-ray crystal structures of *E. coli* RecA complexed with

ADP or AMP-PNP, a non-hydrolyzable analog of ATP, have been determined²⁵⁻²⁷. YADH-I is a member of zinc-containing alcohol dehydrogenases. It contains four identical subunits, and each subunit harbors an active site. The active site binds one NAD and a zinc atom, which is essential for the oxidation of alcohols to aldehydes^{28, 29}. The X-ray crystal structure of YADH-I has been released very recently (PDB entry: 2HCY).

Similar as what we found for the reaction with lysine, the reaction of the acyl phosphate probe with proteins occurs at lysine residues as well. The difference lies in that the latter reaction is site-directed because the probe distinguishes many amino groups in the protein chain and reacts with a specific lysine residue in the two proteins.

We identify the probe labeling site(s) by digesting the RecA and YADH-I after the labeling reactions, treating the digestion mixtures with streptavidin-immobilized magnetic beads, eluting the bound peptides from the beads, and subjecting the peptide mixtures to LC-MS/MS analysis. The LC-MS/MS results showed that only one probe-labeled peptide, IVEIYGPESGK*TTLTLQVIAAAQR, was present in the tryptic digestion products of the reaction mixture of RecA (The asterisk symbol designates the labeled lysine residue, and MS/MS for the modified peptide is shown in Figure 2A). In this respect, the mass difference between the native and the probe-labeled peptide is 297.1 Da, which is consistent with the monoacylation of this peptide segment. In addition, the amide bond on the C-terminal side of the labeled lysine residue was no longer susceptible to trypsin cleavage. Therefore, this peptide with one internal lysine residue, which is K72, can be identified. This conclusion is further substantiated by the MS/MS results (Figure 2A).

In keeping with our observations here, studies on K72R mutant of RecA showed that K72 is critical for ATP hydrolysis^{30, 31}. The X-ray crystal structure of RecA-MgADP complex revealed that the phosphates of ADP form hydrogen bonds with the side chains of S70, K72, T73 and T74, and K72 is believed to stabilize the transition state of the ATP hydrolysis reaction mediated by RecA²⁵⁻²⁷. Moreover, the crystal structure of RecA-MnAMP-PNP complex from the same report showed that K72 contacts directly with the γ phosphate of AMP-PNP, a non-hydrolyzable ATP analog. Site-directed mutagenesis experiments and X-ray crystal structures of RecA also demonstrated that K72 is critical for ATP binding and the subsequent hydrolysis reaction. Different from these previous studies, our method facilitates the identification of the probe-labeled K72 residue, which might serve as direct evidence supporting that K72 is involved in ATP binding and hydrolysis.

The structure of the RecA-ADP complex (Figure 3A) shows that, among all 26 lysine residues in this protein, only K72 is in close proximity to the β phosphate component of ADP (the distance between the ϵ -amino function of K72 and the β phosphate component of ADP is 3.6 Å). In this context, it is worth noting that the solvent accessible surface area of K72 is the smallest among all lysine residues [determined by using GETAREA³², http://www.scsb.utmb.edu/cgi-bin/get_a_form.tcl]. Therefore, the specificity of the labeling reaction between the biotin-ATP probe and K72 is attributed to the binding of the acyl nucleotide probe to the ATP binding site of the protein.

We next asked whether adenosine nucleotide-binding proteins other than ATP-binding proteins can also be labeled by the biotin-ATP probe. To this end, we employed YADH-I, an NAD-binding protein, to carry out the labeling reaction. The LC-MS/MS results revealed again the presence of a single, probe-labeled peptide with the sequence of VLGIDGGEGK*EELFR in the MB-enriched peptide mixture. In this regard, we found a mass difference of 425.2 Da, corresponding to the mass of the labeled lysine residue, between the y_5 and y_6 ions in the MS/MS of this peptide (Figure 2B), supporting that this lysine residue is modified. The labeled

lysine was K206, which, among all the lysine residues, is the closest to NAD according to the X-ray crystal structure of YADH-I (Figure 3B, PDB entry: 2HCY).

It is worth noting that short reaction time and low concentration of biotin-ATP probe (10 μ M probe and 1-hr reaction time at room temperature) were employed for the above labeling experiments. When the labeling reaction was carried out with a higher concentration of the biotin-ATP probe and a longer incubation time, e.g., 1 mM probe and 4-hr incubation, however, the labeling may occur at K225 as well. We again calculated the total solvent accessible areas of all lysine residues in YADH-I (subunit A) and it turned out that, among all the lysine residues, K225 assumes the largest surface area (209.8 $\text{\AA}^2$). In contrast, K206 has only a small area exposed to solvent (15.3 $\text{\AA}^2$). Nevertheless, at low probe concentrations, the lysine residue at the nucleotide-binding site of adenosine nucleotide-binding proteins can be labeled specifically. In this regard, NAD and ATP share the same adenosine diphosphate moiety, which accounts for the reactivity between the acyl nucleotide probe and the NAD-binding protein, YADH-I. This result showed that, to achieve labeling with high specificity, the concentration of the acyl nucleotide probe should be kept as low as feasible.

Affinity Purification and Enrichment of the Labeled Peptides

To demonstrate the potential application of the biotin-ATP probe for more complex samples, we examined the reaction of the probe with a protein mixture containing both RecA (10 μ g) and BSA (from 10 μ g to 1 mg). After the labeling reaction, we again digested the protein mixture with trypsin, subjected the peptide mixture to enrichment with magnetic beads as described above, and analyzed the resulting peptide mixture by LC-MS/MS. The LC-MS/MS results allowed us to identify, without ambiguity, the labeled K72-bearing peptide with the sequence of IVEIYGPESGK*TTLTLQVIAAAQR in all samples containing up to 1 mg BSA. We, however, did not identify any probe-labeled peptide from BSA, demonstrating the specificity of the reaction of the probe with nucleotide-binding proteins. This result also highlights that the affinity purification procedure allows for the enrichment of biotin-bearing peptide from a large quantity of peptide mixture.

To examine the effectiveness of the MBs in the enrichment of the probe-bearing peptides, we also analyzed directly, by LC-MS/MS, the tryptic digests of YADH-I after labeling reaction but without MB purification. We then evaluated the efficiency of the enrichment procedure by comparing the ion intensity for the probe-bearing peptide with respect to two other peptides which are not susceptible to reaction with the biotin-ATP probe, i.e., SIGGEVFIDFTK (m/z 656.8) and DIVGAVLK (m/z 814.5). Figure 4 depicts the selected-ion chromatograms (SICs) for these three peptides based on the LC-MS/MS analyses of the digestion mixture with (Figure 4B) or without (Figure 4A) MB enrichment. The data revealed that the enrichment procedure leads to significant decrease in the ion intensity for the unmodified peptides. In this respect, we estimated that the enrichment procedure allows for the selective enrichment for the probe-bearing peptide by at least 300 fold.

Identification of Biotin-ATP Probe Labeled Proteins in Whole Cell Lysates

From the above experiments with RecA and YADH-I, we showed that the biotin-ATP probe exhibits high specificity for adenosine nucleotide-binding proteins, and the affinity purification strategy can allow for the selective enrichment of the biotin-labeled peptides from a digestion mixture. To assess the performance of the biotin-ATP probe for real complex samples, we employed this strategy to the whole cell lysates of HeLa-S3 and WM-266-4 cells. The results are summarized in Figure 5. Overall, we identified probe-labeled peptides of 58 and 77 proteins from the whole cell lysates of HeLa-S3 and WM-266-4 cells, respectively, and 15 common proteins were found in both cell lines. The identified peptides from these two cell lines, as well as the labeling site(s) are listed in the Supporting Information.

Among these targets, 22 nucleotide-binding proteins were identified from the cell lysate of either HeLa-S3 or WM-266-4 cells (Figure 5A). Most of these identified nucleotide-binding proteins have clear adenosine nucleotide-binding ability. For example, adenylate kinase 2 is a ubiquitous enzyme which acts as a phosphotransferase catalyzing the production of ATP from ADP, a process involved in cellular energy homeostasis³³. We identified one peptide, AVLLGPPGAGK*GTQAPR, with labeling by MS/MS (Figure 6A). The labeled lysine residue, K28, resides at the active site of this enzyme (Figure 6C). Phosphoglycerate kinase 1 (PGK1) is a transferase enzyme involved in glycolysis. It employs the energy released in ATP hydrolysis to catalyze the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate. Recent work has revealed that PGK1 is overexpressed in pancreatic ductal adenocarcinoma³⁴. The X-ray crystal structure of the yeast enzyme reveals that ATP can bind to the enzyme at the active site, where lys residues are present (Figure 6D). Our MS/MS result also confirmed that K216, within peptide ALESPERPFLAILGGAK*VADK, at the active site reacted with the biotin-ATP probe (Figure 6B).

Actins and heat shock proteins are two families of abundant ATP-binding proteins. Some of these proteins have been identified with more than one labeling sites. The multiple labeling of these proteins may be attributed to the high abundances of these proteins, which result in the non-specific labeling of the proteins. Alternatively, the labeling at multiple sites might also be originated from the interactions of actins and heat shock proteins with other nucleotide-binding proteins. The latter might be particularly true for heat shock proteins, which are molecular chaperones and have many interaction partners.

Our results also revealed that, other than ATP-binding proteins, some nucleotide analogue-binding proteins can also couple with the probe. As shown above, YADH-I could react with the biotin-ATP probe specifically via lysine residue at the NAD-binding sites. With respect to cell lysates, 7 and 5 NAD(P)-binding proteins were identified in HeLa-S3 and WM-266-4 cells, respectively (Figure 5A). Most of these proteins are dehydrogenases, which oxidize the substrate by transferring one or more protons and a pair of electrons to an acceptor, usually NAD/NADP or a flavin coenzyme such as flavin adenine dinucleotide (FAD). For example, lactate dehydrogenase A was identified in both cell lysates. The X-ray crystal structure revealed that it was labeled at one of the two conserved active site lys residues (PDB entry, 1I10). Similarly, lactate dehydrogenase B (PDB entry, 1T2F), glyceraldehyde-3-phosphate dehydrogenase (PDB entry, 1ZNQ) and catalase (PDB entry, 1DGB) were identified with specific labeling of the lys residues at the nucleotide analog-binding sites, which is in keeping with the available X-ray crystal structures.

In addition to the identified nucleotide-binding proteins, we also detected probe-labeled peptides from several RNA/DNA-binding proteins and proteins whose nucleotide-binding ability remains unknown. To the best of our knowledge, some of these proteins are actin binders, phosphate binders, kinase substrates, ribosomal proteins and proteins without binding ability being described. Many of these proteins are abundant, and the surface lys residues in these proteins might be involved in the labeling reaction with the biotin-ATP probe. These results, therefore, indicate that the biotin-ATP probe may not be highly specific, especially when the sample is as complex as a whole proteome. Similar results were described in recent reports^{12, 14}. In this context, it is important to note that some kinase substrates could also be labeled in a “crossover” manner¹². Thus, some proteins which are capable of interacting with nucleotide-binding proteins can also be modified by the probe without binding directly to the probe. In most cases, however, we could not differentiate these proteins involved in the “crossover” labeling from those being labeled nonspecifically because of the lack of protein-protein interaction information.

CONCLUSIONS

In this report, we demonstrated the site-directed labeling of nucleotide-binding proteins using a biotin-ATP probe. Results from the study of two adenosine nucleotide-binding proteins, RecA and YADH-I, demonstrated that the probe can allow for the identification of the lysine residue residing at the nucleotide-binding sites of these proteins. In this respect, LC-MS/MS analysis enabled us to identify the nucleotide-binding sites in proteins in a short period of time. K72, playing an important role in ATP hydrolysis for RecA, was labeled with the probe, providing direct evidence supporting that this residue is involved in ATP hydrolysis. K206 in YADH-I was also identified unambiguously by the labeling method. This strategy is relatively simple, fast and straightforward, which involves labeling reaction, enzymatic digestion, affinity purification and LC-MS/MS analysis. The method should be generally applicable for the identification of lysine residues at the binding sites of proteins that can bind to adenosine nucleotide and its analogs. The application of the biotin-ATP probe to cell lysates has shown a great potential in the activity-based functional study of other nucleotide-binding proteins, as well as in the profiling of nucleotide-binding proteins in complex samples. Quantitative analysis of the expression of nucleotide-binding proteins in cells with drug treatment or under different stress conditions may also be achieved by employing the acyl nucleotide probe and incorporating stable isotope labeling techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Negative-ion ESI-MS of 4-hr reaction mixture of biotin-ATP probe and lysine after dilution. The biotin-ATP conjugate of lysine, the product of this reaction, has an m/z of 442 in negative-ion ESI-MS. (B) Product-ion spectrum of the ion of m/z 442 observed in (A).

Figure 2.

The product-ion spectra of the probe-labeled peptides. (A) The identified peptide with the sequence of IVEIYGPESGK*TTLTLQVIAAQR (m/z 981.5, triply charged) from RecA with site-directed labeling at K72; (B) the identified peptide with the sequence of VLGIDGGEGK*EELFR (m/z 958.4, doubly charged) from YADH-I with site-directed labeling at K206. The labeled lysine is designated with “*”, and its structure is shown in (A).

(A)

(B)

Figure 3.

X-ray crystal structures of RecA and YADH-I. The bound ADP and NAD molecules and all lysine residues are shown in stick mode, whereas the lysine residues of the probe-labeling sites are shown in space-filling mode. (A) Structure of RecA-ADP complex (PDB entry, 1XMV); (B) Structure of YADH-I complexed with NAD (PDB entry, 2HCY; only subunit A is shown). RasMol (Version 2.7.2.1.1, <http://www.openrasmol.org/>) was used to display the structures.

(B)

Figure 4.

Selected-ion chromatograms (SICs) of ions, generated from LC-MS/MS analyses, corresponding to the biotinylated peptide, VLGIDGGEGK*EELFR (m/z 958.4), and two internal standard peptides, SIGGEVFIDFTK (m/z 656.8) and DIVGAVLK (m/z 814.5). The internal standard peptides were chosen from the unlabeled peptides identified in LC-MS/MS analysis of the tryptic digestion mixture of YADH-I without (A) and with (B) magnetic beads purification.

(A)

(B)

Figure 5.

Biotin-ATP probe-labeled proteins in HeLa-S3 and WM-266-4 cells. (A) The identified proteins were classified according to their nucleotide-binding ability. AXP: adenosine nucleotide; GxP: guanosine nucleotides; NAD(P): nicotinamide adenine dinucleotide (phosphate); RNA/DNA and others. (B) The Venn diagram shows the numbers of proteins and the overlap (intersection) of proteins identified from both cell lines.

Figure 6.

The product-ion spectra of the probe-labeled peptides from adenylate kinase 2, ALESPERPFLAILGGAK*VADK (m/z 944.1, doubly charged) with labeling site of K28 (A) and phosphoglycerate kinase 1, ALESPERPFLAILGGAK*VADK (m/z 827.2, triply charged) with labeling site of K216 (B). The Structures of adenylate kinase 2 complexed with bis (adenosine)-5'-tetraphosphate (PDB entry, 2C9Y) and yeast phosphoglycerate kinase 1 complexed with ATP (PDB entry, 3PGK) are shown in (C) and (D), respectively. In (D), K213 at the ATP binding site of yeast PGK1 corresponds to K216 in the human counterpart.

Scheme 1.

Reagents: (a) (i) isobutylchloroformate, tri-*n*-butylamine, DMF; (ii) H₂NCH₂CH₂COOH. (b) (i) ethylchloroformate, tri-*n*-butylamine, CH₂Cl₂/DMF; (ii) ATP, CH₂Cl₂/DMF.