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Phosphorylation of structural protein catalyzed by rat-liver mitochondria

Mitochondria are able to catalyze the incorporation of $^{32}P_i$ into exogenous phosphoproteins, such as casein[1] and phosvitin[2,3]. Such a phosphorylation takes place through a reaction catalyzed by a mitochondrial protein kinase (EC 2.7.1.37) requiring the availability of ATP outside the mitochondria[4].

Recently a protein fraction containing radioactive phosphorylserine and having similar characteristics to so-called "structural protein" has been isolated from mitochondria incubated with $^{32}P_i$ (ref. 5). This communication describes experiments which show that structural protein after solubilization can be phosphorylated by mitochondria in the same manner as the foreign proteins, casein and phosvitin. The structural protein was prepared by two methods: (A) the procedure of CURTIS AND WADKINS, which is described in ref. 5, and (B) the procedure of RICHARDSON, HULTIN AND FLEISHER[6]. Both preparations were rendered 70–80 % soluble in water by treatment with 0.2 M 2-mercaptoethanol at pH 8.5 in 8 M urea, after either succinylation[7] or carbamylation. Urea was removed by dialysis (see ref. 5).

The results reported in Table I show that the solubilized structural protein, added to respiring mitochondria with $^{32}P_i$ and ADP, is phosphorylated. The labelling of structural protein is not significantly affected by the different procedures used for its preparation and solubilization. The radioactive protein was found to behave homogeneously through several tests ($(NH_4)_2SO_4$ and pH_i precipitation, paper and sepraphore electrophoresis, gel filtration). These findings would seem to rule out the presence of contaminant radioactive proteins, suggesting that the structural protein itself is the phosphate acceptor.

The phosphorylation process is ATP dependent (Table II). In fact addition of

TABLE I

PHOSPHORYLATION OF SOLUBILIZED STRUCTURAL PROTEIN IN THE PRESENCE OF RESPIRING MITOCHONDRIA

5 mg of the solubilized protein were incubated for 60 min at 27° in 3 ml of a medium containing: rat-liver mitochondria equivalent to about 40 mg protein; potassium glutamate, 60 μmoles; P_i, 3 μmoles, containing 30 μC of $^{32}P_i$; ADP, 2 μmoles; $MgCl_2$, 12 μmoles; Tris–HCl buffer (pH 7.4), 100 μmoles; sucrose, 293 μmoles. The reaction was stopped by addition of 30 μmoles of EDTA. Mitochondria were removed by centrifugation and the protein recovered by precipitation in 15 % satd. $(NH_4)_2SO_4$. The protein was then washed 5 times by dissolving in 5 ml of 0.1 M phosphate followed by precipitation with $(NH_4)_2SO_4$, dissolved in 5 ml of 8 M urea and dialyzed 3 times in 2 l of 0.1 M phosphate (pH 7.5) and once in water. Finally its absorbance at 280 and 260 mμ was determined and its radioactivity measured in a counter. (A) Structural protein isolated according to the method of CURTIS AND WADKINS (see ref. 5). (B) Structural protein isolated according to the method of RICHARDSON, HULTIN AND FLEISHER[6].

Incubation medium	*Protein preparation*	*Counts/min per mg protein*
Protein omitted	—	78
Mitochondria omitted	Average 4 different preparations	180 (± 40)
Complete	(A) Carbamylated	1773
Complete	(A) Succinylated	1907
Complete	(B) Carbamylated	1571
Complete	(B) Succinylated	1686

TABLE II

ATP DEPENDENCE OF ^{32}P INCORPORATION INTO STRUCTURAL PROTEIN

General conditions and incubation medium as in Table I except ADP was omitted. Structural protein Prep. A, solubilized by carbamylation, was used. The amount of phosphate incorporated into the protein and into nucleotides was determined by assuming that it had the same specific radioactivity as $^{32}P_i$. $^{32}P_i$ specific radioactivity at the end of incubation was determined in the deproteinized $(NH_4)_2SO_4$ supernatant by measuring the phosphomolybdate complex and the radioactivity in the isobutanol–benzene phase[9]. The radioactivity still present in the aqueous phase after three extractions with isobutanol–benzene was assumed to be all due to nucleotides.

Additions	*^{32}P incorporated into structural protein (mμmoles/22 mg)*	*^{32}P incorporated into extramitochondrial nucleotides (μmoles)*
—	2.98	0.262
Atractyloside (0.8 μmole)	0.09	0.023
ADP (0.8 μmole)	6.96	1.207
ADP (1.6 μmoles)	9.80	1.786
ADP (1.6 μmoles) + atractyloside (0.8 μmole)	0.71	0.028

ADP greatly increases the incorporation of ^{32}P both into extramitochondrial nucleotides (which can be assumed to be mainly [^{32}P]ATP) and into structural protein. Moreover atractyloside, which is known to abolish the appearance of endogenous ATP in the medium outside mitochondria[4,8], completely prevents, as in the case of phosvitin[4], the phosphorylation of structural protein.

That serine is the amino acid residue mainly involved in the phosphorylation process is supported by the finding that [^{32}P]phosphorylserine, which almost quantitatively accounts for the radioactivity covalently bound to the protein, can be isolated from acid hydrolysates of ^{32}P-labelled structural protein prepared by both procedures. Paper electrophoresis of the acid hydrolysates (see Fig. 1) gives 3 radioactive peaks:

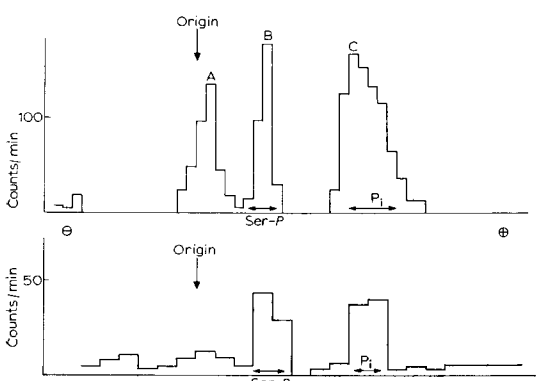

Fig. 1. Isolation of radioactive phosphorylserine from acid hydrolysates of ^{32}P-labelled structural protein. 5 mg of ^{32}P-labelled structural protein (Prep. B, solubilized by carbamylation) were hydrolysed in 2 M HCl at 100° for 10 h. The hydrolysate, with added carrier phosphorylserine and orthophosphate, was subjected to electrophoresis in 20% formic acid (pH 1.4) for 60 min at 45 V/cm on Whatman 3 MM paper (top figure). Peak A from top figure, after elution and further acid hydrolysis in 2 M HCl for 10 h at 100°, has been submitted again to paper electrophoresis, for 60 min at 45 V/cm (bottom figure). Phosphorylserine was detected by ninhydrin reaction. Radioactivity was determined by stepwise counting of the strip.

B and C correspond, respectively, to phosphorylserine and to P_i; Peak A corresponds to a phosphopeptide which gives rise to roughly the same amounts of B and C upon further acid hydrolysis. Under these conditions a 50 % breakdown of phosphorylserine occurs. Therefore, since the recovery of radioactivity present in A *plus* B is not significantly lower than the radioactivity converted into inorganic orthophosphate (Peak C), the above results indicate that [^{32}P]phosphorylserine accounts for at least 80–90 % of the protein-bound radioactivity.

The identification of Peak B as [^{32}P]phosphorylserine has also been confirmed by paper descending chromatography of radioactive band B, eluted from the paper electrophoregram, mixed with authentic phosphorylserine (solvents: (1) isopropyl-ether–*n*-butanol–90 % formic acid (3:3:2, by vol.); (2) *n*-butanol–ethanol–0.005 M HCl (3:2:2, by vol.)).

So far it has been shown that, among the several proteins tested, only casein and phosvitin are able to be phosphorylated by protein kinase[1,10,11].

The present results showing that mitochondrial structural protein can be phosphorylated through the same mechanism indicate such a protein as a possible substrate for mitochondrial protein kinase.

It has been suggested[11,12] that the peculiar property of casein and phosvitin to be phosphorylated (unlike other phosphoproteins) by protein kinase is dependent on the presence of blocks of several phosphorylserine residues in a row, found only in these two proteins[12] and not in many other phosphoproteins[13,14]. Experiments are in progress to investigate whether structural protein also contains such blocks of phosphorylserine residues.

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