ACTION OF DRUGS ON MITOCHONDRIAL PHOSPHOPROTEIN METABOLISM

K. AHMED and J. D. JUDAH

The Wistar Institute of Anatomy and Biology, Philadelphia 4, Pennsylvania

PREVIOUS studies[1,2,3] have shown that mitochondrial swelling and contraction are closely associated with phosphoprotein turnover, and that the inhibition of one process results in the inhibition of the other. Antihistamine drugs, Benadryl and Phenergan, which protect cells from various types of injury [1,2,4], inhibit water movements *in vitro* both in whole cells and mitochondria. These drugs also prevent phosphoprotein phosphorylation. The present communication deals with the effect of antihistamine drugs and the inhibitors of electron transport and oxidative phosphorylation on ^{32}P turnover in rat liver mitochondria, under conditions where no significant changes in the levels of various mitochondrial fractions occurred. The results confirm the locus of action of the antihistamine drugs and suggest a mechanism for the action of the antihistamine drugs and suggest a mechanism for the action of compounds such as antimycin A, amytal and cyanide on mitochondrial swelling. The mechanism of mitochondrial phosphoprotein turnover is also discussed.

METHODS

Rat liver mitochondria were prepared by the method of Schneider[5]. 0·25 M sucrose containing 10^{-3} M ethylenediaminetetra-acetic acid (EDTA) pH 7·2 was used to homogenize liver and for subsequent washings of the mitochondrial pellet. Mitochondria were finally suspended in 0·25 M sucrose — 0·02 M Tris buffer (pH 7·2) or in 0·125 M KCl — 0·02 M Tris buffer (pH 7·2) so that 1 g original liver was equivalent to 1 ml. of mitochondrial suspension. Incubation was carried out at room temperature in a medium of 0·125 M KCl — 0·02 M Tris or 0·25 M sucrose — 0·02 M Tris, pH 7·2. ^{32}P was added as sodium phosphate to give a final concentration of 10^{-4} M P_i. 10^{-3} M EDTA was added to the medium to prevent swelling of the mitochondria. Incubations were carried out in air at room temperature (20 °C). Phosphoprotein was estimated by alkaline hydrolysis or by acid hydrolysis and isolation of P-serine by the method of Heald[6]. ATP was determined by adsorption on charcoal as described by Crane and Lipmann[7].

^{32}P TURNOVER IN RAT LIVER MITOCHONDRIA

Mitochondra suspended in 0·25 M sucrose — 0·02 M Tris or 0·125 M KCl — 0·02 M Tris, pH 7·2, containing 10^{-3} M EDTA and ^{32}P rapidly take up the tracer into ATP and phosphoprotein (Fig. 1). Under these conditions no other acid insoluble fractions (e.g. phospholipids) are labeled. A higher turnover of the phosphoprotein fraction is observed

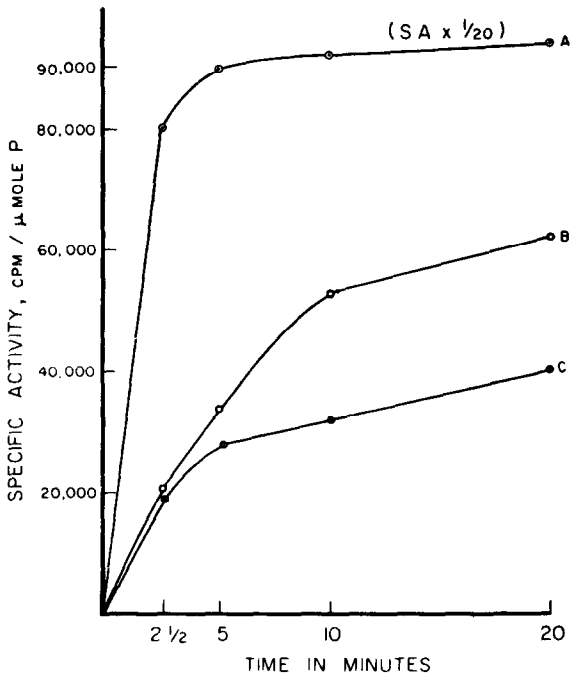

FIG. 1. Radioactivity of mitochondrial ATP and phosphoprotein. Curve A, ATP (specific activity/20); curve C, phosphoprotein, both derived from mitochondria incubated in the presence of ethylenediamine tetra-acetic acid, 10^{-3}M in medium. Curve B, phosphoprotein derived from mitochondria incubated in the absence of EDTA.

when mitochondria are prepared in versene-free 0·25 M sucrose and the final incubation medium is also versene-free. There is no significant change in the level of various fractions during the incubation. Within 2·5 min the specific activity of the phosphoproteins has almost reached its maximum figure, but continues to increase at a slower rate thereafter; that of ATP is at its maximum within 5 min and does not increase thereafter. It is to be noted that the inclusion of versene, which prevents mitochondrial swelling depresses the phosphoprotein turnover.

The effect of inhibitors of oxidative phosphorylation (e.g. 2,4 DNP, azide, amytal) and of antihistamine drugs (Phenergan and Benadryl which have no effect on oxidative phosphorylation) is shown in Table I.

TABLE I

Effect of various inhibitors on phosphoprotein turnover in rat liver mitochondria

Exp. No.	System	Specific activity, cpm/μmole P of	
		ATP	Phosphoprotein
1	Control	1,100,000	175,000
	2:4,Dinitrophenol (10^{-4}M)	1,080,000	41,500 (76%)
	Azide (10^{-3}M)	1,250,000	44,000 (75%)
	Amytal (10^{-3}M)	1,030,000	71,000 (60%)
2	Control	605,000	29,000
	Phenergan (2×10^{-4}M)	671,000	14,000 (52%)
	Benadryl (5×10^{-4}M)	630,000	16,500 (43%)

^{32}P was added as a solution giving 10^{-4}M P_i final concentration with $1\text{-}3\times10^6$ cpm.

TABLE II

Effect of various inhibitors on mitochondrial phosphoprotein turnover

System	Total cpm in the alkali-labile phosphoprotein P	Inhibition %
Control	2420	—
2:4,Dinitrophenol (10^{-4}M)	171	93
Amytal (10^{-3}M)	287	88
Antimycin A (1·5 μg/ml)	159	94
Phenergan (2×10^{-4}M)	785	68
Benadryl (5×10^{-4}M)	1200	51

^{32}P was added as a solution giving 10^{-3}M P_i final concentration with 9×10^6 cpm.

It is of interest to note that there is no effect on the turnover of mitochondrial ATP, whereas the phosphoprotein turnover is strongly inhibited. These effects are observed at low phosphate concentration in the medium (10^{-4} M P_i) as well as at higher P_i concentration (10^{-3} M) (Table II). The insensitivity of intramitochondrial ATP turnover towards the inhibitors discussed above is striking and is observed even when mitochondria are preincubated with DNP before adding ^{32}P. The identity of ATP has also been confirmed by chromatographing the trichloracetic acid extracts of mitochondria. However, when ATP is added to the medium, the exchange of ^{32}P into the added ATP is strongly inhibited as expected. Benadryl has relatively little effect on this system.

EFFECT OF OXIDATION-REDUCTION STATE OF RESPIRATORY CARRIERS ON PHOSPHOPROTEIN TURNOVER IN MITOCHONDRIA

The inhibition of phosphoprotein turnover by compounds well known as inhibitors of oxidative phosphorylation or electron transport suggests that phosphoproteins play a significant role in mitochondrial metabolism. Wadkins and Lehninger[8] have suggested that the oxidized form of respiratory carriers is the state richest in energy, and have shown that reduction of the respiratory chain leads to an inhibition of ATP-$^{32}P_i$ exchange reaction. It was of interest to investigate the effect of reduction of respiratory carriers on phosphoprotein turnover. In confirmation of the observations of Wadkins and Lehninger[8], we found

TABLE III

Effect of oxidation reduction state of respiratory carriers on phosphoprotein radioactivity

System	cpm in phosphoprotein	Specific activity, cpm/μmole P of mitochondrial ATP
Oxidized	2300	$8{\cdot}1 \times 10^6$
Reduced	1400	$8{\cdot}6 \times 10^6$

Reduction of the mitochondrial system was effected by addition of Glutamate, 5×10^{-3}M and KCN, 2×10^{-3}M.

that the exchange reaction is inhibited when reduction of the carriers is caused, but only when ATP is present in the medium; i.e. only the "extramitochondrial" ATP-^{32}P exchange. The labeling of intramitochondrial ATP is insensitive to changes in oxidation-reduction states of the respiratory carriers, but phosphoprotein turnover is inhibited when reduction of the respiratory carriers is caused (Table III); the inhibition is of the same order as the inhibition of ATP-^{32}P exchange.

Identification of Phosphoprotein Fraction

Phosphoproteins in the previous experiments were assayed as the alkali-labile phosphorus. Kennedy and Smith[9] and Heald[6] have shown that acid hydrolysis of crude phosphoproteins from ascites cells and brain slices yields phosphoserine of high specific activity and could be used as a sensitive method for the identification of the phosphoprotein fraction. We have observed that phosphoproteins of liver mitochondria also yield phosphoserine on acid hydrolysis, the specific activity of the amino acid being much higher than the specific activity of alkali-labile phosphate. The results indicate (Table IV) that there is no doubt of the nature of the fraction inhibited by the compounds described. The degree of inhibition of the tracer in the P-serine derived from phosphoprotein is even greater than that previously observed.

TABLE IV

Identification of the phosphoprotein fraction affected by various inhibitors

Exp. No.	Additions	Specific activity. cpm/μmole P of	
		ATP	Phosphoserine
1	Nil	—	1860
	2:4,Dinitrophenol (10^{-4}M)	—	420 (80%)
	Benadryl (5×10^{-4}M)	—	200 (85%)
2	Nil	—	2045
	Azide (2×10^{-3}M)	—	106 (95%)
	Phenergan (2×10^{-4}M)	—	298 (85%)
3	Nil	22×10^{6}	1640
	KCN (10^{-3}M)	19×10^{6}	1100 (33%)
	Antimycin A (2 μg/ml)	18×10^{6}	196 (88%)

^{32}P was added as a solution giving 10^{-4}M P_i final concentration and 56×10^{6} cpm. Figures in parentheses refer to percent inhibition.

DISCUSSION

The insensitivity of the intramitochondrial ATP-P_i exchange is puzzling. Chiga and Plaut[10] have described systems prepared from mitochondria which carry out ATP-P_i exchange insensitive to DNP. It appears that the turnover of mitochondrial ATP is not connected with the mechanisms of respiratory chain phosphorylation, especially in view of the fact that its turnover is not affected by oxidation reduction state of the respiratory carriers. Some of the inhibitors studied may be inhibiting phosphoprotein turnover by interfering between ATP and Protein-P. But the evidence presented makes such a conclusion unlikely, as it will be difficult to understand the inhibitory effect of compounds such as antimycin A. The dependence of Protein~P turnover on the oxidation-reduction state of respiratory carriers suggests that Protein~P must be directly reacting with a phosphorylated intermediate of the electron transport system. These possibilities are represented in Fig. 2, where X~P is an intermediate of the oxidative phosphorylation system. Two loci of action of various inhibitors may be considered. Amytal, antimycin A, etc. may inhibit phosphoprotein turnover by preventing a reaction between electron transport system and X~P, and also between X~P and Protein~P. In the case of antihistamine drugs which have no effect on oxidative phosphorylation and little on ATP-P_i exchange (e.g. Benadryl), the inhibition of reaction between X~P and

Protein~P appears most likely. Phenergan, however, may also react in other ways, being an inhibitor of flavoproteins[11]. It is suggested that during mitochondrial swelling, the turnover between Protein~P and X~P is greatly increased and inhibitors of swelling act by way of depressing this turnover. The present work explains the mechanism by which the inhibitors of electron transport (Antimycin A, cyanide) and of oxidative phosphorylation (Azide, DNP) inhibit mitochondrial swelling. This is to be based upon the ability of these compounds to prevent the turnover of Protein~P.

Our observation that mitochondrial phosphoprotein may accept~P from an intermediate of oxidative phosphorylation may be of greater consequence. It is to be speculated from the scheme, suggested above, that phosphoproteins may act as intermediates in oxidative phosphorylation reaction. That phosphoproteins appear to have somewhat low specific activity does not necessarily eliminate this possibility, because of the inhomogeneity of the fraction concerned. Rabinowitz and Lipmann's observations[12] on this subject are of great interest. They have shown that different P groups of phosphoprotein may turn over at different rates, that phosphoprotein phosphokinase reaction is reversible and that phosphorylation of ADP with Protein~^{32}P can result in ATP of specific activity many times higher than the phosphoprotein. It should be borne in mind, therefore, that an overall measurement of the specific activity of phosphoprotein P groups may be misleading. The other possibility is that phosphoproteins may not lie on the direct

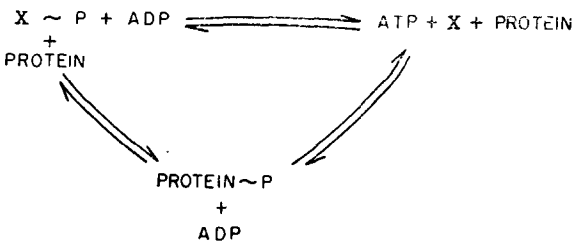

FIG. 2. Scheme for participation of mitochondrial phosphoprotein in oxidative phosphorylation.

pathway of oxidative phosphorylation but may form part of a cyclical mechanism as shown in Fig. 2. At the present time, it is thought likely that the phosphoprotein pathway is not of great importance in the oxidative phosphorylation of normal mitochondria. This conclusion is based partly on the fact that benadryl greatly inhibits ^{32}P uptake into phosphoprotein, while having little effect on oxidative phosphorylation.

Experiments with submitochondrial fragments, obtained by digitonin treatment of mitachondria, seem to confirm this view, since we find little or no labeling of phosphoproteins in such preparations under

a variety of conditions. Further work on these and other systems will perhaps clarify the matter.

Another possibility is that the system $ATP \rightleftharpoons P{\sim}Protein \rightleftharpoons X \sim P \downarrow P_i$ may act as an ATP-ase. In support of this view, we find that certain drugs inhibit ATP hydrolysis during mitochondrial contraction[3] while having no effect on DNP stimulated ATP-ase.

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