ENDOGENOUS PROTEINKINASE-DEPENDENT PHOSPHORYLATION OF RAT LIVER MITOCHONDRIAL MEMBRANES

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Summary: Isolated rat liver mitochondrial membranes are found to contain tightly bound protein substrate(s) which can be phosphorylated in the presence of ATP by protein kinase(s) previously extracted with 0.7 M NaCl from the membranes themselves and by phosvitin kinase purified from liver cytosol.
Such proteinkinase-dependent phosphorylation, which seems to be cyclic AMP-independent, involves the seryl and threonyl residues of the protein substrate(s)

Introduction

During the past few years there has been an ever increasing interest in the study of the phosphorylation of membrane protein components (from a wide variety of mammalian sources) by protein kinase(s) associated to the membrane structure itself.

Previously we had demonstrated that protein substrates (so called "structural proteins") prepared from rat liver mitochondrial membranes can be phosphorylated when added to fresh liver mitochondria in the presence of inorganic phosphate (1).

The present paper gives evidence that also in mitochondrial membranes as well as in other biological membranes, tightly bound protein component(s) can be phosphorylated in the presence of ATP by endogenous protein kinase(s) associated to the membrane structure itself. However such phosphorylation, unlike that evidenced in the other mammalian membranes, appears to be independent of cyclic AMP.

Materials and Methods

Preparation of sonic subparticles from purified rat liver mitochondria: A typical preparation was as follows: rat liver mitochondria prepared according to Schneider (2) were purified by centrifugation in a sucrose density gradient (3). Once recovered by mean of J shaped pipette, purified mitochondria were diluted with ice cold distilled water to lower the sucrose concentration

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A preliminary report of these findings has been given at the 9th FEBS Meeting (Budapest 1974).

to 0.25 M, and centrifuged for 15 min at 15,000 x g. The supernatant was discarded and the mitochondrial pellets, suspended with 25 ml of 10 mM Tris-HCl pH 8, were sonicated four times for 20 sec (Branson sonifier B-12) in ice to prevent the temperature from rising above 2-4°. Not disrupted mitochondria were precipitated by centrifugation at 15,000 x g for 20 min, while sonic particles were recovered by centrifugation at 105,000 x g for 40 min. The precipitated particles were suspended with 4 ml of distilled water and stored at -20°C.

Preparation of "NaCl washed" mitochondrial membranes: Mitochondrial sonic particles (equivalent to 120 mg protein) were firstly suspended by homogenization in 4 ml of distilled water and precipitated by 40 min centrifugation at 105,000 x g. The supernatant was referred to as "H_2O soluble fraction".

The precipitated particles were then extracted with 4 ml of 0.7 M NaCl for 30 min by stirring and recovered by centrifugation at 105,000 x g. The extraction of the particles with 0.7 M NaCl was repeated 2 more times to obtain the first, second and third "NaCl soluble fraction" and a final pellet referred to as "NaCl washed membranes". This latter was resuspended with 4 ml of distilled water. All operations were done in a cold room at 0-4°C and both "soluble fractions" and "washed membranes" were stored at -20°C.

Preparation of cytosol proteinkinase from rat liver: Cytosol proteinkinase has been purified according to the procedure previously described (4).

Assay of proteinkinase activity: Proteinkinase activity (in the different fractions) was determined by incubating at 37°C for 30 minutes in a final volume of 0.2 ml: 10 µmoles Tris-HCl buffer pH 7.0; 1.2 µmoles $MgCl_2$; 1 nmole of [^{32}P]ATP (2.6 x 10^6 cpm/nmole) and 420 µg of mitochondrial "soluble fraction" or 9 µg of purified cytosol kinase.

Incubation was stopped by addition of 0.1 ml of 50% trichloroacetic acid and 1 mg serum albumin, followed by centrifugation. The supernatant was discarded and the precipitate washed 4 times with 5 ml of 10% trichloroacetic acid containing 1 M H_3PO_4. During the last washing the suspended membranes were kept at 100°C in boiling bath for 20 min before centrifugation. The precipitates were finally dissolved in Instagel scintillation liquid and counted in a Packard Tri-Carb mod. 3375. When the phosvitin kinase activity was tested, the mitochondrial "NaCl washed" membranes were replaced by 0.5 mg of phosvitin as substrate and [^{32}P]ATP added was 25 nmoles (7.2 x 10^4/nmole). The remaining conditions were identical.

Other experimental procedures: Labeled membrane proteins were hydrolyzed for 10 h in 2 N HCl at 105°C. After removal of HCl under vacuum over P_2O_5 and NaOH, the hydrolysates were dissolved in a small volume of water and subjected to high voltage paper electrophoresis on Whatman 3 MM paper strips using formic acid: acetic acid (2.5%:7.8%) as buffer. The electrophoresis was run for 135 min at a constant voltage of 130 V/cm. Carrier phosphorylserine and phosphorylthreonine were detected by ninhydrin reaction. The radioactivity on the electrophoretic strip was determined with a Packard Radiochromatogram Scanner mod. 7201. Protein was determined by the method of Lowry et al. (5) with bovine serum albumin as a standard. Phosvitin was prepared from egg yolk according to Mecham and Olcott (6). [γ-^{32}P]ATP was purchased from Radiochemical Centre.

Results and Discussion

As previously reported (7) mitochondrial sonic sub-particles contain a tightly bound proteinkinase activity (tested with phosvitin as substrate) which is not removed by isotonic sucrose or by H_2O from the membranes to which it is associated. However it is extractable by washing with high ionic strength solutions (0.7 M NaCl).

Table I - SOLUBILIZATION OF PHOSVITIN KINASE FROM MITOCHONDRIAL MEMBRANES

	Phosvitin kinase activity (cpm incorporated into phosvitin)
Water soluble fraction	180
1st NaCl soluble fraction	7,730
2nd " " "	1,470
3rd " " "	320
NaCl washed membranes	000

The preparation of "water soluble" and "NaCl soluble" fractions and the experimental conditions for testing the phosvitinkinase activity are described in "Materials and Methods".

As shown in Table I, three successive washings with 0.7 M NaCl are enough to deplete almost completely the membrane fragments of the proteinkinase activity, which is recovered in the supernatant fractions (referred to as the "NaCl soluble fractions").

The remaining "NaCl washed membranes", once preincubated at 70° for 2-3 min, are able to be phosphorylated by proteinkinase(s) previously extracted from the membranes themselves ("NaCl soluble fractions"), indicating that the washed membranes are containing protein-bound phosphorylable sites.

As shown in Fig. 1 curve a, the phosphorylation after reaching a maximal level, decreases slowly afterwards, likely because of the presence of mitochondrial proteinphosphatase (8) and of other interfering enzymes present in the "NaCl soluble fraction". In fact, different levels of phosphorylation are obtained when the same membranes are incubated in the presence of protein kinase purified from rat liver cytosol (4) (fig.1 curve b).

When the mitochondria utilized for the membrane preparations are previously purified by sucrose gradient centrifugation, an increase of their membranes phosphorylation level by the cytosol kinase is observed. This would indicate that the protein substrates phosphorylable by this enzyme are indeed located in the mitochondrial membranes rather than in other contaminating subcellular particles.

The protein-bound ^{32}P transferred from [^{32}P]ATP by both the proteinkinases is alkali-labile (N NaOH at 100° for 15 min) and acid-stable (N HCl at 100° for 10 min). It resulted to be involving the phosphorylation of protein-bound seryl and threonyl residues of the phosphate acceptor protein(s).

Accordingly, both labeled phosphorylserine and phosphorylthreonine, besides ^{32}Pi, can be isolated from ^{32}P-labeled membranes upon hydrolysis in 2 N HCl

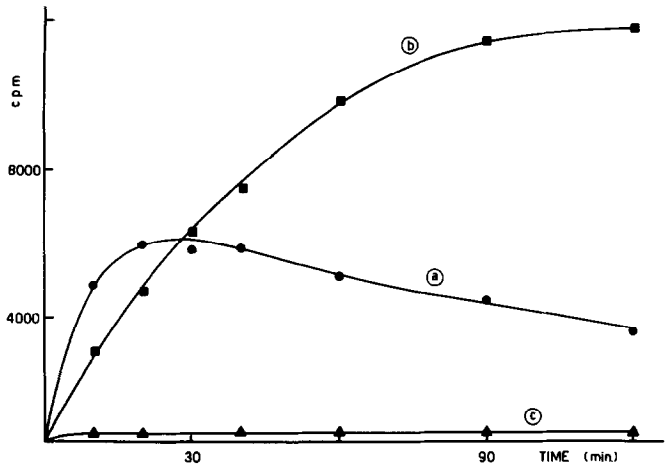

Fig. 1 - Time course of phosphorylation of preheated "NaCl washed" membranes by: ●—● proteinkinase(s) (420 µg of protein) extracted from the membranes themselves ("NaCl soluble fraction); ■—■ phosvitinkinase (9 µg of protein) purified from liver cytosol; ▲—▲ membrane phosphorylation control in the absence of proteinkinase (blank experiment). Incubation medium and the other experimental conditions are described in "Materials and Methods".

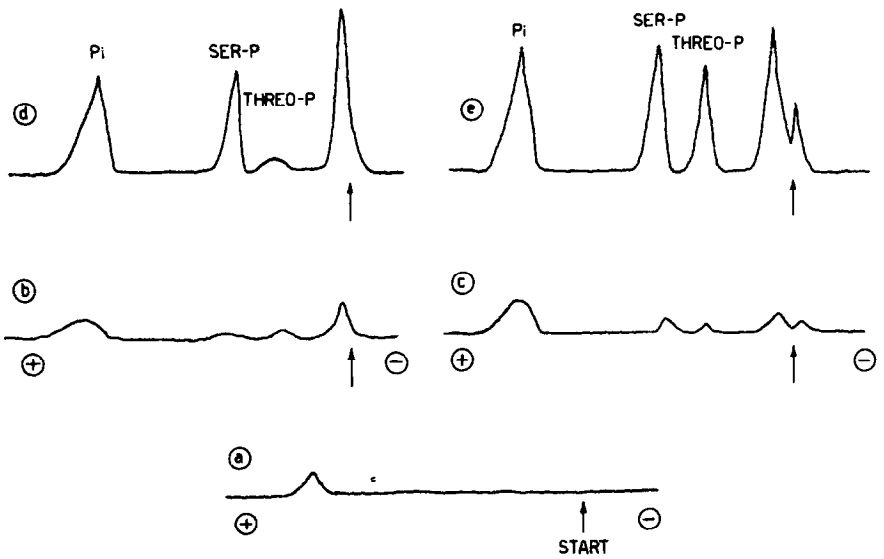

Fig. 2 - Electrophoretic pattern of acid hydrolysate of preheated membranes labeled by [^{32}P]ATP in the presence of: d) proteinkinase(s) extracted from membranes ("NaCl soluble fraction"); e) purified cytosol proteinkinase. The patterns a), b) and c) are obtained from hydrolysate of the following controls: a) preheated membranes; b) "NaCl soluble fraction"; c) cytosol proteinkinase.

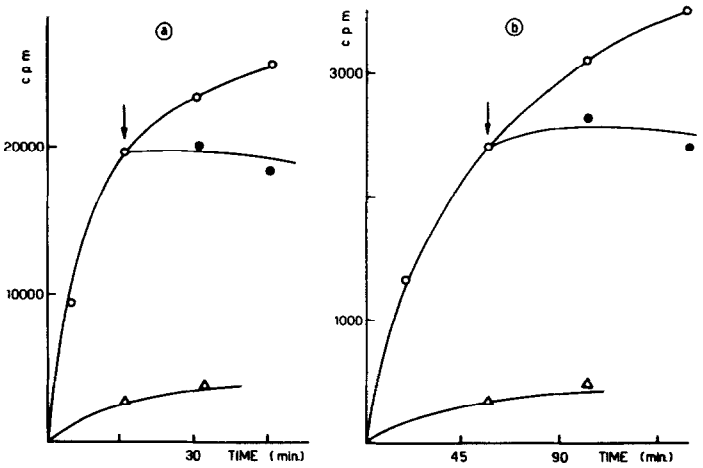

Fig. 3 - Time course of the phosphorylation of phosvitin (a) and of preheated membranes (b) by cytosol proteinkinase in the presence and in the absence of native "NaCl washed" membranes.
o—o in the absence of washed membranes; ●—● in the presence of wa - shed membranes added at the time indicated by the arrow; Δ—Δ in the presence of washed membranes added at zero time.

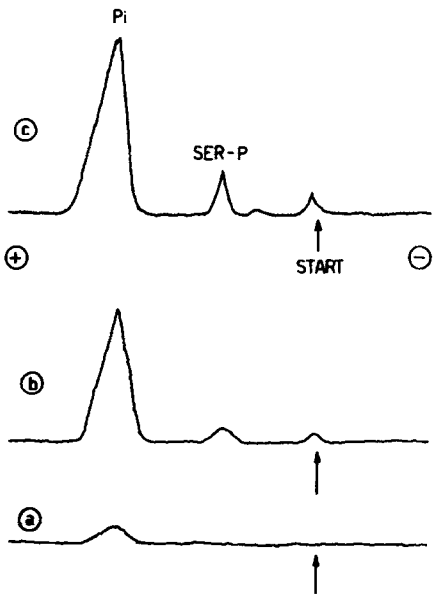

Fig. 4 - Electrophoretic patterns of acid hydrolysates of native "NaCl washed" membranes labeled without preheating.
a) cytosol proteinkinase alone; b) native washed membranes alone;
c) native washed membranes plus cytosol proteinkinase.
Experimental conditions of acid hydrolysis and electrophoresis as described in "Materials and Methods".

at 105° for 10 hrs, followed by high voltage electrophoresis (fig. 2).

Taking into account the hydrolytic loss of serine- and threonine-bound P (about 50%) occurring during such acid hydrolysis, the pattern of fig.2 would indicate that most, if not all, the ^{32}P incorporated by both proteinkinases into preheated membranes is accounted for by phosphorylserine and phosphoryl-threonine.

Moreover the comparison of the two electrophoretic patterns (fig. 2d and 2e) shows that the ratio $\frac{\text{seryl}}{\text{threo}}$ is different in the two hydrolysates.

It is tempting to speculate that the phosphorylation by the two protein-kinases may involve different membrane proteins or different sites of the same membrane protein(s). However, since the used mitochondrial kinase preparation, unlike the cytosol enzyme, is not purified, we are aware that the different pattern ($\frac{\text{seryl}}{\text{threo}}$ ratio) can be due to the presence in the crude mitochondrial "NaCl soluble fraction" of some components (enzymatic or not) selectively affecting the phosphorylation of protein-bound phosphorylserine and phosphoryl-threonine sites.

The phosphorylation catalyzed by both protein kinases appear to be independent of the cyclic AMP: no stimulation by cyclic nucleotide could be found in any condition followed by other investigators in order to evidence the cAMP-dependence of phosphorylation of other biological membranes.

The protein kinase-dependent phosphorylation of the seryl and threonyl sites in the "NaCl washed membranes" is obscured when thermal pretreatment is omitted, probably due to the presence of interfering enzymes or of other inhibitory termolabile factor(s), tightly bound to the membrane structure. Such idea is supported by the finding that the proteinkinase-dependent phosphory - lation of phosvitin (fig. 3a) and of preheated membranes (fig. 3b) is strongly reduced, if not discharged, when native "washed membranes" are added.

Also the native "washed membranes" are rapidly labeled by [^{32}P]ATP when the "NaCl soluble fraction" or cytosol kinase were added.

However such rapid labeling involves some other phosphorylable sites of the membrane structure, as indicated by the electrophoretic pattern obtained from acid hydrolysate (2 N HCl at 105° for 10 hrs) of the labeled membranes.

As shown in the fig. 4, in this case only a minor fraction of the incorporated radioactivity is accounted for by labeled phosphorylserine and phos - phorylthreonine while most of it is alkali-stable and converted into [^{32}P]Pi under acid hydrolysis.

In conclusion: the isolated mitochondrial membranes contain tightly bound protein substrate(s) which can be phosphorylated by associated mitochondrial proteinkinase and by proteinkinase from cytoplasm. Such a protein-kinase-dependent phosphorylation is quite difficult to be directly evidenc -

ed unless the mitochondrial membranes are previously heated to inactivate interfering enzymes or some other inhibitory factors bound to the membrane structure itself. Whether or not these proteinkinases are the same enzyme and are phosphorylating the same membrane-bound proteins is under investigation.

Acknowledgments

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