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The Nature of Absorbance Changes following the Addition of ADP to Mitochondria: ATP Synthesis from Intramitochondrial Inorganic Phosphate

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Abstract

The transient absorbance increase induced by ADP in phosphate-loaded respiring mitochondria becomes stable and greatly amplified by inhibitors of phosphate transport. The absorbance changes are sensitive to oligomycin and to aurovertin and their extent is proportional to the amount of ADP added. Simultaneously with the ADP-dependent increase in absorbance the inorganic phosphate and K^+-ion content of the matrix decreases. It is concluded that the optical change reflects contraction of the matrix compartment secondary to intramitochondrial solute changes.

Introduction

Oxidative phosphorylation is catalyzed by the inner membrane of mitochondria. It was shown earlier by Packer [1, 2] that addition of inorganic phosphate and of ADP to respiring mitochondria resulted,

respectively, in decreased and increased light scattering and absorbance. These changes were considered to indicate alteration of the inner membrane structure associated with "membrane energization" or, according to Hackenbrock [3, 4] "mechanochemical ultrastructural transformation." The data presented herein leads to the conclusion that the increase of absorbance on ADP addition is due to shrinkage of the inner mitochondrial compartment, a secondary consequence of depletion of P_i within the matrix.*

Materials and Methods

Rat liver mitochondria prepared according to Johnson and Lardy [5] were used. Optical changes were followed by recording absorbance at 520 or 546 nm at room temperature using the Beckman DK2A spectrophotometer. Mitochondrial suspension, 50 µl, containing 50 or 60 mg protein/ml was diluted with 3 ml medium at pH 7.0, which contained 235 mM sucrose, 5 mM Tris-HCl, 1 µM rotenone, and either Na-EDTA (0.5 or 1 mM) or in some experiments with valinomycin, 3 mM $MgCl_2$. Potassium phosphate in concentrations between 1.6 and 16 mM, Tris- or sodium succinate (to 1.3 mM), ADP, mersalyl, NEM or DTNB, and (in some experiments) valinomycin (3.3 or 10 ng/mg protein) were added later during the recordings as indicated. (All aqueous solutions added were previously adjusted to pH 7.0.) When intramitochondrial ions were determined the movement of P_i was stopped by mersalyl, and the suspension was then rapidly chilled and centrifuged immediately at 4°C for 4 min at 15,000 × *g* (approximately). The tubes were drained by inversion, wiped dry from the adhering fluid, and the pellet was extracted with trichloroacetic acid. Phosphate was determined colorimetrically [6], potassium by flame photometry. For ATP determinations an aliquot from the spectrophotometer cell was deproteinized with trichloroacetic acid, centrifuged, the supernatant was neutralized immediately, and the ATP was determined enzymatically with the luciferin–luciferase assay system [7].

Results

In order to study oxidative phosphorylation and the effect of ADP on absorbance (with and without a functional system for transport of P_i) mitochondria were first preloaded aerobically with P_i. Respiring mitochondria take up potassium phosphate from the medium with simultaneous swelling indicated by a decrease of the apparent absorbance of the

* Abbreviations: P_i = inorganic phosphate, NEM = *N*-ethylmaleimide, DTNB = 5,5′-dithiobis-(2-nitrobenzoate).

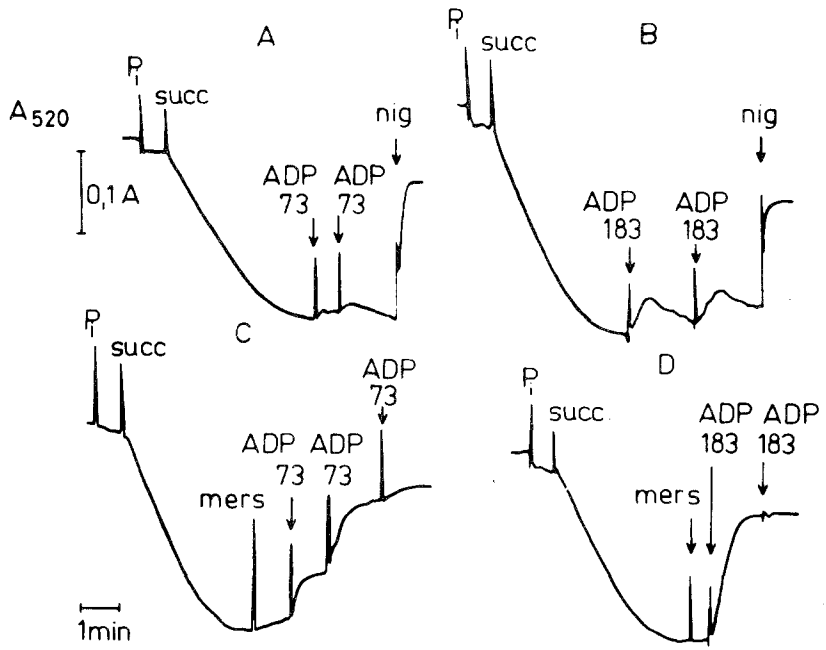

Figure 1. Absorbance changes of mitochondria on addition of ADP. The medium contained 1 mM EDTA. Additions: P_i, 16.6 mM potassium phosphate; succ., 1.3 mM Tris-succinate; mers., 13.3 µM mersalyl = 13.3 nmole/mg protein; ADP, Na-ADP, the number refers to micromolar final concentration; nig., 0.33 µg nigericin/ml.

suspension. The uptake requires either the presence of valinomycin (and in this case the potassium phosphate concentration of the medium may be lower than 2 mM) or, with no valinomycin added, a higher potassium phosphate concentration and the presence of EDTA. The swelling was dependent on the addition of a respiratory substrate (Fig. 1). It was inhibited by uncoupling agents, by nigericin, and by SH-group reagents which inhibit P_i transport, i.e., by mersalyl, NEM, or DTNB. After the swelling reached its maximum a steady state was established at pH 7.0; at higher pH oscillations occurred. The swelling was reversed by anaerobiosis or inhibitors of respiration, by uncouplers, and by nigericin: the reversal caused by any of these agents was prevented when any of the inhibitors of P_i transport was added at the maximum of swelling because accumulated P_i could not leave the mitochondria [8, 9].

ATP synthesis takes place with a theoretical P:O ratio even in the presence of valinomycin, provided the amount of valinomycin added and the K^+ concentration of the medium are below a certain level [10, 11] (see

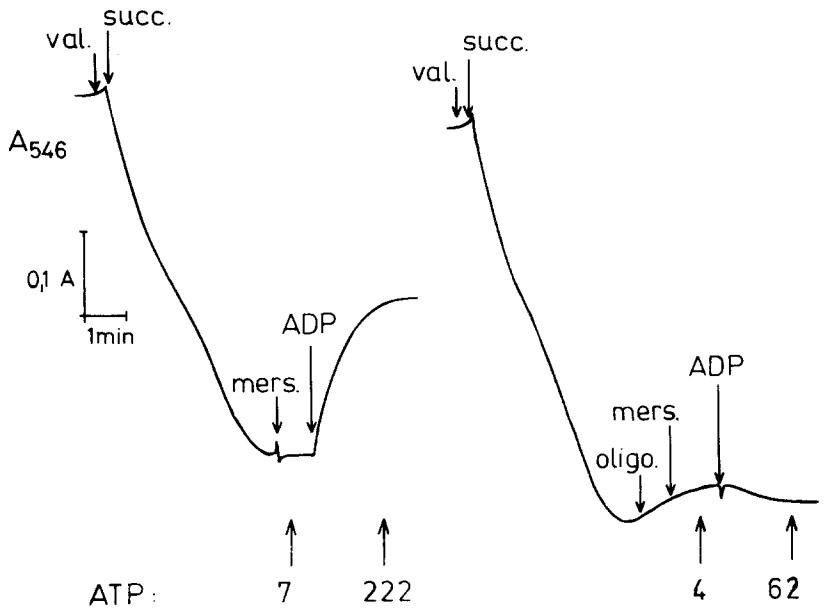

Figure 2. ATP synthesis during ADP-induced shrinkage: The effect of oligomycin. The medium contained 0.5 mM EDTA. Additions: 1 mM potassium phosphate; val., 3.3 ng valinomycin/ml (=3.3 ng/mg protein); succ., 1.3 mM sodium succinate; mers., 10 μM mersalyl (=10 nmole/mg protein); ADP, 300 μM Na-ADP (=330 nmole/mg protein); oligo., 1 μg oligomycin/ml (=1 μg/mg protein). The values for ATP are given as nanomoles per milligram of protein.

Fig. 2). Valinomycin uncouples oxidative phosphorylation only if the K^+ concentration of the medium is higher than in the experiments reported here and more valinomycin is added [10, 11, unpublished results]. In Fig. 2 it is shown that ATP synthesis did occur from added ADP and intramitochondrial P_i and the synthesis was sensitive to oligomycin. The residual synthesis of ATP in the presence of oligomycin was presumably due to the action of mitochondrial adenylate kinase, this latter not completely inhibited by 0.5 mM EDTA.

Addition of ADP at the maximum of swelling, i.e., when P_i uptake and efflux were in the steady state, caused a relatively small, temporary increase of absorbance, subsequently followed by a decrease to the former steady state level. These cycles could be repeated several times by successive additions of small amounts of ADP (Fig. 1). The ADP-induced cycles were completely prevented by inhibitors of oxidative phosphorylation, either oligomycin or aurovertin (not shown in the figures).

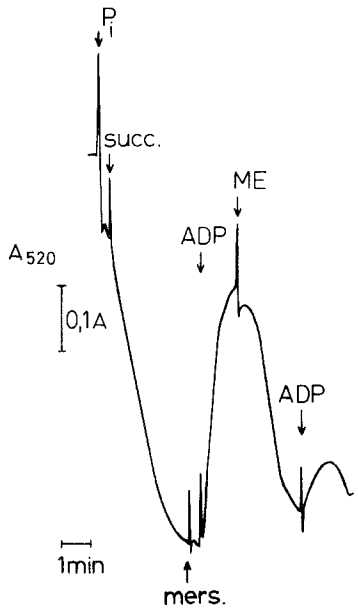

Figure 3. Effect of restoration of P_i transport on the absorbance of mitochondria. The medium contained 1 mM EDTA. Additions: P_i, 16.6 mM potassium phosphate; succ., 1.3 mM Tris-succinate; mers., 11.6 µM (=11.6 nmole/mg protein); ADP, 366 µM Na-ADP; ME, 660 µM 2-mercaptoethanol.

To inhibit P_i transport after P_i accumulation, the SH-group reagent mersalyl (in Figs 1–3), NEM, or DTNB (these latter not shown) were added in the steady state. Inhibition of P_i transport amplified greatly the optical response to ADP addition and abolished the subsequent decrease in absorbance seen in the absence of SH-group reagents. It was possible to obtain graded responses with repeated additions of small amounts of ADP. In such cases the optical changes were proportional to the ADP added and the effects of successive aliquots were additive until the absorbance had increased almost to its initial level (Fig. 1). This final level of absorbance was also reached following a single larger addition of ADP. However, even in the presence of P_i transport inhibitors, ADP-dependent absorbance changes were completely abolished by the prior addition of either oligomycin (Fig. 2) or by aurovertin (not shown).

The low molecular weight thiol compound, 2-mercaptoethanol, restored P_i transport and, when added following an ADP-induced absorbance change, immediately caused a decrease of absorbance to a level approaching that of the initial steady state (i.e., before the addition of

mersalyl). Subsequent addition of ADP then caused only a small and transient cycle of absorbance changes (Fig. 3).

It is noteworthy that under conditions corresponding to those in the experiments illustrated in Fig. 1, ADP caused no change in absorbance if the mitochondria accumulated arsenate in place of phosphate. This finding is in contrast to the results on corn mitochondria reported by Bertagnolli and Hanson [12], and can be interpreted to indicate that in the case of liver mitochondria, any ADP-arsenate formed coupled to respiration is rapidly hydrolyzed in the matrix, so that ADP addition causes no change in the free arsenate content of the intramitochondrial compartment.

The P_i content of the mitochondria decreased substantially when ADP was added after inhibition of P_i transport by mersalyl (Table I). Concomitantly the intramitochondrial potassium-ion content also decreased, the change being comparable to the decrease in the P_i content. In the experiment shown in Table I the addition of ADP in the presence of mersalyl caused (a) incorporation of intramitochondrial P_i into intramitochondrial ATP with a resulting decrease in matrix P_i and (b) the exchange of extramitochondrial ADP^{3-} against intramitochondrial ATP^{4-}, an electrogenic process. The resulting charge differences are probably compensated by the efflux of K^+ ions, the latter, as seen in Table I, corresponding to the disappearance of P_i.

The amount of ATP synthesized in the experiment shown in Fig. 2 was greater than the amount of P_i that disappeared from the matrix in Table I.

TABLE I. Inorganic phosphate and K^+-ion content of mitochondria: Effect of ADP after inhibition of P_i transport [a]

Sequence of additions	P_i (nmole/mg protein)	K (ng ions/mg protein)
20 μM mersalyl	26.1	36.6
1.3 mM succinate, 20 μM mersalyl	63.6	109.3
1.3 mM succinate, 20 μM mersalyl, 183 μM ADP	26.6	65.6

[a] Mitochondria (3 mg protein) were incubated in the spectrophotometer cell in 3 ml of medium of composition 235 mM sucrose, 5 mM Tris-HCl, 3 mM $MgCl_2$, and 1 μM rotenone at pH 7.0. The absorbance was recorded: potassium phosphate (to a final concentration of 1.6 mM), 30 ng valinomycin (=10 ng/mg protein), and the additions indicated in the table were then successively added. (Concentrations quoted are the final ones in the reaction mixture; 20 μM mersalyl=20 nmole/mg protein.) When the change of absorbance, following the final addition in each case, became complete the suspension was transferred to a centrifuge tube, chilled, and treated further as described under Materials and Methods.

However, the conditions were different in the two types of experiments. The amount of the ions accumulated and therefore the amplitude of the resulting swelling depend on the amount of valinomycin added and also on the presence or absence of Mg^{2+} ions (unpublished results, manuscript in preparation). In the absence of Mg^{2+} less valinomycin is required to give a certain level of ion uptake than in its presence. In the experiment shown in Fig. 2 no Mg^{2+} was present, but 0.5 mM EDTA was added to decrease the adenylate kinase activity: 3.3 ng/mg protein valinomycin caused much larger swelling ($\sim$0.4 *A*) than 10 ng/mg valinomycin added in the presence of 3 mM $MgCl_2$ in the experiment of Table I ($\sim$0.2 *A*). This might explain the discrepancy between the two types of experiments.

We attribute the absorbance change following ADP addition to "shrinkage" of the mitochondria caused by partial depletion of intramitochondrial (matrix) P_i. This depletion of matrix P_i would be expected to occur under conditions in which its uptake from the external medium cannot keep pace with its utilization in ATP synthesis. The absorbance changes following ADP addition are therefore attributable simply to transient or (in the presence of mersalyl) permanent reversal of the osmotic swelling of the matrix space associated with the aerobic accumulation of P_i. Electron microscopical data support this conclusion [13; Fonyó, Hajós, and Csillag, manuscript in preparation].

Discussion

There are two different types of ADP-induced absorbance changes occurring in mitochondria. The first of these is that recorded in the present experiments and which was previously attributed to change in "membrane energization" or "membrane conformation" [1–4]. Our data confirm the experimental observations of Packer and Hackenbrock. However, on the basis of additional experiments reported herein we conclude that the increase of absorbance following ADP addition in the experiments just quoted is attributable to partial depletion of P_i within the matrix. A similar conclusion was apparently reached, on the basis of similar experiments, by Munn and Blair, although full details of this work have yet to be published [14].

An apparently different phenomenon is that attributed to conformational changes of the adenine nucleotide carrier of the membrane [15–17]. In these latter experiments ADP-dependent absorbance changes persisted in the presence of oligomycin and uncouplers. The complete sensitivity of the absorbance change in our experiments to oligomycin and to aurovertin rules out the participation of the adenine nucleotide carrier and its reaction with ADP in the optical changes recorded.

It is also apparent from our data that oxidative phosphorylation can occur even if the P_i carrier of the membrane is inhibited, provided sufficient P_i is already present in the matrix. Similar observations have been previously reported for corn mitochondria by Hanson et al. [18]. The phosphorylation system within the inner membrane has thus direct access to the P_i within the matrix and no carrier-mediated transport is required from the matrix to the ATP synthetase. Accordingly the synthetase is asymmetrically shielded from its cosubstrate, P_i, by the inner membrane structure.

McGivan et al. [19] suggested that the exchange between extramitochondrial ADP and intramitochondrial ATP is linked to simultaneous P_i transport in order to compensate for the electrogenicity of the adenine nucleotide exchange. This is probably the mechanism of charge compensation under conditions in which the K^+-ion permeability of the membrane is severely restricted. However, when the membrane is made artificially permeable for K^+ ions, either by EDTA or by valinomycin, K^+-ion movement may effectively compensate the difference of charge between ADP^{3-} and ATP^{4-}. We have observed oxidative phosphorylation of extra mitochondrial ADP when the transport of the latter could not be linked to transport of P_i and the electroneutrality was maintained by movement of other ions such as potassium.

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