Vol. 82, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1978 Pages 648-654

THE INFLUENCE OF NAD^+-LINKED SUBSTRATES ON ENERGY CONSERVATION AT SITES 2 AND 3 IN MITOCHONDRIA TREATED WITH INORGANIC ARSENATE.

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Received March 30,1978

SUMMARY. Exposure of rat liver mitochondria to inorganic arsenate followed by reisolation and washing to remove the added arsenate results in uncoupled respiration with succinate and ascorbate (ADP/O=0), but ADP/O and ATP/O values of 1.3 to 1.6 with 3-hydroxybutyrate or glutamate. ADP/O and ATP/O values greater than 1.0 with NAD^+-linked substrates arise as a result of partial reactivation of coupling at sites 2 and 3 by these substrates. In the presence of rotenone, NAD^+-linked substrates can still reactivate coupling with succinate or ascorbate at these sites. The extent of reactivation in the presence of rotenone by 3-hydroxybutyrate is decreased by simultaneous addition of acetoacetate. The results suggest that the coupling at sites 2 and 3 is amenable to control through changes in the reduction state of some specific components of the respiratory chain located remotely from these sites.

A previous communication from this laboratory demonstrated that incubation of respiring rat liver mitochondria with inorganic arsenate for 20 minutes at 2^o followed by reisolation and washing to remove the added arsenate resulted in uncoupled respiration with succinate (1). Substitution of P_i for arsenate or inclusion of both P_i and arsenate in the initial incubation system had only minimal effect on the coupling capacity for mitochondria oxidizing succinate. The results, therefore, were interpreted to indicate that the uncoupling effect was specific for arsenate and could be effectively counteracted by the simultaneous inclusion of P_i. In view of the fact that the bulk of the added arsenate had been removed by washing prior to assay for oxidative phosphorylation, it was concluded that the uncoupled oxidation of succinate by arsenate-treated mitochondria was not due to an arsenolytic process as proposed for triosephosphate dehydrogenase (2,3).

Data presented in the earlier communication (1) also demonstrated some peculiar properties of arsenate-treated mitochondria that were not readily re-

0006-291X/78/0822-0648$01.00/0

conciliable in terms of a total lack of coupling with succinate. For example, arsenate-treated mitochondria were capable of a limited coupling ability on oxidation of glutamate plus malate or 3-hydroxybutyrate. The ADP/O values with these substrates were between 1.3 and 1.6 as opposed to the total lack of coupling with succinate. Since energy coupling at site 1 can yield a maximal ADP/O value of only 1.0, it would seem totally inconsistent that arsenate-treated mitochondria were capable of yielding ADP/O values greater than 1.0 with NAD^+-linked substrates under conditions that result in a complete lack of coupling with succinate. The studies to be described here show that the ADP/O ratios noted with NAD^+-linked substrates are related to an unusual and hitherto unrecognized influence of these substrates on the energy conserving sites 2 and 3 and that this unusual influence is observable even under conditions wherein no net oxidation of NAD^+-linked substrates occur.

METHODS: Preparation of mitochondria from rat liver, estimation of protein concentration, polarographic evaluation of oxidative phosphorylation and determination of ADP/O and respiratory control ratios (RCR) were as described earlier (1). ATP was estimated using the luciferin-luciferase system described by Strehler (4). Oxidative phosphorylation was also evaluated by measuring the amount of [^{32}P] labeled organic phosphates formed either in presence of excess ADP or in presence of a glucose-hexokinase trap. The [^{32}P] labeled organic phosphates were separated from the inorganic phosphate by an adaptation of the Berenblum-Chain method described previously (1). Reduction of NAD^+ at 340-374 nm (5) was monitored using an Aminco DW-2 Spectrophotometer.

Arsenate-treated mitochondria were prepared in the following manner: Mitochondria equivalent to 30 mg protein were suspended in 3.0 ml media containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 1.0 - 2.0 mM Na-arsenate for 20 minutes at 0^o after which 7.0 ml of ice-cold 0.25 M sucrose was added, mixed and centrifuged at 10,000 x g for 10 minutes at 4^o. The supernatant fraction was discarded and the pellet resuspended in 10 ml of 0.25 M sucrose at 4^o and centrifuged as before. The resulting pellet was resuspended in 1 ml of 0.25 M sucrose and maintained at 0^o until assayed for oxidative phosphorylation. The controls - namely P_i-treated and arsenate plus P_i-treated preparations - were also carried out as outlined above, except 5 mM P_i replaced arsenate in the first and 5 mM P_i was added together with 1 mM arsenate in the latter.

To determine the extent of removal of the added arsenate by the washing procedure, [^{74}As]arsenate was added to the incubation system. The radioactivity of each supernatant fraction and the resulting mitochondrial pellet present as inorganic arsenate soluble in 5% trichloroacetic acid was estimated by an adaptation of the Berenblum-Chain method described previously (1). Greater than 97% of the added arsenate could be removed from the mitochondria by washing two to three times with 0.25 M sucrose alone. The recovery of arsenate in all fractions compared to that present in the initial reaction system was 98%. Approximately 0.3% of the added arsenate was retained by the mitochondrial pellet and could not be released even by trichloroacetic acid treatment.

RESULTS AND DISCUSSION. Arsenate-treatment of rat liver mitochondria results in uncoupled respiration with succinate (ADP/O = 0) but a limited coupling with NAD^+-linked substrates (ADP/O = 1.3 - 1.6) (1). Recently, we have found that these mitochondria exhibit uncoupled respiration with ascorbate plus N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) as well (ADP/O = 0). The coupling of oxidative phosphorylation indicated by ADP/O ratios for these mitochondria with NAD^+-linked substrates as opposed to total lack of coupling with succinate and ascorbate prompted us to reevaluate the coupling ability by assaying for ATP synthesis. ATP synthesis was monitored either by the luciferin-luciferase technique (4) or by the formation of [^{32}P] labeled organic phosphates. Data in Table I reveal that arsenate-treated mitochondria oxidizing succinate yield ATP/O and $^{32}P/O$ values of 0 and 0.16 respectively. However, on oxidation of NAD^+-linked substrates, these mitochondria yield $^{32}P/O$ values of 1.4 consistent with values reported earlier (1). $^{32}P/O$ values greater than 1.0 with NAD^+-linked substrates for arsenate-treated mitochondria persist even in the presence of a glucose-hexokinase trap. Consistent with earlier reports (1), mitochondria treated with either P_i or arsenate plus P_i, synthesize ATP very well with all substrates. From these results we conclude that arsenate-treatment described here decreases preferentially the coupling capacity of rat liver mitochondria at sites 2 and 3 even after the bulk of the added arsenate has been removed (see "Methods" section).

Uncoupling at sites 2 and 3 due to arsenate-treatment likely occurs by a mechanism other than arsenolysis. Site 1 is still coupled in these mitochondria, whereas arsenolysis has been shown to uncouple all three sites (6-12). Besides, the presence of 5 mM P_i in the assay system for oxidative phosphorylation should effectively counteract any arsenolytic effect due to any arsenate that still remains in the mitochondria after the washing procedure (7-9, 11, 12).

The high ADP/O and $^{32}P/O$ values with NAD^+-linked substrates for arsenate-treated mitochondria under conditions that result in uncoupled oxidation with

Table I. Phosphorylation Coupled to the Oxidation of Various Substrates by Arsenate-treated Mitochondria

Treatment	Respiratory Substrate	$^{32}P/O$	ATP/O
As_i	Succinate	0.16	0
$As_i + P_i$	Succinate	1.48	1.30
P_i	Succinate	1.61	
As_i	3-Hydroxybutyrate	1.41	1.43
$As_i + P_i$	3-Hydroxybutyrate	2.44	
P_i	3-Hydroxybutyrate	2.45	
As_i	Glutamate + Malate + Malonate	1.39	1.48
$As_i + P_i$	Glutamate + Malate + Malonate	2.44	
P_i	Glutamate + Malate + Malonate	2.30	

Mitochondria (2 to 3 mg protein) were added to 1.5 ml of medium saturated with air at 28^0 and containing 0.23 M mannitol, 0.07 M sucrose, 0.02 M Tris-HCl, 0.02 mM EDTA and 5 mM P_i, all at a final pH of 7.2. The following substrates were used in individual experiments: 7 mM succinate plus 10 µM rotenone, 7 mM 3-hydroxybutyrate, and 7mM glutamate plus 1.4 mM malate plus 1.4 mM malonate. ATP and $^{32}P/O$ syntheses were monitored after addition of 96 nmoles ADP. The values are the mean of three independent measurements. As_i, stands for Na-arsenate. Other conditions were as described in Methods.

succinate and ascorbate plus TMPD can now be traced to an unusual property of NAD^+-linked substrates on energy conservation sites 2 and 3. The data presented in Table II indicate that following the addition of 3-hydroxybutyrate or glutamate in the presence of rotenone (or amytal), the oxidation of succinate and ascorbate by arsenate-treated mitochondria was associated with much higher ADP/O values and greatly increased respiratory control than those in which the NAD^+-linked substrates were omitted. Estimation of ATP/O as well as $^{32}P/O$ was consistent with an improvement of coupling capacity as revealed by the higher ADP/O values. It should be emphasized that arsenate-treated mitochondria did not synthesize any detectable ATP when exposed to NAD^+-linked substrates alone in presence of rotenone or amytal. At this stage we can only suggest that a component of the respiratory chain on the substrate side of the

Table II. Effect of NAD^+-linked Substrates plus Rotenone on ADP/O and RCR for Succinate and Ascorbate Oxidation by Arsenate-treated Mitochondria

Further Additions to System A or B	State 3 ngatom O/min/mg Protein	RCR	ADP/O
System A			
Glutamate	7	-	0
Succinate	102	1.0	0
Glutamate + Succinate	87	1.5	0.5
3-Hydroxybutyrate	0	-	0
Succinate	93	1.0	0
3-Hydroxybutyrate + Succinate	91	1.7	0.6
System B			
Ascorbate + TMPD	116	1.0	0
3-Hydroxybutyrate + Ascorbate + TMPD	108	1.2	0.3

System A contained 1 to 2 mg mitochondrial protein, 10 μM rotenone and 5 mM P_i. System B was identical to System A except 0.52 μM antimycin A was also present. Where indicated, the other additions were: respiratory substrates, 7 mM of each and TMPD, 100 μM. Abbreviations used are: RCR, respiratory control ratio; and TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine. Other conditions were described in Table I.

rotenone site can, in its reduced state, influence the coupling capacity of sites 2 and 3.

This concept would seem to be substantiated by the data reported in Table III. Inclusion of equimolar concentrations (2mM) of acetoacetate and 3-hydroxybutyrate in presence of rotenone decreased the $^{32}P/O$ obtained with succinate oxidation. This could, however, be counteracted by increasing the 3-hydroxybutyrate concentration to 8 mM, while keeping the acetoacetate concentration fixed at 2 mM. Data presented in Table III show that the oxidation-reduction states of NADH in arsenate-treated mitochondria are predictably altered by the imposed change of the acetoacetate/3-hydroxybutyrate ratio. The correlation

Table III. Effect of 3-Hydroxybutyrate and Acetoacetate plus Rotenone on the Coupling ability of Arsenate-treated Mitochondria Oxidizing Succinate

Further Additions to the Basic System	$^{32}P/O$	ATP/O	NAD^+-Reduced (%)
Succinate	0	0.1	15
3-Hydroxybutyrate (2mM) + Succinate	0.9	0.9	100
3-Hydroxybutyrate (2mM) + Acetoacetate (2mM) + Succinate	0.5	0.3	46
3-Hydroxybutyrate (8mM) + Acetoacetate (2mM) + Succinate	0.8	0.5	73

The basic system contained 2 to 3 mg mitochondrial protein; 10 μM rotenone and 5 mM P_i. Other additions were: succinate, 7 mM and ADP 250 nmoles. Assay conditions were as described in Table I and in Methods.

between extent of NAD^+ reduced and increased $^{32}P/O$ and ATP/O with succinate suggests that coupling of sites 2 and 3 is influenced by the level of reduction of NAD^+ or some other substance on the substrate side of the rotenone block.

Some observations in the literature suggest that reduction of components at one region can regulate the electron transport rate at steps located remotely from this region in mitochondria and submitochondrial particles. Gutman et al (13) observed that reduced coenzyme Q can activate succinic dehydrogenase in submitochondrial particles even in the presence of sufficient thenoyltrifluoro acetone to block electron transfer between the dehydrogenase and coenzyme Q. Likewise Foucher et al (14) observed that acetylcolletotrichin, a phytotoxic agent, inhibits succinate oxidation in rat liver mitochondria by a process that is reversed by uncoupling agents. Moreover, the addition of glutamate or malate in the presence of rotenone decreased the inhibition of succinate oxidation by this agent. The authors suggested that reduction of a component of the respiratory chain on the substrate side of the rotenone block might influence, by an allosteric mechanism, the ability of acetylcolletotrichin to in-

teract with a carrier at the cytochrome b-c level. The possibility exists that glutamate or 3-hydroxybutyrate plus rotenone could in a like manner reduce the affinity of a cytochrome component for a substance generated during respiration in the presence of arsenate. Definitive effects of arsenate-treatment as well as of conditions that restore coupling on the oxidation-reduction behavior of cytochromes b_K and b_T will be described in a subsequent communication. The results presented in this communication suggest that the reduction state of some specific components of the NADH dehydrogenase segment may have a similar relationship on the coupling process at sites 2 and 3 as well.

The exact mechanism of this unusual influence of NAD^+-linked substrate couple on energy coupling at sites 2 and 3 is yet unclear. Nevertheless, this phenomenon may explain the observations that arsenate-treated mitochondria produce ADP/O and $^{32}P/O$ values close to zero with succinate and ascorbate plus TMPD yet values of 1.3 to 1.6 with NAD^+-linked substrates.

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