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PURIFICATION OF NUCLEOTIDE-LINKED PEPTIDE

VIRENDRA N. PANDEY and MUKUND J. MODAK*
Department of Biochemistry, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, 185 South Orange Avenue, Newark, NJ 07203-2757 (U.S.A.)

SUMMARY

Affinity labeling of nucleotide-binding enzymes/proteins with ^{32}P-labeled nucleotides is a powerful technique to identify nucleotide-binding proteins as well as to radiolabel the specific binding site. We have used this approach for labeling a nucleotide-binding domain in DNA polymerase and have isolated peptides bearing the linked nucleotides. The method used for separating tryptic peptides on hydrophobic matrices with an acetonitrile gradient in 0.1% trifluoroacetic acid as eluent results in loss of radioactivity, presumably through dissociation of the cross-linked nucleotide. This can be averted by the use of a non-acidic medium in the peptide purification protocol. We have devised a relatively simple procedure to concentrate the nucleotide-linked peptides by chromatography on DEAE-Sephadex A25. Most neutral and basic peptides as well as free nucleotides are removed by eluting the DEAE-Sephadex column with 0.2 *M* ammonium bicarbonate. The nucleotide-linked peptide is then eluted with 0.6 *M* ammonium bicarbonate. Radioactivity in the collected fractions is conveniently determined by scintillation counting. Labeled peptide in the 0.6 *M* ammonium bicarbonate eluate can be purified on a C_4 reversed-phase column with an acetonitrile gradient in phosphate buffer (pH 6.8). By this procedure, ^{32}P-labeled nucleotide linked with protein/peptide can be quantitatively purified with minimum loss.

INTRODUCTION

Affinity labeling of enzymes with radioactive nucleotide triphosphates (dNTPs) is a direct approach in the identification of nucleotide-binding proteins as well as identification of binding sites in enzymes which use nucleotides as the substrate. Thus, *Escherichia coli* DNA polymerase I (*E. coli* pol I) and calf thymus terminal deoxynucleotidyl transferase (TdT) are successfully labeled with ^{32}P-labeled deoxynucleoside triphosphate (dNTP) as well as 8-azido-ATP[1–3]. The specificity of labeling reaction in both of these enzymes was demonstrated by the fact that labeling occurs only under those conditions which favour the formation of enzyme–substrate (E–S) complex. Similarly, labeling of a wide variety of proteins which contain the nucleotide-binding sites has been successfully and specifically achieved using photoaffinity analogues[4–8]. In most cases, the specificity of photoinsertion of nucleotide analogues is

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well documented although subsequent peptide mapping and actual demonstration of the site of attachment of nucleotide have been difficult. The standard peptide separation protocols, which include two to three steps of high-performance liquid chromatography (HPLC) on various reversed-phase matrices, are not always satisfactory for the purification of nucleotide linked peptides[2,3]. The main reason for the unsuitability of standard protocols is the employment of trifluoroacetic acid (TFA)-based gradients of acetonitrile as peptide eluting system. Although this is a widely used solvent system for effective separation of peptides, exposure of nucleotide-linked peptides to an acidic medium results in dissociation of linked nucleotide. This reaction probably occurs through a well known depurination type of reaction. Thus, the ability to identify the specific peptide which contains labeled nucleotide is lost. We find that substitution of the acidic medium with phosphate buffer at or near neutral pH, in the eluent, preserves the nucleotide label associated with peptides without significantly affecting their separation. Furthermore, a selective concentration of nucleotide-linked peptides has also been achieved by using an anion-exchange chromatography step prior to hydrophobic chromatography. We have used *E. coli* pol I and TdT as test enzymes for affinity labeling with [α-^{32}P]dTTP and/or [α-^{32}P]ATP and have isolated a peptide fraction that contains the covalently linked nucleotide substrate. Details of the involved procedures are the subject matter of this manuscript.

EXPERIMENTAL

α-^{32}P-labeled ribonucleoside triphosphates (rNTPs) and dNTPs were purchased from New England Nuclear (Boston, MA, U.S.A.) and from Amersham (Arlington Heights, IL, U.S.A.). Non-radioactive dNTPs and rNTPs were purchased from P.L. Biochemicals (Milwaukee, WI, U.S.A.); tosylphenylalanine chloromethyl ketone-treated trypsin was obtained from Worthington Biochemical (Freehold, NJ, U.S.A.) and diethylaminoethyl (DEAE)-Sephadex A25 from Pharmacia (Piscataway, NJ, U.S.A.). Sodium dodecyl sulfate (SDS) gel electrophoresis reagents were obtained from Sigma (St. Louis, MO, U.S.A.). TdT was purified to homogeneity from calf thymus as described previously[9]. The large fragment of *E. coli* DNA pol I was purified from a high producer *E. coli* strain as described by Joyce and Grindley[10].

UV-mediated photoaffinity labeling with ^{32}P-labeled NTP of the nucleotide-binding domain of enzyme proteins

UV-mediated labeling of calf thymus TdT protein[1,11] and of *E. coli* pol I[3,12] was carried out as follows: ^{32}P-labeled NTP was converted to its metal chelate form prior to use for affinity labeling. This was achieved by incubating an equimolar mixture of NTP and Mg^{2+} (or Mn^{2+}) in 50 m*M* Tris–HCl (pH 7.5) at 30°C for 10 min. A typical irradiation mixture contained 50 m*M* Tris–HCl (pH 7.5), 0.1 m*M* dithiothreitol, 10% glycerol, and the required concentration of enzyme protein and the metal chelate form of α-^{32}P-labeled NTP. Usually a 1:3 ratio of enzyme protein to the metal chelate of α-^{32}P-labeled NTP was used for preparative enzyme modification. The complete irradiation mixture was incubated on ice for 2–6 h, and irradiation was performed in a shallow polypropylene vessel, using a high-intensity UV lamp at a distance of 10 cm (1300 μW/cm^2/output at 15 cm) for 12 min. After irradiation, the solution was transferred to a dialysis bag and electrodialyzed to remove free α-

^{32}P-labeled NTP and then lyophilized. An aliquot of the labeled protein was subjected to SDS polyacrylamide gel electrophoresis (PAGE) to determine the extent of labeling[1].

Tryptic digestion of modified enzyme protein

The lyophilized [^{32}P]NTP-labeled enzyme protein (50–100 nmol) was dissolved in 200–300 μl of 8 *M* urea in 0.1 *M* Tris–HCl (pH 8.0) and then diluted to 2 *M* urea with the same buffer. Trypsin digestion was carried out initially at a protein:enzyme ratio of 50. The mixture was incubated at 30°C for 4 h. Then a second aliquot of trypsin (protein:enzyme ratio, 50) was added and the incubation was continued overnight. Digestion was stopped by quick freezing at −80°C.

Purification of [^{32}P]NTP-labeled tryptic peptide on a DEAE-Sephadex A25 column

Tryptic digest containing [^{32}P]NTP-linked nucleotide was applied to a 0.5–1 ml DEAE-Sephadex A25 column, equilibrated with 50 m*M* ammonium bicarbonate. This column is conveniently made from a 1-ml disposable Eppendorf pipet tip (Ulster Scientific, High Land, NY, U.S.A.). After the tryptic digest had been adsorbed on the column, the total ^{32}P-radioactivity was determined by placing the column in a counting vial and then counting scintillation directly. The column was washed with the starting buffer until the radioactivity in the wash reached background level. It was then successively washed with 100 ml of each of 0.1 *M*, 0.15 *M* and 0.2 *M* ammonium bicarbonate to remove any free [^{32}P]NTP. After each step, the radioactivity remaining bound to the column and that which was eluted was determined by scintillation counting. The labeled peptide was then eluted quantitatively with 3–4 ml of 0.6 *M* ammonium bicarbonate and lyophilized. Residual ammonium bicarbonate was removed by adding 2 ml of methanol and evaporating and repeating this twice. The residue was then dissolved in 0.5 ml of 20 m*M* sodium phosphate buffer (pH 6.8), containing 4 *M* guanidine–HCl.

C_4 HPLC purification of covalently linked [^{32}P]NTP–peptide complex

The α-^{32}P-NTP-labeled tryptic peptides eluted from the DEAE-Sephadex column were dissolved in 4 *M* guanidine–HCl and chromatographed on a Vydac C_4 reversed-phase column (Fydack, Hesperia, CA, U.S.A.), connected to a Varian Vista 5500 HPLC system, equipped with a Varian Polychrome 9060 diode-array detector (Varian, Sunnyvale, CA, U.S.A.). Elution of the labeled peptide was effected with a linear gradient of eluent B (70% aq. acetonitrile) in eluent A [20 m*M* sodium phosphate (pH 6.8)]: 0–5 min (0% B), 5–15 min (0–5% B), 15–107 min (5–25% B), 107–140 min (25–50% B). Fractions of 1 ml were collected every minute and counted directly.

Desalting of labeled peptide fraction for amino acid composition and sequencing

In order to obtain the labeled nucleotide–peptide complex for amino acid analysis and sequencing, it is necessary to remove the salts from the peptide containing fraction, since the presence of trace amounts of salts interferes with the acid hydrolysis of the peptide material. This was achieved by chromatography on a 0.5-ml column of Vydac RP (reversed-phase material, Vydac Cat. No. 221). The Vydac RP column was made from a small Pasteur pipet, plugged with glass wool and pre-

viously heated at 500°C for more than 12 h. The column was activated by washing under suction with 3 ml of 60% aq. acetonitrile, followed by 4 ml water. The fraction from the C_4 column containing labeled NTP–peptide complex was then diluted four-fold with water and applied to the Vydac RP column under mild suction. After washing the column with 6 ml water the [^{32}P]NTP-labeled peptide was eluted with 30–35% of aq. acetonitrile. Quantitative elution of the peptide is generally achieved by using a concentration of acetontrile 1.5- to 2-fold higher than that at which it was eluted from the C_4 reversed-phase column. Fractions of 200 μl were collected in previously heated tubes, counted, and then lyophilized. Peptide obtained in this manner is suitable for amino acid composition and gas phase sequencing[11,12].

RESULTS AND DISCUSSION

Purification of nucleotide cross-linked peptide from E. coli DNA pol I labeled with [α-^{32}P]dTTP

Earlier attempts to resolve 8-azido-ATP and dTTP-labeled peptides from the tryptic digest of *E. coli* pol I on a C_{18} reversed-phase column had resulted in the loss of radioactive label[3]. Since exposure of nucleotide-linked proteins or peptides to an acidic medium was found to result in the dissociation of radiolabeled nucleotide, we altered the elution conditions by employing phosphate buffer of pH 6.8 instead of 0.1% TFA. However, since it was necessary to resolve a large number of peptides, an intermediate step to partially purify the nucleotide-linked peptides was desirable. Chromatography on DEAE-Sephadex was found to fulfill this need. Chromatography of tryptic peptides derived from *E. coli* pol I on DEAE-Sephadex, followed by a single-step C_4 HPLC purification, provided an excellent method for purification of peptides containing the covalently linked nucleotides. The nucleotide linked peptides, by virtue of their increased negative charge, bind tightly to the DEAE-Sephadex matrix, which permits selective concentration of these peptides. Washing of the column with ammonium bicarbonate solutions of up to 0.2 *M* completely removes the last traces of free radioactive dNTP. Desired peptides are then eluted by increasing

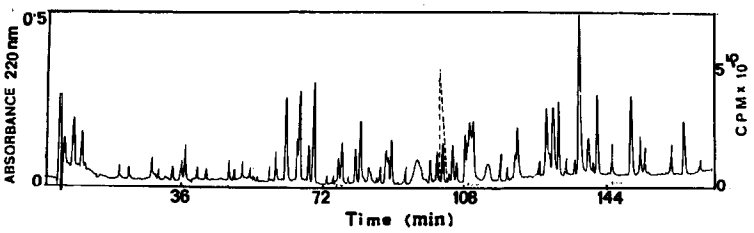

Fig. 1. HPLC profile of tryptic peptide of *E. coli* DNA pol I (large fragment), cross-linked with ^{32}P-dTTP-labeled. Photochemical cross-linking and preparation of a tryptic digest of cross-linked protein were carried out as described under Experimental. A tryptic digest, equivalent to 2 nmol of pol I protein was applied to a Vydac C_{18} reversed-phase column and eluted with a linear gradient of eluent B (70% acetonitrile) in eluent A [20 m*M* sodium phosphate buffer (pH 6.8)]: 0–5 min (0% B), 5–115 min (0–24% B), 115–170 min (24–80% B). Fractions were collected every minute (1 ml/min) and counted. (———) Absorbance of peptide peaks monitored at 220 nm; (– – – –) ^{32}P-radioactivity in each fraction.

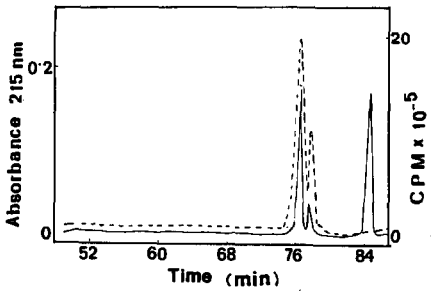

Fig. 2. HPLC purification of [^{32}P]dTTP-labeled tryptic peptide of *E. coli* pol I (large fragment). The labeled tryptic peptides eluted from the DEAE-Sephadex column were further purified on a Vydac C_4 column, as described in Experimental. (———) Absorbance of peptide peaks monitored at 215 nm; (– – – –) ^{32}P-radioactivity in each fraction.

the ammonium bicarbonate concentration to 0.6 *M*. The large fragment of *E. coli* pol I that we have used in these studies contains 36 arginine and 38 lysine residues[13]. A total of 75 peptides may be expected to result from a complete digestion of this protein with trypsin. The separation of tryptic peptiedes obtained from labeled enzyme, before and after DEAE-Sephadex step, is shown in Figs. 1 and 2. While peptide separation without the DEAE-Sephadex step does produce good resolution, further purification of labeled peptide is still required. In contrast, only three peptides were retained on DEAE-Sephadex as judged by their separation of the C_4 reversed-phase HPLC column (Fig. 2) and one of them contained the majority of the covalently-linked [α-^{32}P]dTTP. The minor radioactivity-containing peptide eluted immediately

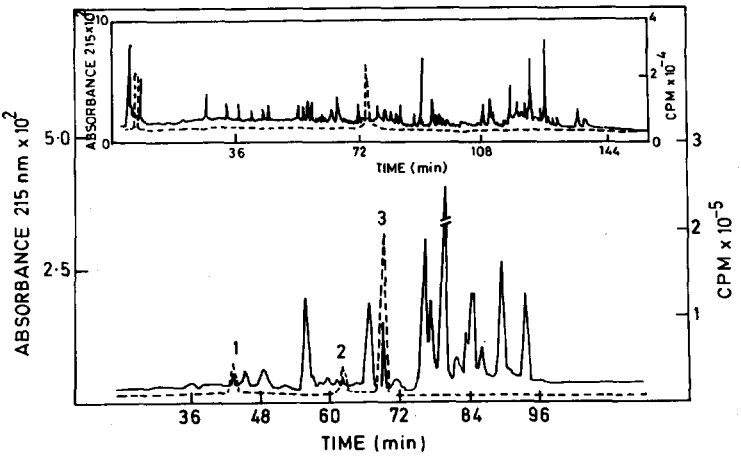

Fig. 3. HPLC purification of [^{32}P]dTTP-labeled peptide of 58-kD TdT. The tryptic peptides labeled with [^{32}P]dTTP, partially purified on DEAE-Sephadex, were resolved on a Vydac C_4 reversed-phase column, as described in Experimental. Figure shown in the inset is the C_{18} HPLC tryptic profile of 1 nmol of TdT cross-linked with [^{32}P]dTTP. A linear gradient was developed of eluent B in eluent A, 0–5 min (0% B), 5–110 min (0–35% B), 110–145 min (35–70% B). Fractions were collected every minute (1 ml/min) and counted. (———) Absorbance of peptides monitored at 215 nm; (– – –) ^{32}P-radioactivity in each fraction.

after the major labeled peak. Amino acid composition analysis of both peptides was found to be identical (data not shown). The apparent difference in the elution time between the two peptides may therefore have resulted from the degradation of covalently linked dTTP to either deoxythymidine diphosphate or monophosphate.

Purification of nucleotide-linked peptide from calf TdT

In order to assess the general applicability of the above protocols, we labeled 58-kD TdT from calf thymus with [α-^{32}P]dTTP and with [α-^{32}P]ATP. Tryptic peptides obtained from the labeled enzyme were separated on reversed phase hydrophobic columns before and after DEAE-Sephadex chromatography. Separation of peptides obtained in this manner is shown in Fig. 3. Similar to *E. coli* pol I, 58-kD TdT has a total of 74 basic amino acid residues (arginine + lysine)[14] and 75 tryptic peptides may be expected from the complete digestion of this enzyme protein. A DEAE-Sephadex purification step followed by separation of peptides on a C_4 reversed-phase matrix revealed that approximately 18 peptides were selectively retained on the DEAE-Sephadex matrix and 3 out of them contained radioactive dTTP (Fig. 3). Further studies of these peptides have indicated that all three peptides were derived from a 29-amino acid-long domain in calf TdT which spanned from residue 221 to 249.

Desalting of peptides

It is our experience that for amino acid composition and sequencing analyses, peptide fractions should be completely free of salt. In the conventional protocol for peptide separation, peptide fractions contain elution solvents (acetonitrile in TFA) which can be easily evaporated via lyophilization. However, when pH 6.8 phosphate buffer is used in the elution solvents, salts present in the peptide fractions must be removed by re-adsorption of the peptide to a small hydrophobic column, followed by elution with acetonitrile alone. A relatively simple protocol to accomplish this is described in Experimental.

CONCLUSIONS

A combination of ion-exchange chromatography together with reversed-phase hydrophobic chromatography that uses pH 6.8 phosphate buffer based solvents provides a simple method to obtain a nucleotide-linked peptide in a nearly homogeneous state. The peptide, upon desalting, is ready for acid hydrolysis (for amino acid composition) or for sequencing analysis using gas phase sequenators.

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