Identification of Serine Phosphate in Mitochondrial Structural Protein[1]

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Previous studies have established that ^{32}Pi is incorporated during coupled respiration of intact mitochondria and phosphorylating sub-mitochondrial particles into a water-insoluble membrane protein fraction which yields radioactive serine phosphate after acid hydrolysis (Ahmed and Judah, 1963, Moret et al., 1963, Wadkins, 1963 and Sperti et al., 1964). Attempts to characterize the labeled mitochondrial protein have been handicapped because of its insolubility. This communication will describe recent studies in which the labeled protein was rendered soluble and purified by conventional methods.

The labeled protein was prepared by incubation of 50-100 mg mitochondrial protein derived from rat liver for 10 minutes at 25° with 10 mM β-hydroxybutyrate, 0.1 M sucrose, 2 mM $MgCl_2$, 0.02 M Tris buffer, pH 7.4, and 0.1 mM potassium phosphate containing 5 x 10^6 c.p.m. Pi^{32} in a total volume of 10 ml. The reaction system was then diluted with an equal volume of water and frozen overnight. The thawed mixture was centrifuged at 20,000 x g for 40 minutes. The residue was suspended in 30 ml 0.05 M sucrose and centrifuged as above. The extraction was repeated twice followed by two extractions with 30 ml of 0.1 M KCl. The final residue was suspended in 30 ml 8 M urea in 1% ammonium carbo-

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nate, allowed to stand at 2° for 30 minutes and centrifuged at 100,000 x g for 40 minutes. The supernatant fraction was dialyzed for 48 hours against 3 changes of 0.05 M Tris-0.05 M KCl pH 7.5 and centrifuged at 10,000 x g for 40 minutes. The precipitated protein was washed at 2° three times with the above Tris-KCl buffer, 2 times with 5% trichloroacetic acid, 3 times with 90% acetone, and 3 times with water in order to remove the bulk of adventitiously bound P^{32}-labeled inorganic phosphate, nucleotides and phospholipid. The acetone extraction and a subsequent extraction with chloroform-methanol removed a total of less than 3% of the radioactivity.

The labeled protein thus prepared was rendered soluble by treating with potassium cyanate in the presence of β-mercaptoethanol. 300-400 mg of the labeled protein (Fraction 1) was suspended in 60 ml of a reaction medium composed of 8 M urea, 0.05 M Tris buffer, pH 8.5, 0.2 M β-mercaptoethanol, and 0.4 M potassium cyanate at room temperature for 6 hours. The mixture was dialyzed for 72 hours against 4 changes of 0.05 M Tris-0.1 M KCl, pH 7.5 and centrifuged at 100,000 x g for 45 minutes. The pH of the supernatant fraction (Fraction 2) was then adjusted to 5.0 and the insoluble protein was removed by centrifugation. The precipitate was dissolved in 5 ml of 0.05 M Tris-0.1 M KCl, pH 7.5 (Fraction 3) and placed on a 1 x 40 cm column of Sephadex G-200, and eluted with the same buffer. The results are shown in Fig. 1. The indicated fractions from peak A were combined, lyophilized, dissolved in 5 ml of water and dialyzed for 24 hours against 2 changes of 0.05 M Tris-0.1 M KCl, pH 7.5 (Fraction 4). The identity of the material present in peak B (cf. Fig. 1) is unknown but its ^{32}P content is not present as inorganic phosphate, as judged by treatment with ammonium molybdate followed by isobutanol-benzene extraction (cf. Wadkins and Lehninger (1963) although it can be converted to inorganic phosphate by treating with 1 N HCl for 10 minutes. Further characterization of this fraction is in progress.

The radioactive protein present in Fraction 4 was subjected to electrophoresis at 200 volts for 1/2 hour on cellulose acetate strips using 0.05 Tris-acetate buffer at several pH values. The position of protein was determined with

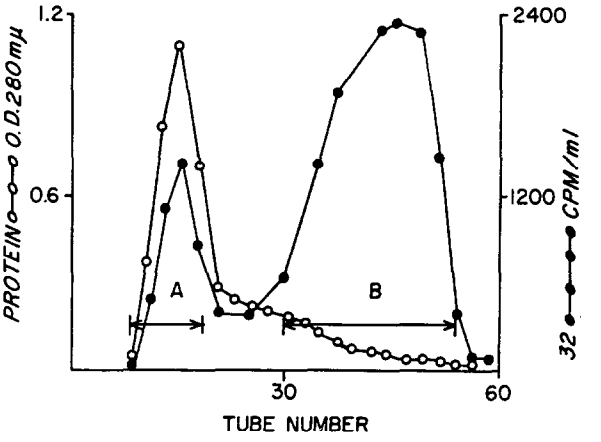

Fig. 1. Purification of soluble phosphoproteins on Sephadex G-200. 15 mg of labeled soluble protein was applied to column and eluted as described in text. 4.0 ml fractions were collected.

1% Amido-Schwartz and the position of the radioactivity was determined with a Vanguard strip scanner and the results are summarized in Table 1. Only single radioactive and protein-containing areas were detected and were found to coincide.

Table 1

Electrophoresis of Soluble Phosphoprotein

Electrophoresis was carried out with 10 mg/ml solution of protein corresponding to Fraction 4 (cf. Table 2) as described in text.

pH	Distance Moved (in mm)	
	Radioactive area	Protein area
8.5	22.0	22.0
7.5	20.0	20.0
7.0	14.0	14.2
6.5	8.8	8.9
6.0	4.0	4.0
5.5	0	0 (insoluble)

The existence of phosphorylated serine residues in the labeled protein was demonstrated by the following procedure. Samples containing 25,000-50,000 c.p.m. were heated at 100° in 2N HCl for 10 hours. 1.0-2.0 μmoles of unlabeled serine phosphate was added and the HCl removed by repeated evaporation with a rotary

evaporator at 50° under vacuum. The residue was dissolved in the minimal volume of 0.05 N HCl and applied to a 1.3 x 55 cm column of Dowex 50, 8x, acid form which had been previously equilibrated with 0.05N HCl. The column was eluted with 0.05N HCl and 3.0 ml fractions were collected. Authentic inorganic phosphate and serine phosphate were found to be eluted at 40 ml and 100 ml respectively. The radioactive fractions derived from the hydrolyzed labeled protein which were eluted at 95-105 ml were combined, dried with a rotary evaporator and dissolved in 0.1-0.2 ml water. The entire amount was placed on Whatman 3 MM paper, saturated with 20% formic acid, pH 1.5 and submitted to electrophoresis at 1500 volts for 1 hour. Serine phosphate was located by spraying with ninhydrin. The radioactive area was located with a Vanguard 4π strip scanner and was found to coincide with the ninhydrin positive zone. Comigration of the radioactive and ninhydrin zones obtained with the experimental sample and with authentic serine phosphate was observed after paper chromatography with isopropyl ether-N-butanol-formic acid (3:3:2 parts by volume). The radioactive area of an unstained electrophoresis strip corresponding to serine phosphate was cut out and eluted with warm 50% acetic acid. The radioactivity corresponding to serine phosphate derived from the several soluble protein fractions as well as from "structural protein" isolated from labeled rat liver mitochondria by the method of Criddle et al. (1962) is presented in Table 2.

Table 2

Fractionation of Mitochondrial Phosphoprotein

Phosphoprotein fractions were derived from 1000 mg rat liver mitochondrial protein as described in text. Serine phosphate values are uncorrected for hydrolytic losses.

Fraction	Protein	Radioactivity	
		Total	Serine phosphate
	mg	c.p.m.	
1	500	1,000,000	9300
2	400	900,000	8900
3	390	850,000	8000
4	350	300,000	8000
"Structural Protein"[1]	220	450,000	5760

[1] Isolated from 800 mg rat liver mitochondria protein previously labeled with Pi^{32} as described in text and isolated as described by Criddle et al. (1962).

Protein bound serine phosphate has also been estimated by the formation of inorganic phosphate during exposure of the protein to 1 N NaOH for 15 minutes at 100° (Ahmed and Judah, 1963; Siliprandi et al., 1966). A comparison of the total protein-bound radioactivity, isolated radioactive serine phosphate, and alkali-labile radioactive inorganic phosphate released by the alkali treatment for several samples of the protein is presented in Table 3. The experimental values for serine phosphate were adjusted for hydrolytic losses and incomplete hydrolysis of the protein by assuming a 30% yield of serine phosphate from phosphoprotein which was determined by application of the same techniques to a purified sample of phosvitin. It is obvious that this constitutes an arbitrary application to the mitochondrial protein and as judged by the data of Table 3 the yield could be as low as 10% which would provide agreement of the serine phosphate values with the alkali-labile phosphate.

Approximately 65% of the total protein-bound radioactivity released from the protein by the alkali treatment appeared in the aqueous phase after treatment with

Table 3

Distribution of Radioactivity in Purified Phosphoprotein

These studies were carried out with 100 mg of the labeled protein corresponding to Fraction 4 (cf. Table 1) as described in text.

Experiment	Radioactivity (c.p.m.)			
	Total	Serine Phosphate	Alkali-labile Pi	Alkali-labile Organic P
1	18,000	2900	7400	8600
2	55,000	8000	21000	28000
3	22,000	2900	7500	14000

molybdate and extraction with isobutanol-benzene and is presumably an organic phosphate compound possibly bound to the original protein through an alkali-labile phosphodiester linkage. Efforts to complete its characterization are in progress.

Amino acid analysis[1] of the labeled soluble protein (Fraction 4) indicate

[1] Unpublished observations of P. Curtis and C. Wadkins

a close similarity to that reported for membrane "structural protein" obtained from beef heart mitochondria (Criddle et al., 1962). Our studies show that 50% of the lysine residues were converted to homocitrulline and that further exposure to potassium cyanate up to 48 hours caused no additional carbamylation. It would appear that only 50% of the epsilon amino groups are reactive under these conditions and that one or more of those influence the solubility properties of the labeled protein. MacLennon et al. (1965) have reported that succinylation of epsilon amino groups of lysine converts the membrane structural protein to a water soluble form.

These studies provide a direct demonstration that the "structural protein" fraction described by Criddle et al. (1962) contains the phosphorylated membrane protein described by other workers. A new technique which does not include the use of detergents has been described which results in solubilization of the bulk of the "structural protein" fraction and for the first time of the membrane phosphoprotein fraction. We have not been able to affect any significant separation of the labeled phosphoprotein moiety from the soluble "structural protein" moiety and conclude on this basis that serine residues of the mitochondrial membrane structural protein can be phosphorylated during coupled respiration of intact mitochondria.

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