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KINETICS OF ADP, ATP TRANSPORT IN MITOCHONDRIA AS STUDIED BY THE QUENCH-FLOW METHOD

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Summary

The kinetics in the millisecond range of ADP, ATP counterexchange in rat liver mitochondria were investigated using a quench-flow apparatus. The exchange was stopped with atractylate and the mitochondria were separated by centrifugation.

1. After correcting for leakage due to flow stress, apparent biphasic exchange kinetics were observed, with a more rapid phase within 100—200 ms, which extends to 10% of the total exchange. In general, the extent of the rapid phase increased when the translocation rate was changed under various conditions, in agreement with the model of the quench mechanism by atractylate.

2. The nature of the "rapid" phase was analyzed in the "steady" and "transient" state of the translocation and was shown to be caused by a delayed binding of atractylate due to competition with ADP. This quench delay results in a residual exchange which could explain the rapid part of the kinetics.

3. Deenergization of mitochondria by valinomycin or by uncoupler largely abolishes the rapid kinetic phase. This is explained by an increased availability of carrier sites at the outer face of the membrane to atractylate in the deenergized state, resulting in a more rapid quench.

4. The interpretation of the rapid exchange phase as a function of carrier sites accessible to atractylate quenching at the outer membrane face was simulated by a computer program based on the reorientating carrier model. With a set of rate constants, an approximate fit for the extent of quench delay with the experimental data is obtained.

Abbreviation: CCP, carbonylcyanide *m*-chlorophenylhydrazone.

Introduction

The kinetics of the ADP, ATP exchange have been of great interest since the discovery of a specific ADP, ATP transport system in mitochondria. The best kinetics resolution was achieved by applying the "inhibitor-stop-method" by which the transport was blocked on addition of an excess of atractylate [1] or carboxyatractylate. This method was combined with a simple sampling method which permitted resolution down to 1.5—2 s. In general, less time resolution was obtained by using Millipore filtration. Some of the kinetic data reported [2—4] are contradictory, since time resolution, temperature control and evaluation of the data have not been mastered sufficiently.

Because of the high exchange rates it seemed desirable to apply more rapid kinetic methods with improved starting and stopping of the reaction. These requirements appeared to be met by the quench-flow method, where the starting reagent ADP and the quenching reagent atractylate are mixed with the mitochondria in a flow system by two mixing chambers. A special flow apparatus adapted for these purposes was built.

With the unprecedented advance into the millisecond range a number of unexpected results and novel problems arose which could be related in part to the quench delay of the transport by atractylate in conjunction with a competition of atractylate and ADP for the transport active sites. The investigation of these problems constitutes an important part of the present report.

Methods

Rat liver mitochondria were prepared as described previously [5]. For the back-exchange experiments the mitochondria were incubated with ^{14}C-labelled ADP at 0° C and then washed as described previously [6]. The stock solution of the labelled mitochondria was diluted to about 1 mg/ml immediately before starting the epxeriment. For the forward exchange experiments the specific activity of incorporated endogenous adenine nucleotides had to be defined. For this purpose, corrections were made for adenine nucleotide contained in the unspecifically permeant space. The size of the matrix space was determined by subtracting the [^{14}C]sucrose permeable space from the total $^{3}H_2O$-permeable space of the mitochondria [7].

In some cases the mitochondrial suspension was partially protected against ageing by the addition of the local anesthetic nupercaine which is an inhibitor of phospholipase A_2. In accordance with Scarpa and Lindsay [9] it was observed that the respiratory control of the nupercaine-treated mitochondria (200 μM) remained unchanged within 5 h. No influence of nupercaine on ADP, ATP translocation activity was noted.

For determination of the adenine nucleotide patterns, small column chromatography was applied [6]. The exchange kinetics at prolonged times were measured either by using the atractylate stop with manual handling, or with a sampling apparatus in which aliquots were injected into small vessels containing atractylate solution.

The quench-flow method for measuring the exchange

For measuring the kinetics in the msec range, a quench-flow apparatus was

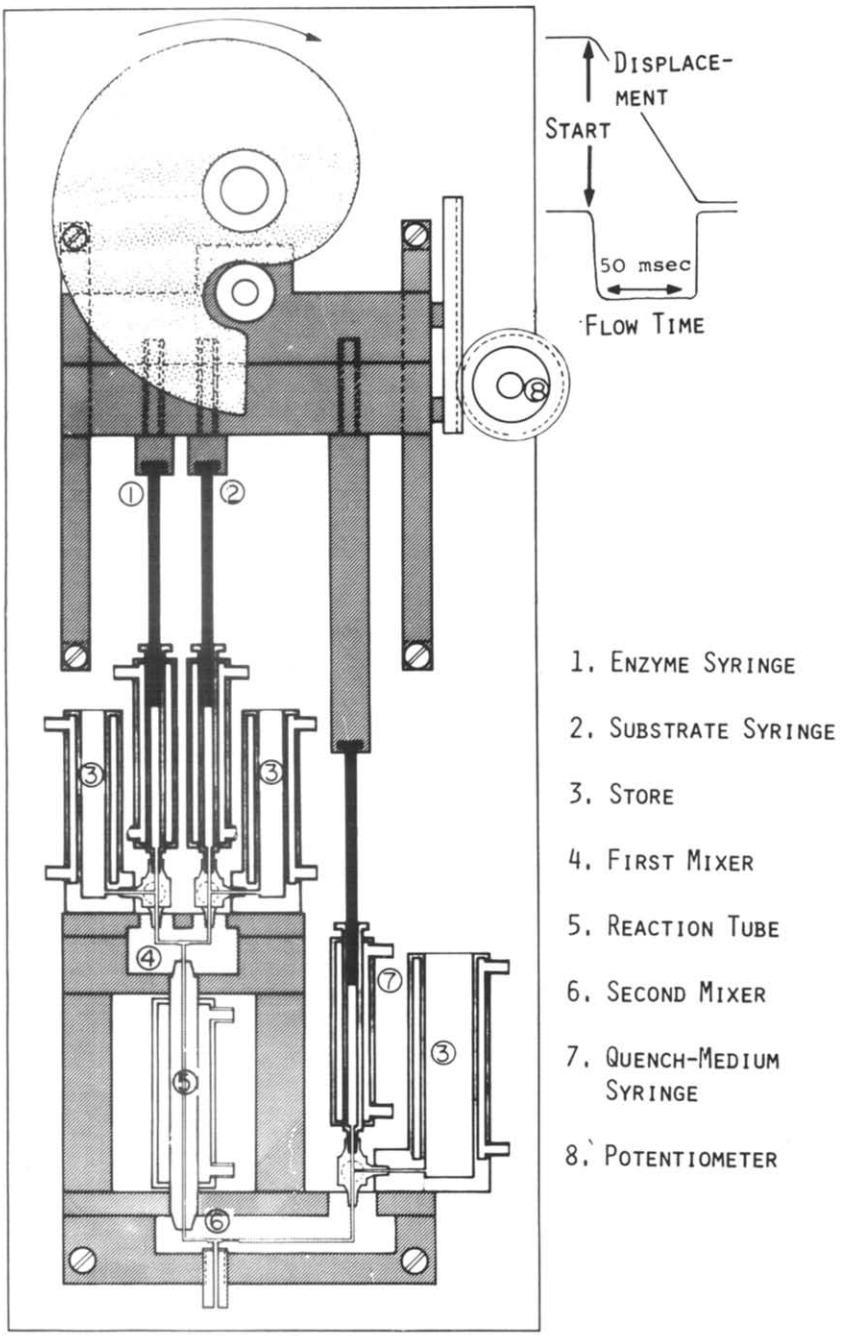

Fig. 1. Quench-flow apparatus designed for studying rapid kinetics of the adenine nucleotide transport. Three 1-ml syringes, which can be filled each by Hamilton 3-way gas-tight valves from a storage reservoir, are discharged simultaneously by a ram connecting the plungers. The rotary motion from the output of a gearbox is converted by the cam (eccentric disc) into linear displacement. The speed of the motor-driven gear is continuously adjustable from 4 to 640 rev./min. The tips of the syringes are fitted to a plexiglass block which conducts the enzyme and substrate syringes to the reaction mixer by two channels. The reactant mixer is 3.34 mm in diameter and 0.5 mm high. Four jets, with a diameter 0.45 mm, representing the channels for reaction, enter the mixer tangentially. Jets in alternating arrangement eject the two reactants into the mixer. The reaction tube following is exchangeable to allow different reaction times. In our experiments a small (43.5-mm length) and a long (120.1-mm length) tube, both 1.5 mm in diameter, were used. To the second mixer (5.32 mm in diameter and 0.7 mm high) the reaction mixture is ejected by two jets and mixed with the quench medium, which is ejected via two other jets. The total diameter of the four jets corresponds to the total diameter of the upper jets. Registration of the derivative signals (discharging time of the syringes, reaction time) shows that at the start the time delay is smaller than 2 ms.

applied. The apparatus shown in Fig. 1 is a modified version of a quench-flow machine described previously [10]. All the syringes and storage vessels are water-jacketed for accurate temperature control. The two mixing chambers are imbedded in metal blocks through which temperature-controlling fluid is also circulated. The temperature of the quench solution and of the second mixing chamber could be adjusted independent from that of the reactant syringes of the first mixing and reaction chamber.

The volume of each of the three syringes was 0.90 ml. The total reaction volume (v_S) determines the reaction time according to the equation $t_R = v_R t_P/v_S$ [10]. The sum of the volumes, v_R, of the first mixing chamber, of the reaction chamber and of the reaction tube of the second mixer is 75 μl. The reaction tube was exchangeable. A larger condutance between the two mixers, amounting to a reaction volume of 212 μl, was used for reaction times between 400 and 1200 ms. The normal version permitted a reaction time between 10 and 400 ms.

The flow rate in the jets at maximum speed is about 23 m/s and the lowest rate approx. 1 m/s. At the highest flow rates, approx 4—8% of the mitochondria were damaged, as judged from the adenine nucleotide leakage.

The flow time, t_P, was varied with a continuously-variable gear box with an electric motor and recording on paper of the plunger displacement. The displacement time is varied from 220 ms, resulting in a reaction time of 10 ms, up to 10.6 s, 1200 ms reaction time.

The performance of the quench apparatus was tested using the dinitrophenol acetate hydrolysis as described previously [10]. The small mixing chamber gives a linear rate up to approx. 440 ms reaction time, the larger reaction chamber up to approx. 1120 ms. At longer reaction times the flow velocities are obviously too small for efficient mixing. The rate constants obtained with the short reaction tube were 49 $M^{-1} \cdot s^{-1}$ and 46 $M^{-1} \cdot s^{-1}$ with the long reaction tube. This was to be expected for good mixing performance of both versions.

Results

The problem of apparent initial rapid kinetics

A typical time dependence is shown in Fig. 2 for the range 25 -400 ms. As is to be expected from previous results [11,12], ADP exchanges nearly twice as fast as does ATP. AMP is nearly inactive. In the initial phase up to 100 ms the exchange appears to be much more rapid, both with ADP and ATP. Fig. 2B gives the subsequent kinetics in the range up to 30 s as determined by stopping the reaction on addition of atractylate by hand. As compared with the rate for this major pool, the exchange obtained with the quench-flow method is about eight times faster within the first 100 ms and then decreases in the range 100—400 ms to a rate which is about two times faster.

For understanding more closely these kinetics, the evaluation of the data obtained with the quench-flow method is demonstrated in another experiment shown in Fig. 3. Meaningful exchange values can be obtained only after subtraction of appropriate blank controls. The control series "simultaneous" and "before" are intended to evaluate the possible quenching artifacts and the

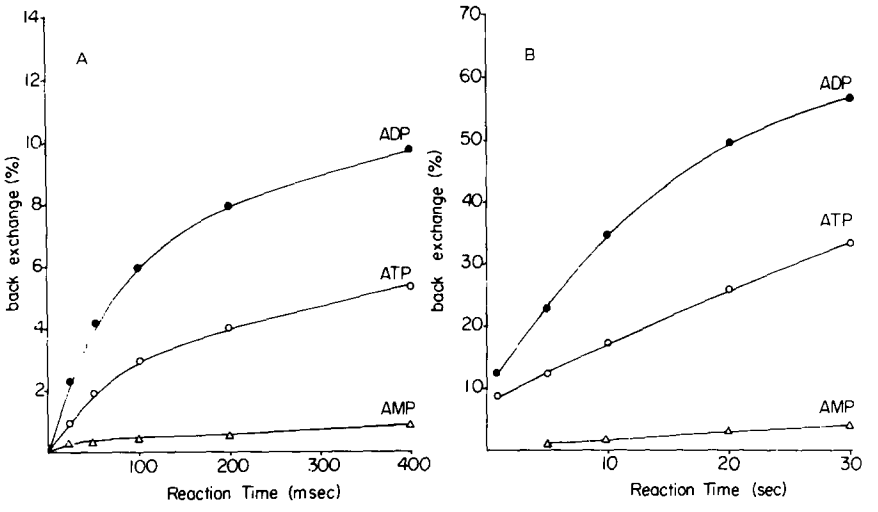

Fig. 2. Comparison of the exchange kinetics for AMP, ADP and ATP with the quench-flow apparatus in the millisecond range (A) and with a simple sampling apparatus [21] for the second range (B). Back-exchange at 10°C, started by addition of (unlabelled) 400 μM AMP, ADP or ATP to the labelled mitochondria and stopped by addition of 70 μM atractylate. The exchange is based on the total labelled endogenous adenine nucleotides, 0.8 mg protein/ml. All curves were corrected for leaked adenine nucleotides, as shown in Fig. 4.

leakage of nucleotides from the mitochondria resulting from the mechanical stress in the flow system. For obtaining this leakage, mitochondria are first incubated with atractylate (A) and then ADP (N) is added, followed by the usual atractylate quench (case[A$\overset{t}{-}$A]). By the addition of both of these, ADP in the first and atractylate in the second chamber, the conditions maintained are consistent with the case [N$\overset{t}{-}$A]. As a control for the quench delay, atractylate was mixed simultaneously with ADP to the mitochondria [A,N]. The difference between the cases [A,N] and [A$\overset{t}{-}$N] should reflect the quench delay. The exchange curve is obtained as the difference between the cases [N$\overset{t}{-}$A] and [A,N].

The question arises as to whether the simultaneous addition of atractylate and ADP (case[A,N]) gives an appropriate correction for the quench delay of atractylate. In particular, the initial fast exchange up to 100 ms may contain some quench artifacts. Therefore, a more detailed investigation of the quench process in the rapid period was necessary. Firstly, dependence on the concentration of atractylate was studied. The exchange after 50 ms, dependent on actractylate concentration from 10 to 90 μM, is shown in Fig. 4 for the exchange [N$\overset{t}{-}$A] with the controls [A,N] and [A$\overset{t}{-}$N]. In the case [A$\overset{t}{-}$N] when atractylate had sufficient time to react with the translocator before addition of ADP, the adenine nucleotide release is nearly independent of atractylate concentration between 10 and 90 μM and corresponds to a "leakage" of endogenous adenine nucleotide.

In the case [N$\overset{t}{-}$A] the release is higher at low concentration, which feature can be attributed to a delay of the quench of the exchange. This is supported by the finding that also in the case of the simultaneous addition [A,N] the release is higher at low atractylate concentration. The delay largely disappears

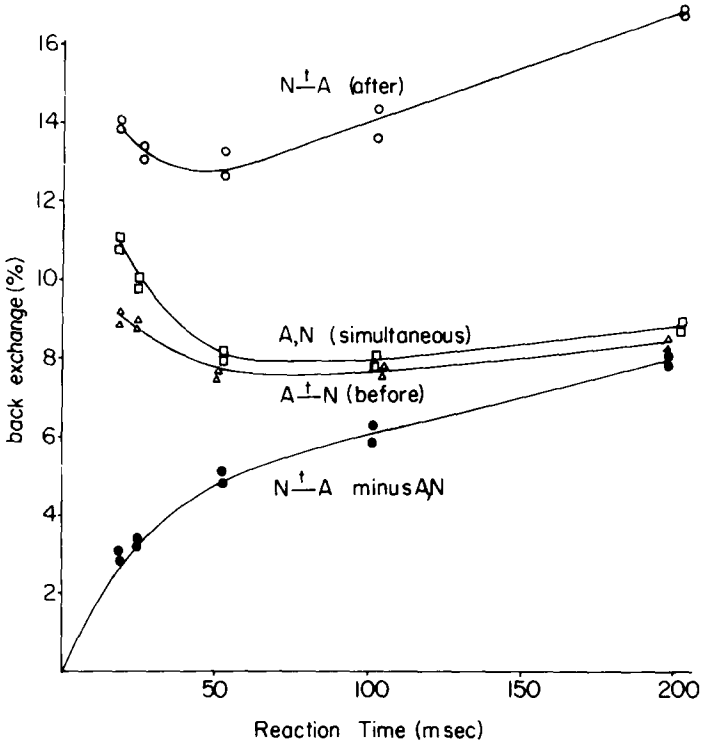

Fig. 3. The biphasic kinetics of the backward exchange with ADP, demonstrating the corrections for adenine nucleotide leakage and atractylate quench. For exchange conditions see legend of Fig. 2. When actractylate was added to the reaction mixture after the addition of ADP [$N\overset{t}{-}A$] (A, atractylate; N, nucleotide; $\overset{t}{-}$ = time interval between the additions), the amount of labelled ADP detected in the supernatant consists of the translocator-mediated transport, a leakage caused by mechanical stress and an additional amount of translocated adenine nucleotides as a result of quench delay. The leakage was given by the exchange of mitochondria which were preincubated with 70 μM atractylate 1 min prior to the addition of ADP [$A\overset{t}{-}N$]. When atractylate and ADP were mixed simultaneously [A,N] the sum of the leakage and the delayed quench is obtained. The exchange mediated by the translocase was estimated by subtraction of curve [A,N] from curve [$N\overset{t}{-}A$]: 10°C, 400 μM ADP, 90 μM atractylate.

above 60 μM actractylate. The presence of ADP added before, or simultaneously with, actractylate delays the quenching, obviously by competing for the carrier sites. Under these circumstances the case [A,N] is not an appropriate control value to be subtracted from the total release.

The quench delay analysis

For understanding this quench delay it is useful to consider the rate and the equilibrium of interaction of the substrate and atractylate with the carrier binding sites.

$$N + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} NC \tag{1}$$

$$A + C \xrightarrow{k_3} AC \tag{2}$$

$$dNC/dt = k_1 \cdot C \cdot N - k_2 NC \tag{3}$$

$$dAC/dt = k_3 \cdot C \cdot A \tag{4}$$

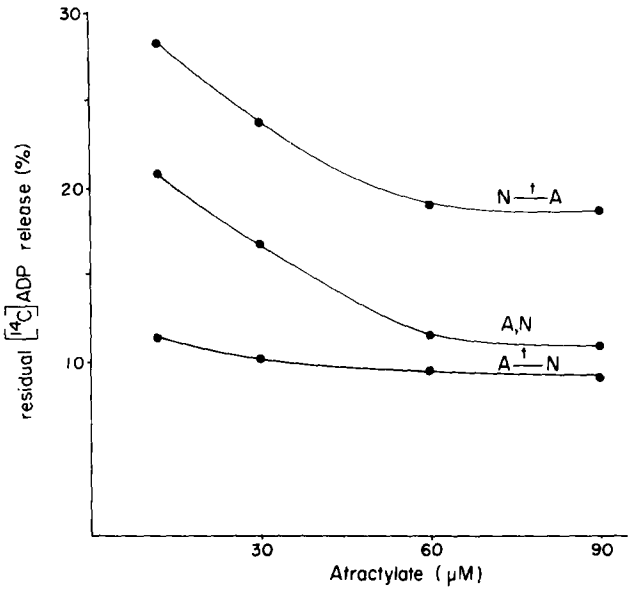

Fig. 4. Influence of atractylate concentration on ADP exchange at 50 ms. "Back-exchange" experiments were performed at 21°C for conditions as described in Fig. 3, with 150 μM ADP to start the reaction. Atractylate was added 1 min before ADP. 0.8 mg protein/ml.

It can be assumed that substrate and inhibitor compete for the same binding site at the carrier (Eqn. 1 and 2). This competition is not so much a problem of the binding constant, considering that atractylate binds $1 \cdot 10^3$ times tighter than ADP to the carrier, but must be interpreted in kinetic terms. Therefore, the rate of the binding of actractylate in comparison to that of the nucleotides may be critical for the quenching rate. This rate should be dependent on the concentration of inhibitor A and of the unoccupied carrier sites C. In the simplest case, the binding rate of the inhibitor actractylate to the carrier is proportional to the product of A and C (Eqn. 2). The concentration of the free carrier sites is dependent on the binding constant of the substrate once equilibrium has been established, such as in the steady-state exchange. However, in the initial period, after addition of ADP, the number of unoccupied sites C decreases with the progress of substrate binding to the carrier. As a consequence, when atractylate is added within this initial period, its binding is expected to be more rapid as compared to the binding when atractylate is added later. The lowest rates of actractylate binding should be found when ADP exchange reaches the steady state.

During the time interval, which is needed after addition of atractylate to block all carrier sites, a residual activity of exchange goes on, which can be called a quench delay. There is no quench delay when atractylate, added before ADP, has time to occupy all sites. When ADP and atractylate are added simultaneously, ADP is exchanged until all sites are occupied by atractylate. Already in this case, the quench is delayed by competition with ADP. The quench delay increases when ADP is added before and reaches a maximum when the steady state of transport is established.

In order to follow the quench delay in a more direct manner, a new approach was worked out. Its principal idea was to set up conditions whereby the nucleotide exchange could be followed at steady state as well as under conditions where the carrier was not yet equilibrated with ADP (transient state). For this purpose it was necessary to study the uptake of ^{14}C-labelled ADP ("forward" exchange) instead of the "back" exchange. The steady state was established by preincubation of the mitochondria with unlabelled ADP. Then the exchange became "visible" when ^{14}C-labelled ADP (in small amounts, scarcely changing the total ADP concentration) was added through the first mixer. Atractylate was added by the second mixer at increasing time intervals, as usual. In this case the incorporation of [^{14}C]ADP into the mitochondria should reflect the equilibration rate of [^{14}C]ADP with the carrier and the endogenous nucleotide pool, by exchange. This was compared with the usual "starting state" experiment, where a mixture of both the unlabelled ADP and [^{14}C]ADP are added to the mitochondria through the first mixer, and again atractylate was added through the second mixer in varying times. The results in Fig. 5A show that with ADP already present (steady state) even at zero reaction time, when [^{14}C]ADP and atractylate were added simultaneously to the mitochondria, a considerable amount of [^{14}C]ADP was incorporated. Without prior ADP incubation (transient state) much less [^{14}C]ADP was found in the mitochondria at zero time. After 400 ms, the same level of exchange as in the steady state is reached.

The results clearly substantiate the proposed quench delay: the considerable uptake of [^{14}C]ADP at time zero in the "steady state" as compared to that in the "transient state" reflects the prolonged time which atractylate needs to inhibit the carrier when its sites are already saturated with ADP. In the "transient state" the retardation of actractylate binding is at first small and increases with the time elapsed between addition of ADP and atractylate. As a result, the difference between both states should disappear once, in the "transient state", ADP had sufficient time to equilibrate with the carrier. It can be concluded that the time delay of approx. 100 ms in the "steady state" represents a maximum that becomes smaller, the shorter the time difference between ADP and atractylate addition (see Fig. 5A). The total exchange, which is the sum of the exchange until atractylate addition and of the subsequent residual delayed exchange, should increase more rapidly in this period.

In further experiments, the quench delay with atractylate was measured directly (Fig. 5B). For this purpose, in a reversed sequence of additions, i.e., first atractylate and then [^{14}C]ADP, the time difference between addition of atractylate and [^{14}C]ADP was varied. Again, "transient" and "steady state" conditions are compared when ADP plus atractylate are added simultaneously in the first mixer and [^{14}C]ADP in the second mixer, and when mitochondria are incubated with ADP, atractylate is added in the first mixer and [^{14}C]ADP in the second mixer.

In these experiments, where [^{14}C]ADP is added after atractylate at varying times a residual exchange becomes visible due to the retarded quench by atractylate. As is to be expected, in the "steady state" the residual exchange is larger as compared to that in the "transient state". This difference decreases with time. The undelayed quenching level is defined by the amount of [^{14}C]-

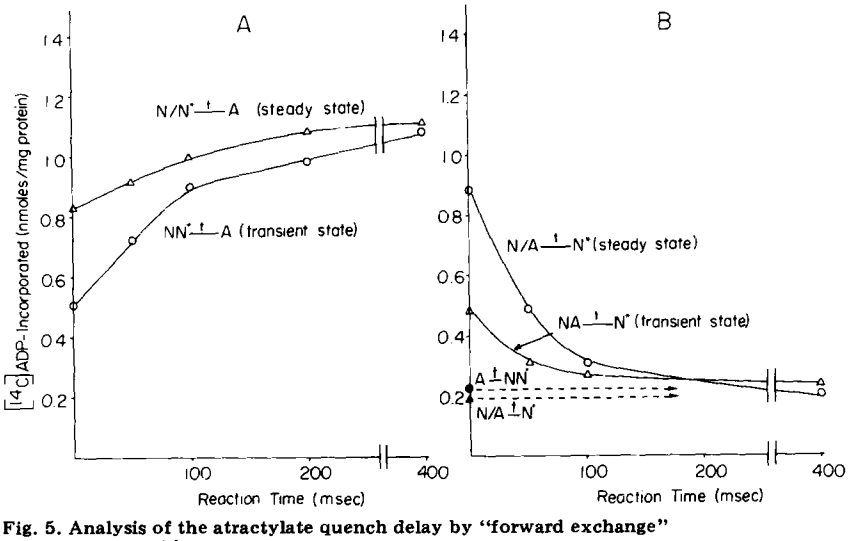

Fig. 5. Analysis of the atractylate quench delay by "forward exchange"

A. Kinetics of [^{14}C]ADP uptake with and without ADP-preequilibration (steady state; transient state). For steady state, 200 μM (unlabelled) ADP were added to mitochondria 2 min before starting the exchange upon addition of [^{14}C]ADP (case N/N*—A) (N* = ^{14}C-labelled nucleotide). The exchange was stopped by atractylate as usual and ADP uptake was determined (see Methods). In the second case (NN*—A) ADP plus [^{14}C]ADP was added simultaneously to initiate the reaction as usual (transient state). The difference between these cases should reflect the competition between atractylate and ADP at the translocator sites. The t = 0 values are obtained with [^{14}C]ADP and actractylate, added together at 50 ms reaction time.

B. Kinetics of the atractylate quench in dependence on the time of preequilibration with ADP. In the case N/A—N* atractylate was added to ADP preequilibrated mitochondria (steady state) in the first mixer and [^{14}C]ADP in the second mixer. In the case NA—N* (transient state) atractylate and ADP (unlabelled) were added simultaneously to mitochondria and then [^{14}C]ADP was admixed in the second mixer. Base lines are obtained by adding atractylate alone in the first mixer and [^{14}C]ADP or ADP plus [^{14}C]ADP in the second mixer (0.6 mg protein/ml).

Conditions: 1. Steady state, mitochondria were incubated with 200 μM ADP 1 min prior to mixing with [^{14}]ADP + 200 μM ADP (A), or mixing with 90 μM atractylate (B) in the first mixer. 2. Transient state, same amount of mitochondrial protein was mixed with 400 μM ADP in the first mixer and 70 μM of atractylate was added in the second mixer (A) or 400 μM ADP plus 90 μM atractylate in the first mixer and [^{14}C]ADP in the second mixer (B).

ADP uptake when atractylate is added with sufficient time before ADP addition. This level is actually reached for both the transient and steady-state conditions when the interval between addition of atractylate and [^{14}C]ADP is allowed to exceed 100 ms.

These results are consistent with the proposed quench delay mechanism. In particular, the direct demonstration of the quench delay in Fig. 5B agrees with the time period of approx. 100 ms for the initial rapid phase observed in the exchange kinetics.

Rapid exchange kinetics under various conditions

The stopped-flow quench method with atractylate was applied to study dependence of the initial exchange rates on various parameters such as temperature, energization at the membrane, concentration of ADP.

The dependence of the initial exchange rate on the concentration of ADP in the quench-flow experiment is shown in Fig. 6. The ADP concentration was varied from 1 to 100 μM. This was of interest because a high ADP concentra-

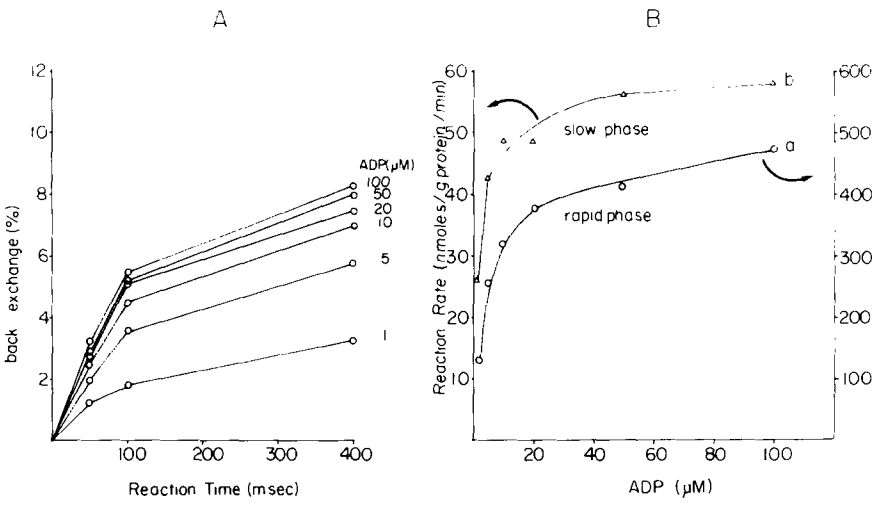

Fig. 6 Dependence of the "back-exchange" kinetics on the ADP concentration. The percent exchange in A is based on the total labelled endogenous adenine nucleotides, the translocation activity in B is calculated from the slopes of A between 0 and 100 (a) and 100 and 400 ms (b) and is based on the exchangeable pool of the endogenous adenine nucleotides (10 μmol/g). Mitochondrial protein was 0.9 mg/ml in all experiments, temperature 10° C, reactions were quenched with 90 μM atractylate.

tion could be expected to increase the quench delay. As shown in Fig. 6, there is a considerable concentration dependence, both of the rapid and the slower phase. The rates deduced from these slopes, as plotted in the accompanying part, increase up to 100 μM. The rapid phase, as well as the slow phase, exhibits a concentration dependence, with a K_m ranging from 2.5 μM for both kinetic phases.

The temperature dependence of the initial exchange rate was followed in the range between 5 and 23° C. For this study, the time range between 25 and 400 ms is covered (experiments not shown). The slopes are evaluated from the values above 100 ms for the exchange rate and plotted against temperature. The results exhibit a more-than-7-fold increase in the exchange rates between 5 and 23° C. This is in agreement with temperature dependence previously reported using a manual stopping method [1].

The influence of the energy state on the initial rates of the ADP exchange was discovered to be particularly interesting. As shown in Fig. 7, under the influence of uncoupler (CCP), and still more with valinomycin, the separation into a rapid phase and subsequent slower phase becomes abolished. The kinetics of these states merge at prolonged time. In terms of the previous discussion the effects of CCP and valinomycin can be interpreted as diminishing the quench delay by atractylate.

Another point of interest in this context was the influence of the endogenous nucleotide pattern, i.e., the phosphorylation state of the endogenous adenine nucleotide pool. With ketoglutarate a particularly high ATP content can be obtained, whereas with arsenate the AMP content is high. As shown in Fig. 8, the quench delay is strong in the energized state with a high ATP content and appears to be considerably smaller in the presence of arsenate with a

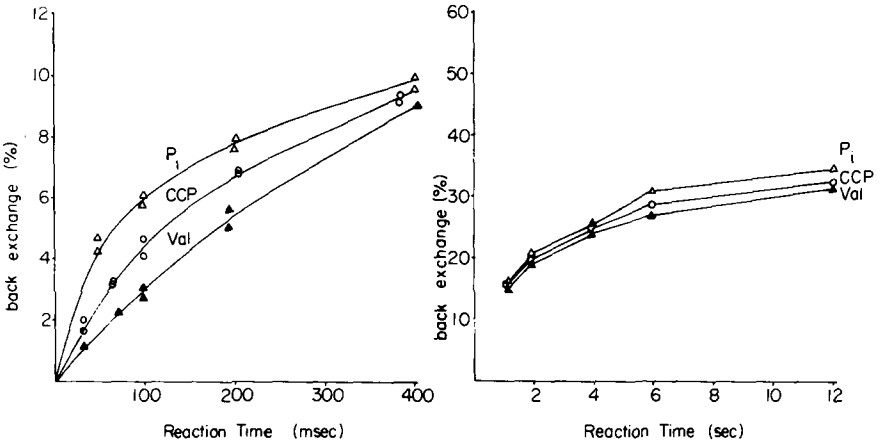

Fig. 7. Influence of various metabolic states on the exchange by ADP. The exchange was measured by "backward exchange" using the quench-flow procedure for fast kinetics (A) and the sampling method (B). All additions to 0.8 mg of mitochondrial protein were performed 1—2 min prior to initiating the exchange reactions which were carried out at 10°C, incubation medium: 130 mM KCl 20 mM Tris-maleate, pH 7.2; plus △, 2 mM P_i; ▲, 1 μg valinomycin (Val)/mg protein; ○, 2.5 nmol CCP/mg protein.

high level of endogenous AMP. In the latter case, the maximum exchange at 6 s is decreased due to the lower level of ADP and ATP. The influence here is mainly to be considered in the decrease of the AMP content, which is also known from other experiments.

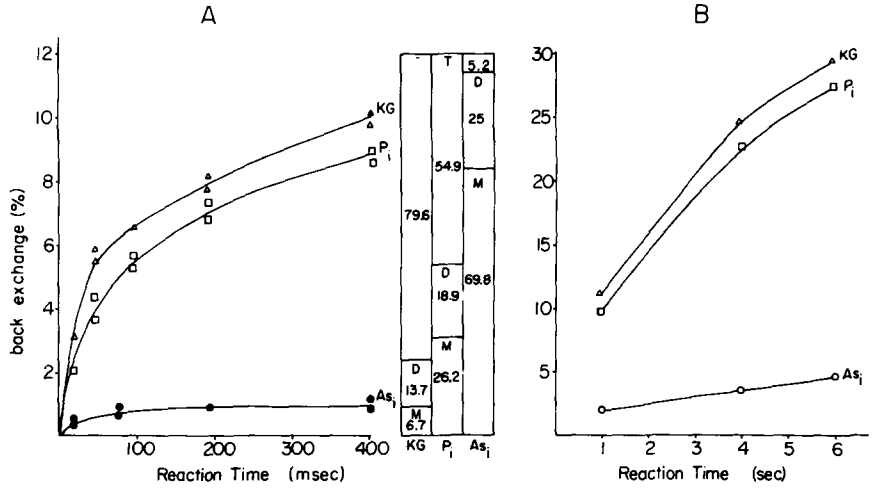

Fig. 8. Comparison of the kinetics of the ADP exchange in energy-rich and energy-depleted mitochondria. The exchange was measured at 10°C as "back-exchange" using the quench-flow apparatus for the ms range (A) and the continuous sampling procedure for the s range (B). Prior to exchange samples were taken for the determination of the pattern of endogenous adenine nucleotides by small-column chromatography [6]. Conditions: 0.8 mg of mitochondrial protein was preincubated with either 2.5 mM ketoglutarate (KG), 1 mM P_i or 10 mM arsenate (As_i) for 4 min at 10°C.

Evaluation and discussion

The attempt described in this paper to resolve the kinetics of the ADP, ATP transport in mitochondria in the millisecond range by a rapid mixing quench flow apparatus resulted in some unexpected phenomena. The main observation was the existence of an initial rapid phase in the first 100 ms, which accounts for approx. 5—10% of the exchangeable pool. After this time the kinetics are relatively uniform and might be approximated by a first-order reaction.

A major question is the nature of the rapid phase. It is all the more interesting since the rapid phase appears to be dependent on the metabolic state of the membrane and disappears in mitochondria which are deenergized by the addition of uncoupler of valinomycin plus K^+. One plausible explanation for the rapid phase may be based on the assumption of a small pool of nucleotides existing separate from the bulk pool. Such a small pool, reflecting the initial rapid phase, might be identical with the nucleotides bound to the carrier sites. The number of carrier binding sites in liver mitochondria should be not larger than 0.2—0.3 μmol/g protein [13,14] if one identifies these sites with the number of atractylate binding sites. However, the rapid pool is considerably larger and reaches (for example, in the experiment of Fig. 2) 6% of approx. 10 μmol/g protein-exchangeable nucleotides, corresponding to approx. 0.6 μmol/g protein.

Accepting some views of the literature, this rapid pool may be interpreted as consisting of nucleotides being in direct contact with the ATP synthase [15, 16]. This assumption seems to agree with the amount of adenine nucleotide pool liganded to the ATPase as calculated from data of Harris et al. [17] and Bertina et al. [18], i.e., 0.6—1.0 μmol/g mitochondrial protein. Such a rapid pool would then bypass the endogenous nucleotide pool in phosphorylation reaction, as has been suggested [15,16]. At any rate, the existence of this rapid pool and the consequent subcompartmentation of the intramitochondrial nucleotide pool must be dependent on the energized state of the mitochondria, since in the presence of CCP and valinomycin the rapid phase is abolished. In this context it might be mentioned that, according to Rosing et al. [19], the nucleotides bound to the ATP synthase are more easily removable by energization of the membrane.

The other possibility, already introduced in the Results section, is quench-delay by atractylate. This interpretation is supported by the "forward" exchange data. Here, indirectly, the speed is measured at which atractylate quenches the exchange. Particularly impressive is the comparison of the steady state in which the carrier is first equilibrated with unlabelled ADP and then [^{14}C]ADP is added, with the case where the exchange is started by both ^{14}C- and unlabelled ADP simultaneously. In the first case the binding sites are already saturated by ADP before starting the kinetics of [^{14}C]ADP uptake, and therefore the quench delay should remain unchanged after [^{14}C]ADP addition, whereas in the second case the binding sites become occupied only after starting the kinetics with [^{14}C]ADP. This implies that binding sites liganded with ADP will bind atractylate more slowly since their availability depends on the dissociation rate of ADP. As a result, the quench delay should increase during the first period after ADP addition when more binding sites become clogged by ADP. This is clearly shown in Fig. 5B.

Parameters varying the rate of the translocation, such as temperature, ADP concentration or the identity of the nucleotides added to initiate the exchange, influence the extent of the rapid phase in a similar manner as they do the rate of translocation. From these observations one may conclude that the rapid phase is a function of the translocation rate. This would not be expected to be the case if the rapid phase were due to nucleotides attached to the ATPase. The correlation of the translocation activity to the size of the rapid phase, is limited, however, only to the "energized" state, since in uncoupled mitochondria the rapid phase is nearly abolished.

The question arises as to whether the interpretation of the rapid phase as a quench delay could conform with its disappearance in the presence of CCP and valinomycin. A possible explanation in line with this proposal would be as follows. The carrier site distribution inside/outside is influenced by the energization of the membrane and is therefore different in the presence of CCP and valinomycin. It is further postulated that in the deenergized state relatively more carrier sites are orientated to the outside than in the energized state. Some support for the assumption comes from the fact that only in the energized state is *N*-ethylmaleimide incorporated to mitochondria [20]. This has been interpreted to indicate that in this case the carrier site is turned mostly towards the inner face ("m" side) [21]. As a result, in the energized state the carrier should be less accessible to atractylate than in the deenergized state, since atractylate can bind to the carrier only when the site is on the outside ("c" state). The increased accumulation of sites outside in the deenergized state would therefore result in a more rapid binding to atractylate and consequently in more rapid quenching.

Appendix *(In collaboration with B. Hess, Max-Planck-Institut für Ernährungsphysiologie, Dortmunt, G.F.R.)*

A model for quantitative evaluation of quench delay

In order to understand more fully the effect of a quench delay on the efflux of nucleotides during the ADP, ATP exchange, a quantitative evaluation of the kinetics involved was attempted, based on the mobile (reorientation) carrier mode, as shown in Fig. 9.

From this scheme the rate equations can be derived for the binding, entry, exit and dissociation of the nucleotides. Atractylate competes with the nucleotides for those carrier sites which are unliganded and appear on the outside. Formation of the atractylate · carrier complex is assumed to be largely irreversible. Differential equations for these reactions can be derived. An analytical solution can be achieved for the formation of atractylate · carrier complex by integration under the assumption of some simplifications. However, a full solution taking into account the efflux of the nucleotides appears to be impossible.

For these reasons a computer solution of the kinetics leading to the quench delay was preferred (Fig. 10). A set of 13 reaction equations was derived from the scheme in Fig. 9 for all the forward and back reactions. With the computer program available at the Max-Planck Institute in Dortmund, the time course of the reaction system could be followed such that each of these twelve variables or reaction species could be printed out as dependent upon time.

In order to simulate the experiments for the computer solutions, the

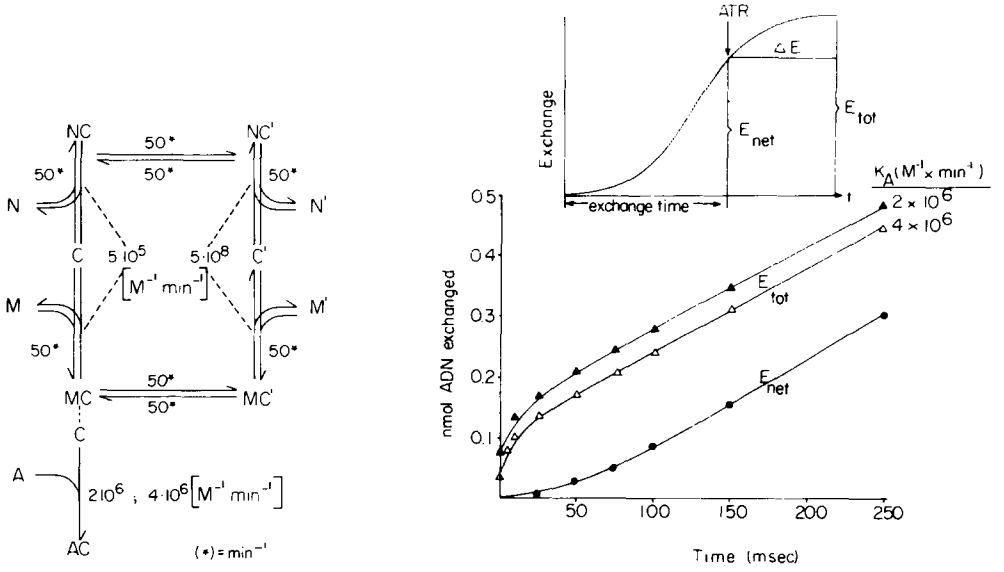

Fig. 9. Schematic presentation of the reaction sequence according to a simple mobile carrier model for analyzing the exchange reaction and the quench by atractylate. The rate constants for the various reaction steps are given in the scheme. They are estimated from rates of swelling, shrinkage induced by ADP or atractylate and overall translocation rate. N, added nucleotide; M, nucleotide originating from the endogenous pool; C, free carrier site; accent (') indicates internal species.

Fig. 10. Simulation of the atractylate (ATR) quench delay by computing the reaction sequence given in Fig. 9 for various exchange times. The net exchange (E_{net}) corresponds to the exchange calculated up to atractylate addition. The residual exchange after atractylate addition due to quench delay (ΔE) added to E_{net} gives E_{total} (E_{tot}). E_{tot} is plotted with two assumed rate constants for atractylate binding (K_A). The values were computed with the "basic enzyme-simulation system, MPD version 7301" at the Max-Planck Institute, Dortmund, on a IBM-360/45 computer.

appearance of endogenous nucleotides was followed before and after atractylate addition. For this purpose the release of nucleotides was integrated up to a certain time when atractylate is added (and the net exchange is obtained (E_{net})). In the second computer run, the residual release of nucleotides after addition of atractylate was calculated (ΔE). This corresponds to the additional nucleotide release due to the quench "delay". The total nucleotides released (E_{tot}) are calculated as the sum of the nucleotides released and of the additional released ADP due to the quench delay. These values are plotted as a function of the time interval up to the actractylate addition. The curves in Fig. 10 are calculated with two different rate constants of atractylate binding.

One observes clearly an apparent rapid phase in the initial 25 ms. Already at the simultaneous addition of atractylate and ADP ($t = 0$) considerable residual exchange takes place. This simultaneous value is subtracted in the experimental data (Fig. 4). With the present set of rate constants is obtained a relatively good fit of the extent of the quench delay with the experimental data, amounting to approx. 0.06 μmol adenine nucleotide/g protein. However, the changeover from the rapid phase to the transition phase often takes place at longer times in the experiments.

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