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Kinetics of Nucleotide Transport in Rat Heart Mitochondria Studied by a Rapid Filtration Technique†

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ABSTRACT: A rapid filtration technique has been used to measure at room temperature the kinetics of ADP and ATP transport in rat heart mitochondria in the millisecond time range. Transport was stopped by cessation of the nucleotide supply, without the use of a transport inhibitor, thus avoiding any quenching delay. The mitochondria were preincubated for 30 s either in isotonic KCl containing succinate, $MgCl_2$, and P_i (medium P) or in isotonic KCl supplemented only with EDTA and Tris (medium K); they were referred to as energized and resting mitochondria, respectively. The kinetics of [^{14}C]ADP transport in energized mitochondria were apparently monophasic. The plateau value for [^{14}C]ADP uptake reached 4-5 nmol of nucleotide·(mg of protein)$^{-1}$. V_{max} values for [^{14}C]ADP transport of 400-450 nmol exchanged·min^{-1}·(mg of protein)$^{-1}$ with K_m values of the order of 13-15 μM were calculated, consistent with rates of phosphorylation in the presence of succinate of 320-400 nmol of ATP formed·min^{-1}·(mg of protein)$^{-1}$. The rate of transport of [^{14}C]ATP in energized mitochondria was 5-10 times lower than that of [^{14}C]ADP. Upon uncoupling, the rate of [^{14}C]ATP uptake was enhanced, and that of [^{14}C]ADP uptake was decreased. However, the two rates did not equalize, indicating that transport was not exclusively electrogenic. Transport of [^{14}C]ADP and [^{14}C]ATP by resting mitochondria followed biphasic kinetics. These consisted of a rapid nucleotide uptake of about 350 nmol·min^{-1}·(mg of protein)$^{-1}$, lasting for about 1 s, up to 1.0-1.2 nmol of [^{14}C]nucleotide taken up·(mg of protein)$^{-1}$, followed by a slow phase leading to a plateau value of 4-5 nmol of [^{14}C]nucleotide·(mg of protein)$^{-1}$, which was attained in 1 min. Depletion of nucleotides in resting mitochondria resulted in a greater decrease in the extent of the slow phase than of the rapid one. In addition, about half of the nucleotides taken up at the end of the rapid phase were not discharged into the medium upon addition of carboxyatractyloside. This suggested that matricial nucleotides are compartmentalized in two pools which are exchangeable at different rates with external nucleotides.

The kinetic data so far reported for the transport of adenine nucleotides by the mitochondrial ADP/ATP carrier have been obtained from "inhibitor-stop" experiments, using the specific inhibitors atractyloside (ATR)[1] or carboxyatractyloside (CATR) when transport was assayed with mitochondria (Vignais et al, 1985; Klingenberg, 1985) and bongkrekic acid (BA) in the case of inverted submitochondrial particles (Lauquin et al., 1977). The temperature was routinely lowered to 0-5 °C to slow down transport. A rapid filtration method has been recently developed (Dupont, 1984) which allows measurement of transport at room temperature in the millisecond time range without the use of an inhibitor to stop the reaction. This technique is based on perfusion of immobilized particles with a solution of labeled substrate and termination of transport by cessation of the perfusion. It has been successfully used to determine the initial rates of Ca^{2+} binding and transport in sarcoplasmic reticulum (Dupont, 1984) and of P_i transport in liver mitochondria (Ligeti et al., 1985). In the present work, we have applied the rapid filtration method to the study of the rapid kinetics of ADP/ATP transport in rat heart mitochondria at room temperature under different metabolic conditions. Some of the results suggest the existence of microcompartmentation of mitochondrial adenine nucleotides.

Experimental Procedures

Materials. Cellulose nitrate filters (0.8 μm, AAWP) were from Millipore. [^{14}C]Nucleotides and [^{3}H]dextran were from Amersham. Tritiated ATR and BA were synthesized as previously described [see Vignais et al. (1985)]. All other reagents were of the highest purity commercially available.

Methods. Rat heart mitochondria were prepared according to the method of Mela and Steitz (1979). Their protein content was determined by the biuret method (Gornall et al., 1949).

Prior to the transport step, the rat heart mitochondria were incubated at room temperature at a concentration of 1 mg/mL, either for 30 s in 125 mM KCl, 10 mM NaP_i, 5 mM $MgCl_2$, and 10 mM sodium succinate, pH 7.3 (medium P),

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[1] Abbreviations: ATR, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; Tris, tris(hydroxymethyl)aminomethane; MOPS, 4-morpholinepropanesulfonic acid; TCA, trichloroacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene; PPO, 2,5-diphenyloxazole.

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or for 15 min in 125 mM KCl, 10 mM Tris, and 1 mM EDTA, pH 7.3 (medium K). Because of the presence of an oxidizable substrate in medium P, these mitochondria were referred to as "energized mitochondria", whereas mitochondria incubated in medium K were called "resting mitochondria".

Time-resolved measurements of ADP transport were carried out with the rapid filtration system described by Dupont (1984) and produced by Bio-Logic Co (Z. A. du Rondeau, 38130 Echirolles, France). Unless indicated, all measurements were carried out at 22–23 °C. The basic principle of the rapid filtration technique is to deliver the substrate to be transported to particles adsorbed onto a nitrocellulose filter. The filter was perfused for a preset period of time with a solution containing the substrate at a fixed concentration, at a rate high enough to keep constant the concentration of the substrate. When transport was measured over periods of time lower than 2 s, the rate of perfusion was 4 mL/s. To spare radioactive ligands when perfusion was performed for more than 2 s, the rate of perfusion was adjusted to values between 4 and 1 mL/s. It was checked that lowering the rate of perfusion had no effect on the amount of ligand taken up. Transport was stopped by cessation of substrate supply and not by the addition of an inhibitor. Consequently, there is no lag due to mixing of reagents and no quenching delay. For each transport measurement, mitochondria (1 mg of protein) were immobilized on a nitrocellulose filter (Millipore AAWP, 0.8 μm) placed on a filter holder below an injection device that contained medium P or medium K with the substrate to be transported, namely, [^{14}C]ADP or [^{14}C]ATP. At zero time, the filter was applied against the injection device and perfused with the substrate medium for a preset period ranging from 10 ms to 10 s. At the end of the transport step, the filter holder was separated from the injection device. The filter on which the mitochondria were retained was removed and dissolved in a scintillation fluid composed of 100 g of naphthalene, 6 g of PPO, and 0.3 g of POPOP per liter of a dioxane/ethanol mixture (95/5, v/v). The radioactivity incorporated into mitochondria was counted in an Intertechnique SL30 scintillation spectrometer. The amount of nonspecifically bound radioactivity was estimated from assays performed with mitochondria preincubated with 10 μM CATR for 1 min. For correction of the background radioactivity retained in the filters, use was made of a nonpermeant marker such as tritiated dextran included in the transport medium. Only the measurements in which the radioactivity incorporated into mitochondria amounted to at least 30% more than the background radioactivity were considered. When transport was assayed over periods of time longer than 10 s, the perfusion of adsorbed mitochondria was performed by manual supply of substrate solution.

The kinetics of binding of ATR or BA to mitochondria were measured with a rapid filtration system in which the injection device was filled with [^{3}H]ATR in medium K or with [^{3}H]BA in a medium consisting of 125 mM KCl, 10 mM MOPS, and 1 mM EDTA, final pH 6.7. In the case of [^{3}H]BA binding, the medium was supplemented with 2 μM ADP, as ADP facilitates the binding of [^{3}H]BA (Lauquin & Vignais, 1976). In order to avoid any limitation of binding due to a lack in the amount of inhibitor supplied during perfusion of mitochondria, care was taken to provide the inhibitor in excess relative to the number of binding sites of the ADP/ATP carrier. The nonspecific binding and the background radioactivity were both estimated from parallel binding experiments using mitochondria incubated with 20 μM CATR for 1 min at room temperature.

The adenine nucleotide content of mitochondria was assayed in neutralized perchloric extracts by the luciferin–luciferase method (ATP monitoring kit, LKB) with an LKB 1250 luminometer. The rate of oxidative phosphorylation of ADP was measured from the incorporation of [^{32}P]P_i in [^{32}P]ATP [cf. Vignais et al. (1975)] or from the phosphorylation of [^{14}C]ADP into [^{14}C]ATP. Mitochondria (50 μL, 50 μg of protein) were added to 50 μL of a phosphorylating medium, pH 7.3, containing 20 mM NaP_i, 20 mM succinate, 10 mM $MgCl_2$, 125 mM KCl, and 20 μM [^{14}C]ADP (10^5 dpm/nmol). The reaction was allowed to proceed at 22 °C for different periods of time and then stopped by the addition of TCA (10% final concentration). Samples were immediately centrifuged at 0 °C, and supernatants were analyzed for their nucleotide contents. Separation of adenine nucleotides was performed by TLC on silica plates (Merck) according to Bronnikov and Zakharov (1983).

The protonmotive force developed by respiring mitochondria consisted mainly of a membrane potential, $\Delta\Psi$, since the pH gradient was largely abolished by the transport of P_i present in the medium (Nicholls, 1982). $\Delta\Psi$ was qualitatively assessed by following the fluorescence intensity of the potential probe, 3,3′-dipropylthiodicarbocyanine, and quantitatively determined by the amount of [^{14}C]tetraphenylphosphonium (TPP^+) incorporated into mitochondria after correction for nonspecifically bound radioactivity.

Results

Control Assays. Controls similar to those used in a previous work on rat liver mitochondria (Ligeti et al., 1985) were carried out on rat heart mitochondria. These controls showed that 95% of the mitochondria deposited onto the nitrocellulose filter (0.8 μm) were adsorbed. The adenine nucleotides released either from mitochondria adsorbed onto the filter or from mitochondria of the same preparation after centrifugation amounted to about 15% of the total nucleotide content in both cases, pointing to the limited damage of mitochondria brought about by adsorption onto the filter. Three independent determinations of intramitochondrial adenine nucleotides were performed in the following conditions: (1) mitochondria adsorbed on the filter; (2) mitochondria adsorbed and perfused for 1 s at the highest flow rate (4 mL/s) with medium K; (3) mitochondria adsorbed and perfused during 3 min with the same medium supplied manually. No significant difference in the amounts of internal nucleotides was observed. A final control tested the ability of mitochondria absorbed on filter to develop a membrane potential upon respiration. In medium P, this membrane potential, measured with TPP^+ (cf. Experimental Procedures) amounted to 180–200 mV, a value similar to that determined in a parallel experiment carried out with the same probe, in which mitochondria were sedimented by centrifugation.

Time Course of Atractyloside (ATR) and Bongkrekic Acid (BA) Binding. ATR (or CATR) and BA have been widely used to terminate transport of ADP or ATP in mitochondria [for a review, see Vignais et al. (1985)]. When kinetics are studied over short periods of time, it is important to determine the quenching efficiency of the inhibitor used to stop the reaction. The data in Figure 1 show the time course for the specific binding of [^{3}H]ATR and of [^{3}H]BA to rat heart mitochondria at room temperature. Both inhibitors were used at a concentration of 10 μM, largely in excess with respect to the K_d value of 0.5 μM [cf. Vignais et al. (1985)]. Half-saturation was attained in approximatively 100 ms. Full saturation corresponding to 1.0–1.2 nmol of [^{3}H]ATR or [^{3}H]BA bound·(mg of protein)$^{-1}$ required more than 300 ms.

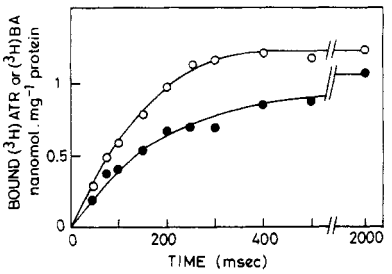

FIGURE 1: Time course of [^{3}H]atractyloside and [^{3}H]bongkrekic acid binding to rat heart mitochondria. Rat heart mitochondria were diluted to 1 mg·mL^{-1} with medium K or with a medium consisting of 125 mM KCl, 10 mM MOPS, and 1 mM EDTA, final pH 6.7 (pH 6.7 medium), when used for [^{3}H]ATR binding or for [^{3}H]BA binding, respectively. One milliliter of the diluted suspension was filtered through a 0.8-μm Millipore filter. Adsorbed mitochondria were immediately perfused for different periods of time, by using the rapid filtration system, either with 10 μM [^{3}H]ATR in medium K (○) or with 10 μM [^{3}H]BA in the pH 6.7 medium (●). The perfusion rate was 4 mL/s. The radioactivity accumulated onto the filter was determined by scintillation counting. The binding curves were corrected for unspecific binding and for background radioactivity both estimated from control experiments in which the mitochondria were incubated for 1 min at 22 °C with 20 μM CATR prior to perfusion with [^{3}H]ATR or [^{3}H]BA.

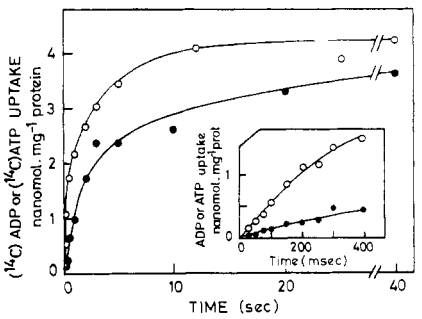

FIGURE 2: Time course of [^{14}C]ADP and [^{14}C]ATP uptake in energized mitochondria. Rat heart mitochondria (1 mL, 1 mg·mL^{-1}) were preincubated in medium P for 30 s at 22 °C. They were then adsorbed onto a Millipore filter placed in the rapid filtration system and perfused with 10 μM [^{14}C]ADP (○) or 10 μM [^{14}C]ATP (●) in medium P for a preset time. The perfusion rate was 4 mL/s when rates were measured for periods of time shorter than 2 s, and between 4 and 1 mL/s for periods of time longer than 2 s (cf. Experimental Procedures). The radioactivity incorporated into mitochondria was measured by scintillation counting. Correction for background radioactivity was performed as described under Experimental Procedures. Inset: initial kinetics of [^{14}C]ADP uptake (○) and [^{14}C]ATP uptake (●) are shown with an expanded time scale.

Clearly, ATR and BA even added in large excess (10 μM) cannot be used safely to quench transport of ADP or ATP in kinetic studies in the millisecond time range at room temperature.

Kinetics of ADP and ATP Transport in "Energized Mitochondria". Figure 2 shows the time course of [^{14}C]ADP or [^{14}C]ATP uptake at 22 °C into rat heart mitochondria preincubated in medium P. The presence of substrate in this medium, together with that of P_i, allowed the generation of a protonmotive force used for ATP synthesis. The [^{14}C]ADP uptake proceeded quasi-linearly with time for about 100 ms, after which the rate of uptake decreased exponentially until isotopic equilibrium was attained, corresponding approximately to 4–5 nmol of [^{14}C]ADP incorporated·(mg of mitochondrial protein)$^{-1}$, depending on the mitochondrial preparation. No lag phase nor initial jump was observed at 22 °C. Omission of $MgCl_2$ from the medium had no effect on the kinetics.

The apparently monophasic kinetics observed at 22 °C became heterogeneous when the temperature was lowered to 3 °C, with a rapid phase lasting for less than 1 s, followed by a much slower phase. In all cases, incubation of mitochondria with 10 μM CATR for a few seconds prior to perfusion with [^{14}C]ADP resulted in full inhibition of transport.

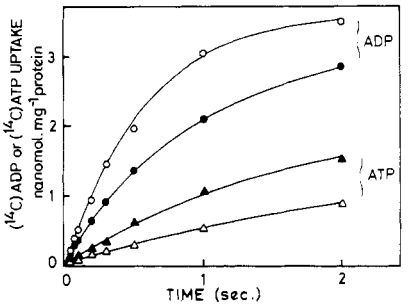

FIGURE 3: Effect of uncoupling on the kinetics of [^{14}C]ADP and [^{14}C]ATP uptake in rat heart mitochondria. Experimental conditions for control ADP uptake (○) and ATP uptake (△) are similar to those described in Figure 2. When indicated, 1 μM FCCP plus 0.3 μg of valinomycin·(mg of protein)$^{-1}$ was added to and incubated with energized mitochondria for 30 s at 22 °C prior to adsorption onto the Millipore filter and perfusion with [^{14}C]ADP (●) or [^{14}C]ATP (▲).

From the double-reciprocal plots of the initial velocity measured for concentrations of [^{14}C]ADP ranging between 1 and 20 μM (three experiments), a V_{max} of 400–450 nmol of [^{14}C]ADP transported·min^{-1}·(mg of protein)$^{-1}$ was calculated. These values were consistent with the rates of phosphorylation [320–400 nmol of ATP formed·min^{-1}·(mg of protein)$^{-1}$] determined in the presence of succinate by the oxygraphic method or directly by incorporation of [^{32}P]P_i (five experiments) (Vignais et al., 1975). The K_m for ADP transport was estimated to be 13–15 μM. This value might be overestimated due to the transphosphorylation of [^{14}C]ADP into [^{14}C]ATP and [^{14}C]AMP catalyzed by the adenylate kinase present in the intermembrane space of mitochondria, and activated by Mg^{2+} ions supplied by the medium. To determine approximately the activity of adenylate kinase, [^{14}C]ADP was added to mitochondria pretreated with CATR, and the transphosphorylation reaction was quenched by perchloric acid after different periods of incubation. We found that only 5–6% of the [^{14}C]ADP initially present was converted into [^{14}C]AMP and [^{14}C]ATP in 1 s.

The rate of [^{14}C]ATP transport in phosphorylating medium was much lower than that of [^{14}C]ADP transport. In six independent experiments in which the rates of transport of [^{14}C]ADP and [^{14}C]ATP were studied comparatively, using nucleotides at the fixed concentration of 10 μM, the rates of [^{14}C]ADP and [^{14}C]ATP uptake were 337 ± 24 and 40 ± 6 nmol.min^{-1}·(mg of protein)$^{-1}$, respectively. Although these values are lower than those of the V_{max}, they clearly point to the differences in the kinetics of [^{14}C]ADP and [^{14}C]ATP uptake. Due to the low rates of [^{14}C]ATP uptake, it was not possible to determine accurately the K_m and V_{max} values for ATP transport, the background radioactivity becoming excessively high at high ATP concentrations.

In agreement with the electrogenic nature of ADP/ATP exchange, it was found that the addition of 1 μM FCCP, or 1 μM FCCP plus 0.3 μg of valinomycin·(mg of protein)$^{-1}$, resulted in a decrease of the initial velocity of [^{14}C]ADP transport and in an increase of that of [^{14}C]ATP transport (Figure 3). In six independent experiments carried out under uncoupling conditions, the initial velocities of [^{14}C]ADP and [^{14}C]ATP uptake were 192 ± 22 and 65 ± 7 nmol·min^{-1}·(mg of protein)$^{-1}$, respectively, compared to 337 ± 24 and 40 ± 6 nmol·min^{-1}·(mg of protein)$^{-1}$ for phosphorylating mitochondria (see above). Due to the presence of P_i in the medium, the protonmotive force generated by the oxidation of succinate

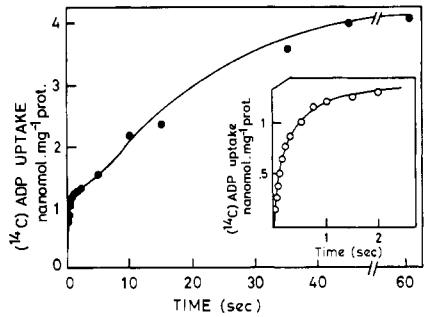

FIGURE 4: Time course of [^{14}C]ADP uptake in resting mitochondria. Rat heart mitochondria (1 mL, 1 mg·mL^{-1}) were incubated in medium K for 15 min at 22 °C. They were assayed for [^{14}C]ADP uptake with the rapid filtration system by perfusion with 10 μM (^{14}C]ADP in medium K as described in Figure 2. The inset shows the initial rapid phase of [^{14}C]ADP uptake with an expanded time scale.

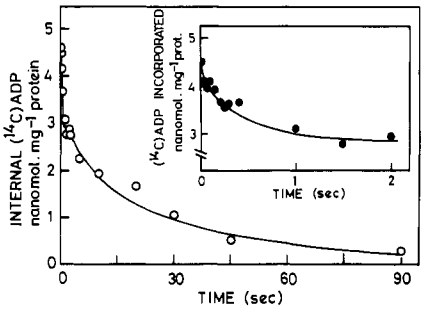

FIGURE 5: Kinetics of back-exchange of [^{14}C]nucleotides in resting mitochondria. Rat heart mitochondria were loaded with [^{14}C]nucleotides as described under Experimental Procedures. Back-exchange of nucleotides was determined by measurement of radioactivity remaining bound to the mitochondria adsorbed onto the Millipore filter after perfusion with 10 μM unlabeled ADP in medium K, for different periods of time as described in Figure 2.

consisted essentially of the membrane potential component, which could be readily assessed by the fluorescence changes of 3,3′-dipropylthiodicarbocyanine. The difference in rates of [^{14}C]ADP and [^{14}C]ATP uptake in energized mitochondria was therefore decreased, but not abolished, when the membrane potential was collapsed. If only the electrogenic nature of the ADP/ATP exchange had been involved, then the rates of ADP and ATP uptake under uncoupling conditions would have been equal. A different behavior is encountered with resting mitochondria subjected to energization and then deenergization (see Kinetics of ADP Transport in Resting Mitochondria).

Kinetics of ADP Transport in Resting Mitochondria. In contrast with the monophasic kinetics of [^{14}C]ADP transport in energized mitochondria, biphasic kinetics were observed with resting mitochondria, i.e., mitochondria preincubated in medium K (cf. Experimental Procedures) (Figure 4). The first phase consisted of a rapid uptake of [^{14}C]ADP, reaching a plateau in 1–2 s, corresponding approximately to 1.0–1.2 nmol [^{14}C]ADP·mg^{-1}. This was followed by a slow, steady uptake of [^{14}C]ADP, reaching a value of 4–5 nmol of [^{14}C]ADP·(mg of protein)$^{-1}$ after 1 min. The plateau value attained after the rapid phase was independent of the concentration of added [^{14}C]ADP, at concentrations up to 100 μM. The initial rates of [^{14}C]ADP and [^{14}C]ATP uptake during the rapid phase were virtually the same, and estimated to be approximately 350 nmol·min^{-1}·(mg of protein)$^{-1}$ (data not shown). This value was similar to that found with energized mitochondria. Biphasic [^{14}C]ADP or [^{14}C]ATP uptake by resting mitochondria did not depend on the composition of the preincubation medium, in particular on the presence of Mg ions. It occurred when resting mitochondria were preincubated in sucrose medium or in medium K supplemented with 6 mM $MgCl_2$. Biphasic kinetics were also observed in back-exchange transport experiments performed on mitochondria first loaded with [^{14}C]nucleotides by incubation with [^{14}C]ADP for 2 min at 22 °C and then resuspended in medium K at a concentration of 1 mg·mL^{-1} after centrifugation (Figure 5). The mitochondria were adsorbed onto the filter and then perfused with a solution of unlabeled ADP in medium K. The back-exchange curve was virtually the mirror image of the direct exchange curve.

Resting mitochondria and energized mitochondria contained similar amounts of adenine nucleotides (Table I). However, their ATP content differed considerably, being much lower in resting mitochondria. Incubation of resting mitochondria for 3 min at room temperature in medium P resulted in an increase in their ATP content, from 0.3 to 1.6 nmol·(mg of protein)$^{-1}$, and in the suppression of the biphasic kinetics of

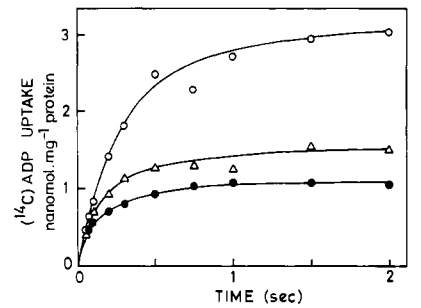

FIGURE 6: Kinetics of initial [^{14}C]ADP uptake in resting mitochondria after energization. Rat heart mitochondria (0.9 mg, 1 mg·mL^{-1}) were incubated in medium K for 15 min at 22 °C and then assayed for [^{14}C]ADP uptake with the rapid filtration system, by perfusion with 10 μM [^{14}C]ADP in medium K (○). In a parallel experiment, resting mitochondria (0.9 mg, 1 mg·mL^{-1}) were supplemented with 0.1 mL of a medium consisting of 20 mM P_i, 70 mM succinate, 50 mM $MgCl_2$, and 0.125 M KCl, final pH 7.3. After a 3-min incubation period, they were assayed for [^{14}C]ADP transport either in the absence (●) or in the presence of 1.5 μM FCCP plus 0.3 μg·mL^{-1} valinomycin (△) added to the mitochondria for 30 s before the transport assay. The perfusion rate was 4 mL/s.

Table I: Comparison of Adenine Nucleotide Contents in Energized and in Resting Mitochondria[a]

	adenine nucleotide content [nmol·(mg of protein)$^{-1}$]			
	AMP	ADP	ATP	total
energized mitochondria	3.80 ± 0.40	2.10 ± 0.50	2.90 ± 0.80	8.80 ± 1.02
resting mitochondria	4.70 ± 0.60	1.02 ± 0.37	0.34 ± 0.16	6.06 ± 0.72

[a] Rat heart mitochondria (1 mL, 1 mg·mL^{-1}) were incubated for 30 s at 22 °C in medium P or for 15 min at 22 °C in medium K. Incubation was terminated by addition of perchloric acid (final concentration 0.2 M). Adenine nucleotides were assayed in the perchloric extracts after neutralization by KOH.

[^{14}C]ADP uptake (Figure 6). The modified kinetics were similar to those observed with energized mitochondria, [^{14}C]ADP being accumulated more rapidly than [^{14}C]ATP (Figure 7A). It was found that resting mitochondria phosphorylated externally added ADP at the same rate as energized mitochondria did, when they were brought to medium P (data not shown). However, in contrast to fresh mitochondria that were directly energized by preincubation in medium P, mitochondria initially in medium K (resting mitochondria) and then energized by transfer to medium P and finally uncoupled were found to transport [^{14}C]ADP and [^{14}C]ATP at the same rate, and the plateau of rapid nucleotide uptake [1 nmol·(mg

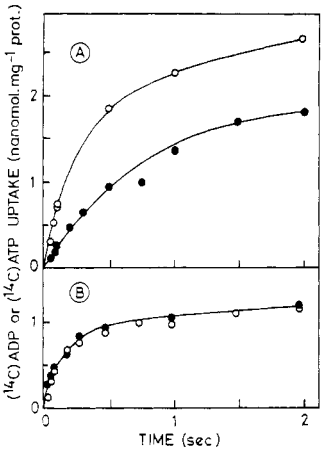

FIGURE 7: Effect of energization and uncoupling on the kinetics of initial [^{14}C]ADP or [^{14}C]ATP uptake in resting mitochondria. Resting rat heart mitochondria (1 mL, 1 mg·mL^{-1}) were energized as described in the legend of Figure 6 and assayed for transport by perfusion with 10 μM [^{14}C]ADP (○) or with 10 μM ATP (●) in the absence (A) or in the presence (B) of 1.5 μM FCCP plus 0.3 μg·mL^{-1} valinomycin added to the mitochondria for 30 s before the transport assay. The perfusion rate was 4 mL/s.

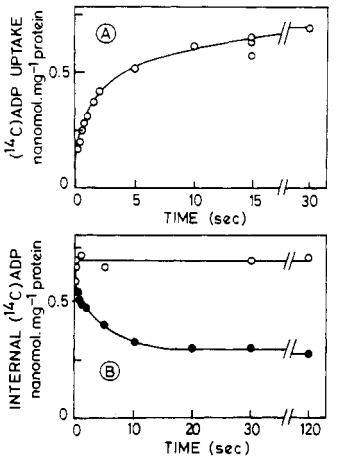

FIGURE 8: Kinetics of [^{14}C]ADP uptake in resting mitochondria at 3 °C and of removal of externally bound [^{14}C]ADP. (A) Resting rat heart mitochondria (1 mL, 1 mg·mL^{-1}) were assayed for [^{14}C]ADP uptake at 3 °C as previously described, by perfusion with [^{14}C]ADP in medium K. (B) Resting mitochondria (1 mg) that were first perfused for 15 s at 3 °C with 10 μM [^{14}C]ADP in medium K were further perfused for different periods of time at 3 °C, as indicated, with the same solution in the absence of CATR (○) or in the presence of 20 μM CATR (●). [^{14}C]ADP uptake was determined as previously described. In (A) and (B), the perfusion rates were as in Figure 2.

of protein)$^{-1}$] was identical with that found in the original resting mitochondria (Figure 7B). In summary, prior to energization, resting mitochondria apparently catalyzed electroneutral ADP/ATP transport. Following energization by transfer to medium P, they catalyzed purely electrogenic ADP/ATP transport.

Heterogeneous Pools of Exchangeable Mitochondrial Nucleotides. Due to the large concentration of ADP/ATP carrier protein in heart mitochondria, the amount of carrier-bound adenine nucleotides corresponds to a substantial fraction of the pool of internal adenine nucleotides. It was therefore interesting to compare the amount of externally bound [^{14}C]ADP to that of [^{14}C]ADP incorporated into mitochondria at the end of the rapid phase in resting mitochondria. Externally bound [^{14}C]ADP was checked by displacement of bound [^{14}C]ADP by CATR. For convenience, the duration of the first plateau was increased by lowering the temperature to 3 °C, and resting mitochondria were assayed for [^{14}C]ADP transport by the perfusion method (cf. Experimental Procedures). At 3 °C, the rapid phase of transport was slowed down, and the first plateau phase lasted for more than 30 s (Figure 8A), thus permitting the investigation of the CATR-dependent displacement of the rapidly incorporated [^{14}C]ADP. In the experiment illustrated in Figure 8B, mitochondria adsorbed on the filter were first perfused manually with 10 μM [^{14}C]ADP in medium K for 15 s at 3 °C. This was followed by perfusion for different periods of time with 10 μM [^{14}C]-ADP in the absence or presence of 20 μM CATR. The amount of incorporated radioactivity in the absence of CATR remained stable, whereas it was depressed by about 50% when the perfusion medium was supplemented with CATR. This means that a significant amount of [^{14}C]ADP was most probably imported into the matrix space of mitochondria and was no longer accessible to the externally added CATR. This experiment was repeated at 22 °C, using mitochondria that had been incubated for a few minutes in medium K supplemented with 10 mM P_i. Mitochondria incubated in a P_i-supplemented medium rapidly lose their internal nucleotides (Wilson et al., 1987). The effect of these incubation conditions on [^{14}C]ADP uptake is illustrated in Figure 9. Rat heart mitochondria were diluted to 1 mg·mL^{-1} in medium K (control) or in medium K supplemented with 10 mM P_i and used after 10 and 20 min of incubation of 22 °C. The extent of

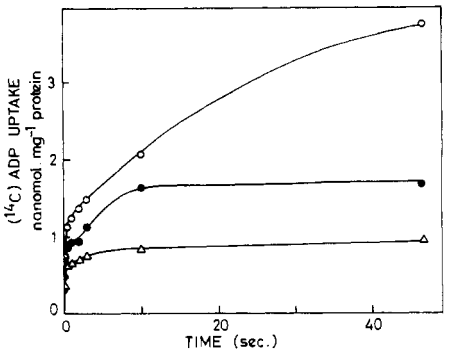

FIGURE 9: Time course of [^{14}C]ADP uptake in depleted mitochondria. Rat heart mitochondria (1 mL, 1 mg·mL^{-1}) were preincubated in medium K for 15 min (○) or in medium K supplemented with 10 mM phosphate for 10 min (●) or 20 min (△). They were assayed for [^{14}C]ADP uptake with the rapid filtration system by perfusion with medium K supplemented with 10 μM [^{14}C]ADP, as in Figure 2.

[^{14}C]ADP uptake was considerably lowered, most probably because it was limited to the small pool of the remaining exchangeable nucleotides. The slow phase of [^{14}C]ADP transport was more affected than the rapid one, suggesting a kinetic compartmentation of adenine nucleotides within mitochondria (see Discussion). The plateau attained after the rapid phase of ADP uptake lasted for several minutes. Removal of externally bound [^{14}C]ADP upon addition of CATR was then assayed. As in the preceding experiment, only half of the [^{14}C]ADP taken up by mitochondria was removed by CATR (Figure 10).

Phosphorylation of ADP in Nucleotide-Depleted Mitochondria. It was interesting to check whether the resting and depleted mitochondria which exhibit only a rapid phase of incorporation of [^{14}C]ADP were competent in the phosphorylation of this [^{14}C]ADP. The aim of the two parallel experiments illustrated in Figure 11 was to compare the kinetics of [^{14}C]ADP uptake and [^{14}C]ADP phosphorylation in these mitochondria, after they were energized in the presence of succinate. In about 1 min, virtually all of the added [^{14}C]ADP was phosphorylated into [^{14}C]ATP (Figure 11B). This demonstrated that the ADP imported during the rapid phase

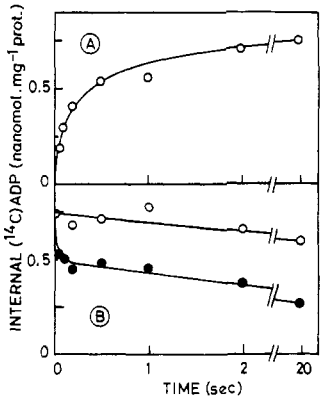

FIGURE 10: Kinetics of [^{14}C]ADP uptake and of displacement of externally bound [^{14}C]ADP by CATR in depleted mitochondria. (A) Rat heart mitochondria (1 mg·mL^{-1}) were first depleted by incubation for 15 min in medium K supplemented with 10 mM P_i and then assayed for [^{14}C]ADP uptake at 22 °C by perfusion with 10 μM [^{14}C]ADP in medium K as previously described. (B) The depleted mitochondria (1 mL, 1 mg·mL^{-1}) were adsorbed onto a Millipore filter and perfused for 20 s at 22 °C with 10 μM [^{14}C]ADP in medium K supplied manually. Then they were immediately perfused for different periods of time in the rapid filtration system with the same [^{14}C]ADP solution in the absence of CATR (○) or in the presence of 20 μM CATR (●). [^{14}C]ADP incorporated into mitochondria was determined as described under Experimental Procedures. In (A) and (B), the perfusion rates were as in Figure 2.

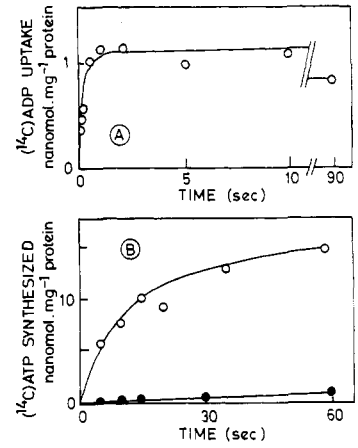

FIGURE 11: Kinetics of ATP synthesis measured with depleted mitochondria. (A) Transport of [^{14}C]ADP. Rat heart mitochondria were first depleted of internal nucleotides by preincubation for 15 min at 22 °C in medium K supplemented with 10 mM phosphate. These depleted mitochondria (0.9 mL) (1 mg/mL) were added to 0.1 mL of a medium consisting of 100 mM P_i, 100 mM succinate, 50 mM $MgCl_2$, and 0.125 M KCl, pH 7.3. After 3 min at 22 °C, they were assayed for [^{14}C]ADP uptake by perfusion with 10 μM [^{14}C]ADP, as described in Figure 2. (B) Phosphorylation of [^{14}C]-ADP. In a parallel experiment, depleted mitochondria obtained as in (A) were assayed for phosphorylation of external [^{14}C]ADP. These depleted mitochondria (50 μL, corresponding to 50 μg of protein) were added to 50 μL of a medium consisting of 20 mM succinate, 20 mM P_i, 10 mM $MgCl_2$, and 125 mM KCl, final pH 7.3, so that the final concentrations of the components were the same as in (A). The reaction was stopped by addition of 30 μL of TCA (50% v/v in water) after different periods of incubation with [^{14}C]ADP. Samples were centrifuged, and [^{14}C]ATP present in the supernatants was determined as described under Experimental Procedures. Control assays (●) were performed with mitochondria preincubated with 20 μM CATR for 30 s at 22 °C prior to phosphorylation.

(Figure 11A) was efficiently phosphorylated by the mitochondrial ATP synthase. As expected, ATP synthesis was completely abolished in control experiments performed in the presence of 20 μM CATR or in the presence of FCCP plus valinomycin (not shown).

Discussion

Validity of the Rapid Filtration Technique for Determination of the Rate of ADP/ATP Transport in Mitochondria. The major advantage of the rapid filtration technique over the inhibitor stop method for rapid kinetic study is the fact that the termination of transport is achieved by the cessation of substrate supply without the addition of an inhibitor to stop the transport process. Although the mitochondria adsorbed on the nitrocellulose filter are subjected to the flux of the transport medium pushed through the filter, no measurable damage was revealed based on the criteria of morphology, permeability, and the ability to generate a membrane potential close to 200 mV.

The "inhibitor-stop" method has been routinely used to measure the rates of anion transport in mitochondria, and in the case of ADP/ATP transport, the favored inhibitors were ATR and CATR for intact mitochondria and BA for inverted submitochondrial particles (Vignais et al., 1985; Klingenberg, 1985). As shown in the present study, when ATR is added at the inhibitory concentration of 10 μM to rat heart mitochondria at room temperature, a period of 200–300 ms is required before full saturation of the ATR binding sites is attained. This delay clearly points to the limitation of the inhibitor-stop technique to study rapid kinetics of transport since very high concentrations of inhibitor (>90 μM) must be used to shorten the quench-delay (Nohl & Klingenberg, 1978; Sluse et al., 1988). At these high concentrations, CATR, which is an amphiphilic molecule, may have deleterious detergent effects on mitochondria.

Another advantage of the rapid filtration technique over the inhibitor-stop method during the initial phase of uptake is that it permits the discrimination between [^{14}C]ADP binding to the cytosolic sites of the ADP/ATP carrier and [^{14}C]ADP transport.

The main limitation of the rapid filtration technique concerns the background radioactivity accumulated into the Millipore filter. This technical problem can be largely overcome by the use of nonpermeant radioactive markers introduced into the perfusion medium (see Experimental Procedures). Nevertheless, the concentration of [^{14}C]ADP or [^{14}C]ATP to be used should not be higher than 100–200 μM. Because of this limitation, the determination of kinetic parameters of the very low ATP transport in energized mitochondria was hardly possible.

When ADP/ATP exchange was studied in rat liver mitochondria using the inhibitor-stop method, biphasic kinetics were observed, which were attributed to a partial inhibition of the initial uptake of ADP due to the quench-delay of atractyloside (Nohl & Klingenberg, 1978). This explanation should be reconsidered in view of our results obtained with the rapid filtration method from rat heart mitochondria (this paper). Complex pre-steady-state kinetics have been observed for ADP/ATP transport measured at low temperature in rat heart mitochondria (Sluse et al., 1988). Kinetics of ADP uptake were modulated by the nucleotide content of mitochondria, but the influence of the metabolic state of mitochondria was not investigated.

The electrogenicity of ADP/ATP transport in mitochondria (Laris, 1977; Lanoue et al., 1978) and inverted submitochondrial particles (Villiers et al., 1979) was demonstrated in experiments performed at low temperatures (0–2 °C) to slow down transport and measure rates accurately at periods of time of a few seconds. Electrogenicity of transport in energized mitochondria was confirmed at 22 °C by using the rapid filtration technique. In fact, ATP uptake was much slower than ADP uptake (Figure 3). When the energized

mitochondria were uncoupled by FCCP and valinomycin, the rate of ADP uptake decreased and that of ADP increased. These results are in accordance with the prediction that, in respiring mitochondria the membrane potential drives the asymmetric exchange of ADP_{ex}^{3-} against ATP_{in}^{4-} and that the entry of ATP_{ex}^{4-} is very slow unless the membrane potential is abolished. It is noteworthy, however, that in fully uncoupled mitochondria the rate of ADP uptake remained significantly higher than that of ATP uptake (Figure 3). This difference in rates suggests that, besides the extrinsic control by membrane potential, ADP/ATP transport is intrinsically regulated by the nature of the transported substrate, i.e., ADP or ATP, as previously suggested (Souverijn et al., 1973), or that ADP/ATP exchange is sensitive to a residual Donnan potential. Different kinetics were obtained with mitochondria that had been preincubated in medium K (resting state) prior to energization by transfer to medium P. Although the rate of ATP transport in these mitochondria was lower than that of ADP transport, the two rates equalized upon uncoupling (Figure 7). A possible explanation for the different behaviors of mitochondria directly energized and mitochondria aged for a few minutes prior to energization is that in aged mitochondria, the electrogenic ADP/ATP transport is solely controlled by the membrane potential. In directly energized mitochondria, other regulatory processes may be superimposed on the control by the membrane potential, for example, a Donnan potential or specific interactions of the transported ADP and ATP with related sites on the carrier protein.

Do the Biphasic Kinetics of ADP Transport in Resting Mitochondria Reflect Microcompartmentation of Intramitochondrial Adenine Nucleotides? Although the kinetics of ADP transport in energized mitochondria at 22 °C are apparently monophasic, they are biphasic when the temperature is lowered to 3 °C; this may be due to lack of resolution at higher temperature, despite the performance of the filtration device. The biphasic kinetics of ADP transport in resting mitochondria at 22 °C are characterized by an initial rapid phase lasting for about 2 s, whose extent is limited to 1.0–1.5 nmol of [^{14}C]ADP incorporated·(mg of protein)$^{-1}$, and a slower phase lasting for more than 1 min during which remaining matricial nucleotides are exchanged. Three possible interpretations can be envisaged:

(a) The initial rapid phase might be due to a preferential electrogenic exchange between external ADP and ATP present in resting mitochondria. The second phase would then correspond to the exchange of external ADP either against internal ADP, which is largely in excess with respect to internal ATP, or against ATP slowly generated by enzymatic phosphorylation of internal ADP. This hypothesis might be supported by the presence of a substantial amount of ATP in resting mitochondria. However, would an electrogenic ATP_i/ADP_{ex} exchange occur, one should expect an inhibitory effect of uncouplers. This is not observed.

(b) The initial rapid phase might result from the binding of external ADP to the binding sites of the ADP/ATP carrier exposed to cytosol, and the second phase would reflect the transport of the externally bound ADP. This hypothesis is supported by the fact that the amount of [^{14}C]ADP incorporated at the end of the rapid phase is close to the number of external ADP/ATP binding sites on the adenine nucleotide carrier protein, irrespective of the external concentration of ADP (up to 100 μM). If this hypothesis were correct, one would expect that the rapidly bound [^{14}C]ADP would be released upon perfusion with CATR, since CATR is known to displace ADP from the external binding sites of the ADP/ATP carrier. Only 50% of the bound [^{14}C]ADP was displaced by CATR (Figures 8 and 10), indicating that the remaining bound [^{14}C]ADP was internalized. That the rapid kinetic phase of [^{14}C]ADP uptake reflects a transport process was conclusively demonstrated by the fact that it led to the phosphorylation of external ADP (Figure 11).

(c) The third explanation for the biphasic kinetics is based on the microcompartmentation of internal nucleotides, the plateau attained at the end of the rapid phase corresponding to the isotopic equilibrium between external nucleotides and nucleotides contained in an internal rapidly exchangeable pool. Addition of CATR at this stage would result in a release of bound ADP from the external sites, which is in fact observed. The rapidly exchangeable pool, distinct from, but in equilibrium with, the pool of endogenous nucleotides, would consist of nucleotides bound to protein species located on the matrix face of the inner mitochondrial membrane. Likely candidates are the ADP/ATP carrier itself and the ATPase complex.

To explain the different kinetics of ADP transport in energized and resting mitochondria at 22 °C, one may imagine that the equilibrium between the rapidly and slowly exchangeable pools of matricial nucleotides is modulated by the energy state of mitochondria and temperature: the two internal pools would be in fast equilibrium (both rapidly exchangeable) in energized mitochondria. In resting mitochondria, one pool would be rapidly exchangeable and the other slowly exchangeable. If the rapidly exchangeable pool consists of nucleotides bound to the ADP/ATP carrier and (or) to the F_1 complex, exchangeability would depend on the affinity of nucleotides for the binding sites which would be higher in energized mitochondria than in resting mitochondria. In agreement with this, a dependence of the exchangeability of F_1-ATPase-bound nucleotides on the energy state of mitochondria was proposed by Rosing et al. (1977). This hypothesis is supported by the fact that resting mitochondria, when energized, exhibit monophasic kinetics for [^{14}C]ADP and for [^{14}C]ATP uptake. Conversely, when energized mitochondria are brought to 3 °C, biphasic kinetics are displayed, which may be explained on the basis of two exchangeable pools as discussed above.

The results of earlier experiments carried out with rat liver mitochondria indicated that F_1-dependent phosphorylation of external ADP entering the matrix space of mitochondria was faster than mixing with internal ADP (Vignais et al., 1975). It was hypothesized that the ADP carrier and the F_1-ATPase either shared the same microenvironment on the matrix side of the inner membrane or interacted directly. In these experiments, a temperature of 4 °C was chosen for better kinetic resolution and more accurate measurements over periods of several seconds. It could not be excluded, however, that low temperature might have influenced the lipid/protein interactions in the inner mitochondrial membrane as suggested by Hartung et al. (1983). The kinetics of ADP transport at room temperature measured by the rapid filtration method support the hypothesis of microcompartmentation of internal nucleotides (Vignais et al., 1976; Out et al., 1976). It must be stressed, however, that the entire pool of internal nucleotides is exchangeable over a sufficient period of time (Duée & Vignais, 1969). For this reason, it is mainly in terms of kinetic compartmentation that the kinetic data reported here are tentatively explained.

As discussed by Murthy and Pande (1985), enzymes which belong to the same metabolic pathway often share the same microenvironment. This results in a high metabolic efficiency and may be advantageous for metabolic regulation. Mito-

chondrial compartmentation is apparently not unique to nucleotides transported by the ADP/ATP carrier. Murthy and Pande (1985) recently reported recycling of transported ADP, carnitine, and acetylcarnitine in a submitochondrial compartment. There are a number of examples of interactions between mitochondrial carriers and metabolizing mitochondrial enzyme systems: the pyruvate carrier and pyruvate dehydrogenase (Pande & Parvin, 1978), the malate carrier and malic enzyme in tumor mitochondria (Moreadith & Lehninger, 1984), the glutamine carrier and P_i-dependent glutaminase in kidney mitochondria (Simpson, 1983), and the glutamate/aspartate carrier and glutamate/oxaloacetate transaminase in liver mitochondria (Duszynski et al., 1978).

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