ENDOGENOUS PHOSPHATE-DEPENDENT PHOSPHORYLATION OF ADP AND OLIGOMYCIN-SENSITIVE ADP–ATP EXCHANGE

A quantitative assessment of their activity in inner membrane matrix particles from rat liver mitochondria

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1. Introduction

Two ADP–ATP exchange reactions, identified in mitochondria, are differentiated on the basis of their sensitivity to oligomycin and dinitrophenol. The first reaction, which is oligomycin- and dinitrophenol-sensitive, seems to be closely associated with the mechanism of energy coupling in oxidative phosphorylation [1–11]. The other, which is oligomycin- and dinitrophenol-insensitive, is catalyzed by an NDP kinase [8–10], external to the inner mitochondrial membrane. Although much experimental evidence for participation of ADP–ATP exchange in oxidative phosphorylation has been reported, conflicting data remain which deserve attention. For instance, it is puzzling that coupled beef heart submitochondrial particles which catalyze Pi–ATP exchange and phosphorylate at three sites do not catalyze a dinitrophenol-sensitive ADP–ATP exchange [12] and that the soluble ATPase which behaves as a coupling factor in oxidative phosphorylation is practically devoid of ADP–ATP exchange activity [13]. It has been argued [10, 11] that detection of ADP–ATP exchange activity requires the structural integrity of highly ordered complexes present in the inner mitochondrial membrane.

The most crucial problem, as suggested by Guillory and Slater [5], is to distinguish between the Pi-dependent phosphorylation of ADP into ATP and the oligomycin-sensitive ADP–ATP exchange. Thus, the effect of added Pi has been investigated [3, 4, 7, 10]. By using inner membrane-matrix particles prepared from rat liver mitochondria, Pedersen and Schnaitman [10] showed that the mitochondrial oligomycin-sensitive ADP–ATP exchange is not affected by Pi at concentrations below 4 mM. This finding was considered to rule out a direct participation of Pi in the oligomycin-sensitive ADP–ATP exchange. However, the possible function of intramitochondrial Pi was not examined. Pi assays carried out in this laboratory on inner membrane-matrix particles similar to those used by Pedersen and Schnaitman [10], yielded values as high as 60 to 100 nmoles of Pi per mg protein. Thus, the present paper describes experiments to detect whether ADP phosphorylation dependent on endogenous Pi could occur when mitochondrial particles are incubated under the assay conditions of the ADP–ATP exchange reaction. The results indicate that the oligomycin-sensitive incorporation of ^{14}C-ADP into ATP (which is the basis of the assay for the ADP–ATP exchange) may be accounted for by direct phosphorylation of ADP into ATP coupled to oxidation of residual endogenous substrates.

2. Materials and methods

^{14}C-ADP was obtained from Schwarz Laboratories, $^{32}P_{\alpha}$-ATP and ^{32}P-phosphate from the Commissariat à l'Energie Atomique, Saclay. Digitonin (A grade)

Table 1

Distribution of ^{32}P and ^{14}C in ATP, ADP and AMP after incubation of mitochondrial inner membrane-matrix particles with ^{14}C-ATP, $^{32}P_{\alpha}$-ATP and ^{32}P-phosphate.

Incubation time	additions	Pi	$^{32}P_{\alpha}$-ATP	$^{32}P_{\alpha}$-ADP	$^{32}P_{\alpha}$-AMP	$^{32}P_{\gamma}$-ATP	^{14}C-ATP	^{14}C-ADP	^{14}C-AMP
(min)		(μmoles)	(%)	(%)	(%)	(nmoles)	(nmoles)	(nmoles)	(nmoles)
	EDTA	0.19	95	5	< 1	19	6	30	2
0.5	EDTA + oligo.	0.18	95	5	< 1	1	2	35	1
	EDTA + FCCP	0.32	82	17	< 1	< 1	< 1	37	< 1
	$MgCl_2$	0.24	95	4	1	5	23	16	< 1
0.5	$MgCl_2$ + oligo.	0.24	98	2	< 1	1	26	15	< 1
	$MgCl_2$ + FCCP	0.28	91	8	1	–	24	15	< 1
	EDTA	0.25	91	8	1	34	8	28	1
1	EDTA + oligo.	0.21	94	5	1	1	1	37	1
	EDTA + FCCP	0.46	64	35	1	2	< 1	38	< 1
	$MgCl_2$	0.28	90	9	1	9	27	13	< 1
1	$MgCl_2$	0.23	96	4	< 1	1	28	11	< 1
	$MgCl_2$	0.35	84	14	2	4	26	15	< 1
	EDTA	0.31	83	15	2	92	14	22	2
3	EDTA + oligo.	0.23	91	8	1	1	3	32	2
	EDTA + FCCP	0.69	28	72	< 1	2	< 1	40	< 1
	$MgCl_2$	0.32	82	16	2	17	28	12	< 1
2	$MgCl_2$ + oligo.	0.25	96	3	1	2	29	10	< 1
	$MgCl_2$ + FCCP	0.43	74	22	4	4	22	16	< 1

The incubation medium contained 0.038 mM ^{14}C-ADP 200,000 cpm, 0.612 mM $^{32}P_{\alpha}$-ATP 240,000 cpm, 0.10 mM ^{32}P-phosphate 240,000 cpm, 100 mM triethanolamine buffer pH 7.7, 2 mg bovine serum albumin and 2 mg of inner membrane-matrix particles in a final volume of 1 ml. Where indicated 1 mM EDTA, 5 mM $MgCl_2$, 10 μg oligomycin and 10 μM FCCP were included in the assay. Incubation was performed at 25°. The reactions were stopped by the addition of 0.1 ml of 30% trichloroacetic acid. After centrifugation, aliquots of supernatant were spotted on paper chromatography for separation of adenine nucleotides as described in Methods. The mean average value of total inorganic phosphate (intra- and extramitochondrial) assayed at zero time and at the end of the incubation period was used to calculate the amount of ^{32}P-phosphate incorporated into ATP. Since the amount of endogenous ATP or ADP found in inner membrane-matrix particles (less than 2 nmoles per mg protein) is much less than the amount of added ADP or ATP, calculation of ^{14}C-ADP incorporated into ATP was essentially based on the value of the specific radioactivity of the added ^{14}C-ADP.

was obtained from Calbiochem, oligomycin from Sigma Chemical Company, nucleotides from PL-Biochemicals. FCCP (carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone) was a gift from Dr. P.G. Heytler.

Digitonin inner membrane-matrix particles were prepared from rat liver mitochondria as described by Schnaitman and Greenawalt [14]. These particles were chosen in preference to mitochondria since they are practically devoid of adenylate kinase and nucleoside diphosphokinase and are able to catalyze oxidative phosphorylation efficiently [14 and personal results].

Conditions for the assay of oxidative phosphorylation are detailed in the legend to tables 1 and 2. Pi, AMP, ADP and ATP in 0.05 ml aliquots of trichloroacetic extracts were separated by paper chromatography as described by Krebs and Hems [15]. Nucleotide spots were located under U.V. light and the respective area cut out. They were placed on aluminium planchets and their radioactivity was counted with a low background gas flow counter. The use of ^{32}P- and ^{14}C-labelled compounds required double isotope analysis. The low ^{14}C energy was eliminated nearly 99% from the higher ^{32}P energy by covering the spots with a thin plastic filter. Distribution of

Table 2
Effect of cyanide on the ATP–Pi exchange and ^{14}C-ADP incorporation into ATP catalyzed by mitochondrial inner membrane-matrix particles.

Additions	$^{32}P\gamma$-ATP (nmoles)	^{14}C-ATP (nmoles)	^{14}C-ADP (nmoles)	^{14}C-AMP (nmoles)
None	52	11	39	1
KCN	21	< 0.1	49	1
$MgCl_2$	24	13	31	3
$MgCl_2$ + KCN	19	15	30	4

The incubation medium contained 0.054 mM ^{14}C-ADP 200,000 cpm, 0.598 mM $^{32}P_\alpha$-ATP 400,000 cpm, 0.10 mM ^{32}P-phosphate 300,000 cpm, 100 mM triethanolamine buffer pH 7.7, 2 mg bovine serum albumin and 2 mg of inner membrane-matrix particles in a final volume of 1 ml. Where indicated 1 mM KCN and 1.5 mM $MgCl_2$ were included in the assay. Incubation was for 5 min at 25°. All other conditions as in table 1.

^{32}P into the P_α, P_β and P_γ atoms of ^{32}P-ATP was determined as follows: an aliquot of 0.2 ml of trichloroacetic extract was spotted on a line (5 cm) on Whatmann no 1 paper. After separation by chromatography [15], the area corresponding to ATP was cut out and ATP eluted with water. The eluate (concentrated by freeze-drying) was incubated with 10 mM glucose, 0.5 mg hexokinase and 10 mM tris-buffer pH 7.8, for 10 min at 30° (volume 0.1 ml). Enzymatic action was stopped by adding 20 µl of 30% (w/w) trichloroacetic acid. After centrifugation, 50 µl of supernatant was used to separate [15] ADP from glucose-6-phosphate formed in the hexokinase reaction. The radioactivity of glucose-6-phosphate corresponds to that of the P_γ atom of ATP. The remaining ADP was labelled in the P_α atom since it originates from $^{32}P_\alpha$-ATP. The labelling of P_β of ADP was assayed by incubation of the eluate with adenylate kinase and 1 mM $MgCl_2$ in the presence of 0.1 M triethanolamine, pH 8. The resulting ATP and AMP were separated by paper chromatography [15] and their ^{32}P-radioactivity determined: in the experiments reported, the radioactivity of ATP was virtually the same as that of AMP, a result which excludes a significant labelling of the P_β of ATP. The same assay performed on ADP initially present in the trichloroacetic extracts gave negligible labelling in P_β. For these reasons, results concerning the ^{32}P labelling were expressed with reference only to the P_α of ATP, ADP and AMP and to the P_γ of ATP.

Pi was estimated by the method of Fiske and Subba Row [16]. Protein was determined by the biuret reaction with bovine serum albumin as standard.

3. Results and discussion

Digitonin inner membrane-matrix particles prepared from rat liver mitochondria were incubated at 25° in a medium supplemented with ^{14}C-ADP, ^{32}P-ATP and trace amounts of ^{32}P-phosphate (100 nmoles). Under these conditions, added ^{32}P-phosphate equilibrated in a few seconds with the inorganic phosphate present in the inner membrane matrix particles (70 nmoles/mg protein) whereas extramitochondrial ^{14}C-ADP and ^{32}P-ATP equilibrated in about one minute with intramitochondrial ADP and ATP (unpublished results).

The following reactions are expected to occur:

$$^{14}\text{C-ADP} + {}^{32}P_\alpha\text{-ATP} \rightleftarrows {}^{14}\text{C-ATP} + {}^{32}P_\alpha\text{-ADP} \quad (1)$$

(NDP kinase or ADP–ATP exchange enzyme)

$$2\text{-}{}^{14}\text{C-ADP} \rightleftarrows {}^{14}\text{C-ATP} + {}^{14}\text{C-AMP} \quad (2)$$

(Adenylate kinase)

$$^{32}P_\alpha\text{-ATP} \longrightarrow {}^{32}P_\alpha\text{-ADP} + \text{Pi} \quad (3)$$

(ATPase)

$$^{14}\text{C-ADP} + {}^{32}\text{Pi} \longrightarrow {}^{14}\text{C},{}^{32}P_\gamma\text{-ATP} \quad (4a)$$

$$^{32}P_\alpha\text{-ADP} + {}^{32}\text{Pi} \longrightarrow {}^{32}P_{\alpha,\gamma}\text{-ATP} \quad (4b)$$

(oxidative phosphorylation)

The relative contribution of each reaction can be assessed by studying the distribution of ^{14}C and ^{32}P in the different labelled products. For instance,

adenylate kinase activity will lead to an accumulation of ^{14}C-AMP and ADP–ATP exchange to an accumulation of ^{14}C-ATP exceeding that of $^{32}P_{\gamma}$-ATP. On the other hand, the distribution pattern of $^{32}P_{\alpha}$-ATP, $^{32}P_{\alpha}$-ADP, $^{32}P_{\alpha}$-AMP will give an indication of ATPase activity of the preparation. As already mentioned in Methods, the P_{β} labelling of ATP and ADP remains negligible, a result which excludes significant contamination by adenylate kinase (reaction 2), and which is confirmed by the low amount of ^{14}C-AMP formed.

As shown in table 1, *in the case of an EDTA-supplemented medium,* accumulation of ^{32}P- and ^{14}C-ATP is inhibited by oligomycin and FCCP and is time-dependent. The amount of accumulated $^{32}P_{\gamma}$-ATP is 3 to 6 times higher than that of ^{14}C-ATP, which suggests that reaction 4b predominates reactions 4a and 1. For initiation, reaction 4b must be coupled to reaction 3, the net results of the two coupled reactions being an ATP–Pi exchange.

$$^{32}P_{\alpha}\text{-ATP} + {}^{32}\text{Pi} \rightleftarrows {}^{32}P_{\alpha,\gamma}\text{-ATP}$$

In other words, ATP–Pi exchange proceeds more readily than ^{14}C-ATP accumulation.

Accumulation of ^{14}C-ATP may occur either via ^{14}C-ADP–ATP exchange or via phosphorylation of ^{14}C-ADP coupled to oxidation of endogenous substrates. Addition of 1 mM cyanide to inner membrane-matrix particles incubated in an EDTA-supplemented medium totally inhibits ^{14}C-ATP accumulation, but only partially inhibits ATP-^{32}Pi exchange (table 2). Since endogenous oxidizable substrates remain in digitonin particles [17], the inhibitory effect of cyanide supports the alternative that ^{14}C-ATP accumulation is mainly due to oxidative phosphorylation of ^{14}C-ADP. The partial inhibition by cyanide of ATP-^{32}Pi exchange and its total inhibition of ^{14}C-ADP phosphorylation fully support the contention that ATP–Pi exchange is a partial reaction of oxidative phosphorylation [18] which is only partially dependent on the oxidation–reduction state of the respiratory carriers [17–20] whereas the oxidative phosphorylation of ^{14}C-ADP is per se entirely dependent on substrate oxidation.

When EDTA is replaced by $MgCl_2$ incorporation of ^{32}Pi into ATP is much slower than that of ^{14}C-ADP (table 1). On the other hand, the Mg^{2+}-dependent incorporation of ^{14}C-ADP into ATP is insensitive to oligomycin, FCCP (table 1) and cyanide (table 2), and is therefore not related to oxidative phosphorylation. It is probably a reflection of a Mg^{2+}-dependent NDP kinase which is external to the inner mitochondrial membrane and may be bound to contaminant fragments of outer mitochondrial membrane [21–24], since its activity is inhibited by EDTA, which does not enter the inner mitochondrial membrane [25].

The lack of a clearly demonstrable oligomycin-sensitive ADP–ATP exchange activity in these experiments is puzzling since inner membrane-matrix particles catalyze an ATP–Pi exchange reaction in which ADP appears to be a necessary intermediate. This is tentatively explained by compartmentalization of ADP formed from ATP during the ATP–Pi exchange. Such compartmentalization may be accounted for by ADP-binding to F_1 factor as suggested by Zalkin et al. [12] to explain the lack of oligomycin-sensitive ADP–ATP exchange in well-coupled beef heart submitochondrial particles.

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