EVIDENCE OF A PHOSPHORYLATED INTERMEDIATE IN MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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SUMMARY

The effects of aurovertin and oligomycin on the rates of oxidative phosphorylation and arsenate-stimulated respiration have been studied over a wide range of concentrations. It has been found that both arsenate and phosphate can react with an energy-rich intermediate of oxidative phosphorylation even when reaction with ADP is prevented by aurovertin. These results suggest that tenable hypotheses of oxidative phosphorylation should include a phosphorylated intermediate.

The continued inability to detect a phosphorylated intermediate in mitochondrial oxidative phosphorylation has led several investigators in the field to propose a concerted mechanism in which ATP is formed as the first covalent product of P_i and ADP (1-5). However, failure to detect a phosphorylated intermediate, $X \sim P$, may be due to its lability under the conditions chosen to quench oxidative phosphorylation and precipitate the mitochondria. If this is the case, then until we learn more of the nature of the intermediate the best opportunity for observing it may be provided by indirect methods. A notable attempt in this direction was made by Ter Welle and Slater (6, 7) in their studies of arsenate uncoupling. Finding that arsenate-induced hydrolysis of ATP (ATPase) is very slow compared to arsenate-stimulated respiration, they suggested that a stable phosphorylated intermediate is formed during arsenate-induced ATPase which is not present during arsenate-stimulated respiration. They also suggested that a concerted mechanism did not explain the inhibition by P_i of arsenate-stimulated respiration. When substrate-binding steps are considered, however, the results of the arsenate-uncoupling studies can also be explained by the competitive binding of P_i and arsenate. That is to say, Ter Welle and Slater's data cannot distinguish between possible presence of an enzyme bound phosphate or a phosphorylated intermediate.

Indirect evidence for the existence of a phosphorylated intermediate can be found in the results of studies with the phosphorylation inhibitors, aurovertin and oligomycin. The difference in their effect on submitochondrial particles (8-11) and binding studies with coupling factors (12-14) support the view of Lee and Ernster (10) that oligomycin inhibits before and aurovertin after the formation of a phosphorylated intermediate, $X \sim P$. Establishing these proposed sites of inhibition would, in effect, show the existence of $X \sim P$.

Although no explanation was offered, Ter Welle and Slater (7) observed that arsenate-stimulated respiration was not inhibited by addition of high concentrations of aurovertin, but was still susceptible to inhibition by phosphate or oligomycin. In the present work, these observations have been extended and results indicate the formation of a phosphorylated intermediate in mitochondrial oxidative phosphorylation.

MATERIALS AND METHODS

Rat-liver mitochondria were prepared according to the method of Johnson and Lardy (15) with the exception of the following modifications: 0.25 M Sucrose + 1 mM Tris buffer (pH 7.4) was used as the isolation medium and mitochondria were collected by centrifugation at 9,000 g for 5 minutes. Protein was determined by the fat-free dry weight method of Slater as described by King (16). Oxygen uptake was followed polarographically using a YSI Model 53 Oxygen Monitor. As lucite was found to absorb appreciable amounts of the antibiotics, a Kel-F probe sheath patterned after the YSI 5093 lucite plunger, and teflon stirring bars were used. The reaction medium contained 15 mM KCl, 50 mM Tris buffer (pH 7.4), 1 mM EDTA, 2.5 mM $MgCl_2$, 60 mM succinate, and 150 X 10^{-12} moles of rotenone, in a total volume of 3.0 ml at 25° C.

Aurovertin was the generous gift of Professor Henry A. Lardy. Rotenone was obtained from S. B. Penick & Co. and oligomycin, ADP, succinate, and dinitrophenol were obtained from Sigma Chemical Co. Aurovertin, rotenone and oligomycin were dissolved in ethanol.

RESULTS

Figure 1A illustrates the well-known characteristics of arsenate uncoupling. Arsenate-stimulated respiration is inhibited by P_i and this inhibition is relieved by ADP. Figure 1B shows that at high concentration (6.3 μg/mg protein) aurovertin has little effect on arsenate-stimulated respiration. Arsenate-stimulated respiration is still inhibited by P_i but the inhibition is no longer relieved by ADP. This suggests that arsenate uncouples in the presence of high concentrations of aurovertin by reacting with a high-energy nonphosphorylated intermediate, $X \sim I$, to give $X \sim As$ which then hydrolyzes rapidly. Added phosphate would compete with arsenate resulting in the formation of $X \sim P$. As phosphate esters are in general much more stable than arsenate esters (17) this would lead to an inhibition of arsenate-stimulated respiration. This suggestion is consistent with the fact that at the same concentration, aurovertin inhibits oxidative phosphorylation (Figure 1C). The fact that ADP does not stimulate respiration in Figure 1B establishes the site of inhibition by aurovertin as between $X \sim P$ and ATP in the phosphorylation sequence proposed by Lee and Ernster (10). The stimulation of respiration by 2,4-dinitro-

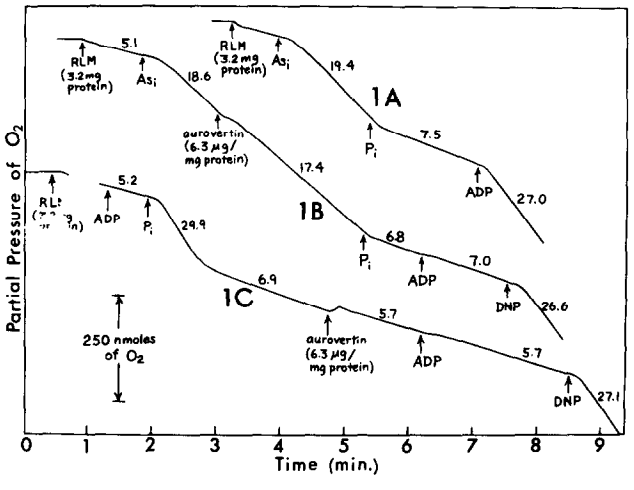

Figure 1. Effect of aurovertin at high concentration on the oxidative phosphorylation and arsenate-stimulated respiration of rat-liver mitochondria (RLM). Composition of the reaction medium is described under Materials and Methods. Additions were made in small volumes, final concentrations were: Arsenate (As_i), 20 mM; P_i, 4 mM; ADP, 0.16 mM; 2,4-dinitrophenol (DNP), 0.032 mM; RLM and antibiotic at the indicated amounts. The slopes are given in numbers representing % O_2 consumed per minute.

phenol, indicates that aurovertin does not have secondary effects on the electron transport chain.

Figure 2A shows that at low concentration (0.12 μg/mg protein) aurovertin partially inhibits arsenate-stimulated respiration. This suggests that when aurovertin is absent or present at low concentration, X ~ As is hydrolyzed slowly and that most of the arsenate-stimulated respiration can be accounted for by the participation of endogenous ADP. Oligomycin (0.125 μg/mg protein) completely inhibits arsenate-stimulated respiration, since Figure 2B shows that P_i has no effect on the oligomycin-inhibited, arsenate-stimulated respiration rate.

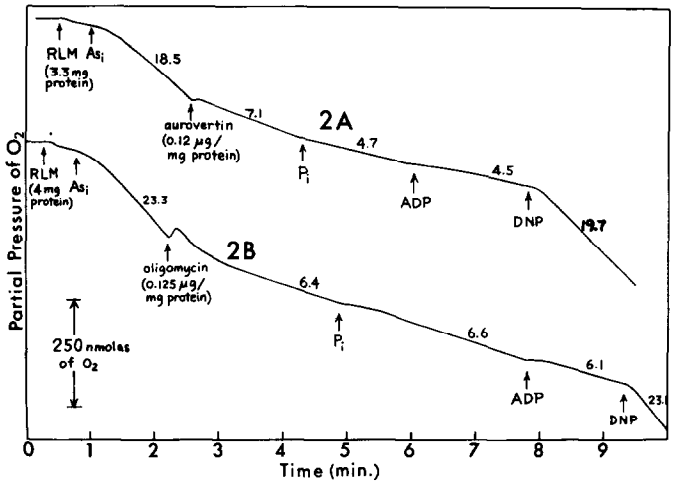

Figure 2. Effects of aurovertin and oligomycin at low concentration on arsenate-stimulated respiration of RLM. Additions of reagents: same as described under Figure 1.

In Figure 3 the rate of arsenate-stimulated respiration is shown as a function of antibiotic concentration. At concentrations greater than 0.10 μg/mg protein both oligomycin and aurovertin inhibit oxidative phosphorylation but only oligomycin completely inhibits arsenate-stimulated respiration. This is consistent with the generally accepted view that oligomycin acts prior to the incorporation of P_i or arsenate into the reaction sequence (18, 19).

The rate of respiration of mitochondria in the resting state was stimulated by

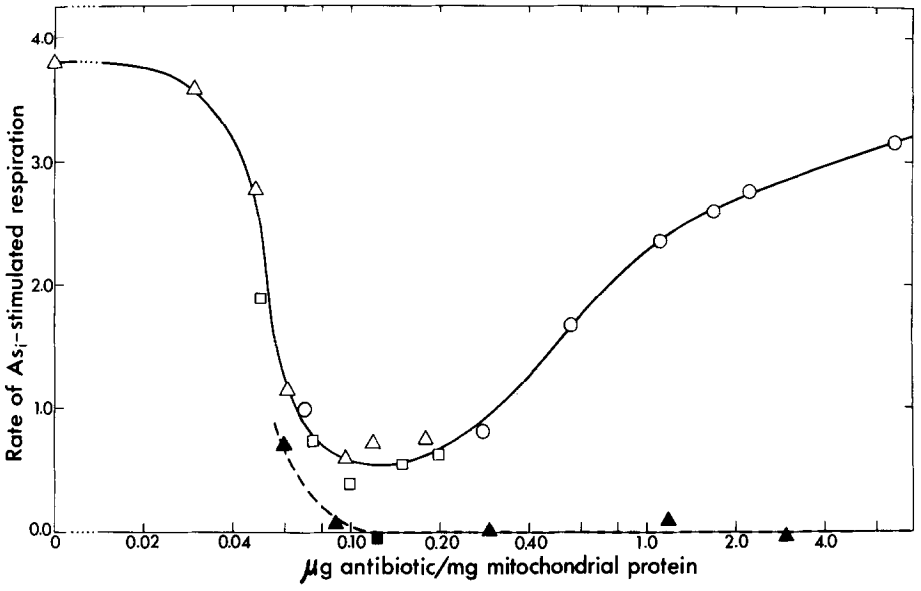

Figure 3. Inhibition of the rate of arsenate-stimulated respiration of RLM by antibiotics. The ordinate represents the rate of arsenate-stimulated respiration per mg protein in the presence of the indicated amount of antibiotic minus the corresponding rate after inhibition by added P_i. △, □, ○ represent experiments with aurovertin; ▲, ■, ● represent experiments with oligomycin. Experimental points of similar shape correspond to the same preparation of RLM. The minimum in the aurovertin curve occurs approximately at 2 moles of aurovertin per mole of cytochrome oxidase. Note that the abscissa is in logarithmic scale; the point at $-\infty$ corresponds to the experiment without added antibiotic.

the addition of very large amounts of oligomycin (40 μg/mg protein). This stimulation, however, was little affected by the addition of arsenate, P_i, or ADP.

DISCUSSION

The data presented in Figure 1 show that both As_i and P_i can react with X ~ I even when reaction with ADP is blocked by aurovertin. These results are not consistent with the suggestion that arsenolysis of X ~ I has an absolute requirement for ADP (20). Furthermore the results are not easily accommodated by concerted mechanisms in which no X ~ P intermediate is formed (1-5).

The data presented in Figures 2 and 3 show that low concentrations of aurovertin, sufficient to inhibit oxidative phosphorylation, only partially inhibit arsenate-stimulated respiration. These results are not in agreement with those reported by Ter Welle and Slater (7) who found low concentrations of aurovertin

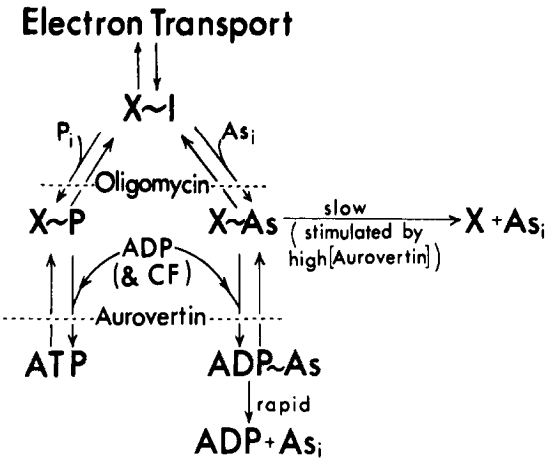

Figure 4. Reaction scheme of P_i, As_i, ADP and antibiotics with the oxidative phosphorylation system.

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and oligomycin equally effective in inhibiting arsenate-stimulated respiration. The present results are consistent, however, with the scheme proposed by Ernster, Lee and Janda (Figure 8D of reference 9) to accommodate kinetic data on arsenate-stimulated respiration, arsenate-induced ATPase and the $As_i \rightleftharpoons H_2O$ exchange. A modification of that scheme is given in Figure 4 which takes into account the effects of aurovertin and oligomycin observed in the present study. X ~ As and ADP ~ As may hydrolyze as indicated or could conceivably participate in a trans-arsenylation reaction resulting in the formation of a stable derivative of some mitochondrial component (21).

The fact that the rate of arsenate-stimulated respiration is slow in the presence of low concentrations of aurovertin but rapid in the presence of high concentrations may be due to a change in the environment of X ~ As on multiple binding of aurovertin by the coupling factor (CF).

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