INACTIVATION OF COUPLED RESPIRATION OF MITOCHONDRIA BY INORGANIC ARSENATE AND PARTIAL RESTORATION BY ATP

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SUMMARY. Incubation of rat liver mitochondria with inorganic arsenate and β-hydroxybutyrate followed by centrifugation and washing with 0.25 M sucrose produced nearly complete uncoupling of respiration with succinate (ADP/O = 0) but somewhat less with β-hydroxybutyrate or glutamate (ADP/O = 0.9-1.6). The ability of the As_i-treated mitochondria to form a respiration-dependent arsenic containing substance (As-X) was also reduced by 60-90%. These several effects were not observed when phosphate replaced arsenate nor when arsenate and phosphate or arsenate plus KCN were included in the initial incubation system. ATP and deoxy-ATP partially restored the coupling activity of the As_i-treated mitochondria; CTP, GTP and their deoxy derivatives and ITP were inactive. These results are not consistent with an arsenolytic action of As_i and suggest that one or more components of coupling sites II and III are inactivated by conditions favorable for As-X formation.

The uncoupling effect of inorganic arsenate (As_i) on oxidative phosphorylation is generally rationalized in terms of formation of an unstable arsenylated analogue of a normally phosphorylated component of the coupling mechanism (1-3). However, recent studies by Mitchell et.al. (4), Huang and Mitchell (5) and by Sandoval et.al. (6) are not necessarily consistent with this mechanism. Previous studies in this laboratory demonstrated that incubation of respiring rat liver mitochondria with ^{74}As-arsenate resulted in the formation of a low molecular weight, acid soluble, arsenic containing substance-As-X (7). The formation of As-X is inhibited by inorganic phosphate (P_i), KCN, oligomycin, and 2,4-dinitrophenol (DNP). We concluded that a functional respiratory chain and at least part of the coupling process of oxidative phosphorylation are required for As-X formation.

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Inasmuch as the conditions of As-X formation correspond to those required for stimulation of respiration and for inhibition of ATP synthesis by As_i (1,2), it seemed possible that the two events could be related. The primary purpose of these studies was to determine the effect on coupled respiration and ATP synthesis of prior exposure of mitochondria to As_i under specific conditions that influence the extent of As-X formation.

MATERIALS AND METHODS

Rat liver mitochondria were prepared by the method of Schneider (8) and were washed once with 0.25 M sucrose.

Prior to assay for oxidative phosphorylation, mitochondria were exposed to an arsenate-containing media. For Method A, approximately 40 mg mitochondrial protein were suspended in 5 ml of 0.18 M sucrose, 1 mM ADP, 1.6 mM $MgCl_2$, 10 mM Tris HCl, pH 7.4, 1 mM β-hydroxybutyrate and 1 mM sodium arsenate for 20 minutes at 25°. The system was chilled in an ice bath and centrifuged at 10,000 x g for 10 minutes at 4°. The pellet was washed twice with 10 ml of 0.25 M sucrose at 2°, resuspended in 1 ml 0.25 M sucrose and assayed for oxidative phosphorylation. A second method - Method B- was essentially identical to Method A except that $MgCl_2$ was omitted, 1 mM EDTA was included, and the sucrose concentration was increased to 0.25 M.

Oxidative phosphorylation was estimated in the presence of a glucose-hexokinase trap by measuring disappearance of P_i as described by Slater (9). Succinate oxidation was measured by the manometric method (9) and β-hydroxybutyrate oxidation by acetoacetate formation (10). Oxidative phosphorylation was also measured polarographically with a Clark Oxygen Electrode (11). A 1.75 ml air-saturated medium containing 0.23 M mannitol, 0.07 M sucrose, 0.02 M Tris HCl, 0.02 mM EDTA, and 5 mM potassium phosphate at pH 7.2 was employed. Respiratory substrates included 7 mM β-hydroxybutyrate, 7 mM glutamate plus 1.4 mM malate plus 1.4 mM malonate, 7 mM succinate plus 8.4 μM rotenone. State 3 respiration was initiated by addition of 200 nmoles ADP. ADP/O and respiratory control ratio - RCR - were estimated as described by Estabrook

(12). ^{74}As-X formation was measured as described by Chan et.al. (7). Protein concentrations were estimated by the biuret method of Layne (13) in 1% sodium deoxycholate.

Rotenone, ATP, ADP, succinic acid, DL-β-hydroxybutyric acid, L-malic acid, malonic acid, DL-glutamic acid, 2,4-dinitrophenol, Tris·HCl and EDTA were obtained from Sigma Chemical Co. CTP, GTP and their deoxy derivatives, and ITP and deoxy ATP were obtained from Schwarz-Mann Co. All other compounds were analytical reagent grades.

RESULTS

The P/O ratios and ^{74}As-X formation capacity were determined using mitochondria that had been previously incubated aerobically according to Method A. The data presented in Table I demonstrate that prior treatment with sucrose, inorganic phosphate (P_i) or P_i plus KCN had very little effect on the ability of the washed mitochondria to carry out oxidative phosphorylation or to form ^{74}As-X. Prior exposure to As_i alone produced a marked reduction of both reactions. The uncoupled state as well as the reduction of As-X formation capacity induced by prior treatment with As_i were prevented by inclusion of P_i or KCN in the initial incubation system. Although significant protection has been observed with lower P_i concentrations (0.5 - 1.0 mM) the optimal protection required $\geq$ 3.0 mM. Addition of cytochrome c or bovine serum albumin did not increase respiration or the P/O ratios. The P/O ratio of As_i-treated mitochondria was routinely 0-0.5 with succinate and 0.9-1.6 with β-hydroxybutyrate.

The respiration of mitochondria previously incubated with As_i or with $As_i + P_i$ as described for Method B was studied by the polarographic method. The results presented in Fig. 1 demonstrate that succinate oxidation by As_i-treated mitochondria was not stimulated by ADP (Curve A), by As_i (Curve B) nor inhibited by oligomycin (Curve A) but some stimulation was produced by 2,4-DNP. Mitochondria that were previously incubated with As_i plus P_i (Curve C) responded to these several additions in a manner comparable to untreated mitochondria (Curve E). Respiration of As_i plus P_i-treated mito-

Table 1

Effect of Arsenate Treatment on Oxidative Phosphorylation

Exp.	Initial Incubation System	Oxidative Phosphorylation		^{74}As-X Formation
		P/O	O natoms/min/mg	nmoles/mg
1	β-hb	2.8	37	2.4
	β-hb + P_i	2.9	34	2.2
	β-hb + As_i	0.9	30	0.6
	β-hb + P_i + As_i	2.4	32	2.0
	β-hb + P_i + KCN	2.6	35	2.0
	β-hb + As_i + KCN	2.5	33	2.1
2	β-hb	1.8	90	2.0
	β-hb + P_i	1.7	85	1.9
	β-hb + As_i	0.2	75	0.5
	β-hb + P_i + As_i	1.5	83	1.8
	β-hb + KCN + As_i	1.5	85	1.9

Initial incubation of mitochondria was carried out as described in Methods (Method A) with indicated additions of P_i, 5 mM; As_i, 1 mM; KCN, 1 mM; β-hydroxybutyrate (β-hb), 1 mM. Following centrifugation and washing, each mitochondrial preparation was assayed for oxidative phosphorylation and ^{74}As-X formation as described in Methods. β-hb was the respiratory substrate in experiment 1 and succinate was used in experiment 2 which were measured by acetoacetate formation and manometry respectively.

Table 2

Uncoupling of Respiration by Arsenate

Exp.	Initial Incubation System	Respiratory Substrate	State 3 Respiration	RCR	ADP/O
			(ngatoms O/min/mg protein)		
1	None	Succinate	115.3	2.6	1.5
	As_i	"	83.2	1.0	0
	P_i	"	114.7	2.0	1.3
	As_i + P_i	"	121.7	2.1	1.3
2	None	Glutamate	81.2	4.3	2.4
	As_i	"	63.6	2.3	1.6
	P_i	"	77.8	3.8	2.2
	As_i + P_i	"	71.9	3.7	2.1
3	None	β-hydroxybutyrate	48.7	2.9	2.2
	As_i	"	35.6	1.8	1.3
	P_i	"	44.1	2.6	2.2
	As_i + P_i	"	40.9	2.3	2.2

The initial incubation was carried out as described for Method B using 1 mM As_i, 5 mM P_i as indicated. Coupled respiration with the indicated substrate was estimated by the polarographic method as described in Methods and In Fig. 1.

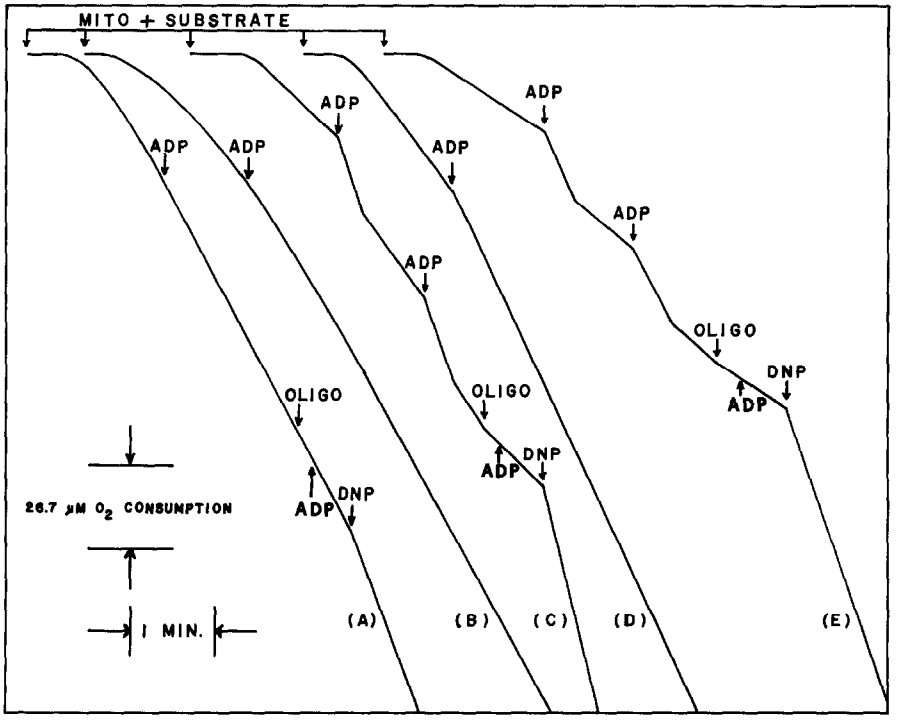

Fig. 1. Succinate oxidation by rat liver mitochondria. Mitochondria were initially exposed to As_i, 1 mM; P_i, 5 mM; or As_i, 1 mM + P_i, 5 mM under aerobic conditions for 20 mins. and subsequently isolated and washed as described for Method B. Where indicated, additions to the polarographic assay system were treated mitochondria, 2.3 mg protein; succinate, 7 mM; rotenone, 8.35 mM; ADP, 73 μM; oligomycin, 31 μM; and 2,4-DNP, 110 μM. Curve A, As_i-treated mitochondria; B, 1.15 mM sodium arsenate added to As_i-treated mitochondria just prior to addition of succinate; C, As_i plus P_i-treated mitochondria; D, 1.15 mM sodium arsenate added to As_i plus P_i-treated mitochondria; E, freshly prepared or untreated mitochondria (1.7 mg protein).

chondria was stimulated by As_i (Curve D). The latter response could be reversed by P_i. Addition of serum albumin to the assay system did not improve the coupling activity of As_i-treated mitochondria.

The degree of respiratory control of mitochondria previously incubated with As_i and As_i plus P_i is summarized in Table 2. It is apparent that As_i treatment results in nearly complete loss of respiratory control with succinate but less than complete loss with β-hydroxybutyrate or glutamate. This loss was not as pronounced when the mitochondria were subjected to prior treatment with As_i plus P_i or when P_i replaced As_i. The ADP/O values

Table 3

Nucleotide Activation of Coupled Respiration

Exp.	Nucleotide	State 3 Respiration -oligo	State 3 Respiration +oligo	RCR	ADP/O
		ng-atoms O/min/mg prot.			
1	None	89.4	89.4	1.0	0
	+ATP (0.15 mM)	93.3	57.5	1.6	1.1
	+GTP (0.30 mM)	85.6	85.6	1.0	0
	+CTP " "	85.6	85.6	1.0	0
	+ITP " "	95.3	-	1.0	0
2	None	81.2	81.2	1.0	0
	+ATP (0.15 mM)	79.5	50.1	1.6	1.0
	+ATP (0.30 mM)	79.5	-	1.6	0.9
	+deoxy ATP (")	79.5	55.3	1.5	1.1
	+deoxy CTP (")	79.5	-	1.0	0
	+deoxy GTP (")	91.6	-	1.0	0

Rat liver mitochondria were exposed to the initial As_i-containing medium (Method B). The indicated nucleotide and 7mM succinate were added to the polarographic assay medium 60 seconds prior to addition of ADP or ADP plus 30 μM oligomycin. State 3 respiration (+ADP), RCR, and ADP/O were determined as described in Methods with 2.1 mg mitochondrial protein.

greater than 1.0 observed with NAD^+-linked substrates but essentially zero with succinate is unexplainable now and is being investigated.

Partial restoration of respiratory control of the As_i-treated mitochondria can be obtained by addition of ATP. The data presented in Table 3 demonstrate that 0.15 mM ATP produced a significant increase of the ADP/O ratio and of RCR with succinate. The increase of RCR was oligomycin sensitive. Higher concentrations of ATP did not completely restore normal ADP/O values. No effect was observed with CTP, GTP, their deoxy forms, nor with ITP. Deoxy ATP was as active as ATP. Stimulation of ADP/O and of RCR of As_i-treated mitochondria by ATP has also been observed with glutamate and with the TMPD-ascorbate system. These results will be described in more detail in a subsequent communication.

DISCUSSION

These results demonstrate that As_i treatment of respiring rat liver

As_i is removed. Since the P_i present in the assay system should reverse the uncoupling effect of As_i (1,14), it seems unlikely that the uncoupled state is due to incomplete removal of As_i from the treated mitochondria by the washing procedure. We conclude that the uncoupling effect described here is not due exclusively to an arsenolytic process.

These results demonstrate that the As_i-treated mitochondria have a markedly reduced capacity to form ^{74}As-X. However, when P_i or KCN was included along with As_i in the original incubation system, the uncoupling effect and loss of ^{74}As-X formation capacity were decreased. Previous studies demonstrated that P_i and KCN inhibit As-X formation (7). We suggest therefore that As-X formation during the initial incubation period is related to the imposition of an uncoupled state of the respiratory chain that also limits the capacity of those mitochondria to subsequently produce additional As-X.

We have been unable to demonstrate the restoration of coupling potential of the As_i-treated mitochondria by addition of purified As-X. However, the studies presented here demonstrate that the coupling potential is increased by addition of ATP or deoxy ATP. Since CTP, ITP, GTP and their deoxy forms were inactive, it is possible that reactivation occurs through reversal of one or more of the terminal transphosphorylation steps of the coupling mechanism. This response indicates that the uncoupling effect of As_i described here is not due to the loss of protein coupling factors. It seems more likely that during respiration, As_i causes inactivation or possibly displacement of a coupling factor from its functional environment of the membrane by an ATP-reversible process.

Whereas succinate oxidation by As_i-treated mitochondria can be completely uncoupled the same mitochondria produced an ADP/O of 1.0-1.6 with β-hydroxybutyrate or glutamate. Moreover, addition of As_i or of ADP to the assay system containing As_i-treated mitochondria produced some stimulation of mitochondria produces an uncoupled state that persists after most of the

respiration with NAD^+-linked substrates but not with succinate. We concluded that coupling sites II and III might be the primary loci for the As_i effect. Studies are in progress to determine directly the efficiency of each coupling site of As_i-treated mitochondria.

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