Acta Chemica Scandinavica B 28 (1974) 1169—1174

Isolation of ^{32}P-Labeled Phosphorylserine and Phosphorylthreonine from Ehrlich Mouse Ascites Tumor Cells Suspended in Different Isotonic Media Containing ^{32}P-Labeled Adenosine Triphosphate

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Intact Ehrlich tumor ascites cells were handled under physiological conditions and incubated for short periods with [^{32}P]ATP in isotonic media of varying cationic composition. Labeled phosphorylserine, phosphorylthreonine as well as three additional labeled fractions were isolated from a partial hydrolysate of the phosphoproteins in the outer layer of the plasma membrane.

The specific ^{32}P radioactivity was about the same for the phosphorylserine and the phosphorylthreonine fractions as well as for an additional one (peak 1, Fig. 1).

A complete amino acid analysis was carried out on the five fractions. As expected serine and threonine were most abundant in the corresponding phosphorylated fractions. Surprisingly, a high amount of proline was found in the sample corresponding to peak 1, having the same high specific radioactivity as found for phosphorylserine and phosphorylthreonine.

Cyclic AMP even in as high a concentration as 1×10^{-4} M did not stimulate a further phosphorylation. Maximal incorporation occurred in the presence of both Na^+ and K^+ as well as Mg^{2+}. If Mg^{2+} was stoichiometrically exchanged for Ca^{2+}, the incorporating activity decreased by more than 80 %. If half the amount of Mg^{2+} was excanged for Ca^{2+}, the corresponding activity was 50 % lower. With only Mg^{2+} and a monovalent cation present choline stimulated more than Na^+, which in its turn was more stimulatory than K^+. Possible functions of the labeled phosphoproteins are discussed.

Previous work from this laboratory has shown that [^{32}P]-labeled phosphorylserine as well as phosphorylthreonine can be isolated from Ehrlich mouse ascites tumor cells incubated under isotonic conditions with [γ^{32}P]ATP or [γ^{32}P]GTP. The extent of phosphorylation is much lower when either of these two nucleotides is replaced by CTP, UTP or pyrophosphate and is negligible when replaced by [^{32}P] orthophosphate. [1–3] It was concluded that the reaction between [γ^{32}P]ATP and the cells took place at the cell surface because ATP does not penetrate the intact cell membrane [4] and the cells remained intact throughout the experiments.

The labeled phosphoryl groups in the two phosphorylated amino acid residues represented only a small fraction of the total amounts of labeled orthophosphate liberated from [γ^{32}P] ATP at the cell surface. This type of phosphorylation may represent either an intermediary phosphorylation of an enzyme or a regulatory phosphorylation of a protein in the membrane. The latter type of reaction is catalyzed by a protein kinase. Furthermore, an intracellular protein kinase has been shown to be sensitive to cyclic AMP stimulation.[5,6]

In the present investigation, we have studied the effect of adding cyclic AMP to the incubation medium and of varying the cationic composition of the isotonic medium on the transfer of phosphate from external ATP to seryl- and threonyl residues of proteins presumably located at the cell surface.

MATERIAL AND METHODS

The Ehrlich mouse ascites tumor cells were grown for 7 – 8 d in 5 week old Swiss albino

mice obtained from the Anticimex breeding farm, Norrviken, Stockholm. The tumor cells were separated by centrifugation of the ascitic fluid, which had been diluted without delay several-fold with icecold Krebs-Ringer bicarbonate medium in order to diminish the tendency of cell agglutination. The cells were washed once in the Krebs-Ringer bicarbonate medium and the final washing was carried out in a medium with the same buffer and cationic composition as the incubation medium. The [γ^{32}P]ATP used was prepared as previously described.[3]

The cells were incubated in a medium containing either 130 mM sodium chloride and 25 mM potassium chloride or 155 mM of one single cationic salt (NaCl, KCl, choline Cl) to maintain isotonicity. The incubation procedure as well as the isolation of the phosphorylated amino acid residues have been described earlier.[1,2] The Schneider protein fraction was isolated from the cell [7] and partially hydrolyzed according to Lipmann. [8] Radioactivity was determined in a Nuclear Chicago Scintillation counter by measuring the Cherenkov radiation.

RESULTS

Fig. 1 illustrates the elution pattern from a typical experiment where 500 mg of a partial hydrolysate of the Schneider protein from incubated Ehrlich cells was separated on a Dowex 50 column. Five peaks can be distinguished. The two main peaks contain [^{32}P] phosphorylserine and [^{32}P]phosphorylthreonine residues. In addition three smaller labeled peaks are distinguished, the first in position

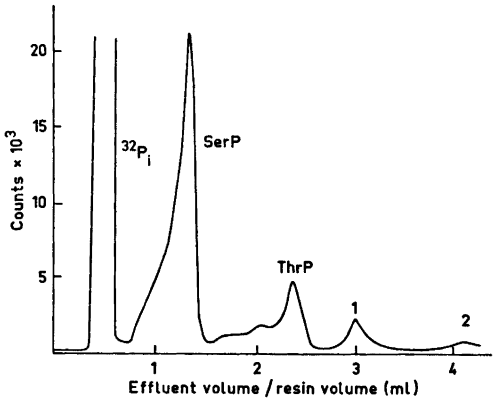

Fig. 1. Elution curve from a partial hydrolysate of the Schneider protein from incubated intact Ehrlich cells. Chromatography was performed on a 50 ml Dowex 50 (8 % DVB) column with 0.01 M HCl as eluent.

Table 1. Total radioactivity of the five fractions collected during chromatography on Dowex 50 (Fig. 1) together with their relative specific activities.

Fraction obtained[a]	Total ^{32}P-radio-activity[b]	Fraction weight[c]	Specific ^{32}P-radio-activity[d]
SerP	8 317	12	100
Peak between SerP and ThrP	630	6	15
ThrP	2 384	3	115
Peak 1	674	1	97
Peak 2	177	3	8

[a] Peak 3, occasionally found, is not included because of its very low specific activity. [b] Activity expressed in counts per min. [c] Schneider protein, mg [d] Expressed as percentage of the specific activity of SerP.

between phosphorylserine and phosphorylthreonine and the other two after the phosphorylthreonine peak. Occasionally a third peak after phosphorylthreonine could be observed.

Table 1 summarizes the total amount of material corresponding to each peak as well as total ^{32}P-activity. It is evident from the table that the specific activity of the [γ^{32}P]-phosphoryl group of ATP incorporated into the five fractions was about the same for the phosphorylserine and phosphorylthreonine fractions and the first fraction after phosphorylthreonine while it was significantly lower for the two other peaks.

Each fraction collected from the column was submitted to total acid hydrolysis and the amino acid content was analyzed. The amino acid content of each of the five fractions are given in Table 2. Serine residues were predominant in the [^{32}P]phosphorylserine fraction as is seen in the table.

Only slight amounts of threonine were detected in that fraction. Glutamic acid was present in a higher amount than any other amino acids with the exception of serine. Likewise, in the small fraction eluted between phosphorylserine and phosphorylthreonine, glutamic acid and serine were predominant.

The threonine content of the phosphorylthreonine fraction was high compared with the amount of serine. Even here as was the case for the phosphorylserine fraction glutamic

Table 2. Amino acid composition of the five radioactive fractions collected during chromatography on Dowex 50 of the partially hydrolyzed Schneider protein from Ehrlich cells.

Amino acid composition	Serine[a]	Threonine	Glutamic acid	Leucine	Isoleucine	Proline	Glycine	Valine	Aspartic acid	Alanine	Cystine	Phenylalanine[c]
SerP	216[b]	5	39	12	13	5	10	6	6	5	<5	<5
Peak between SerP and ThrP	30	9	34	8	5	11	9	12	<5	7	<5	5
ThrP	8	27	17	<5	<5	12	7	<5	<5	<5	<5	<5
Peak 1	13	5	24	8	10	126	6	14	<5	<5	5	6
Peak 2	9	<5	11	5	5	5	5	<5	<5	<5	<5	<5

[a] Amino acid analysis was carried out according to Stein and Moore using a Biocal BC 200 analysator. [b] Figures denote nmol per 100 mg Schneider protein from Ehrlich cells. [c] Tyrosine, methionine, arginine, histidine, and lysine were all less than 5 nmol per 100 mg Schneider protein in all fractions.

Table 3. Incubation of Ehrlich cells for 30 s at 37 °C with [γ^{32}P]ATP in a medium made isotonic by 130 mM NaCl, 25 mM KCl, and 2 mM $MgCl_2$.

Medium[a]	SerP	ThrP	Peak 1
1. Na^+, K^+, Mg^{2+}	100[b]	100	100
2. As in 1 plus 3′,5′-AMP (10^{-4} M)	90	90	84
3. As in 1 plus 3′,5′-AMP (10^{-6} M)	87	92	83

[a] Unlabeled orthophosphate concentration was 1×10^{-3} M in all media. The incubation was terminated with trichloroacetic acid giving a final conc of 5 %. [^{32}P]phosphorylserine (SerP), [^{32}P]phosphorylthreonine (ThrP), and [^{32}P]-labeled peptide material (Peak 1) were isolated from the acid insoluble material as described in Methods. [b] The figures given denote percentage values of phosphoryl group incorporation obtained in a medium containing Na^+, K^+ and Mg^{2+} but lacking 3′,5′-AMP. All figures are average values from two experiments.

acid was present in a comparatively high amount. The fraction corresponding to peak No. 1, Fig. 1, contained both serine and threonine in a relationship of 2.6 to 1. The amounts of glutamic acid in this fraction were again relatively high. Proline, however, was the predominant amino acid of this fraction exceeding by nearly ten times the amount of serine.

In order to elucidate the effect of cyclic AMP on the phosphorylation of the different membraneous protein fractions, experiments with [γ^{32}P]-labeled ATP were carried out with the addition of two different concentrations of cyclic AMP (10^{-6} M and 10^{-4} M, respectively) to the isotonic medium. As is seen in Table 3 cyclic AMP, even at the higher concentration, does not stimulate the phosphorylation by [γ^{32}P]ATP.

The effects of different cations on the phosphorylation of the phosphorylserine and phosphorylthreonine residues are given in Table 4.

Table 4. Incubation of Ehrlich cells for 1 min at 37 °C with [γ^{32}P]ATP in an isotonic medium with variation of the cationic composition.

Medium[a]	SerP	ThrP	Peak 1	Preak 2
1. Na^+, K^+, Mg^{2+}[b]	401.6[c] (100)[d]	100.9 (100)	31.2 (100)	13.8 (100)
2. Na^+, K^+, Mg^{2+}, Ca^{2+}	210.3 (52.4)	39.3 (39.9)	11.4 (36.5)	0
3. Na^+, K^+, Ca^{2+}	71.2 (17.7)	14.0 (13.9)	0	0
4. Na^+, Mg^{2+}	189.8 (47.3)	47.8 (47.4)	14.7 (47.1)	5.4 (39.5)
5. K^+, Mg^{2+}	142.2 (35.4)	32.0 (31.7)	12.2 (39.2)	0
6. $Choline^+$, Mg^{2+}	293.3 (73.0)	69.1 (68.5)	21.7 (69.7)	0

[a] Unlabeled orthophosphate concentration was 1×10^{-3} M in all media. The incubation was terminated with trichloroacetic acid giving a final conc of 5 %. [^{32}P]Phosphorylserine (SerP), [^{32}P]phosphorylthreonine (ThrP) and [^{32}P]-labeled peptide material (Peak 1 and Peak 2) were isolated from the acid insoluble material as described in Methods. [b] 1. 130 mM Na^+, 25 mM K^+ and 2 mM Mg^{2+}; 2. Na^+ and K^+ as in 1, with 1 mM Mg^{2+} and 1 mM Ca^{2+}; 3. Na^+ and K^+ as in 1 with 2 mM Ca^{2+}; 4. 155 mM Na^+ and 2 mM Mg^{2+}; 5. 155 mM K^+ and 2 mM Mg^{2+}; 6. 155 mM $choline^+$ and 2 mM Mg^{2+}. [c] Figures denote [^{32}P]phosphoryl groups incorporated in pmol per 100 mg Schneider protein from Ehrlich cells. [d] Figures in brackets denote percentage value of that obtained under standard incubation conditions (1. Na^+, K^+, Mg^{2+}).

When the Mg^{2+} concentration and the ATP/-Mg^{2+} ratio are kept constant, the phosphorylation is greatest when both Na^+ (130 mM) and K^+ (25 mM) are present. If all Mg^{2+} is replaced stoichiometrically by Ca^{2+} under otherwise similar experimental conditions the phosphorylation observed represents only 18 % of the maximum phosphorylation. This phosphorylation can be increased by replacing half the amount of Ca^{2+} by Mg^{2+}.

Experiments were also made in the presence of Mg^{2+} and [γ^{32}P]ATP as described above, but in the presence of only *one* monovalent cation (155 mM). The monovalent cations tested were: sodium, potassium, and choline. Most phosphorylation was obtained with choline (73 % of maximum value), whilst with sodium and potassium phosphorylation proceeded to the extent of 47 % and 35 %, respectively, of the maximum value.

The amount of labeled phosphorylthreonine residue was in all experiments 4 – 5 times smaller than that of the phosphorylserine residue [3] regardless of extracellular ionic conditions.

DISCUSSION

The time interval between the removal of the tumor cells from the donor animals and the incubation never exceeded 50 min. In addition, the cells were handled in a physiological buffer at 4 °C during preparation. We therefore have every reason to believe that the cells were intact during the incubation. In addition, no macroscopic signs of cell damage, *e.g.* any tendency of agglutination, were observed during the incubation. Since [γ^{32}P]ATP does not penetrate an intact plasma membrane [4] we conclude that the phosphorylation reactions observed occur at the cell surface.

Phosphorylserine and phosphorylthreonine residues from a protein hydrolysate are indicative of either an intermediate phosphorylation of certain enzymes [9,10] or a regulatory phosphorylation catalyzed by protein kinase.[6]

Alkaline phosphatase, an enzyme forming an intermediate which is phosphorylated at a phosphorylserine residue,[3] has been shown by de Thé [11] to be associated with the outer surface of the plasma membrane of thymomas of leukemic mice. Thus, alkaline phosphatase activity might also be associated with the outer surface of the plasma membrane of Ehrlich tumor cells. However, Engström [8] has shown that alkaline phosphatase is also phosphorylated with [^{32}P]orthophosphate as substrate. But since [^{32}P]orthophosphate does not act as a phosphoryl donor at the Ehrlich cell surface [3] and labeled phosphorylthreonine has never been isolated from alkaline phosphatase (Engström, personal communication 1974), we consider it unlikely that the observed labeling involves an alkaline phosphatase on the cell surface. In addition, although the experimental conditions have been varied as regards substrates,[3] ionic composition of the incubation medium as well as the incubation time the ratio between phosphorylserine and phosphorylthreonine has remained constant. This fact favors the view that one single entity of protein(s) is phosphorylated with [γ^{32}P]ATP.

Other enzymes (*e.g.* phosphoglucomutase) are probably also phosphorylated exclusively at a serine residue in the active centre. The hypothesis of an intermediate enzymatic phosphorylation would therefore appear untenable.

The phosphorylation studied here might well represent a regulatory phosphorylation catalyzed by one or more protein kinases localized at the surface of the plasma membrane. In this connection it may be mentioned that Matsumura and Takedo [12] have observed that a phosphorylation probably representing a regulatory function occurs in the cytosol of rat liver cells. Furthermore, they were able to separate two main fractions one catalyzing mainly the phosphorylation of a seryl residue, the other mainly a threonyl residue. The importance of the phosphorylation of the threonyl residue in our study is emphasized by the fact that the specific activities of the phosphorylserine and phosphorylthreonine residues were about the same (Table 1).

We made the interesting observation that cyclic AMP even at high concentrations (1×10^{-4} M) did not stimulate the assumed protein kinase reaction. This does not exclude the possible existence of a regulatory phosphorylation system localized at the surface of the cell membrane. This behavior might reflect an adaptation to the physiological conditions existing in the plasma membrane architecture when adenyl cyclase is believed to be located

on the inner surface of the plasma membrane.[13] Adenyl cyclase activity has never been possible to demonstrate at the outer surface of several types of tumor cells.[14] The possible existence of external ATP as a substrate in a "membraneous metabolic pool" has been discussed earlier.[15] Recent reports concerning the effects of *extracellular* ATP on different cells [16–19] might be explained by protein kinase action at the outer surface of the cell.

Our results demonstrate the presence of metabolically active proteins on the outside of the plasma membrane. In accordance with this observation it is interesting to note the recent finding of the occurrence of surface peptides on intact Ehrlich cells.[20]

Acknowledgements. We are indebted to Ing. S. Eklund, Mr. C. Wernstedt and Miss H. Grundberg for skilful technical assistance. We also thank Professor J. Sjöquist of this institute for kindly carrying out the amino acid analyses.

This investigation was supported by a grant from the Swedish Medical Research Council (project B73-13X-228-10B).

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Received June 6, 1974.