DETECTION OF A PHOSPHORYLATED INTERMEDIATE IN MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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SUMMARY

A greater incorporation of ^{32}P-labeled inorganic phosphate is observed when aurovertin is used to block the phosphorylation sequence of rat-liver mitochondria than when oligomycin is used. The ^{32}P-labeled intermediate is rapidly discharged by 2,4-dinitrophenol and is rapidly diluted on addition of unlabeled P_i. In the presence of succinate plus rotenone, the increased incorporation amounts to approximately 0.05 nmol per mg protein. The results warrant the tentative conclusion that a phosphorylated intermediate of oxidative phosphorylation has been detected.

INTRODUCTION

Despite many intensive efforts over the past thirty years to identify intermediates of mitochondrial oxidative phosphorylation, the molecular mechanism has remained largely unresolved. Evidence has accumulated, however, to support the idea that a nonphosphorylated and a phosphorylated intermediate participate in the reaction sequence. The existence of a nonphosphorylated intermediate, X ~ I, common to all coupling sites, is suggested by the following observations: Energy derived at one or more coupling sites of the respiratory chain from the oxidation of substrate can be used (a) to reverse electron flow through another coupling site (1), (b) to drive the energy-linked pyridine nucleotide transhydrogenase reaction (2,3), or (c) to provide energy for the translocation of ions (4). Each of these processes is insensitive to oligomycin when energy is supplied by substrate oxidation and is not dependent on the presence of inorganic phosphate.

The existence of a phosphorylated intermediate, X ~ P, is suggested by the following observations: (a) The terminal bridge-oxygen of ATP formed during oxidative

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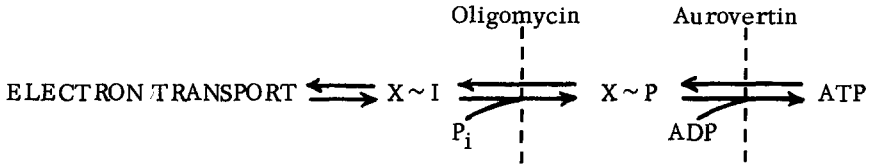
phosphorylation is provided by ADP rather than by P_i which indicates that P_i enters the phosphorylation sequence prior to or at the same time as ADP (5). (b) Studies of the inhibition of submitochondrial particles by aurovertin and oligomycin (6-9) and of the binding of these reagents to coupling factors (10-12) suggest that the inhibitors block different steps in the phosphorylation sequence with oligomycin inhibiting before aurovertin and prior to incorporation of P_i. (c) Oligomycin appears to inhibit before and aurovertin after the formation of an arsenylated intermediate, as evidenced by the full inhibition of arsenate-stimulated respiration by oligomycin but only partial inhibition by aurovertin at concentrations just sufficient to completely inhibit oxidative phosphorylation (13). This last observation was interpreted as strong support for the following proposed reaction sequence (8, 13):



The present study was undertaken to determine whether or not aurovertin and oligomycin, used to block selected steps in the phosphorylation sequence, might aid in the isolation of a phosphorylated intermediate. Inhibition by aurovertin may effect a higher steady-state concentration of X~P than that attainable during controlled respiration. Also, inhibition by oligomycin prior to addition of $^{32}P_i$ offers a convenient control with which to compare the incorporation of $^{32}P_i$ by aurovertin-blocked mitochondria. Finally, these inhibitors may prevent enzymatic discharge of X~P during the quenching process.

MATERIALS AND METHODS

Rat-liver mitochondria were prepared as described previously (13). Respiratory control ratios of mitochondria were normally above 5 with succinate as substrate in the presence of rotenone. Protein was determined by the fat-free dry weight method of Slater as described by King (14).

A mitochondrial suspension was prepared consisting of 32 ml of medium (0.25 M Sucrose, 20 mM Tris pH 7.4, 2 mM $MgCl_2$, and 10 mM KCl), 5 ml mitochondria (mito-

chondria from 30 grams liver diluted to 5 ml with 0.25 M Sucrose, 1 mM Tris pH 7.4), and 0.2 ml of 3 x 10^{-4} M rotenone. Aliquots of 2.0 ml each were transferred to 30 ml Corex centrifuge tubes containing a small teflon stirring bar and stored on ice. The tubes were brought to room temperature by immersion, with stirring, in a temperature bath for 150 seconds. Additions were then made as described in the tables. Five seconds before stopping the reaction the tubes were removed from magnetic stirring plates and placed on vortex mixers. While vortexing, 15 ml of cold 0.4 M perchloric acid (PCA) was added and mixed for 10 sec. The tubes were then rapidly cooled to 0°C by immersion for 5 seconds in a Dry Ice - acetone bath.

The following washing procedure was carried out at 1 to 4°C. Protein-lipid precipitates were collected by centrifugation at 16,000 g for 10 minutes. The supernatant was carefully decanted and the precipitate dispersed with a glass stirring rod in 20 ml of 0.4 M PCA. The precipitate was collected as above and the washing cycle was repeated two more times.

The washed precipitates were dissolved in 1.5 ml Soluene 100 (Packard) and transferred quantitatively to nylon counting vials using 15 ml of a toluene-based scintillation solution. An average standard deviation of 5% was obtained for replicate determinations in a number of experiments. Error estimates given in the tables represent a 95% confidence level (i.e., $\pm 2\sigma_{mean} = 2\sigma / \sqrt{\text{number of samples}}$).

Endogenous inorganic phosphate was estimated by isotopic dilution to be approximately 100 nmol per mg protein.

Aurovertin was the generous gift of Professor Henry A. Lardy. Carrier-free $H_3{}^{32}PO_4$ was obtained from New England Nuclear and purified as described by Boyer and Bieber (15). Aurovertin, rotenone and oligomycin were used in ethanolic solutions.

RESULTS

Table I shows the effect of aurovertin and oligomycin on the incorporation of $^{32}P_i$ by mitochondrial protein-lipid. At zero seconds the sample is inhibited by aurovertin and the control is inhibited by oligomycin. Substrate and phosphate are added as indicated. At 100 seconds, in order to minimize differences in background labeling, oligomycin is added to the sample and aurovertin to the control. The reaction is

TABLE I

Effect of Aurovertin and Oligomycin on ^{32}P Incorporation into Protein-Lipid of Rat Liver Mitochondria

		Sample	Control
Time of Additions (sec)	0	Aurovertin	Oligomycin
	20	Succinate	Succinate
	40	Carrier P_i	Carrier P_i
	80	$^{32}P_i$ (carrier-free)	$^{32}P_i$ (carrier-free)
	100	Oligomycin	Aurovertin
	120	$HClO_4$	$HClO_4$
^{32}P Incorporated	CPM[a]	2630 ± 130	1700 ± 85
	% ^{32}P per mg protein	0.00743 ± .00037	0.00481 ± .00024
	Δ % ^{32}P per mg protein	0.0026 ± .0006	
	nmol X~P per mg protein	0.05 ± .01	

Tubes containing mitochondria equivalent to 17 mg protein in 2.0 ml were brought to room temperature as described under methods. Additions were made in small volumes; final concentrations were: 1.2 μg aurovertin or oligomycin per mg protein, 7.5 mM succinate, and 0.1 mM carrier P_i. At 80 seconds 2.08×10^6 cpm of $^{32}P_i$ (carrier-free) was added. At 100 seconds 15 ml of ice-cold 0.4 M PCA was added. Endogenous P_i was estimated to be 0.8 mM giving a final concentration of P_i of 0.9 mM. (In similar experiments, inhibitor concentrations were varied between 0.5 and 1.2 μg per mg protein. The results were similar to data reported in this table).

[a]Each value represents the average of four identical experiments.

TABLE II

Rapid Discharge of the Intermediate by an Uncoupler and Rapid Dilution of Label by Addition of Carrier P_i.

Time of Additions (Sec)	Samples A,B,C	Controls A,B,C
0	Aurovertin	Oligomycin
20	Succinate	Succinate
40	Carrier P_i	Carrier P_i
80	$^{32}P_i$ (carrier-free)	$^{32}P_i$ (carrier-free)

^{32}P Incorporated	Sample A	Control A	Sample B	Control B	Sample C	Control C
130	—	—	P_i	P_i	DNP	DNP
150	$HClO_4$	$HClO_4$	$HClO_4$	$HClO_4$	$HClO_4$	$HClO_4$
CPM[a]	3570 ±250	2340 ±170	2400 ±170	2060 ±150	2030 ±140	2360 ±170
% ^{32}P per mg protein	0.0119 ±.0008	0.0078 ±.0006	0.0080 ±.0006	0.0069 ±.0005	0.0068 ±.0005	0.0079 ±.0006
Δ% ^{32}P per mg protein (Sample - Control)	0.0041 ±.0014		0.0012 ±.0010		-0.0011 ±.0010	
nmol X ~ P per mg protein	0.053 ±.018		0.015 ±.013		-0.014 ±.013	

Tubes containing mitochondria equivalent to 12.2 mg protein in 2.0 ml were brought to 21° C as described under methods. Additions were made in small volumes; final concentrations were: 0.82 μg aurovertin or 1.64 μg oligomycin per mg protein, 7.5 mM succinate, and 0.1 mM carrier P_i. At 80 seconds 2.46×10^6 cpm of $^{32}P_i$ (carrier-free) was added. Endogenous P_i was estimated to be 0.55 mM giving a final $[P_i] = 0.65$ mM. In Experiments B unlabeled P_i was added at 130 seconds to give a five-fold dilution of the specific activity of $^{32}P_i$. In Experiments C dinitrophenol was added to a final concentration of 0.25 mM at 130 seconds.

[a]Each value represents the average of two identical experiments.

stopped at 120 seconds by acid precipitation. A greater incorporation of $^{32}P_i$ by the sample is observed.

All samples and all controls in Table II are treated similarly up to and including addition of $^{32}P_i$ at 80 seconds. In experiment B a five-fold dilution of the specific activity of $^{32}P_i$ is obtained on addition of P_i at 130 seconds. By comparison of Samples A and B it is observed that addition of unlabeled P_i results in a rapid loss of ^{32}P from the intermediate. By comparison of Samples A and C it is found that addition of dinitrophenol at 130 seconds results in the rapid discharge of the phosphorylated intermediate Dinitrophenol causes no detectable change in the amount of $^{32}P_i$ incorporated by Control C as compared with Control A.

DISCUSSION

The 0.05 nmol X~P per mg protein detected probably represents the minimum amount of intermediate present, because the control may contain some X~P due to labeling by a small amount of endogenous ATP and the isotopic dilution method used probably underestimated the amount of endogenous P_i. On this basis, the amount of X~P detected represents at least 18% of the estimated number of coupling sites, assuming two sites per cytochrome oxidase in the presence of succinate and rotenone (16). The amount of the intermediate detected is thus consistent with its participation in oxidative phosphorylation.

The rapid discharge of the intermediate by dinitrophenol and the rapid exchange of the phosphoryl group with the unlabeled P_i are also consistent with its being on the main phosphorylation path rather than resulting from a leak through or around the aurovertin block.

Finally, in view of the fact that the sample and control in Table I both contain the same amount of each inhibitor, only the order of addition with respect to $^{32}P_i$ being different, and the evidence supporting the proposed sites of action of oligomycin and aurovertin, it seems reasonable to expect that any difference in the amount of $^{32}P_i$ incorporated would reflect the presence of X~P.

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