Functioning of the Adenine Nucleotide Transporter in the Arsenate Uncoupling of Corn Mitochondria

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ABSTRACT

Arsenate uncouples mitochondrial respiration in a process stimulated by ADP, inhibited by oligomycin, and competitively inhibited by inorganic phosphate. If mersalyl is added to corn mitochondria to block further transport of accumulated arsenate, the uncoupled respiration continues unabated due to recycling of matrix arsenate. Addition of ADP now inhibits rather than promotes respiration and the mitochondria shrink. It is established by arsenate analyses that arsenate is removed from the matrix. Oligomycin or atractyloside block the removal by inhibiting ADP-arsenate formation or transport, respectively. It is deduced that ADP-arsenate is stable in the membrane and is transported outward for hydrolysis in the external aqueous phase. Hence, ADP-arsenate formed in oxidative phosphorylation is not directly released to the matrix, and a mechanism must exist for its direct transfer to the transporter.

The findings that arsenate uncoupling of mitochondrial respiration is oligomycin-sensitive (4, 7, 8, 14, 15), ADP-stimulated (4, 6–9, 15, 21), and competitively inhibited by Pi (1–3, 5–8, 21) has led to the opinion that instability of the ADP-As
bond formed in oxidative phosphorylation is responsible (7). It appears implicit in this view that the F1-ATPase forms the arsenylated analog of ATP which is hydrolyzed either in the membrane or on release to the matrix, recycling As and ADP as acceptor. However, Ernster et al. (7) found intramitochondrial ADP concentrations relatively static under a variety of experimental conditions and concluded that the respiratory release by As in the absence or in the presence of added ADP was not rate-limited by intramitochondrial ADP. They proposed a slower rate of X-As hydrolysis compared to ADP-As hydrolysis as responsible, thus supporting their concept of a dual pathway of As uncoupling. In contrast, Mitchell et al. (17) favors a nonhydrolytic mode of action of As and would explain the observations on kinetic grounds rather than as alternative energy-transfer pathways.

In a recent publication on the phosphate release of acceptorless respiration (10), we reported that As-loaded, mersalylblocked corn mitochondria respond to addition of ADP by shrinkage and decline in respiration rate. This was the same response obtained with phosphate where ATP formation could be demonstrated in amounts equivalent to intramitochondrial PI. We speculated that hydrolysis of ADP-As did not occur until exit from the membrane, thus removing As from the matrix space.

We report here on further studies of this phenomenon. The initial supposition is supported. The ADP-stimulation of arsenate uncoupling involves the AdN transporter, and it appears that oxidative phosphorylation—or arsenylation in this case—is accomplished without direct flux of adenine nucleotides in and out of the matrix.

MATERIALS AND METHODS

Mitochondria. Corn mitochondria (Zea mays L. WF9(Tms)-xM14) were isolated, and recordings of oxygen consumption and percentage transmission were as previously described (10). Final suspension of the mitochondria (10–15 mg protein/ml) was in the same medium as used for the basic reaction mixture: 200 mM sucrose, 10 mM TES buffer, 1 mM MgSO4, and 1 mg/ml bovine serum albumin, adjusted to pH 7.6 with KOH. Including a correction for the bovine serum albumin of the basic reaction mixture, protein concentrations were determined by the method of Lowry et al. (16). All preparations of mitochondria were checked for tight coupling by determining respiratory control and ADP/O ratios.

Arsenate Transport. Radioactive "As (Amersham/Searle as arsenic acid in 0.04 M HCl) was mixed with potassium arsenate stock as carrier. For each experiment, a 0.1-ml aliquot of mitochondrial suspension was added to 0.9 ml of basic reaction mixture (28–29 C) with aeration by stirring. After additions (Table II), 100-μl aliquots (about 0.15 mg of protein) were withdrawn, and the mitochondria were collected on 0.45-μm pore size Millipore filters with suction being applied for 15 sec. The filters were dried, toluene based scintillation fluid (toluene-PPO-POPOP) was added, and the vials were counted.

Atractyloside, oligomycin, and mersalyl were from Sigma Chemical Company.

RESULTS

Respiratory and Swelling Contraction Studies. Addition of mersalyl blocks As transport as shown by the lack of swelling and respiratory release (Fig. 1A). As previously reported (10) addition of ADP to arsenate-loaded mitochondria which have had further transport blocked by mersalyl leads to active shrinkage (Fig. 1B). Concurrently there is a decline in respiration rate. The shrinkage is in two phases: a very abrupt initial phase lasting a few seconds, followed by a slow phase lasting for about 1 min. Subsequent addition of atractyloside causes an equally abrupt swelling of about the same magnitude as the

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ADP shrinkage, but there is no second phase. Uncoupling with dinitrophenol appears to release a small amount of additional solute, giving shrinkage.

If oligomycin is added to inhibit phosphorylation—or arsenylation in this case—the respiratory release on addition of arsenate is greatly inhibited as is the second phase of shrinkage with ADP (Fig. 1C). However, the abrupt shrinkage with ADP and the abrupt swelling with atractyloside are not affected. Uncoupling with DNP now gives greater shrinkage, presumably due to loss of arsenate by pathways other than the Pi-OH transporter. It is interesting that oligomycin somewhat increases the rate and extent of As swelling.

Figure 2, A and B, compare the responses to ADP addition as affected by the presence of mersalyl. When mersalyl is not used, allowing continuous operation of the Pi-OH transporter, ADP gives the customary increase in respiration rate but only the abrupt shrinkage is found. Addition of atractyloside reduces respiration to about the rate obtained before ADP addition.

Figure 2 also shows a commonly observed phenomenon: the release of respiration after addition of As gradually increases with time, which is not true for addition of phosphate (Fig. 7, A and B, in ref. 10). This delayed respiratory release is greater with higher As concentrations (not shown). Azzone and Ernster (1) also reported the uncoupling by As in a time-dependent manner as a function of the concentration of As. They attributed this increase in respiratory rate with time as due to the gradual loss of Pi from the mitochondria. The increase in respiration with ADP addition (Fig. 2B) can be made more striking if the addition is made before the arsenate respiration is fully developed (data not shown; see ref. 15).

When ADP is added initially, there is an abrupt and large release of respiration on addition of As which does not increase in time (Fig. 3). Mersalyl will completely block the As-induced increment in respiration over a period of 15 to 20 sec. Under these conditions, the amplitude of As swelling before mersalyl and the shrinkage after mersalyl is much reduced. Evidently, the As uncoupling in the presence of ADP is dependent on continuous As transport through the Pi-OH transporter. When As transport is blocked atractyloside no longer has an effect, although the abrupt increase in transmission is still found indicating atractyloside reaction with the membrane. Collectively, the data of Figures 2 and 3 suggest that As uncoupling in the presence of ADP involves cyclic As transport: in through the Pi-OH transporter, out through the ADN transporter as ADP-As.

**Arsenate Determinations.** It was essential to confirm that the swelling shown by the transmission trace on addition of As represented As uptake by the mitochondria, and that the
corresponding shrinkage on addition of ADP represented As loss. A great deal of exploratory work with both centrifugation and filtration techniques showed that the arsenate is held by the mitochondria in a very labile fashion. Any manipulation which removed the mitochondria from substrate (NADH), produced anoxia or reduced external As concentrations resulted in rapid As efflux, vitiating results. Mersalyl did not block the loss, and concentrations above 20 to 25 μM seemed to enhance leakage in some preparations, probably due to the enhanced permeability mercurials can produce (18). The only technique which would give reproducible results was rapid filtration without any washing. Unfortunately, this technique gives a high "blank" value for As held with the mitochondria on the filter, and to minimize the blank relative to active uptake or loss of As, it was necessary to use relatively low concentrations of As (1 mM). Table I shows representative data illustrating the loss of As accompanying washing on the filter, and the lack of change in As content without substrate but with arsenate, mersalyl, and ADP.

Table II gives the results from eight determinations of the increase in As that accompanies active swelling and the decrease which follows addition of ADP. Clearly, ADP removes most of the As from the mitochondria, just as indicated by the transmission traces. We were not able to determine directly what share, if any, of this loss was due to the abrupt shrinkage. However, when oligomycin was added the addition of ADP did not lead to a loss of As (Table II). It appears that the abrupt shrinkage which still occurs in oligomycin-blocked mitochondria (Fig. 1C) is not due to loss of As from the matrix. Very recently, Stoner and Sirak (19, 20) have reported contraction of the inner membrane of heart mitochondria due to the binding of adenine nucleotides and reversal with atracyloside. This contraction produces optical changes comparable to those we report here.

![Fig. 2. Respiratory and swelling-shrinkage responses. Conditions and additions as in Figure 1, except 2.5 mM As was used. A: mersalyl added before ADP; B: no addition of mersalyl before ADP.](image)

**Table I. Effect of Washing and of Absence of Exogenous Substrate on the Arsenate Content of Corn Mitochondria**

Sequential additions were made in the order shown to mitochondria suspended in the basic reaction medium, using 3.5 μmoles of NADH, 1 mM As (34As), 18 μM mersalyl, and 75 μM ADP. One minute elapsed between additions and between final addition and filtration with the exception of only a 10-sec period after mersalyl. Filtration of 100-μl aliquots was on 0.45-μm Millipore filters without washing (see text), except as indicated where the filtered mitochondria were washed with 0.5 ml of basic reaction medium.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Not Washed</th>
<th>Washed</th>
<th>Not Washed</th>
</tr>
</thead>
<tbody>
<tr>
<td>As (control)</td>
<td>68</td>
<td>9</td>
<td>61</td>
</tr>
<tr>
<td>As + NADH + Mersalyl</td>
<td>96</td>
<td>16</td>
<td>(−NADH) 63</td>
</tr>
<tr>
<td>As + NADH + Mersalyl + ADP</td>
<td>75</td>
<td>11</td>
<td>(−NADH) 59</td>
</tr>
</tbody>
</table>

**Atracyloside Inhibition.** When the Pi-OH transporter is blocked by mersalyl the only avenue for As removal from the matrix would be as ADP-As via the AdN transporter (other than nonspecific leakage). Blocking the AdN transporter with atracyloside should be effective in preventing such efflux, and this proved to be true (Table II).

Chappell and Crofts (4) proposed that the enhanced rate of As uncoupling of respiration by external ADP involved atracyloside-sensitive ADP-As transport, whereas the rate-limiting reaction in the absence of added ADP was the spontaneous hydrolysis of internal ADP-As (atracyloside insensitive). However, with or without mersalyl present corn mitochondria show a 25 to 35% suppression of As-uncoupled respiration on addition of atracyloside (Fig. 4, A and B). If mersalyl was not present, the subsequent addition of ADP completely reversed the effect of atracyloside and enhanced respiration. The same results were obtained if the atracyloside was added before the As (Fig. 5, A and B): respiratory uncoupling by As was diminished, but this was reversed if ADP were added. Again, mersalyl blocked the ADP response.

Apparently, a minor part of the As-uncoupled respiration is mediated through endogenous AdN, and cyclic transport of As out through the AdN transporter and back in through the Pi transporter is involved. Blocking the AdN transporter with atracyloside inhibits this cyclic component, but addition of ADP must place atracyloside partially and allow some ADP-As to exit. With mersalyl to block reentry of As there...
Table II. Uptake of Arsenate during Respiration and Loss of Arsenate on Addition of ADP; Effect of Oligomycin and Atractyloside on Loss of Arsenate

Experiments were performed as in Table I with the addition of 5 μM oligomycin and 50 μM atractyloside as indicated. One minute elapsed between additions and between final addition and filtration with the exception of a 10-sec period after mersalyl, oligomycin and atractyloside.

| Addition                  | As Content | ΔAs  
|---------------------------|------------|------
| As (control)              | 56 ± 4     |      |
| As + NADH + Mersalyl      | 84 ± 5     | +28  |
| As + NADH + Mersalyl + ADP| 65 ± 4     | -19  |
| As (control)              | 49         |      |
| As + NADH + Mersalyl + Oligomycin | 74      | +27  |
| As + NADH + Mersalyl + Oligomycin + ADP | 81 | +9   |
| As (control)              | 62         |      |
| As + NADH + Mersalyl + Atractyloside | 92     | +27  |
| As + NADH + Mersalyl + Atractyloside + ADP | 104 | +10  |

1 Standard error of the mean for eight experiments. Gain in As with respiration and loss of As with ADP addition are significant at the 0.001 level of significance by the paired t-test.

with a countertransport of ATP from the matrix to the external aqueous phase. The ATP formation occurs with Pi and ADP drawn from the matrix. Heldt (13) mentions that the endogenous adenine nucleotides seem to be in solution, confined by the inner membrane which surrounds the matrix space, and that the amount of endogenous adenine nucleotide is kept constant. These observations support the concept that the atractyloside-sensitive AdN transporter functions in oxidative phosphorylation by maintaining matrix levels of ADP.

However, our experiments with arsenate-loaded, mersalyl-blocked corn mitochondria suggest that ADP-As formed in oxidative phosphorylation is not directly delivered to the matrix when the transporter is functioning. If it were, the ADP-As should hydrolyze in the aqueous matrix, recycling ADP and As for reaction at coupling sites. Instead, the ADP-As seems to be transported out of the inner membrane, hydrolyzing in the intermembrane aqueous phase. Only in this way is it possible to account for the loss of arsenate from the matrix on addition of ADP. Both arsenate analyses and the decline in respiration support the transmission traces in showing that arsenate is removed from the matrix on addition of ADP. Of course, this can only be demonstrated if the reentry of As into the matrix via the Pi-OH transporter is blocked.

The mitochondria must have some means of transporting ADP into the site where ADP-As is formed and then transporting it back out without meeting an aqueous phase. To accomplish this there must be a transfer site associated with the F1-ATPase which can interact with the AdN transporter. A hypothetical mechanism is illustrated in Figure 6. Continued operation of the AdN transporter in mersalyl-blocked mitochondria would give the observed withdrawal of As from the matrix. Hydrolysis of ADP-As would occur after exit into

**DISCUSSION**

It seems to be widely assumed that the formation of ATP (ADP-As here) results from transport of ADP into the matrix

![Fig. 4. Inhibition by atractyloside of As-uncoupled respiration and reversal by addition of ADP as affected by mersalyl. Conditions as in Figure 1 except 1 mm As was used. A: mersalyl added before atractyloside; B: no mersalyl added before atractyloside.](image)

is a slow shrinkage (Figs. 4A and 5B) indicative of partial AdN transporter operation. The responses in Figures 4 and 5 are qualitatively similar to those where atractyloside is absent, with the quantitative aspects apparently reflecting the efficiency of atractyloside in competing for sites on the AdN transporter.

![Fig. 5. Effect of atractyloside on As-uncoupled respiration and ADP-induced shrinkage in the presence and absence of mersalyl. Conditions and additions as in Figure 5, except that atractyloside was added initially. A: no addition of mersalyl before ADP; B: addition of mersalyl before ADP.](image)
The fast reaction involves the AdN transporter (4), but via a transfer mechanism which bypasses release of ADP-As to the matrix. The slow reaction in the absence of exogenous ADP may involve hydrolysis at the level of this transfer mechanism, and a minor contribution might be made through transport of endogenous AdN. As previously pointed out (10) the slow pathway, as we visualize it, requires a finite permeability of the inner membrane to H+ or K⁺.

**LITERATURE CITED**


