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Carrier-free 8-azidoadenosine 5′-[γ-^{32}P]triphosphate

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We found 8-azidoadenosine 5′-diphosphate to be a phosphoryl acceptor in the enzymatic conversion of 1,3-diphosphoglyceric acid to 3-phosphoglycerate. This has allowed us to synthesize in a single-step procedure carrier-free 8-azidoadenosine 5′-[γ-^{32}P]triphosphate, requiring no further purification of the end product. The synthesized 8-azidoadenosine 5′-[γ-^{32}P]triphosphate has been characterized and shown to meet all the criteria for a specific photoreactive ATP analogue.

1. INTRODUCTION

The identification of membrane bound protein kinases, ATPases [1,2] and autokinase properties of receptors [1,3,4] is of high significance in the investigation of cell regulatory processes. ATP binding proteins are unambiguously identified by their binding of photoaffinity derivatives of ATP [5]. The low abundance of e.g. receptors in membranes, in the case for steroid hormones [6] or MSH [7] in the order of 10^4 per cell, requires that such photoaffinity label be of very high specific radioactivity. Such a (γ-^{32}P)-photoaffinity derivative of ATP appears not to be commercially available on a regular basis.

The chemical synthesis of azido photoaffinity analogues of adenine nucleotides is well documented [8]. The chemical synthesis of N_3ATP is a multistep process resulting in a relatively low yield (30–50%) of the desired product.

Enzymatic methods for the synthesis of [γ-^{32}P]N_3ATP have been described [9,10]. They are based on exchange reactions [9] or require subsequent chromatographic purification of the product [10] and thus do not lend themselves easily to routinely producing carrier-free products.

We present the enzymatic synthesis which yields, in a single step, carrier-free [γ-^{32}P]N_3ATP which does not require further purification.

2. MATERIALS AND METHODS

Glycolytic enzymes were purchased from Boehringer Mannheim. [^{32}P]Orthophosphate was purchased from Amersham. Unless specified all other reagents were purchased from Sigma.

Incubation medium A: 50 mM Tris-HCl; 25 mM KCl; 5 mM $MgCl_2$. Incubation medium B: 50 mM Tris-HCl, pH 7.4; 25 mM KCl; 5 mM $MgCl_2$; 30 mM NaCl.

2.1. *Synthesis of [γ-^{32}P]N_3ATP*

The synthesis was performed in subdued light and is a modification of the method of Johnson and Walseth [11] for the synthesis of [γ-^{32}P]ATP. To 40 μl reagent mixture (125 mM Tris-HCl, pH

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Abbreviations: N_3ATP, 8-azidoadenosine 5′-triphosphate; N_3ADP, 8-azidoadenosine 5′-diphosphate; NAD, nicotineamide adenine dinucleotide; ME, β-mercaptoethanol; DDT, dithiothreitol

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9.0; 30 mM $MgCl_2$; 4.5 mM spermine; 0.3 mM L-α-glycerolphosphate; 0.5 mM NAD; 0.125 mM N_3ADP and 2.5 mM pyruvate) was added 20 μl of the glycolytic enzyme mixture (glycerolphosphate dehydrogenase, 0.34 U; triosephosphate isomerase, 0.1 U; glyceraldehyde-3-phosphate dehydrogenase, 0.16 U; 3-phosphoglycerate kinase, 0.09 U, and lactate dehydrogenase, 1.9 U, in 50 mM Tris-HCl, pH 9.0, and 2 mM ME). To this mixture was added 50 μl [^{32}P]orthophosphate. After 30 min incubation, the level of incorporation of ^{32}P into N_3ADP was determined by an activated charcoal assay [11]. The reaction was terminated by addition of an equal volume of Tris-saturated phenol (pH 8.0). The aqueous phase was then extracted with chloroform and the resultant product in the aqueous layer analysed by thin-layer chromatography. Storage was at −20°C.

2.2. *Photoaffinity labelling*

Protein, dissolved at various concentrations in 250 μl medium A, pH 9.0, was added to [γ-^{32}P]N_3ATP in quartz silica cuvettes and irradiated by means of five 10 ms flashes of a quartz xenon lamp connected to a 500 V capacitor discharge circuitry. When labelling creatine kinase, 5 mM creatine was included in the incubation medium. Labelled proteins were trichloroacetic acid precipitated at 10% final concentration and the resulting precipitate neutralized and solubilized for PAGE analysis in SDS sample application buffer. Solubilized samples were subjected to one-dimensional SDS-polyacrylamide electrophoresis in 10% gels [12] and the resulting labelled band excised and its radioactivity determined in a Packard Scintillation counter. Photoaffinity labelling of rat liver nuclear envelope prepared by the modified method of Bornens and Courvalin [13] (50–100 μg in 250 μl medium A, pH 7.5) was performed as above except that labelled nuclear envelope was pelleted by centrifugation.

2.3. K_m *determination*

0.02 U (Na+K)-ATPase in 250 μl medium B, pH 7.4, was incubated with varying amounts of [γ-^{32}P]N_3ATP and [γ-^{32}P]ATP for 10 min. Rates of hydrolysis of ATP were determined by the activated charcoal assay [11]. K_m determination was by Lineweaver-Burk analysis.

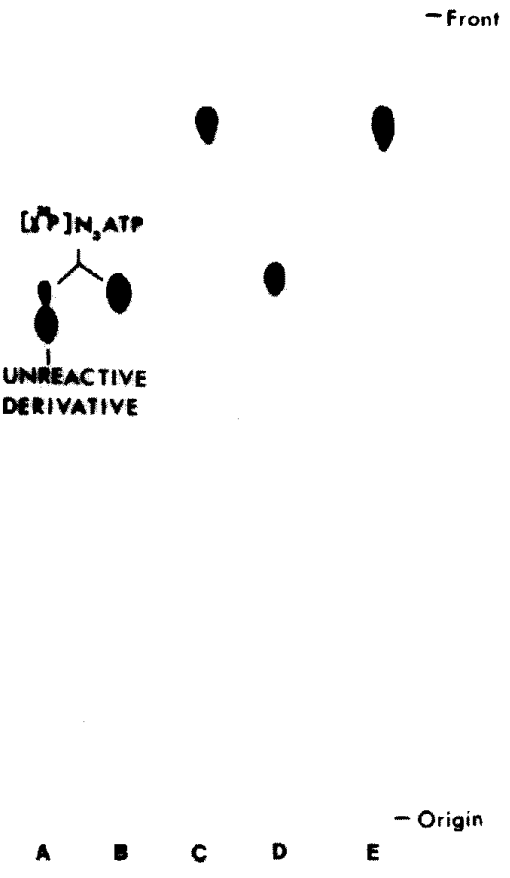

Fig.1. Thin layer chromatography of products in the enzymatic synthesis of [γ-^{32}P]N_3ATP. (A) Enzymatic reactions in the presence of 6 mM ME or 6 mM DTT; (B) enzymatic reactions in the presence of 370 μM ME; (C) enzymatic reactions in the absence of ME; (D) [γ-^{32}P]ATP; (E) [^{32}P]orthophosphate.

Table 1

Specificity and photoreactivity of [γ-^{32}P]N_3ATP

Substrate	UV radiation	Incorporation (cpm)
Creatine kinase	+	6500
Creatine kinase	−	250
Bovine serum albumin	+	600
Concanavalin A	+	600
Carbonic anhydrase	+	450
Soybean trypsin inhibitor	+	650
Alcohol dehydrogenase	+	350

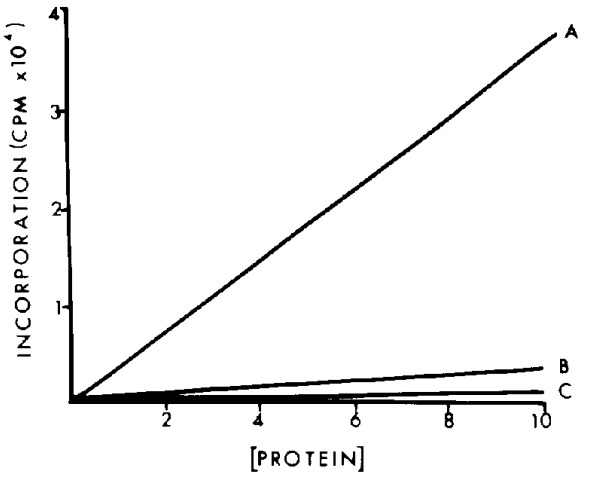

Fig.2. The effect of increasing protein concentration on specific (A) and non-specific (B,C) labelling by [γ-^{32}P]N_3ATP. (A) Creatine kinase; (B) bovine serum albumin; (C) concanavalin A. Incubation was in the presence of UV radiation; [protein] = μM.

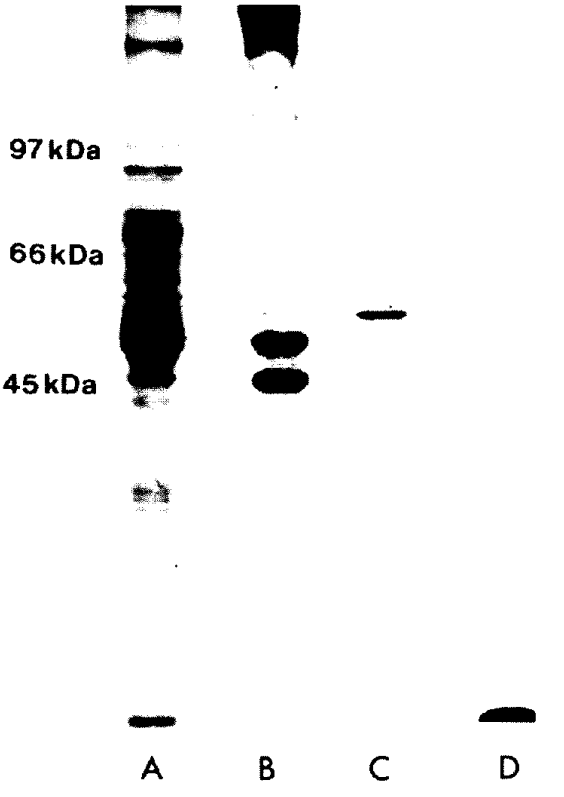

Fig.3. Photoaffinity labelling of rat liver nuclear envelope. (A) Coomassie stain; (B) nuclear envelope incubated in the presence of [γ-^{32}P]N_3ATP (+ UV radiation); (C) nuclear envelope incubated in the presence of [γ-^{32}P]N_3ATP (− UV radiation); (D) nuclear envelope incubated in the presence of [γ-^{32}P]N_3ATP and cold ATP (+ UV radiation).

2.4. *Analytical methods*

Thin layer chromatography was performed on PEIE cellulose plates (Merck) and developed in 1.8 M lithium chloride. After drying, the plates were subjected to autoradiography. The activated charcoal assay was performed by adding an aliquot of the solution in question to 1 ml of 50 mM potassium dihydrogen phosphate. After vortexing, 25 μl was counted. To the remainder of the solution was added 5 mg activated charcoal. After thorough mixing and centrifugation, 25 μl was counted (unbound $^{32}P_i$). In this manner, the ratio of organic to inorganic ^{32}P can be determined [11].

3. RESULTS

The enzymatic synthesis of [γ-^{32}P]N_3ATP was initially hindered by the presence of mercaptoethanol or DTT (6 mM). At this concentration conventionally used in reactions involving the respective glycolytic enzymes [11] the major product (80%) is not [γ-^{32}P]N_3ATP but a derivative which is not photoreactive (fig.1). There is a direct relationship between decrease in mercaptoethanol concentration and decrease in the quantity of the derivative. This decrease is accompanied by a proportional increase in [γ-^{32}P]N_3ATP. Optimal mercaptoethanol concentration is 370 μM when 99.5% of the [^{32}P]orthophosphate is incorporated into [γ-^{32}P]N_3ADP. The non-photoreactive derivative is not formed at this mercaptoethanol concentration (fig.1).

This enzymatically synthesized [γ-^{32}P]N_3ATP binds specifically to ATP-binding proteins such as creatine kinase and labelling is dependent on photoactivation (table 1). The non-specific binding is independent of the ratio of basic to acidic amino acid residues. Whereas further increas in protein concentrations does not result in significantly increased non-specific labelling (fig.2), the creatine kinase labelling increases as expected.

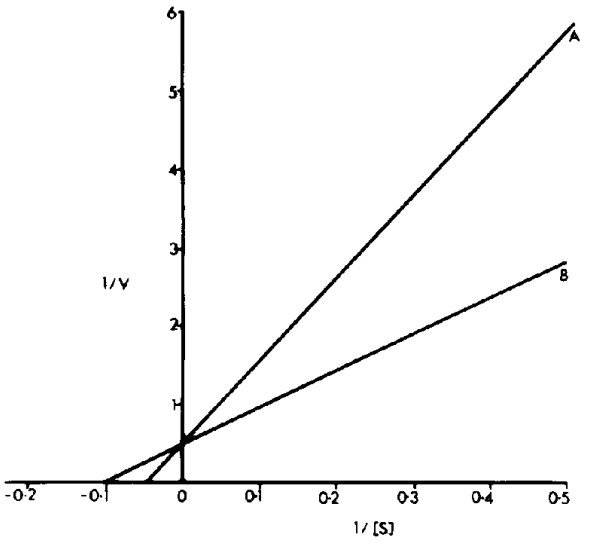

Fig.4. Determination of K_m of hydrolysis of [γ-^{32}P]N_3ATP by (Na + K)-ATPase in medium B, pH 7.4. (A) [γ-^{32}P]N_3ATP + 20 μM cold ATP, [S] = μM; (B) [γ-^{32}P]N_3ATP, [S] = μM. All reactions were performed in subdued light (− UV). V = nmol $^{32}P_i$ released/0.02 U (Na + K)-ATPase per 10 min.

Specificity of binding was also demonstrated in rat liver nuclear envelope (fig.3). The labelling pattern is similar to that obtained by Clawson et al. [2]. The labelled 46 kDa protein has been identified as a nuclear envelope ATPase [2]. The labelling was inhibited by addition of excess carrier ATP (fig.3).

The synthesized [γ-^{32}P]N_3ATP was shown to be a substrate for (Na + K)-ATPase (fig.4) with a K_m of hydrolysis of 10 μM. Hydrolysis was competitively inhibited by cold ATP.

4. DISCUSSION

Carrier-free [γ-^{32}P]N_3ATP has been prepared for the identification of low abundance ATP binding proteins. We found it essential to keep the mercaptoethanol concentration at 370 μM otherwise the azido grouping in the molecule is converted resulting in a photo-insensitive derivative of [γ-^{32}P]N_3ATP, which requires separation by chromatography [10].

The enzymatically synthesized [γ-^{32}P]N_3ATP is free of the derivative and meets all the criteria of a specific, photoreactive ATP analogue, i.e. labelling is specific and photo-dependent. It acts as a substrate for both kinases and ATPases, its K_m of hydrolysis is comparable to that reported [14] and hydrolysis is competitively inhibited by carrier ATP indicating binding of [γ-^{32}P]N_3ATP to a specific ATP-binding site.

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