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Thiophosphate Labelling of Mitochondria – Lack of Evidence for an Acyl-Phosphate Intermediate in Oxidative Phosphorylation

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Abstract

In the presence of substrate and oxygen, aurovertin-inhibited rat-liver mitochondria incorporate 0.27 ± 0.02 nanomoles of [^{35}S] thiophosphate per mg protein into an acid-precipitable fraction. This incorporation is not prevented by uncouplers of oxidative phosphorylation. Furthermore, there is no significant difference in thiophosphate incorporation by aurovertin- or oligomycin-inhibited mitochondria.

Since acyl-phosphate intermediates in other energy-transducing membrane systems are stable to acid precipitation and since acyl thiophosphates are anticipated to be more stable than acyl phosphates, these results support previous indications that acyl phosphates do not participate as intermediates in oxidative phosphorylation.

Introduction

In 1953, Slater [1] proposed a mechanism for energy coupling in oxidative phosphorylation based upon the known mechanism of ATP formation accompanying the oxidation of glyceraldehyde-3-phosphate [2]. An essential feature of this chemical coupling hypothesis is the formation of an energy-rich, covalent intermediate coupled to oxidation-reduction reactions of the respiratory chain. Shortly after publication of Slater's proposal, Lehninger [3] suggested that the hypothetical, covalent intermediate might undergo phosphorolysis to give an energy-rich phosphorylated intermediate that, in a subsequent reaction, would transfer its phosphoryl group to ADP.

Since the time it was first proposed, this theory has served as one of the most widely accepted working hypotheses in the field of oxidative phosphorylation. However, direct evidence for the existence of the proposed intermediates has not been obtained despite major efforts on the part of numerous laboratories. One such approach has been to attempt to label the hypothetical phosphorylated intermediate using ^{32}P-labeled inorganic phosphate. In 1966, Bieber and Boyer [4] showed that the phosphohistidine, phosphoserine, and phosphorylated lipid fractions that are labelled in mitochondria from P_i all fail to meet reasonable criteria for participation as intermediates. In 1973, using procedures which had been successful in demonstrating acyl-phosphate intermediates in microsomal $(Na^+ + K^+)$–ATPase [5, 6] and the Ca^{2+}-dependent ATPase of sacroplasmic reticulum vesicles [7, 8], Cross and Boyer [9] were unable to detect any acyl-phosphate compounds in mitochondria under conditions designed to favour the accumulation of energy-rich intermediates of oxidative phosphorylation.

Obviously, failure to detect a phosphorylated intermediate in oxidative phosphorylation does not prove that one does not exist. Under the reaction conditions used, it is possible that such an intermediate might be sufficiently labile to escape isolation. With this point in mind, we have attempted in the present work to detect energy-linked formation of acyl-phosphate-like compounds using [^{35}S] thiophosphate. In model enzyme [10] and mitochondrial studies Tavares de Sousa, Packer, and Schonbaum [11] found that thiophosphoryl transfer is much slower than the rate of phosphoryl transfer, e.g., causing synthesis of ATPγ(S) instead of ATP in mitochondria. This is consistent with the prediction that replacement of one of the three oxygen atoms of a phosphoryl group by a less electronegative sulfur atom will result in a phosphorus atom less susceptible to nucleophilic attack. The rationale behind the present work is that any thiophosphorylated analogue of the hypothetical phosphorylated intermediate in oxidative phosphorylation might be expected to be more stable and therefore more easily detected.

Materials and Methods

Preparation of [^{35}S] *thiophosphate*

[^{35}S] PO_3Na_3 was prepared essentially as described by Åkerfeldt [10]. 0.26 mmoles of [^{35}S] PCl_3, 19.8 mCi/mmoles, (Amersham-Searle, Illinois, U.S.A.) were diluted tenfold with carrier and refluxed with a solution of NaOH. The products were collected by crystallization at 4°C and desiccated. The molarity of the working solution was determined by ferricyanide oxidation at pH 7.0.

Preparation and Assay of Mitochondria

Mitochondria were prepared from rat-liver essentially as described by Johnson and Lardy [12]. Mitochondria gave respiratory-control ratios of 5 or above when assayed polarographically using a Clark-type electrode at 25°C. Protein was determined by the Lowry method using bovine serum albumin as a standard. Labelling experiments were carried out as described in the caption to Table I.

Results and Discussion

An important implication of the work of Lee and Ernster [13] and of Robertson *et al* [14] is that oligomycin inhibits the phosphorylation sequence of oxidative phosphorylation at least one step before aurovertin. In view of the evidence favoring oligomycin inhibition at a point prior to entry of P_i, these investigators have suggested that a phosphorylated intermediate might exist between the sites of action of oligomycin and aurovertin. Evidence supporting this view was obtained by Cross and Wang [15], who found that at concentrations which completely inhibit oxidative phosphorylation, oligomycin completely inhibits arsenate-stimulated respiration, while aurovertin only partially inhibits this activity. These results were interpreted as indicating the formation of an unstable arsenylated compound in the presence of aurovertin, but not in the presence of oligomycin.

In the present studies, we have measured the incorporation of [^{35}S] thiophosphate by aurovertin-inhibited mitochondria in order to determine whether or not a stable thiophosphorylated analogue of the hypothetical phosphorylated intermediate can be detected. In samples A and B of Table I, aurovertin-inhibited mitochondria were incubated for one minute with [^{35}S] thiophosphate in the presence of substrate and oxygen. Reactions were stopped by addition of perchloric acid containing unlabelled thiophosphate and the precipitates were collected by centrifugation. After four wash cycles, the precipitates were assayed for ^{35}S. In order to determine the amount of ^{35}S incorporated by non-energy-linked processes, either an uncoupler of oxidative phosphorylation was added at the same time as [^{35}S] thiophosphate (Control A) or oligomycin was used instead of aurovertin (Control B). As can be seen in Table I, similar amounts of ^{35}S were incorporated by samples and controls. Thus, no acid-precipitable, energy-linked thiophosphate incorporation is observed in aurovertin-inhibited mitochondria.

Our results were in accord with recent studies of Cross and Boyer [9] in which $^{32}P_i$ was used as a probe for energy-rich ATP precursors, stable

TABLE I. Incorporation of [^{35}S] thiophosphate into acid-precipitated material from mitochondrial suspensions

Time (Seconds)	Sample A	Control A	Sample B	Control B
-90	Aurovertin	Aurovertin	Aurovertin	Oligomycin
0	Succinate	Succinate	Succinate	Succinate
60	[^{35}S] P_i + H_2O	[^{35}S] P_i + DNP	[^{35}S] P_i	[^{35}S] P_i
120	$HClO_4$ + SP_i	$HClO_4$ + SP_i	$HClO_4$ + SP_i	$HClO_4$ + SP_i
nmoles/ mg protein Precipitate	0.261	0.259	0.295	0.286
nmoles/ mg protein (Sample-control)	0.00 ± 0.01		0.01 ± 0.01	

Aliquots of 1 ml each of 0.25M sucrose, 20 mM tris, 10 mM KCl, 2 mM $MgCl_2$ (pH 7.4) containing rotenone-inhibited, rat-liver mitochondria equivalent to 6.5 mg of protein were brought to 21°. Additions were made in small volumes; final concentrations were: 0.77 μg aurovertin or oligomycin per mg protein, 7.5 mM succinate and 0.8 mM [^{35}S] thiophosphate (910 cpm per nmole). In control A, 2,4-dinitrophenol was added at the same time as [^{35}S] thiophosphate to a final concentration of 200 μM. Reaction was stopped by addition of 4 volumes of 0.375 M $HClO_4$ plus 1.25 mM thiophosphate ($HClO_4$ + SP_i) and the precipitate collected by centrifugation. The acid-precipitable material was washed at 0° by dispersion in 0.3 M $HClO_4$, 1 mM SP_i followed by centrifugation. After 4 wash cycles, the precipitates were dissolved and counted by liquid scintillation. In a blank reaction, all additions were the same as in Sample A, except that the [^{35}S] thiophosphate was added at the time of acid denaturation. The values listed have been corrected for a blank of 0.009 nmoles per mg protein and represent averages of duplicate determinations.

to acid precipitation. They were unable to detect any energy-linked acyl-phosphate formation in rat-liver mitochondria. Since acyl phosphate microsomal ($Na^+ + K^+$)-ATPase [5, 6] and Ca^{++}-dependent ATPase of sarcoplasmic reticulum vesicles [7, 8] are stable to acid precipitation and since acyl thiophosphates are expected to be more stable than acyl phosphates, our results give further indication that acyl phosphate does not participate as an intermediate in oxidative phosphorylation in mitochondria.

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