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Reversal of oxidative phosphorylation in submitochondrial particles using glucose 6-phosphate and hexokinase as an ATP regenerating system

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Soluble F_1-ATPase and submitochondrial particles are able to bind and to hydrolyze ATP formed from the reversal of the reaction catalyzed by hexokinase:

glucose 6-phosphate + ADP ⇌ ATP + glucose ($K_{eq} = 5 \times 10^{-4}$)

During steady-state, the P_i released in the medium is derived from glucose-6-phosphate which continuously regenerates the ATP hydrolyzed. A membrane potential ($\Delta\Psi$) can be built up in submitochondrial particles using glucose-6-phosphate and hexokinase as an ATP-regenerating system. The energy derived from the membrane potential thus formed, can be used to promote the energy-dependent transhydrogenation from NADH to $NADP^+$ and the uphill electron transfer from succinate to NAD^+. In spite of the large differences in the energies of hydrolysis of ATP (ΔG° = −7.0 to −9.0 kcal/mol) and of glucose-6-phosphate (ΔG° = −2.5 kcal/mol), the same ratio between P_i production and either NADPH or NADH formation were measured regardless of whether millimolar concentrations of ATP or a mixture of ADP, glucose-6-phosphate and hexokinase were used. Rat liver mitochondria were able to accumulate Ca^{2+} when incubated in a medium containing hexokinase, ADP and glucose-6-phosphate. The different reaction measured with the use of glucose-6-phosphate and hexokinase were inhibited by glucose concentrations varying from 0.2 to 2 mM. Glucose shifts the equilibrium of the reaction towards glucose-6-phosphate formation thus leading to a decrease of the ATP concentration in the medium.

Submitochondrial particle; Membrane potential; Glucose 6-phosphate; Transhydrogenation; $NADP^+$; NADH

1. INTRODUCTION

In aqueous solutions the standard free energy of hydrolysis of ATP varies between −7 and −9 kcal/mol. It is generally accepted that in metabolic processes, ATP is regenerated by phosphate compounds having a standard free energy of hydrolysis higher than that of ATP itself, such as creatine phosphate (ΔG° = −10 kcal/mol).

creatine phosphate + ADP ⇌ creatine + ATP
(K_{eq} 2 × 10^2)

The standard free energy of hydrolysis of glucose-6-phosphate (ΔG° = −2.5 kcal/mol) is smaller than that of ATP [1–4]. Therefore it is not readily apparent that the reaction catalyzed by hexokinase:

glucose-6-phosphate + ADP ⇌ ATP + glucose
($K_{eq} = 5 \times 10^{-4}$)

may play a role in regeneration of ATP from ADP in biological systems, because after the reaction reaches equilibrium, the concentration of ATP formed is too low.

In this report it is shown that soluble F_1 and submitochondrial particles can use glucose-6-phosphate and hexokinase as an ATP-regenerating system. The K_a for ATP binding at the catalytic site of these enzymes is 10^{-12} M [5,6]. This affinity for ATP is sufficiently high to permit the formation of the enzyme–substrate complex in the presence of very small concentrations of ATP formed from ADP and glucose-6-phosphate. After each catalytic cycle, the ATP hydrolyzed by the F_1-ATPase and submitochondrial particles is rephosphorylated by glucose-6-phosphate in order to maintain the equilibrium concentration of ATP. Thus, submitochondrial particles are able to both form a membrane potential and to promote the uphill electron transfer from succinate to NAD^+ at the expense of glucose-6-phosphate cleavage.

2. MATERIALS AND METHODS

Submitochondrial particles and soluble F_1 ATPase without inhibitory protein were prepared from bovine heart mitochondria as previ-

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ously described [7–9]. The submitochondrial particles were suspended in 0.25 M sucrose solution and stored at −70°C.

ATPase activity and hydrolysis of glucose-6-phosphate were assayed measuring the $^{32}P_i$ produced from [γ-^{32}P]ATP and [^{32}P]glucose-6-phosphate with ammonium molybdate and a mixture of isobutyl alcohol and benzene [10].

Membrane potential was monitored by fluorescence quenching using 1–3 μM oxonol-V and 20 μg/ml of submitochondrial particles protein. Excitation and emission wavelength were 580 and 628 nm, respectively [11,12].

The transhydrogenation from NADH to NADP$^+$ and electron transfer from succinate to NAD$^+$ were assayed as previously described [13–16]. The extinction coefficient used for NADPH and NADH absorbance at 340 nm was 6,220 $M^{-1} \cdot cm^{-1}$.

Ca^{2+} uptake by liver mitochondria was measured with the use of ^{45}Ca and Millipore filters [10]. Rat liver mitochondria were freshly prepared before measurements [17].

Hexokinase (ATP:D-hexose-6-phosphotransferase; EC 2.7.1.1), sulphate free, lyophilized powder from bakers' yeast and 400 units/mg protein, creatine kinase (EC 2.7.3.2), salt free, from rabbit muscle and alcohol dehydrogenase (EC 1.1.1.1) from bakers' yeast were purchased from Sigma.

3. RESULTS AND DISCUSSION

3.1. *Cleavage of glucose 6-phosphate*

Soluble F_1-ATPase catalyzed the cleavage of glucose-6-phosphate when both hexokinase and ADP were included in the assay medium (Fig. 1). There was no measurable cleavage when one of these two components was omitted from the assay medium. The rate of cleavage varied depending on the concentrations of hexokinase, glucose-6-phosphate and of ADP used (Fig. 1). The same results were obtained when submitochondrial particles were used (data not shown).

The equilibrium concentration of ATP which can be formed from glucose-6-phosphate and ADP decreased when glucose was added to the medium [2,18]. Accordingly, increasing concentrations of glucose inhibit the cleavage of glucose-6-phosphate promoted by both the soluble F_1-ATPase and by submitochondrial particles (Fig. 2). Glucose had no effect on the rate of P_i production when 3 mM ATP was used as substrate (Fig. 2).

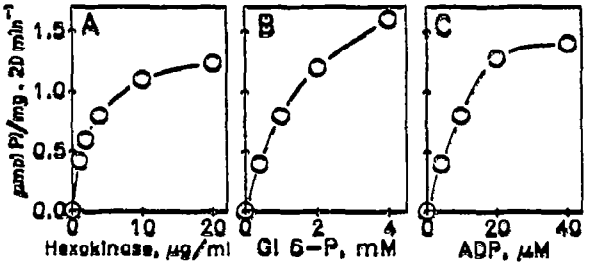

Fig. 1. Cleavage of glucose-6-phosphate by soluble F_1 ATPase. The assay medium composition was 50 mM MOPS-Tris buffer pH 6.0, 1 mM $MgCl_2$, 0.03 mg/ml soluble F_1 and either (A) 0.04 mM ADP, 2 mM [^{32}P]glucose-6-phosphate and the concentrations of hexokinase shown in the figure; (B) 20 μg/ml hexokinase, 20 μM ADP and the concentrations of [^{32}P]glucose-6-phosphate shown in the figure; or (C) 20 μg/ml hexokinase, 2 mM [^{32}P]glucose-6-phosphate and the concentrations of ADP shown in the figure. The reaction was started by the addition of soluble F_1 and quenched after 20 min at 35°C with a 20% TCA solution containing 2 mM P_i.

The findings of Figs. 1 and 2 indicated that the true substrate used by F_1-ATPase and by submitochondrial particles was the very low ATP concentration available in the medium and that the radioactive P_i released into the medium was derived from radioactive glucose-6-phosphate which continuously regenerates the ATP hydrolyzed.

3.2. *pH and Mg^{2+} dependence*

In the presence of 3 mM ATP, the ATPase activity of both soluble F_1-ATPase and of submitochondrial particles increased as the pH of the medium was raised from 6.0 to 9.0. A different pH profile was observed when glucose-6-phosphate and hexokinase were used as an ATP-regenerating system. In this case the activity was maximal in the pH range of 6.0–7.0 and decreased abruptly when the pH was raised from 7.0 to 8.0. The concentration of ATP which can be obtained after the ATP-regenerating reaction reaches equilibrium, will vary depending on the pH value of the medium [2–4]. Thus, the pH dependence observed with the use of glucose-6-phosphate is probably not related to an effect on the mitochondrial ATPase, but rather to the concentrations of ATP in the medium.

At pH 7.0, the maximal rate of glucose-6-phosphate cleavage was obtained in the presence of 0.4 mM $MgCl_2$. With the use of 3 mM ATP, the maximal rate of hydrolysis was observed in the presence of 2 mM $MgCl_2$ both at pH 7.0 and 8.0.

3.3. *Membrane potential*

The experiments in Fig. 3 show that the energy derived from the steady-state hydrolysis of glucose-6-phosphate was sufficient to form and to maintain a membrane potential in submitochondrial particles similar to that observed with the use of millimolar concentrations of ATP. In the presence of hexokinase, the addition of glucose-6-phosphate and ADP promoted a quenching of oxonol-V fluorescence indicating that a

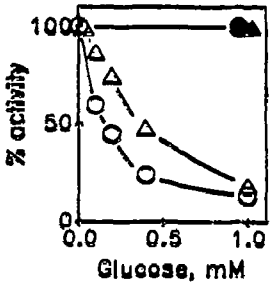

Fig. 2. Inhibition of glucose-6-phosphate hydrolysis by glucose. The assay medium composition was 50 mM MOPS-Tris buffer pH 6.5, 2 mM $MgCl_2$ and either (●,▲) 3 mM ATP or (○,△) 0.1 mM ADP, 0.05 mg hexokinase/ml and either 2 mM (○) or 4 mM (△) [^{32}P]glucose-6-phosphate. The reaction was performed at 35°C and it was started by the addition of either soluble F_1 (○,●) to a final concentration of 10 μg/ml, or by the addition of submitochondrial particles (△,▲) to a final concentration of 0.9 mg/ml. In the figure 100% activity corresponds to (○) 0.1, (△) 0.012, (●) 10.6 and (▲) 0.17 μmol P_i/mg protein · min^{-1}.

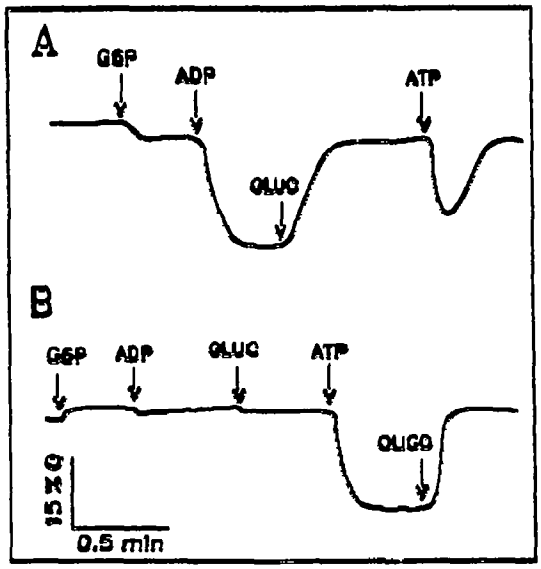

Fig. 3. Formation of membrane potential in submitochondrial particles. The assay medium composition was 50 mM MOPS-Tris pH 6.5, 0.4 mM $MgCl_2$, 2 μM Oxonol-V and 20 μg/ml of submitochondrial particles. In (A) 30 μg/ml of hexokinase was included in the assay medium. In (B) hexokinase was not added. Arrows indicate the additions of (G6P) 10 mM glucose-6-phosphate; (ADP) 20 μM ADP; (Gluc) 20 mM glucose; (ATP) 0.4 mM ATP; and (oligo) 2 μg/ml oligomycin. The assay was performed at room temperature.

membrane potential was formed across the submitochondrial particles membrane [11,12]. The membrane potential formed with the use of ADP and glucose-6-phosphate was only observed in the presence of hexokinase (compare Fig. 3A,B) and was abolished when glucose was added to the medium. In the conditions of Fig. 3A, the addition of ATP promoted a transient formation of a membrane potential. Immediately after its addition, ATP is used by both the submitochondrial particles and by hexokinase to form glucose-6-phosphate. In the subsequent incubation intervals, all the added ATP is converted into glucose-6-phosphate and the equilibrium concentration of ATP remaining in the medium is much smaller than that needed to maintain the potential due to the excess of glucose available in the medium. In absence of hexokinase, the potential formed by added ATP is sustained during several minutes, both in the presence (Fig. 3B) or in the absence (data not shown) of glucose. The membrane potential formed by either added millimolar ATP concentrations (Fig. 3B) or by the small ATP concentration formed from ADP and glucose-6-phosphate (data not shown) are both impaired by oligomycin, an antibiotic that inhibits the mitochondrial F_1-ATPase. The glucose-6-phosphate, ADP and the pH dependence for the formation of the membrane potential are essentially the same as those observed for glucose-6-phosphate hydrolysis (data not shown). The adenylate kinase inhibitor P^1,P^5-di(adenosine 5')pentaphosphate, up to a concentration of 0.1 mM, has no effect on either the hydrolysis of glucose-6-phosphate or formation of the membrane potential in submitochondrial particles (data not shown), thus excluding a possible interference of adenylate kinase in the experiments shown in Figs. 1–3.

3.4. *ATP dependence for the formation of the membrane potential*

This was found to be the same regardless of the ATP regenerating system used (Fig. 4). With the use of glucose-6-phosphate the ratio ADP/ATP in the medium varied from 5 (Fig. 4B) up to 330 (Fig. 4A) while with the use of creatine phosphate, practically all the nucleotides of the medium were in the form of ATP. The apparent K_m for ATP was found to vary from 10 to 16 μM regardless of the ATP-regenerating system used. These data indicate that the formation of the membrane potential is not impaired by the excess of ADP found in the medium when glucose-6-phosphate is used. Fig. 4A shows that the membrane potential and the rate of

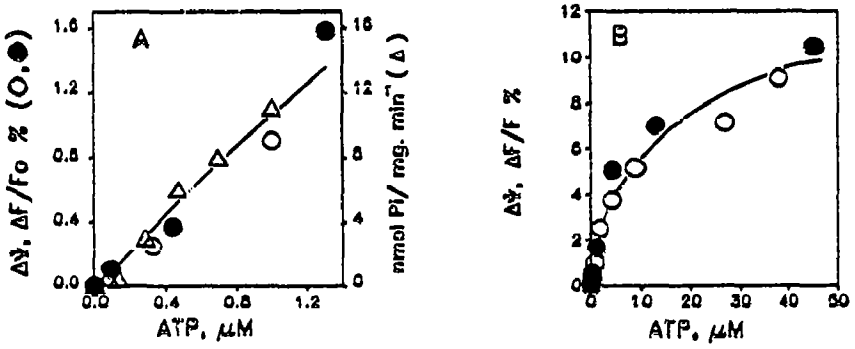

Fig. 4. ATP dependence for membrane potential formation. The assay medium composition was 50 mM MOPS-Tris buffer pH 6.8, 0.1 mM P^1,P^5-di(adenosine 5')pentaphosphate, 2 μM Oxonol-V, 20 μg SMP/ ml and (●) 1 mM $MgCl_2$, 2 mM creatine phosphate, 30 μg/creatine kinase and ADP concentrations varying from 0.09 up to 46 μM; (○) 0.4 mM $MgCl_2$, 0.1 mM (A) or 0.2 mM (B) ADP and glucose-6- phosphate concentrations varying from 0.5 mM up to 35 mM (A and B); (Δ) as in (○) but using [^{32}P]glucose-6-phosphate. With the use of glucose-6-phosphate, the ATP concentration available during the steady state was calculated as described in [14]. For creatine phosphate, the ATP concentrations in the medium were the same as those of ADP added.

glucose-6-phosphate cleavage increased to the same extent as the concentration of ATP available in the medium was raised from 0.3 up to 1.0 μM.

3.5. *Utilization of the energy derived from the membrane potential*

This can be studied in submitochondrial particles measuring the energy-dependent transhydrogenation from NADH to NADP$^+$ and the uphill electron transfer from succinate to NAD$^+$ [13–16]. The ΔG° of these two reactions are 5.7 and 16.2 kcal/mol, respectively. Both reactions were observed when submitochondrial particles were incubated in media containing either 0.1 mM ATP or a mixture containing hexokinase, ADP and glucose-6-phosphate (Table I). Under these later conditions, there was no formation of NADPH or of NADH, if hexokinase or glucose-6-phosphate were not included in the assay medium. The formation of NADPH and of NADH were impaired by oligomycin, regardless of whether 0.1 mM ATP or a mixture of ADP and glucose-6-phosphate were used as substrate. Glucose impaired the reduction reactions measured in presence of hexokinase and glucose-6-phosphate (Table I) but had no effect when 0.1 mM ATP was used. An intriguing finding was that in spite of the large differences in the energies of hydrolysis of ATP and glucose-6-phosphate, the ratio between the rates of P_i production and that of either NADPH or NADH were practically the same when measured using either 0.1 mM ATP or the glucose-6-phosphate system (Table I).

3.6. *Ca^{2+} uptake*

Intact mitochondria isolated from rat liver are able to accumulate Ca^{2+} using the proton gradient formed during hydrolysis of ATP [19,20]. A small, but significant uptake of Ca^{2+} was measured in presence of ADP, glucose-6-phosphate and hexokinase (Table II).

The large difference between the amount of Ca^{2+} accumulated in the presence of 1 mM ATP and that measured using glucose-6-phosphate (Table II) is perhaps related to the translocation of ATP from the assay medium to the mitochondrial matrix. In submitochondrial particles, the F_1-ATPase faces the outer surface of the vesicles and is in direct contact with the ATP available in the assay media. In intact mitochondria, the ATP in solution must cross the mitochondrial membrane in order to reach the ATPase, using the ADP/ATP carrier [21,22]. With the use of hexokinase, the large ADP/ATP ratio in the medium does not favor the translocation of ATP into the mitochondrial matrix. Thus, it is probable that during steady state, the concentration of ATP inside the mitochondria would be smaller than that found in the assay medium.

4. CONCLUSION

With the use of glucose-6-phosphate and hexokinase as an ATP-regenerating system, the concentrations of ATP and ADP remain constant during the steady state and the work done by the ATPase of submitochondrial particles to form and to maintain a membrane potential,

Table I

Energy-dependent transhydrogenation from NADH to NADP$^+$ and uphill electron transfer from succinate to NAD$^+$

	(n)	Rates, nmol/mg prot · min^{-1}		Ratio A/B
		A P_i production	B reduction	
(A) Transhydrogenation				
ATP	5	162.4 ± 20.8	95.5 ± 8.1	1.7 ± 0.1
Glucose-6-P + ADP	7	13.5 ± 2.6	16.9 ± 2.7	0.9 ± 0.2
Glucose-6-P + ADP + glucose	7	0	0	--
(B) Electron transfer from succinate to NAD$^+$				
ATP	3	49.1 ± 8.1	6.8 ± 0.4	7.2 ± 0.9
Glucose-6-P + ADP	3	71.9 ± 3.7	11.5 ± 1.3	6.6 ± 0.8
Glucose-6-P + ADP + glucose	3	0	0	-

For the transhydrogenation reaction (K_{eq} = 6.7 × 10^{-5}; ΔG° = 5.7 kcal/mol) the assay medium composition was 50 m M MOPS-Tris buffer pH 7.0, 2 mM $MgCl_2$, 15 μM rotenone, 258 mM ethanol, 0.1 mg/ml alcohol dehydrogenase, 1 mM NADP$^+$, 0.05 mM NADH, 0.2 mM P^1, P^5-di(adenosine-5') pentaphosphate, 0.2 mg/ml submitochondrial particles protein and either 0.1 mM ATP or 0.1 mM ADP, 10 mM glucose-6-phosphate and 30 μg hexokinase/ml. When used, the glucose concentration was 10 mM. This mixture was used in the two cuvettes of the spectrophotometer. The reaction was started by adding to the test cuvette either ATP or glucose-6-phosphate. For the reaction for the electron transfer from succinate to NAD$^+$ (K_{eq} = 1.2 × 10^{-12}, ΔG° = 16.2 kcal/mol) the assay medium composition was 50 mM MOPS-Tris buffer pH 7.0, 125 mM KCl, 2 mM $MgSO_4$, 1.6 mM KCN, 1 mM NAD$^+$, 5 mM succinate, 0.1 mM P^1, P^5-di(adenosine-5') pentaphosphate, 0.2 mg/ml submitochondrial particle protein and either 0.1 mM ATP or 0.1 mM ADP, 10 mM glucose-6-phosphate and 30 μg hexokinase/ml. When used, the glucose concentration was 10 mM. The reaction was started by the addition of either ATP or glucose-6-phosphate. The rates of P_i production were measured in parallel experiments using either [γ-^{32}P]ATP or [^{32}P]glucose-6-phosphate. The values shown in the table are means ± S.E. of the number of experiments shown in parentheses.

Table II

Ca^{2+} uptake in intact mitochondria

Additions	nmol Ca^{2+}/mg prot.
(1) ATP	26.8 ± 1.1 (5)
ATP + oligomycin	1.1 ± 0.9 (3)
(2) ADP	0.4 ± 0.1 (5)
ADP, glucose-6-P, hexokinase	3.3 ± 0.3 (7)
ADP, glucose-6-P, hexokinase, glucose	0.5 ± 0.1 (6)
ADP, glucose-6-P, hexokinase, oligomycin	0.5 ± 0.1 (5)

The assay medium composition was 10 mM HEPES/Tris buffer pH 7.0, 130 mM KCl, 2 mM P_i, 2 mM $MgCl_2$, 0.06 mM $^{45}CaCl_2$, 5 μM rotenone, 0.2 mM P^1, P^5-di(adenosine-5') pentaphosphate, and the additions shown in the Table to the final concentrations of: ATP, 1 mM; oligomycin, 10 μg/ml; ADP, 0.1 mM; glucose-6-phosphate, 10 mM; hexokinase, 30 μg/ml and glucose, 20 mM. The reaction was started by the addition of mitochondria, to a final concentrations of 1 mg/ml and filtered after 1, 2 and 4 min. The filters were washed 3 times with 10 ml samples of 130 mM KCl and counted in a scintillation counter. Maximal value of Ca^{2+} uptake were attained after the first minute incubation and remained constant during the subsequent incubation intervals. The values shown in the table are means ± S.E. of the number of experiments shown in parentheses.

is coupled to a decrease of the glucose-6-phosphate concentration, a compound which has a **lower** energy of hydrolysis than ATP. This is an unusual thermodynamic situation, different from that observed with the use of creatine kinase, where work is coupled with the decrease of phosphocreatine concentration, a compound which has a **higher** energy of hydrolysis than ATP. In a recent report [18], a similar situation was described for the Ca^{2+}-ATPase of sarcoplasmic reticulum. This raises the possibility that under steady-state conditions, different enzymes involved in energy transduction, may use phosphate compounds of low energy of hydrolysis to perform the work which is usually associated to the consumption of ATP. The use of glucose-6-phosphate and hexokinase as an ATP-regenerating system is limited to ATPases with a K_m for ATP smaller than 0.05 mM. Enzymes with a lower affinity for ATP cannot use this ATP regenerating system because they cannot form an enzyme–substrate complex in the presence of the very small concentration of ATP formed from ADP and glucose-6-phosphate [18].

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