A GENERAL METHOD FOR THE ISOLATION OF LABELLED PEPTIDES FROM AFFINITY-LABELLED PROTEINS

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Received January 7, 1970

SUMMARY

A general method for specific isolation of ligand-bound peptides from affinity-labelled proteins is described. The method makes use of the affinity of the native protein to the ligand that was used for labelling of the same protein. Thus the labelled peptide can be isolated in one step in a very rapid procedure. The method was applied successfully to peptides from an antibody and from ribonuclease.

INTRODUCTION

Site-specific reagents are used extensively for affinity labelling of the active site of proteins (see ref. 1 for review). In these studies analogues of the substrate (in the case of enzymes) or of the haptens (in the case of antibodies) that possess a chemically reactive group are reacted with the protein. The specific and reversible binding of the analogue at the combining site of the protein is followed by the formation of a covalent bond, whereby a residue at or near that site is labelled. The isolation of the labelled peptide from the protein digest is usually performed by the conventional techniques of peptide fractionation and requires many steps. This presents special difficulties in cases of proteins whose sequence is not fully known (e.g. antibodies).

We present a general method for the isolation of such peptides by affinity chromatography where the labelled peptide could be isolated in essentially one step. The method is based on the fact that the native protein (enzyme or antibody) can be used to bind, specifically, the labelled peptide by virtue of the ligand which labels it. When the native protein is covalently attached to Sepharose (2) the ligand-coupled peptide is the only one in the digest which is bound to the protein-Sepharose column. After washing the column, the labelled peptide may be eluted under conditions that

dissociate the protein-ligand bonds. Two examples are given: 1) the isolation of a uridine diphosphate-labelled peptide from bovine pancreatic ribonuclease (RNase) by RNase-Sepharose column; 2) the isolation of a dinitrophenyl (DNP)-labelled peptide from antibody to DNP by an anti DNP-Sepharose column.

MATERIALS AND METHODS

Affinity labelling. RNase (5 times crystallized, Sigma) was labelled by the diazonium derivative of 5'-(4-aminophenyl phosphoryl) uridine 2'(3')-phosphate (PUDP) (3). This reagent reacted with RNase in a 1:1 molar ratio and labelled specifically a tyrosyl residue. The conditions and specificity of the reaction will be described elsewhere.

Goat anti-DNP antibodies (prepared from goat serum by affinity chromatography on DNP-ovalbumin-Sepharose column) was labelled by ^{14}C, 1-bromoacetyl, 1'-DNP ethylenediamine (BADE) as previously described (4). The labelled antibodies were reduced and alkylated and the heavy and light chains were separated on a Sephadex G-100 column, equilibrated with 8M urea - 1M propionic acid.

Protein-Sepharose columns. The conjugates of RNase, or anti-DNP with Sepharose were prepared as follows: 4.5 g (wet weight) of washed Sepharose 4B (Pharmacia, Uppsala) were suspended in 15 ml water and 0.5 g CNBr (Eastman, Kodak) was added. The pH of the suspension was adjusted to pH 11.0 and was kept at this pH for 8 min. by the addition of 2N NaOH. The reaction was terminated by filtration and washing three times with cold water (100 ml). The activated Sepharose was suspended in a 30 ml solution of 0.1 M $NaHCO_3$ which contained 150 mg of either RNase or anti-DNP. After 16 hr. of stirring at 4^0 the suspension was filtered and washed several times with 0.1 M $NaHCO_3$ until the absorbence at 280 nm of the wash was less than 0.02. The anti-DNP-Sepharose was equilibrated with 0.14 M NaCl-0.01 M phosphate buffer, pH 7.4 (PBS). In the case of anti-DNP the coupling of the antibodies to Sepharose was also performed in the presence of 0.01 M DNP-OH in order to protect the binding site of the antibody. The bound DNP-OH was removed from the anti-DNP-Sepharose by 0.1 M acetic acid followed by equilibration with PBS. This preparation will be referred to as protected anti-DNP-Sepharose. From the absorbance of the protein solution before and after the coupling to the Sepharose, it was estimated that 97% of the protein became covalently bound to the Sepharose. The protein-Sepharose conjugate was packed to a column of 1 cm i.d. with a layer of about 0.5 cm height of Sephadex G-15 (Pharmacia) on top of it.

RESULTS

DNP peptides from BADE labelled anti-DNP antibodies. The capacity of anti DNP-Sepharose column (containing 40 mg of bound antibody) was tested with the hapten ^{14}C, ε-DNP lysine (8×10^{-5}M in 0.05 $NaHCO_3$ pH 8). The column absorbed 0.36 μmole of the DNP derivative, corresponding to 1.3 moles of the hapten per mole of antibody bound to Sepharose and no radioactivity was detected in the effluent until this point of saturation was reached. After washing with water the column was washed successively with 0.1 M acetic acid, 5% formic acid and 20% formic acid. Only the last solvent was found to be effective in eluting the hapten from the column and the recovery of the hapten was 80% . The column was immediately washed with PBS and tested again for charging and eluting (20% formic acid) of the DNP derivative, followed by three more such cycles. After the first absorption and elution the column lost about 40% of its capacity to bind the hapten but no further decrease of activity was noticed in the case of the protected anti-DNP-Sepharose. On the other hand, anti-DNP-Sepharose that was prepared without protection lost activity more rapidly after the acid step. It is worth mentioning that the whole operation is very fast. The column could be charged with hapten at a flow-rate of 200 ml per hr. and due to its small size the equilibration after acid elution is also very fast. Thus, the antibody is exposed to 20% formic acid for no more than 10-15 min. Fig. 1 depicts the use of the anti-DNP-Sepharose column for the isolation of DNP peptides from a tryptic digest of light chain derived from affinity-labelled goat anti-DNP. The digest was first run on a Sephadex G-50 column equilibrated with 0.05 M NH_4OH. A major fraction from this column was pooled (100 ml) and was adjusted to pH 8. The peptide mixture was run at room temperature through the anti-DNP-Sepharose column and the DNP peptide was accumulated as a yellow band on the column. No radioactivity emerged with the effluent whereas the absorbance of the effluent at 220 nm was very close to that of the peptide mixture (Fig. 1). After washing with water the DNP-peptide was eluted at room temperature with different concentrations of formic acid. The eluted peptide moved on the column as a yellow band and was collected in 2-3 ml in a recovery of 75% of the radioactivity that was applied to the column. The column was immediately equilibrated with PBS. The eluted yellow DNP-peptide was lyophilized, dissolved in water and purified by paper electrophoresis at pH 3.5 to yield a lysyl peptide of 12 residue which contained the DNP labelled tyrosine (4) whose sequence is now under investigation. The

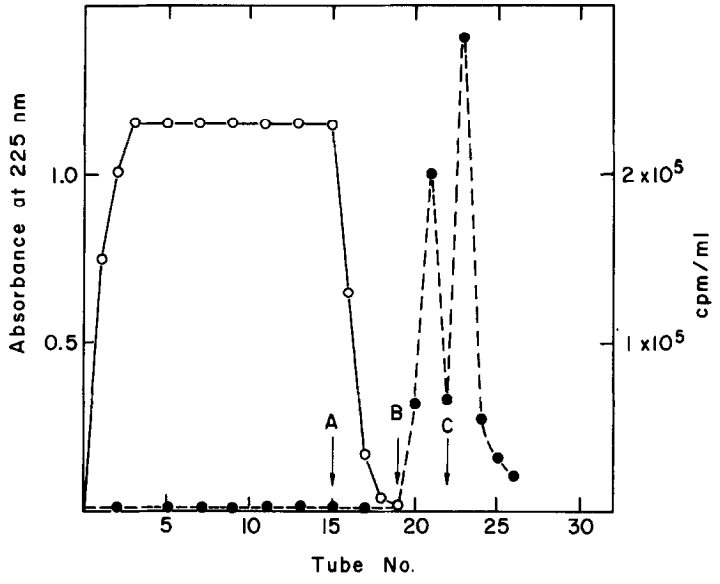

Fig. 1. Isolation of ^{14}C-labelled DNP-peptide on anti DNP-Sepharose. A Sephadex G-50 fraction (100 ml) of tryptic digest of light chain derived from goat anti DNP antibodies that were affinity labelled with BADE (4) was run through the anti DNP-Sepharose column (1 x 2.5 cm). The antibody column contained 25 mg of antibody. The absorbance at 225 nm of the peptide fraction was 1.25 and it contained 10^6 cpm. Tube 1-19 and 20-26 contained 6 ml and 1 ml respectively. A - wash with water. B - elution with 10% formic acid. C - elution with 20% formic acid. The yield of cpm eluted with formic acid was 75%. ○ - absorbance at 225 nm. ● - cpm.

final step of purification by electrophoresis or by other means seems necessary in all cases, mainly because some of the protein that was bound to the Sepharose is released under the conditions of elution.

PUDP-peptide from PUDP-labelled RNase. PUDP-RNase (15 mg) was reduced and carboxymethylated in 8M urea - 1 M Tris-HCl pH 8.6 and the reagents were removed on a Sephadex G-25 column. The alkylated protein was digested with trypsin at pH 8.5, 30^0 for four hrs. The digest was brought to pH 5.0 (conditions under which the binding of PUDP to RNase is maximum) and was run through an RNase-Sepharose column that contained 50 mg of bound RNase. Fig. 2 demonstrates that no peptide labelled with PUDP (that absorbs at 342 nm due to the azo linkage) emerged from the column, whereas most of the digest passed through the column. The column was washed with water followed by Na acetate buffers (pH 5.0) which removed some more peptides that did not contain the PUDP ligand.

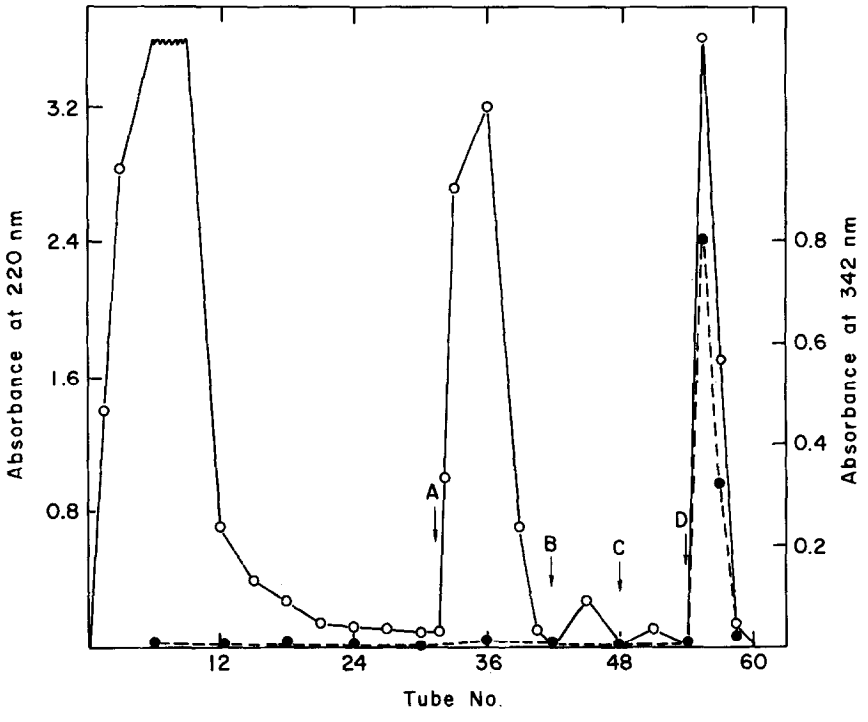

Fig. 2. Isolation of PUDP-peptide on RNase-Sepharose column. Tryptic digest of 15 mg PUDP-labelled RNase in water was applied to an RNase-Sepharose column (1 x 4 cm) that contained 50 mg RNase. The flow rate was 80 ml per hr. A - wash with 0.02 M Na acetate pH 5.0. B - wash with 0.05 M Na acetate pH 5.0. C - wash with 0.1 M Na acetate pH 5.0. D - elution with 0.8 M NH_4OH. The recovery of the PUDP-peptide eluted with 0.8 M NH_4OH, as measured by the absorbance at 342 nm, was 60%. ○ - absorbance at 220 nm. ● - absorbance at 342 nm.

The yellow PUDP peptide which was bound to the column was eluted with 0.8 M NH_4OH as a sharp yellow-band (Fig. 2). The yield of the PUDP-peptide thus obtained was 60% from the amount that was applied to the column as judged from the absorbance of 342 nm. After one paper electrophoresis the acidic PUDP-peptide was freed from minor impurities. Analysis of this peptide showed that it is identical to PUDP-peptide previously isolated by several steps of chromatography and electrophoresis and that this peptide corresponds to a region between residues 67 and 85 in the sequence of RNase. The exact location of the labelled tyrosyl residue will be described elsewhere. After elution the RNase-Sepharose column was washed immediately with water and was used again. The capacity of the column decreased by 20% but the column was still efficient in binding the PUDP-peptide.

DISCUSSION

The method described in this communication makes use of the affinity of a protein to its ligand in order to "fish out" a peptide labelled with this ligand. It is therefore obvious that in any case of affinity labelling of a protein, the same protein in its native state may be used to purify the affinity labelled peptide. This method was suggested previously for antibodies (5) but was used until now only in solution. The preparation of a protein-Sepharose column as a specific tool for the purification of such peptides provides some advantages. The operation is very fast, the peptide could be concentrated from big volumes and the column is rapidly recovered from the conditions of elution. Thus the column could be used several times with only small changes in its capacity. However, the conditions for preparation of the Sepharose conjugate as well as for elution of the bound peptide should be investigated for each protein. Thus it seems that antibody Sepharose column will be more stable if prepared in the presence of a protector which is a low-affinity analogue of the hapten. Since antibody could be prepared against very many chemical structures it is possible to introduce insoluble antibody columns as a general tool to purify peptides from proteins that undergo any kind of chemical modifications. The method is not limited to antibodies and its applicability to other proteins is demonstrated by the example of PUDP-RNase where RNase was used to isolate the PUDP-peptide. A similar approach was also tried successfully on affinity labelled peptides derived from Staphylococcal nuclease (M. Wilchek, in preparation). In most studies of affinity chromatography (6) a ligand-Sepharose column was used to purify the protein which has an affinity to this ligand. In this study the reverse approach was taken and a protein-Sepharose column was used to purify a ligand-bound peptide. This could of course be applied to other problems of purification of small molecules which possess affinity to macromolecules attached to an insoluble carrier.

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