Binding of the intramitochondrial ADP and its relationship to adenine nucleotide translocation

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Received 15 April 1982

1. INTRODUCTION

It is well established that the main source of energy production in all eukaryotic cells is oxidative phosphorylation. This process which converts ADP and P_i to ATP is located within the mitochondrial matrix; hence the synthesis of ATP from cytosolic ADP is linked to a transport reaction in which ADP is moved inwards and the newly synthesized ATP is moved outwards. Transport of the adenine nucleotides involves, what is now known to be, a 1:1 exchange catalysed by a transmembrane protein, the adenine nucleotide translocase (reviews [1–3]). Early studies showed that if ADP was added to suspensions of mitochondria which were actively phosphorylating, the extramitochondrial [ATP]/[ADP] could exceed the intramitochondrial ratio by a substantial amount. This was interpreted as indicating that translocation of adenine nucleotides normally takes place against the chemical potential gradient. The energy thought to be required for this process has been attributed to translocase-catalysed electrogenic exchange of ATP^{4-} against ADP^{3-} which couples the exchange to the trans-mitochondrial membrane potential [1,2,4].

Unfortunately values of intramitochondrial [ATP]/[ADP] measured under conditions producing maximal phosphorylation of extramitochondrial ADP (state 4) differ markedly from laboratory to laboratory (table 1 and citations therein). Even more importantly, little is known about the extent to which intramitochondrial adenine nucleotides are attached to internal binding sites or how far the thermodynamically relevant ratio of the free nucleotides may differ from that calculated from the measured total ATP and ADP. Without this information the energetics of nucleotide translocation and its role in the overall process of oxidative phosphorylation remains unknown.

Here, we have addressed these two points and shall present evidence that, contrary to the currently accepted views, the intramitochondrial free adenine nucleotide ratio, $[ATP]_f/[ADP]_f$, may be actually equal to or greater than the extramitochondrial [ATP]/[ADP]. If this is generally the case, it will necessitate a re-evaluation of the role that adenine nucleotide translocase may play in mitochondrial oxidative phosphorylation.

2. METHODS

2.1. *Isolation of mitochondria*

Mitochondria were isolated from livers of 24-h starved rats in 0.225 M mannitol, 0.075 M sucrose, 0.2 mM EDTA (pH 7.2) medium essentially as in [5]. For experiments with detergents, the mitochondria were subjected to an additional (third) wash in 0.1 M KCl, 0.02 M Tris–Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 6.8) medium. The final pellet was suspended in the same medium at 100 mg protein/ml.

2.2. *Incubation conditions*

These are described in detail in the legends to tables 1 and 3.

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2.3. *Analytical measurements*

ATP and creatine phosphate were measured in the same sample as in [6] and [7], respectively, and ADP and creatine as in [8] and [9], respectively. Adenine, guanine and uridine nucleoside di- and triphosphates were determined in the same neutralized perchloric acid (PCA) extracts with high-pressure liquid chromatography (HPLC, Perkin-Elmer, series 2 liquid chromatograph). Chromatographic separations were carried out on an EM Regents' Hibar EC-RT 125 mm × 4.0 mm column packed with 5 μM Lichrosorb NH_2. Nucleotide concentrations were determined by relative peak areas, using a Perkin-Elmer LC-75 spectophotometric detector (254 nm) that was coupled to a Hewlett Packard 3390A reporting integrator. A monobasic ammonium phosphate (Fisher Scientific) binary gradient (pH 3.1) was used consisting of solutions: (A) 0.025 M $NH_4H_2PO_4$; (B) 0.7 M $NH_4H_2PO_4$. Initially 10%B/90%A was run isocratically at a flow rate of 1.5 ml/min. After 5 min, gradient elution was initiated and the % of B was increased at 5%/min until 100% B was attained. Typical retention times for nucleoside di- and triphosphates were 9.4 min ADP, 10.9 min UDP, 12.8 GDP, 18.8 min ATP, 19.7 min UTP and 22.1 min GTP.

2.4. *Protein*

Protein was determined by the biuret reaction [10] with bovine serum albumin as a standard.

2.5. *Evaluation of the relationship of $[ATP]_t/[ADP]_t$ to $[ATP]_f/[ADP]_f$*

Binding of the adenine nucleotides was evaluated by comparing the measured $[ATP]_t/[ADP]_t$ to that calculated from equilibration with nucleotide substrates for which binding should be minimal. The mitochondria were lysed with detergents to remove diffusional barriers and then supplemented with enzymes catalyzing reactions of the type:

$$A{-}P + ADP \rightleftharpoons ATP + A$$

where the equilibrium constant is:

$$K = \frac{[A]\,[ATP]}{[A{-}P]\,[ADP]}$$

If the reactants A and A–P are not bound in significant amounts, at equilibrium, $[ATP]_f/[ADP]_f$ can be calculated from [A]/[A–P] and K. Here, two enzymes were used, creatine phosphokinase (CPK) and nucleoside diphosphate kinase (NDPK). The equilibrium constants for the reactions catalyzed by these enzymes were independently measured in the mitochondrial lysate. The values for the equilibrium constants were 109 ± 16 (± SD) for CPK and 1.0 for NDPK at pH 7.0 and 22°C; in the pH range employed in this work only the former constant is pH dependent [11,12]. The measured equilibrium constant for CPK is in good agreement with literature values for this temperature and our estimated $[Mg^{2+}]_f$ of 0.5–2.0 mM [11,12].

3. RESULTS

3.1. *The intramitochondiral $[ATP]/_t[ADP]_t$ in respiring suspensions of rat liver mitochondria*

Table 1 shows the calculated [ATP]/[ADP] in quenched, neutralized suspensions of respiring

Table 1

Phosphorylation values of intramitochondrial adenine nucleotides during oxidative phosphorylation by isolated rat liver mitochondria

Ref.	Substrate	$[P_i]_e$	$[ATP]_i/[ADP]_i$
[13]	succ. + rot.	1.45	2.8
[14]	succ. + rot.	10	4
[15]	glut. + mal.	5	4.8
[16]	glut. + mal.	12	2.7
		2	1.28
[17]	glut. + mal.	2	4
[4]	succ. + rot.	0.48	3.9
This work	glut. + mal.	3	8–12

Experimental conditions for the data reported in this work were as follows. Mitochondria suspended in mannitol, sucrose, EDTA medium at ~80 mg protein/ml were diluted 4-fold with 0.1 M KCl, 20 mM Tris–Hepes buffer (pH 7.2) and supplemented with 10 mM glutamate + malate and 3 mM phosphate. After 3–5 min aerobic incubation with constant shaking, 1.8 ml samples were withdrawn and rapidly injected into 0.7 ml stirred perchloric acid solution maintained at −6°C. The final concentration of perchloric acid was 6%. The quenched mixtures were centrifuged to remove the precipitated proteins and the supernatant neutralized with a mixture of potassium carbonate and triethanolamine base (legend of table 2). ATP and ADP were measured in the neutralized extracts by both enzymatic assays and HPLC

freshly prepared rat liver mitochondria with malate + glutamate as the substrate. The total adenine nucleotide content was 12 nmol/mg protein and the $[ATP]_t/[ADP]_t$ ratios were 8–12 at 3 mM extracellular phosphate. The mean [ATP]/[ADP] value (± SD) measured by enzymatic analysis was 9.39 ± 1.22 (n = 16) in excellent agreement with that of 9.45 ± 1.0 (n = 12) obtained by HPLC.

Table 1 gives some representative [ATP]/[ADP] ratios. (Since it has been observed by Kunz et al. [18] and by ourselves that the intramitochondrial [ATP]/[ADP] ratios are dependent on extramitochondrial $[P_i]$, concentrations of the latter are included in table 1.) It is interesting to note that the values range from 1.2–12, even at very similar concentrations of P_i. It should be stressed that in our experiments in which high values were observed, the utmost care was taken to optimize quenching conditions: the volume of the quenching mixture was relatively large, the flasks containing the quench solution were precooled to – 6°C and the samples were injected rapidly with energetic agitation.

3.2. *The effect of nucleotide binding on the relationship between $[ATP]_t/[ADP]_t$ and $[ATP]_f/[ADP]_f$ in the mitochondrial matrix*

Preparations of mitochondria at 50–80 mg protein/ml (final conc.) were lysed by detergents (cf. legend to table 2) and supplemented with CPK, NDPK and oligomycin. The reaction was started by addition of creatine phosphate (CrP), UDP and GDP, conditions which lead to very rapid synthesis of ATP from the endogenous adenine nucleotide pool and, in turn, synthesis of GTP and UTP from the added GDP and UDP. The activities of CPK and NDPK were sufficiently high that maximal synthesis of ATP, UTP and GTP was attained in < 30 s. The triphosphates and creatine phosphate were then slowly hydrolized over 25–50 min, depending on the preparation, due to the presence of a low level of oligomycin-insensitive ATPase activity. When aliquots of the incubation mixture were quenched at various time intervals and assayed for CrP, creatine (Cr), ATP, ADP, GTP, GDP, UTP and UDP the pattern shown in table 2 was observed. It can be seen that at all [CrP]/[Cr] values the

Table 2

Interrelationships of the creatine phosphokinase and nucleoside diphosphate kinase metabolites in detergent lysates of rat liver mitochondria

No.	Time (s)	CrP (mM)	Cr (mM)	CrP/Cr	Adenine nucleotides			Uridine nucleotides			Guanine nucleotides		
					ATP (mM)	ADP (mM)	ATP/ADP	UTP (mM)	UDP (mM)	UTP/UDP	GTP (mM)	GDP (mM)	GTP/GDP
1	150	5.56	5.70	0.975	1.20	0.066	18.2	1.42	0.026	54.6	1.90	0.036	52.8
2	300	4.28	6.81	0.63	1.16	0.063	18.4	1.40	0.032	43.7	1.90	0.043	44.2
3	400	3.59	8.67	0.41	1.20	0.075	16.0	1.44	0.045	32.0	2.02	0.060	33.7
4	500	2.96	8.67	0.34	1.15	0.095	12.1	1.37	0.053	25.8	1.93	0.072	26.8
5	600	2.25	9.73	0.23	1.19	0.106	11.2	1.40	0.075	18.7	1.94	0.112	17.3
6	700	1.60	9.86	0.16	1.09	0.123	8.86	1.28	0.098	13.1	1.69	0.128	13.4
7	800	1.28	10.22	0.13	1.05	0.159	6.60	1.23	0.128	9.60	1.64	0.168	9.8

Mitochondria were suspended at 80–100 mg protein/ml in 0.150 M KCl, 0.02 M Tris–Hepes medium and incubated for 3 min at 25°C to equilibrate the temperature. The suspension was supplemented with 10% Triton and 10% sodium deoxycholate to give a final concentration of each detergent of 1%. The incubation mixture was then supplied with magnesium 5.8 mM; oligomycin, 0.2 mg; nucleoside diphosphate kinase 400 units; GDP and UDP (solid) ~2 mM each; creatine phosphokinase, 600 units and creatine phosphate, 12 mM. The reaction was started by the addition of the nucleoside diphosphates and the pH measured (pH 7.26). At times indicated 300 μl samples were withdrawn and rapidly diluted 5-fold into a stirred, 8% solution of perchloric acid maintained at – 6°C. The precipitated protein was separated by centrifugation and the supernatants neutralized carefully with a mixture of 3 M K_2CO_3 and 0.5 M triethanolamine base. Concentrations of ATP, ADP creatine phosphate and creatine were determined within 2 h

[GTP]/[GDP] and [UTP]/[UDP] ratios were the same, within experimental error, but significantly higher than the [ATP]/[ADP] ratios. The apparent equilibrium constant for the CPK-catalyzed reaction calculated from the guanine and uridine nucleotides was found to be 74.0 ± 9.1 (table 3), a value very close to the reported equilibrium constant for this pH and temperature [11,12]. In contrast, the mass action ratio calculated from the adenine nucleotides was less than the equilibrium constant by a factor of 4.0 when the [CrP]/[Cr] ratio was near 1.0 and this factor fell to 1.2 when the [CrP]/[Cr] decreased to 0.13.

Fig.1 summarizes the results from 8 independent expt. carried out under similar conditions. All data points with similar [CrP]/[Cr] ratios were combined and subjected to statistical analysis. The resulting curve is drawn through the points representing means ± SEM for the number of data points indicated. The curve shows an upward trend so that the differences between K_{eq} and K_{app} become greater at higher [CrP]/[Cr] ratios and smaller at their lower values.

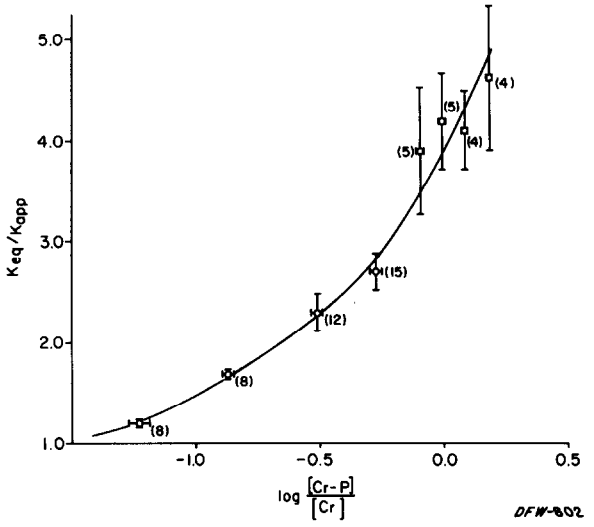

Fig.1. Dependence of the measured K_{app} for creatine phosphokinase on the [CrP]/[Cr] ratio. The data from 8 independent expt. of the type shown in table 2 were collected in groups with similar [CrP]/[Cr] ratios. These values ± SEM were then plotted as K_{eq}/K_{app} *vs* log [CrP]/[Cr]

Table 3

The apparent equilibrium constant for creatine phosphokinase and nucleoside diphosphate in the presence of mitochondrial lysate

No.	K_{app}			K_{eq}/K_{app}	
	CPK AN	NDPK UN/AN	NDPK GN/AN	NDPK	CPK
1	18.7	0.32	0.33	3.1	3.96
2	29.3	0.42	0.42	2.4	2.53
3	39.0	0.5	0.47	2.1	1.90
4	35.6	0.47	0.45	2.2	2.08
5	48.8	0.60	0.65	1.6	1.52
6	55.3	0.68	0.66	1.5	1.34
7	50.7	0.69	0.67	1.5	1.46

The equilibrium constant for creatine phosphokinase was calculated from the uridine and guanine nucleotides to be 74.0±9.1 (± SD). The equilibrium constant for nucleoside diphosphate kinase was found to be 1.0 in the presence and absence of mitochondrial lysate. Data taken from table 2

4. DISCUSSION

The observation that the intramitochondrial $[ATP]_t/[ADP]_t$ ratio is between 8–12 under conditions of maximal ATP synthesis represents a significant increase in this value to previous reports. Nevertheless, 8–12 is markedly lower than the maximal extramitochondrial [ATP]/[ADP] of 100–200 attained under the same conditions. However, it is possible that these high values measured by us are still lower than the true intramitochondrial $[ATP]_t/[ADP]_t$ because ATP hydrolysis in a dense suspension of intramitochondrial proteins is faster than the quenching process.

The measurements of the CPK and NDPK equilibria in suspensions of detergentlyzed mitochondria reported in this work provide a first approximation of the effect of binding on the intramitochondrial adenine nucleotide pool. The data show that the measured mass action ratios for these enzymes, written in the direction of ATP synthesis, are less than the true equilibrium constants. In the case of CPK (fig.1), the mass action ratio becomes progressively smaller relative to the equilibrium constant as the [CrP]/[Cr] increases. When the latter

has attained a value of 1.58, equivalent to an $[ATP]_f/[ADP]_f$ of 109 at pH 7.2, the mass action ratio is less than K_{eq} by 4.5-fold and the measured $[ATP]_t/[ADP]_t$ is < 20. The observed relationships are consistent with ADP being bound to a site which is present at $\sim 0.4-1.0$ nmol/mg protein and which binds ADP with $K_d < 10\ \mu M$ and ATP with a $K_d \geq$ 100-times that for ADP. This is not an unreasonable number of binding sites as both the adenine nucleotide translocase and ATP synthase are present each at ~ 0.4 nmol/mg protein. Moreover, adenine nucleotide translocase has been reported to bind ADP with $K_d = 1-2\ \mu M$ and ATP with $K_d \geq 50\ \mu M$ [1,2].

In view of the untercainty concerning the effectiveness of current quenching techniques, it is not yet possible to quantitate the extent to which the 'excess' ADP measured by $[ATP]_t/[ADP]_t$ may be due to ATP hydrolysis during quenching and how much may be caused by its preferential binding. However, these data strongly suggest that the intramitochondrial $[ATP]_f/[ADP]_f$ is much larger than the intramitochondrial $[ATP]_t/[ADP]_t$ and may well equal or even exceed the extramitochondrial [ATP]/[ADP]. If this is true, the translocation of ADP into and ATP out of the mitochondria may occur down rather than up its chemical activity gradient.

It is evident that a re-evaluation of mitochondrial adenine nucleotide translocation is needed in order to clarify its energetics and answer the questions raised above. Moreover, the preferential binding of ADP by the mitochondrial contents, presumably proteins, casts doubt on any interpretation of results based on total ATP and ADP content, including the kinetics of adenine nucleotide translocation.

ACKNOWLEDGEMENT

This work was supported by grant GM12202 from the United States of America Public Health Service.

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