ACTA CHEMICA SCANDINAVICA 25 (1971) 2931–2934

Isolation of ^{32}P-Labelled Phosphorylserine and Phosphorylthreonine from Ehrlich Mouse-ascites Tumour Cells, Suspended in an Isotonic Medium, Containing ^{32}P-Labelled Nucleoside Triphosphates or Inorganic Pyrophosphates

GUNNAR ÅGREN and GUNNAR RONQUIST

Institute of Medical Chemistry, University of Uppsala, Box 551, S-751 22 Uppsala 1, Sweden

It was shown that extracellular [γ-^{32}P] labelled nucleoside triphosphates, containing a purine or a pyrimidine nucleus and also inorganic pyrophosphate, could donate a terminal phosphoryl group to serine or threonine residues of proteins located at the cell surface. Adenosine and guanosine triphosphates were the most rapid, reaching a maximal effect after less than 30 sec while the pyrimidine triphosphates reached a maximum after 60 sec and inorganic pyrophosphate after 5 min or more. At maximal transfer time of phosphoryl groups the purine triphosphates were from 8 to 20 times more active as phosphoryl group donors than the corresponding pyrimidine compounds or inorganic pyrophosphate. A method is described to prepare pure labelled purine and pyrimidine triphosphates with high specific activity.

It was recently reported that intact Ehrlich tumour ascites cells were capable of forming extracellular ATP in an isotonic medium containing the necessary substrates and cofactors. During efforts to find out possible functions for the *de novo* formed ATP* in enzyme reactions at the cell surface, [^{32}P]phosphorylserine, as well as [^{32}P]phosphorylthreonine, were isolated from the tumour cells.[2,3] The amount of isolated labelled phosphorylserine was five times that of labelled phosphorylthreonine.

The labelled phosphoryl groups in the two phosphorylated amino acids represented only a small part of the total amounts of labelled ortophosphate liberated from [γ-^{32}P]ATP at the cell surface. It was of interest to find out if other energy rich phosphoryl compounds could function more effectively as donors of phosphoryl groups to serine and threonine residues at the cell surface.

* *Abbreviations.* ATP adenosine triphosphate. GTP guanosine triphosphate. CTP cytosine triphosphate. UTP uridine triphosphate. UV ultraviolet.

In the present paper it is shown that [^{32}P]labelled GTP, CTP, UTP, as well as labelled inorganic pyrophosphate, could transfer their terminal phosphoryl groups to serine and threonine residues isolated from surface located proteins.

METHODS

The details of the incubation procedure have been described previously.[2,3] The isolation and identification of phosphorylserine and phosphorylthreonine have also recently been described.[2,3]

[γ-^{32}P]labelled ATP and other nucleoside triphosphates of high purity were prepared according to a method described by Pfleiderer.[4] It has been found necessary to modify the method in several respects and at present the labelled nucleotides are prepared in the following way. 30 mCi carrier-free [^{32}P]orthophosphate in 3 ml water was purified by chromatography using a 2 × 1 cm column of Dowex 50 (8 % cross-linking, 250 – 500 mesh) to remove impurities which had some influence on the enzymatic reaction. The phosphate was completely eluted by washing with an additional volume of 4 ml water.

The [^{32}P]labelled ATP was formed in an incubation mixture containing the purified carrier-free [^{32}P]phosphate in a volume of 7 ml. To this was added 0.8 ml of a 1 M solution of a Tris-acetic acid buffer, pH 7.5, 0.2 ml of 1 M Tris solution, 0.2 ml of 0.5 M magnesium acetate, 5 mg of NAD^+ in 0.1 ml of water, 5 mg of ADP* in 0.1 ml of water, 20 mg fructose-1,6-diphosphate in 0.1 ml of water, 0.05 ml of an aldolase solution containing 10 mg of enzyme per ml, 5 mg glyceraldehyde-3-phosphate dehydrogenase in 0.1 ml solution, and 0.5 mg of phosphoglycerate kinase in 0.05 ml of water.

The enzymatic reaction was performed in a shake incubator at 37° for 2 h and was terminated by heat coagulation at 100°C for 1 min. 0.1 mg unlabelled ATP and 2 mg of unlabelled orthophosphate were added and the incubation mixture was applied to a 2 × 27 cm Dowex 1 formate column at 20°C with a linear gradient elution, using 4 M formic acid in 0.2 M ammonium formate ⟶ 4 M formic acid in 0.6 M ammonium formate. The volume in the mixer was 1400 ml. The chromatographic separation was followed directly on the column wall by means of a Panax GM Monitor and by measuring the radioactivity of the different fractions from column chromatography on stainless steel planchettes with a Tracerlab Superscaler.

In this way a highly active ATP preparation was obtained free from all impurities present in the commercial labelled orthophosphate solution.[5] The fractions containing the labelled ATP was evaporated to dryness *in vacuo* at less than 30°C using repeated additions of water to remove free formic acid.

Column chromatography through Dowex 50 was then used in order to purify the ATP from ammonium ions. 17 g of Dowex 50 is known to absorb 1 g of ammonia. Twice this amount was packed in the column. A filter paper was placed at the bottom of the column to prevent any Dowex particles migrating out of the column. The resin was pre-treated by washing with 2 M formic acid followed by water until neutral reaction in the eluate. The ATP solution was applied to the column and washed out with water.

The ATP solution was concentrated to a small volume, 2 – 3 ml, as described above. In order to remove impurities such as phosphate, sulphate, and formate ions from the ATP fraction the following procedure was developed. A column maintained at 4°C was packed with Sephadex G – 10 and equilibrated with an ethanol-water solution in proportions 5 to 95, which caused a decrease in volume of the Sephadex particles. This facilitated a separation of ATP from the anions present which were retarded in the gel. Orthophosphate was detected by its radioactivity, and sulphate by precipitation with barium chloride.

The ethanol water solution of ATP eluted from the column was evaporated to dryness *in vacuo*. The pH was neutral during this process. The specific activity of the ATP obtained in this way was usually between 1.5 to 3 mCi per μmol. The colourless ATP solution showed expected UV ratios.[6] This extensive way of purification was found

* ADP was exchanged for GDP, CDP, and UDP, respectively, during the preparation of the other nucleoside triphosphates.

necessary in order to obtain optimal conditions for the present experiments. Usually, the whole procedure took about 10 days.

[^{32}P]Tetrasodium pyrophosphate was purchased from the Radiochemical Center, Amersham, Bucks., England, with a specific activity of 70.5 mCi/mM. It was used without further purification. Unlabelled tetrasodium pyrophosphate was from E. Merck AG., Darmstadt, West Germany. Fructose-1,6-diphosphate (sodium salt), ADP and NAD^+, as well as aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.6), glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12), and phosphoglycerate kinase (ATP:D-3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3) were obtained from Boehringer and Soehne, Mannheim, West Germany.

Table 1. Incorporation of labelled phosphoryl groups from the extracellular donor compounds into a seryl residue by intact Ehrlich mouse-ascites tumour cells.

Ehrlich mouse-ascites tumour cells incubated for various times under isotonic conditions at 37°C in N_2-atmosphere with different labelled compounds. The orthophosphate concentration in all medium was 10^{-3} M. The incubations were terminated with perchloric acid, and [^{32}P]phosphorylserine (SerP) was isolated from the acid-insoluble material as given in Methods. The figures denote the phosphoryl incorporation into phosphorylserine in μmol $\times 10^{-6}$ per mg dry weight (Schneider protein).[7]

Labelled compound in incubation medium	5 sec SerP	30 sec SerP	60 sec SerP	5 min SerP
[^{32}P]ATP	0.95	2.91	2.54	2.65
[^{32}P]GTP	1.10	2.80	3.06	2.90
[^{32}P]CTP	0.04	0.12	0.32	0.26
[^{32}P]UTP	0.05	0.09	0.24	0.17
[^{32}P]inorganic pyrophosphate	0.02	0.06	0.17	0.27
[^{32}P]orthophosphate	< 0.01	< 0.01	< 0.01	0.02

Table 2. Incorporation of labelled phosphoryl group from the extracellular phosphoryl compound into a threonyl residue by intact Ehrlich mouse-ascites tumour cells.

Ehrlich mouse-ascites tumour cells incubated for various times under isotonic conditions at 37°C in N_2-atmosphere with different labelled compounds. The orthophosphate concentration in all mediums was 10^{-3} M. The incubations were terminated with perchloric acid and [^{32}P]phosphoryl threonine (ThreP) was isolated from the acid-insoluble material as given in Methods. The figures denote the phosphoryl incorporation into phosphoryl-threonine in μmol $\times 10^{-6}$ per mg dry weight (Schneider protein).[7]

Labelled compound in incubation medium	5 sec ThreP	30 sec ThreP	60 sec ThreP	5 min ThreP
(^{32}P)ATP	0.17	0.52	0.46	0.44
(^{32}P)GTP	0.24	0.56	0.67	0.57
(^{32}P)CTP	0.01	0.02	0.06	0.07
(^{32}P)UTP	0.01	0.02	0.03	0.02
(^{32}P)inorganic pyrophosphate	< 0.01	0.01	0.02	0.04
(^{32}P)orthophosphate	< 0.01	< 0.01	< 0.01	0.01

RESULTS AND DISCUSSION

From Table 1 it is quite clear that all nucleoside triphosphates, as well as inorganic pyrophosphate, are able to rapidly transfer a phosphoryl group to seryl residues of proteins at the cell surface. It is well known that nucleotides do not penetrate the cell membrane.[1,8] Therefore, it can be concluded that this transfer reaction occurs at the cell surface.

The low values for [^{32}P]phosphate regardless of time excludes the possibility that the nucleoside triphosphates have been hydrolyzed into diphosphates and labelled orthophosphate which after penetration of the membrane could react with intracellular proteins also yielding phosphorylserine.

It is also evident from Tables 1 and 2 that labelled ATP and labelled GTP were more active in donating their terminal phosphoryl group into seryl and threonyl residues of proteins at the cell surface. Only small amounts of labelled phosphoryl groups were transferred into seryl and threonyl residues of surface located proteins when CTP, UTP, or inorganic pyrophosphate were donor compounds of phosphoryl groups. It is also of interest to point out that ATP and GTP as purine containing nucleotides were more active as donor compounds than the corresponding pyrimidine nucleotides as well as inorganic pyrophosphate.

A comparison between the values in Tables 1 and 2 also shows that the specific activity of the phosphorylserine and phosphorylthreonine reaches a maximum already after 30 sec of incubation. This was not the case in the reactions when CTP and UTP were donor compounds. They seemed to reach a maximum first after 60 sec. Inorganic pyrophosphate seemed to function still slower and it was not clear whether the reaction had reached a maximum after 5 min.

A comparison of data for phosphorylserine (Table 1) with those for phosphorylthreonine (Table 2) again showed that on an average the amount of the isolated phosphorylserine was about four to five times that of phosphorylthreonine, irrespective of which type of donor compound used.

Acknowledgement. We are indebted to Ing. S. Eklund and Miss M. Hellström for skilful assistance. This investigation was supported by a grant from the *Swedish Medical Research Council* (project No. B71 – 13X – 228 – 07C).

REFERENCES

1. Ågren, G. and Ronquist, G. *Acta Physiol. Scand.* **75** (1969) 124.
2. Ågren, G. and Ronquist, G. *Acta Physiol. Scand.* **79** (1970) 125.
3. Ronquist, G. and Ågren, G. *Acta Chem. Scand.* **24** (1970) 728.
4. Pfleiderer, G. *Biochim. Biophys. Acta* **47** (1961) 389.
5. Ronquist, G. *Acta Chem. Scand.* **21** (1967) 1484.
6. Pabst Laboratories, Division of Pabst Brewing Co., Milwaukee, Circular OR – 7, March 1955.
7. Schneider, W. C. *J. Biol. Chem.* **161** (1945) 293.
8. Ronquist, G. *Acta Physiol. Scand.* **74** (1968) 594.

Received January 18, 1971.