A simple, rapid preparation of α -[^{32}P]-labelled adenosine diphosphate

I. L. Cartwright and D. W. Hutchinson*

Department of Molecular Sciences, University of Warwick, Coventry, CV4 7AL, UK

Received 3 June 1977

ABSTRACT

Hexokinase (EC 2.7.1.1) will convert commercially available α -[^{32}P]-labelled ATP into α -[^{32}P]-labelled ADP. A simple, rapid isolation procedure for the α -[^{32}P]-labelled ADP is described and this synthetic method can be used for the preparation of other α -[^{32}P]-labelled nucleoside diphosphates.

INTRODUCTION

During our studies on the synthesis of polynucleotides from ribonucleoside diphosphates using polynucleotide phosphorylase (EC 2.7.7.8), it was necessary, for the purpose of nearest neighbour analysis, to use α -[^{32}P]-labelled nucleoside diphosphates. Although both α -[^{32}P]-labelled nucleoside mono- and triphosphates are readily available from commercial sources, α -[^{32}P]-labelled nucleoside diphosphates are not. The chemical¹ and combined chemical and enzymic² syntheses of α -[^{32}P]-labelled nucleoside diphosphates from the parent nucleosides have been described, but these methods are time-consuming and give only moderate yields of the diphosphates. Recently, a completely enzymic method for the synthesis of radioactively labelled ADP and ATP from adenosine has been published³. This method makes use of an adenosine kinase from Saccharomyces cerevisiae which is not commercially available and which has an unknown substrate specificity.

We have chosen commercially available α -[^{32}P]-ATP and yeast hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) as starting materials for a simple, rapid synthesis of α -[^{32}P]-ADP. This method can be extended to the synthesis of α -[^{32}P]-CDP, -GDP, and -UDP by slight adjustment of the reaction conditions.

MATERIALS AND METHODS

α -[32 P]-ATP was obtained from the Radiochemical Centre, Amersham. Yeast hexokinase was obtained from the Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey and was used without further purification. Polynucleotide phosphorylase (EC 2.7.7.8) was isolated from E.coli as previously described⁴. The radioactivity of samples in solution was determined in a scintillation medium made up of toluene (600 ml), 2-ethoxyethanol (400 ml), PPO (4 g), POPOP (0.2 g), while the radioactivity of samples on glass fibre discs was determined in a medium made up of toluene (1000 ml), PPO (4 g), POPOP (0.1 g).

PREPARATION OF α -[32 P]-ADP

α -[32 P]-ATP (0.25 mCi, specific activity 10.3 Ci/mmole) was transferred to a small reaction vessel containing unlabelled ATP (1 μ mole) by washing with water. The ATP was lyophilised and the following reagents were added in order: 0.05 M sodium phosphate buffer pH 7.5 (0.728 ml), 0.1 M $MgCl_2$ (0.067 ml) and 0.5 M glucose in 0.05 M sodium phosphate buffer pH 7.5 (0.2 ml). After 5 minutes incubation at 25 $^{\circ}$, yeast hexokinase (0.005 ml, 8 units) was added and the incubation continued for an hour when cellulose tlc (isobutyric acid/ NH_4OH/H_2O ; 66/1/33) showed that complete conversion to the diphosphate had occurred. Ethanol (1 ml) was added and the precipitated protein removed by centrifugation. The supernatant was applied to a column of DEAE Sephadex A25 (HCO_3^- form, 25 x 1.1 cm) which was then washed with water. The column was eluted with a linear gradient of triethylammonium bicarbonate pH 8.9 (0.15-0.35 M, 500 ml total). The fractions containing the peak radioactivity were eluted at 0.26 M triethylammonium bicarbonate, these being pooled and evaporated to dryness under reduced pressure. All traces of triethylammonium bicarbonate were removed by several evaporations of methanol solutions of the residue. Exchange of the cation was achieved with SE Sephadex C-25 (Na^+ form, 17 x 0.75 cm). Lyophilisation of the radioactive eluate gave α -[32 P]-ADP in 88% yield (specific activity 0.23 mCi/ μ mole). This was stored frozen in water at -20 $^{\circ}$ until used.

When CTP, GTP or UTP were used in the above incubation mixture with 16 units of hexokinase, after 3 hr at 50 $^{\circ}$ complete conversion to the diphosphates was

observed (silica tlc. isobutyric acid/ammonia/water, 57/4/39). If the reaction was carried out with 16 units of hexokinase at 37⁰, complete conversion of UTP to UDP was observed after 3 hr but only partial conversion of CTP and GTP took place. At 25⁰ with 32 units of hexokinase only partial conversion to the diphosphates was observed with all three triphosphates.

RESULTS AND DISCUSSION

The synthetic scheme outlined above makes use of the first step in glycolysis when ATP is used as a cofactor for the phosphorylation of glucose by the enzyme hexokinase:

Other nucleoside triphosphates can function as a cofactor in this reaction though the rate of phosphoryl transfer is greatly reduced⁵. We have found that CTP, GTP and UTP are all efficiently converted to the corresponding diphosphates when a higher concentration of hexokinase and an increase in temperature is used in the reaction.

The α -[³²P]-ADP runs with the same R_F as ADP on paper chromatography in isobutyric acid/NH₄OH/water and 0.1 M phosphate pH 6.8/ammonium sulphate/n-propanol (100/60/2). There is no contamination by ATP and by only a few per cent of AMP. The latter is probably an artefact of the paper chromatography as the column chromatographic procedure separates ADP completely from AMP.

The isotopic purity and positional analysis of the α -[³²P]-ADP was checked by the preparation of high molecular weight poly A using polynucleotide phosphorylase (Table 1). The amount of orthophosphate liberated from the radioactive ADP matches within experimental error the amount of [³²P] incorporated into poly A. If the α -[³²P]-ADP contained any impurity or if there had been any scrambling of the label, then the two amounts would not match.

The experimental procedure outlined above provides a simple, rapid method for the synthesis of α -[³²P]-ADP and other nucleoside diphosphates. The procedure could be adapted for the synthesis of α -[³²P]-nucleoside diphosphates of high specific radioactivity.

TABLE 1
POLYMERISATION OF α -[32 P]-LABELLED ADP

% phosphate released from ADP	% of input counts incorporated in poly A
30.6	31.4

The assay mixture containing the following: ADP (5.3 mM), $MgCl_2$ (3 mM) Tris-HCl pH 9.0 (50 mM), polynucleotide phosphorylase (0.3 U⁴) and α -[32 P]-ADP (5.5×10^4 cpm) was incubated at 45° for 30 minutes. Phosphate release was assayed colorimetrically⁶. Poly A production was assayed by pipetting aliquots of the reaction mixture on to Whatman GF/A glass fibre discs, washing the discs with 5% trichloroacetic acid and then briefly with ethanol followed by ether. The radioactivity on the discs was determined in the toluene-based scintillation medium.

We wish to thank the Wellcome Foundation for financial support.

* To whom correspondence should be addressed

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