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MITOCHONDRIAL PHOSPHOPROTEIN METABOLISM

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SUMMARY

1. Rat-liver mitochondria incubated at 20° in a medium of KCl, Tris, Versene, and containing ^{32}P, rapidly take up the tracer into ATP and phosphoprotein fractions. Phospholipids are not labelled under these conditions.

2. Inhibitors of electron transport and of oxidative phosphorylation reduce the rate of labelling of the phosphoproteins. In the presence of these inhibitors the phosphoproteins fail to reach control levels of radioactivity at any time.

3. The turnover of mitochondrial ATP is also reduced by inhibitors of electron transport and oxidative phosphorylation. In contrast to the phosphoproteins, mitochondrial ATP reaches the same isotopic equilibrium both in the presence and absence of inhibitors within a few minutes.

4. Oligomycin, however, does not inhibit phosphoprotein turnover at concentrations at which the labelling of ATP is greatly reduced.

5. It is proposed that mitochondrial phosphoproteins lie on a cyclic pathway between intermediates of the oxidative phosphorylation system and ATP.

INTRODUCTION

In previous work[1,2] we attempted to correlate mitochondrial phosphoprotein phosphorylation with water transport in these particles. Many inhibitors of electron transport and oxidative phosphorylation are known to affect water shifts also[3-5]. In this paper we examine work which suggests that the phosphoproteins may in fact accept phosphate from an intermediate of the oxidative phosphorylation chain rather than from ATP. The results given here support this view, though the nature of the problem excludes a definitive solution at the present time. However, the use of certain inhibitors together and separately allows accumulation of fairly strong circumstantial evidence that phosphoproteins take part in a cyclical reaction involving ATP and intermediates of the oxidative phosphorylation reaction.

METHODS

Animals

Sprague Dawley male rats of about 200 g body weight were used throughout. They were maintained on a standard laboratory cube diet, with a supplement of vitamin E (α-tocopherol acetate) 10 mg by mouth thrice weekly. This is required for

the maintenance of normal sodium transport by liver slices[6] and may be needed for mitochondrial transport functions also.

Mitochondria were prepared as already described[1]. Phosphoproteins were determined either as alkali-labile P (*i.e.*, inorganic P liberated from the proteins by digestion at 38° in 0.2 N KOH for 15 h) or as phosphorylserine after acid hydrolysis and chromatography as described earlier[7] as was ATP. ATP was also characterized by chromatography on Dowex-1 (see ref. 8) or by paper chromatography[9]. [^{32}P]ATP was prepared by oxidative phosphorylation in a medium containing ^{32}P-labeled inorganic orthophosphate (see ref. 8). [^{32}P]P_i as obtained was impure and was hydrolysed in 1 N HCl for 30 min at 100°. It was then precipitated as the magnesium salt as already described[10].

Nucleotides and coenzymes were obtained from Sigma Chemical Company. We are indebted to Dr. B. CHANCE for samples of antimycin A and to Dr. B. C. PRESSMAN for samples of oligomycin. "Digitonin particles" were prepared from mitochondria by an unpublished technique[11].

RESULTS

Time course of ^{32}P uptake in ATP and phosphoproteins and the effect of inhibitors

Rat-liver mitochondria isolated from 0.25 M sucrose containing 1 mM EDTA were incubated in 0.125 M KCl, buffered with 0.02 M Tris–HCl buffer (pH 7.4) and containing ^{32}P at the concentrations shown in the tables, but with no other additions

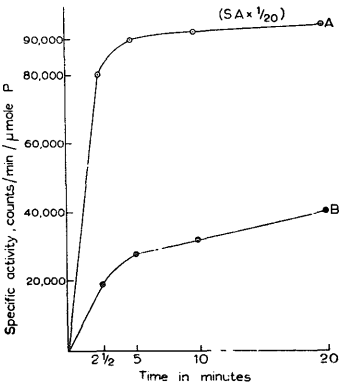

Fig. 1. Time course of ^{32}P turnover in ATP and phosphoproteins. Mitochondria equivalent to 500 mg original rat liver were incubated in a medium of 0.125 M KCl, 0.02 M Tris (pH 7.4), 1 mM Versene and inorganic phosphate $2 \cdot 10^{-4}$ M. ^{32}P was present at a specific activity of $3 \cdot 10^6$ counts/min/µmole P. The final volume was 2 ml and incubation temperature 20°. A, ATP × 1/20; B, phosphoprotein.

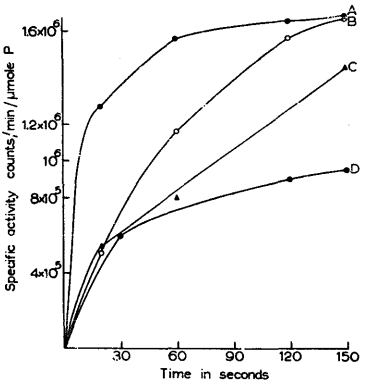

Fig. 2. Effect of various inhibitors on ^{32}P turnover in ATP. The experimental conditions were exactly the same as described in Fig. 1. A, control; B, dinitrophenol (10^{-4} M) or azide (2 mM); C, antimycin A (2 µg/ml); D, oligomycin (1.5 or 2.5 µmoles/g protein).

except as shown. Under these conditions, the mitochondrial ATP and phosphoprotein were labelled rapidly, but the isotope passed into no other fractions. Fig. 1 shows the time course of the incorporation into ATP and phosphoprotein. The former labels with great rapidity and reaches equilibrium within 2 min, while the phosphoproteins do not reach this point until some 5 min have elapsed. In Table I we show the effects of a number of inhibitors which affect either electron transport or oxidative phosphorylation. The uptake of ^{32}P into the phosphoproteins is greatly reduced by all these agents. It can be seen that the specific activity of ATP is, however, unaltered in the presence or absence of the inhibitors. This is because the experiments were carried on to a point where isotopic equilibrium had been reached in all the systems. The point is made clear in Fig. 2, where the time course of ^{32}P uptake into ATP is shown, together with the effects of dinitrophenol (10^{-4} M), azide ($2 \cdot 10^{-3}$ M), antimycin A (2 μg/ml) or oligomycin (1.5 or 2.5 μmoles/g protein). It is seen that all 3 inhibitors greatly reduce the rate of turnover of mitochondrial ATP, but that the levels of radioactivity approach isotopic equilibrium in their presence. The effect of oligomycin, however, is far greater than that of dinitrophenol or antimycin A. It is dealt with in a separate section below.

TABLE I

EFFECT OF VARIOUS INHIBITORS ON PHOSPHOPROTEIN AND ATP TURNOVER IN RAT-LIVER MITOCHONDRIA

Mitochondria equivalent to 1.8 g liver suspended in 0.125 M KCl, 0.02 M Tris (pH 7.4) were incubated in 6 ml for 30 min at 20°. The reaction was terminated by the addition of 6 ml 10% trichloroacetic acid. ATP and phosphoserine were assayed as described in METHODS. Specific activity of added P_i $60 \cdot 10^6$ counts/min/μmole. The figures in parentheses represent percent inhibition.

Experiment No.	*Additions*	*Specific activity (counts/min/μmole P) of*	
		ATP	*Phosphoserine*
1	Nil	$23 \cdot 10^6$	2045
	2,4-Dinitrophenol (10^{-4} M)	$22.6 \cdot 10^6$	420 (80%)
	Azide ($2 \cdot 10^{-3}$ M)	$29 \cdot 10^6$	106 (95%)
2	Nil	$22 \cdot 10^6$	1640
	KCN (10^{-3} M)	$19 \cdot 10^6$	1100 (33%)
	Antimycin A (2 μg/ml)	$18 \cdot 10^6$	196 (88%)
	Amytal (10^{-3} M)	$21 \cdot 10^6$	190 (90%)

Fig. 3 shows the effects of dinitrophenol (10^{-4} M), azide ($2 \cdot 10^{-3}$ M), antimycin A (2 μg/ml) or oligomycin (1.5 and 2.5 μmole/g protein) on the turnover of phosphoprotein. The most striking thing about these curves is that in the presence of DNP, azide or antimycin A the radioactivity of the phosphoproteins never reaches control levels. Consideration of oligomycin is postponed to a later section below.

The identity of the mitochondrial ATP in all the systems tested was established by chromatography on paper. In all the experiments reported here, there was no change in the level of mitochondrial ATP nor in the level of phosphoprotein. However, if incubation were carried on for long periods (longer than 30 min), falls in mitochondrial ATP and phosphoprotein may be observed, either in the presence or absence of inhibitors.

Effect of oligomycin

Three concentrations of oligomycin were used, 10 μg, 20 μg and 33 μg/g original liver. This represents a titre of 1.5 μmoles, 3 μmoles and 5 μmoles/g protein if we follow the notation previously used[12]. The lowest concentration is enough to inhibit oxidative phosphorylation by over 80 % in our system when β-hydroxybutyrate is the substrate. All 3 levels of oligomycin inhibit incorporation of isotope into mitochondrial ATP to the same extent, but the lowest level (1.5 μmoles/g protein) has no effect upon phosphoproteins (Fig. 3). At 2.5 μmoles/g protein oligomycin reduces the rate of phosphoprotein turnover markedly, but the counts increase steadily with time until

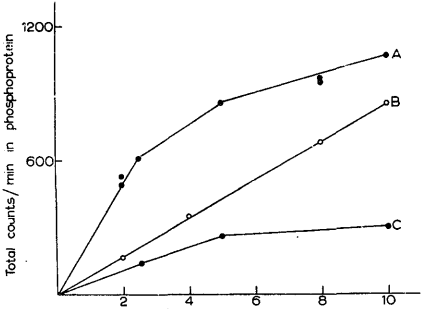

Fig. 3. Effect of inhibitors on ^{32}P turnover in phosphoproteins. The experimental conditions were same as described for Fig. 1. A, control and oligomycin (1.5 μmoles/g protein); B, oligomycin (2.5 μmoles/g protein); C, dinitrophenol (10^{-4} M) or azide (2.0 mM) or antimycin A (2μg/ml).

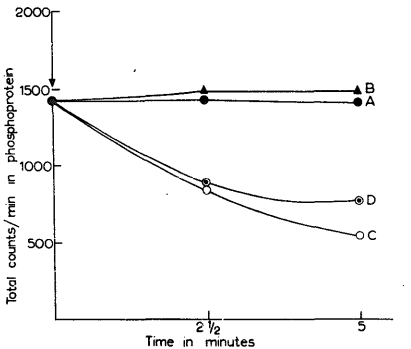

Fig. 4. Effect of dinitrophenol and oligomycin on the phosphoprotein radioactivity of mitochondria prelabelled with ^{32}P. Mitochondria were labelled with ^{32}P for 10 min according to conditions described in Fig. 1. Additions of dinitrophenol or oligomycin were made as shown at the end of 10 min. A, Control; B, oligomycin (5 μmoles/g protein); C, dinitrophenol (10^{-4} M); D, dinitrophenol plus oligomycin.

control levels have been reached by the end of 10 min incubation. This is in marked contrast to the other inhibitors tested, *e.g.*, DNP, azide. It is to be noted that the inhibition of ATP turnover is also in contrast to that by other inhibitors, for radioactivity does not reach control levels during the course of the experiment. It may be inferred from this experiment that the ^{32}P incorporated into phosphoprotein does not come from ATP.

Effect of DNP and oligomycin on mitochondria prelabelled with ^{32}P

To test the idea that mitochondrial ATP does not necessarily label the phosphoprotein, the following experiment was undertaken. As illustrated in Fig. 4, mitochondria were prelabelled with ^{32}P for 10 min, thus ensuring a plateau level of radioactivity in ATP and phosphoprotein. Addition of dinitrophenol, 10^{-4} M, at this stage causes the radioactivity of the prelabelled phosphoprotein to be discharged. The addition of oligomycin at a level of 5 μmoles/g protein failed to influence this loss, even when the concentration of dinitrophenol was reduced to $3.3 \cdot 10^{-5}$ M. Oligomycin itself at the concentration used had no effect upon the radioactivity of the prelabelled phosphoprotein. It may, therefore, be concluded that discharge of the phosphoprotein-P could not have been via ATP, but must have been by some more direct route.

Effect of thyroxine on mitochondrial phosphoprotein

Thyroxine at $5 \cdot 10^{-5}$ M inhibited the incorporation of ^{32}P into the phosphoproteins (Table II), but only in the absence of EDTA. In the presence of the latter,

TABLE II

EFFECT OF $5 \cdot 10^{-5}$ M THYROXINE ON ATP AND PHOSPHOPROTEIN

The experimental conditions were the same as described for Fig. 1 except that in Expt. No. 1, EDTA was also absent during the isolation of mitochondria. Incubation time 10 min at 20°.

Experiment No.	*mM EDTA*	*$5 \cdot 10^{-5}$ M Thyroxine*	*Total counts/min in phosphoprotein*	*ATP (counts/min/μmole P)*
1	—	—	992	$1.88 \cdot 10^6$
	—	+	500	$1.71 \cdot 10^6$
2	+	—	801	$2.67 \cdot 10^6$
	+	+	913	$2.84 \cdot 10^6$

TABLE III

EFFECT OF TWO ANTIHISTAMINE DRUGS ON PHOSPHOPROTEIN AND ATP TURNOVER

Mitochondria equivalent to 1.8 g liver suspended in 0.125 M KCl, 0.02 M Tris (pH 7.4) were incubated in 6 ml for 30 min at 20°. The reaction was terminated by the addition of 6 ml 10% trichloroacetic acid. ATP and phosphoserine were assayed as described in METHODS. Specific activity of added Pi $60 \cdot 10^6$ counts/min/μmole. The figures in parentheses represent percent inhibition.

Additions	*Specific activity (counts/min/μmole)/P of*	
	ATP	*Phosphoserine*
Nil	$23 \cdot 10^6$	1860
Promethazine·HCl ($2 \cdot 10^{-4}$ M)	$25 \cdot 10^6$	298 (84%)
Diphenylhydramine·HCl ($5 \cdot 10^{-4}$ M)	$24 \cdot 10^6$	200 (85%)

all effect of thyroxine was abolished. This is in marked contrast to the other inhibitors tested, all of which worked as well or better in the presence as in the absence of EDTA.

Thyroxine was also capable of bringing about a loss of radioactivity from the phosphoproteins of mitochondria prelabelled with ^{32}P. This discharge was not so great as that caused by DNP and was not further investigated.

Effect of two antihistamine drugs

The effects of diphenhydramine and promethazine on mitochondrial ATP and phosphoprotein turnover are shown in Table III. Both these drugs have been shown to inhibit mitochondrial swelling[1], neither has any significant effect on oxidative phosphorylation at the concentrations used. The two drugs show no inhibitory effect on mitochondrial ATP turnover but powerfully inhibit the labelling of phosphoprotein. In other work[7] it has also been demonstrated that these drugs inhibit sodium-activated increase in phosphoprotein turnover in whole cells.

TABLE IV

EFFECT OF Mg^{2+} ON REACTION OF ADDED [^{32}P]ATP WITH MITOCHONDRIAL PHOSPHOPROTEIN

Rat-liver mitochondria suspended in 0.125 M KCl–0.02 M Tris were added in a volume of 0.3 ml (equivalent to 600 mg original liver) to a medium containing 0.125 M KCl, 0.02 M Tris, 10^{-3} M Versene and 4 mM $MgSO_4$ where shown. The reaction was started by the addition of labelled [^{32}P]ATP, mixed with carrier ATP (total 1.3 μmoles), final volume of the reaction mixture being 1 ml. Temperature 20°. Reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. Counts/min in phosphoprotein represents total counts recovered in alkali-labile phosphate. Specific activity of ATP added was $3.2 \cdot 10^6$ counts/min/μmole P.

Time	$MgSO_4$ *(4 mM)*	*Counts/min in phospho-protein*
45 sec	−	74
	+	504
90 sec	−	129
	+	800
16 min	−	169
	+	1600

Labelling of mitochondria with [^{32}P]ATP

The experiments cited above suggest that the mitochondrial ATP plays little part in the labelling of the mitochondrial phosphoproteins. To investigate this point, mitochondria were incubated with [^{32}P]ATP for various times. The results are shown in Table IV. There is little transfer of ^{32}P to the proteins unless Mg^{2+} or Mn^{2+} are present. To show that this result was not due to failure of the [^{32}P]ATP to mix with the mitochondrial ATP, mitochondria were incubated with [^{32}P]ATP and then diluted with suspending medium and centrifuged out; they were resuspended and washed in medium twice more. The particles were then extracted with 5% trichloroacetic acid and the trichloroacetic acid extract analysed for ATP. It was found that the ATP had the same specific activity as calculated for complete mixing of the external and internal pools (Table V). DNP and Mg^{2+} had no effect on mixing of internal and external ATP. It may be concluded that the protein phosphokinase known

to be present in mitochondria is not active in these preparations unless Mg^{2+} is also added (Table IV).

Apart from the necessity for Mg^{2+}, a problem of accessibility is also involved. This is shown in Table VI. The preincubation of mitochondria in hypotonic solutions prior to addition of [^{32}P]ATP and Mg^{2+} causes a great increase in transfer of radioactivity to the proteins.

TABLE V

MIXING OF ADDED [^{32}P]ATP WITH MITOCHONDRIAL ATP

Mitochondria equivalent to 600 mg liver were incubated for 45 sec at 20° in the medium as described for Table IV. The calculated specific activity for complete mixing of the external and internal ATP was 695 000 counts/min/μmole P. The conditions of the experiment are described in the text.

$MgSO_4$ 4 mM	DNP 10^{-4} M	Specific activity of total ATP (counts/min/μmole P)
−	−	620 000
+	−	695 000
−	+	690 000
+	+	560 000

TABLE VI

EFFECT OF SWELLING ON LABELLING OF MITOCHONDRIAL PHOSPHOPROTEINS WITH [^{32}P]ATP

Mitochondria equivalent to 600 mg rat liver were used. The conditions and incubation medium for unswollen mitochondria were the same as described for Table IV. In the case of swollen mitochondria, Versene was omitted throughout and mitochondrial pellet was suspended in water. KCl was omitted from the incubation medium but 15 mM Tris was present. Final volume of the incubation media in both cases was 1 ml. 3 mM $MgSO_4$ was present in each case. The reaction was started by the addition of [^{32}P]ATP having a specific activity of $1.2 \cdot 10^6$ counts/min/μmole P. Incubation temperature 20°. The reaction was terminated by the addition of 1 ml 10% trichloroacetic acid. The specific activity or the levels of ATP did not change in case of unswollen mitochondria but in the case of swollen mitochondria 50–60% ATP was hydrolysed at the end of 8 min incubation without change in its specific activity.

Time (min)	Total phosphoproteins (counts/min)	
	Unswollen mitochondria	Swollen mitochondria
1	462	1300
4	810	1950
8	920	2400

Experiments with "submitochondrial" particles

These were of two types, one using mitochondria treated with digitonin[11], and in the other, using mitochondria disrupted by sonic oscillation. In neither case did we observe any labelling by the use of ^{32}P even when conditions for oxidative phosphorylation were employed. This was investigated in detail in preparations obtained by sonic oscillation. From these, two types of particles were obtained by differential centrifugation, both capable of ^{32}P–ATP exchange, but in neither of these was any trace of activity ever seen in the phosphoprotein fraction.

DISCUSSION

The present investigation supports the view that phosphoproteins of mitochondria may accept phosphate from an intermediate of oxidative phosphorylation. The evidence for this is as follows: (a) Inhibitors of electron transport and oxidative phosphorylation depress the uptake of ^{32}P into the proteins. In the presence of dinitrophenol, azide, antimycin A and amytal the radioactivity of the phosphoproteins reaches and maintains a level of about 25 % of that of the control. The radioactivity of ATP, however, reaches the same point as the controls, though at a slower rate. (b) Oligomycin which is known to interact between formed intermediates of oxidative phosphorylation and ATP does not greatly reduce the uptake of ^{32}P into phosphoprotein, even at concentrations at which ATP synthesis in the usual oxidative phosphorylation system is reduced by 80 %. At high concentration, oligomycin does diminish the rate of turnover of the phosphoprotein, but in contrast to the other inhibitors, the labelling of the proteins increases steadily with time until it is the same in the presence as in the absence of the oligomycin. (c) Dinitrophenol causes a rapid discharge of radioactivity from the mitochondrial phosphoproteins, which is unaffected by high concentrations of oligomycin. This suggests that the discharge is not mediated by ATP, *e.g.*, by reversal of protein phosphokinase in combination with an ATPase. (d) Evidence is presented which supports the view that mitochondrial ATP is ineffective in labelling the mitochondrial phosphoproteins in the systems used by us

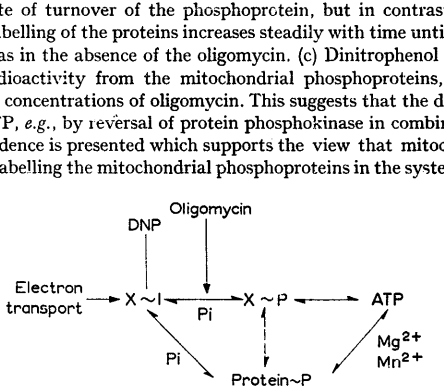

Fig. 5. Proposed mechanism for the interaction of phosphoprotein, inorganic phosphate and ATP in mitochondria.

The strongest argument that can be brought against the proposal of direct reaction between protein and an unknown intermediate is that the protein is in fact labelled by a small pool of ATP which is inaccessible to the inhibitors but not to the ^{32}P which is added. However, this hypothetical pool would also have to be inaccessible to added [^{32}P]ATP. While such arguments can be brought, there is no evidence to support them. On the contrary, our experiments show that added [^{32}P]-ATP mixes freely with the mitochondrial nucleotides. We, therefore, propose the mechanism in Fig. 5 for the interaction of phosphoprotein, inorganic phosphate, and ATP.

In this scheme, we suggest that phosphorylation of protein may be brought about by interaction first with a primary intermediate of the oxidative phosphorylation system. This results in an "energizing" of the protein, and is followed by phosphorolysis. The resulting phosphoprotein may then come into equilibrium with other intermediates of the reaction.

This scheme explains the lack of inhibition by oligomycin. It also explains the the effect of dinitrophenol in discharging the radioactivity of the phosphoprotein and the failure of oligomycin to block this. Alternately, the hypothesis that the primary intermediate is not phosphorylated[12, 13] is untenable.

The points of action of the inhibitors of electron transport and oxidative phosphorylation need not be discussed further here.

The drugs promethazine and diphenhydramine which inhibit mitochondrial swelling are somewhat different. It has been shown that promethazine has some effects on the respiratory chain[14, 15], but diphenhydramine does not. It is thought probable that these compounds inhibit the energy transfer to the proteins. They have no effect upon the phosphoprotein kinase of the mitochondria[10].

We do not believe that phosphoproteins are intermediate in the actual oxidative phosphorylation reaction for two reasons. First of all, compounds such as the antihistamine drug diphenhydramine do not inhibit oxidative phosphorylation, but do inhibit phosphoprotein turnover. Secondly, the rate at which the phosphoproteins reach isotopic equilibrium is many times slower than that at which the mitochondrial ATP reaches isotopic equilibrium.

In spite of this, the phosphoproteins may be intermediates in another type of synthesis of ATP. It has been shown[16] that purified alkaline phosphatase may react with P_i to give a phosphoprotein, hydrolysis of which yields phosphoryl serine. If such a reaction occurs in the case of the mitochondrial phosphoproteins, it could yield ATP by reversed action of the protein phosphokinase[17].

Finally, previous work[1,2] has suggested a relationship between phosphoprotein and mitochondrial water shifts. A role in ion transport in whole cells has also been ascribed to phosphoproteins[7, 18]. We thus have the attractive idea of a single group of compounds mediating water and ion transport in whole cells and their separate parts, a suggestion which is supported by the inhibiting effect of the drugs promethazine and diphenhydramine upon mitochondrial water movements and upon ion transport in whole cells[19,20].

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