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Once Again about the Functional Coupling between Mitochondrial Creatine Kinase and Adenine Nucleotide Translocase

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Abstract—The synthesis of creatine phosphate (CP) by mitochondrial creatine kinase during oxidative phosphorylation was terminated when the mass action ratio of the creatine kinase reaction $\Gamma = [ADP]\cdot[CP][ATP]\cdot[Cr]$ became equal to the apparent equilibrium constant (K_{eq}^{app}) of this reaction. Subsequent excess of Γ over the K_{eq}^{app} was due to an increase in the ADP concentration in the medium. A comparable increase in the ADP concentration also occurred in the absence of creatine (Cr) in the incubation medium. Increase in the ADP concentration was shown to be associated with a decrease in the rate of oxidative phosphorylation and with a relative increase in the ATPase activity of mitochondria during the incubation. A low concentration of ADP (<30 μM) and relatively high concentrations (1-6 mM) of other components of the creatine kinase reaction prevented the detection of the reverse reaction within 10 min after Γ exceeded the K_{eq}^{app}, but the reverse reaction became evident on more prolonged incubation. The reverse reaction was accompanied by a further increase in Γ. Low ADP concentration in the medium was also responsible for the lack of an immediate conversion of the excess creatine phosphate added although $\Gamma > K_{eq}^{app}$. The findings are concluded to be in contradiction with the concept of microcompartment formation between mitochondrial creatine kinase and adenine nucleotide translocase.

Key words: mitochondrial creatine kinase, physiological role, adenine nucleotide translocase, functional coupling

According to a current concept, a compartment in the cell need not be delimited by membranes, e.g., as mitochondria are. A microenvironment in the region of firmly or even weakly interacting proteins and also near the intracellular surfaces can form a compartment. These are so-called "metabolic" or "functional" compartments [1]. The interaction of proteins which have substrates in common and form a metabolic compartment is suggested to produce a functional coupling, i.e., the compartment constituents prefer to use the substrates generated inside the compartment and not substrates from the outside.

The problem of the functional coupling of mitochondrial creatine kinase (mtCK) and adenine nucleotide translocase (ANT), the structural basis and physiological role of this phenomenon, has been under discussion for more than 25 years [2]. Some authors believe that functional coupling between mtCK and ANT is structurally based on microcompartment formation during the interaction of these proteins as a result of collision of their molecules during oxidative phosphorylation on the mitochondrial inner membrane [3]. This provides a direct exchange of adenine nucleotides between active sites of the two proteins [4], i.e., ATP produced during oxidative phosphorylation directly enters the active site of mtCK from the active site of ANT without mixing with ATP of the medium, whereas ADP produced during the creatine kinase reaction is transfered in the reversed direction. As a result, in the region of the mtCK active site a high concentration of ATP and a low concentration of ADP are maintained that promotes mtCK to synthesize more creatine phosphate (CP) than could be synthesized in the absence of the structural interaction between mtCK and ANT [4] (on the suggestion that the compartment is permeable for creatine (Cr) and creatine phosphate). And a high concentration of ADP and a low concentration of ATP are maintained in the region of the active site of ANT. This is favorable for a high respiration rate at low ADP concentration in the medium, i.e., mtCK amplifies the control of respiration by ADP [4].

Data of thermodynamic analysis are considered to prove the existence of the mtCK—ANT compartment. The idea of these experiments was as follows. Obviously, creatine phosphate in the mtCK—ANT compartment

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could be synthesized until the mass action ratio (Γ) of the creatine kinase reaction components ($\Gamma = [ADP]\cdot[CP][ATP]\cdot[Cr]$) in the region of the mtCK active site became equal to the apparent equilibrium constant (K_{eq}^{app}) of this reaction. It is impossible to determine concentrations of the reaction components inside the microcompartment. They can be determined only in the incubation medium after precipitation of mitochondria. However, if during oxidative phosphorylation the ADP concentration in the region of the mtCK active site is really lower and the ATP concentration is higher than in the incubation medium, then at the mass action ratio of the reaction components (determined by their concentrations in the incubation medium) getting equal to the K_{eq}^{app}, this ratio in the microcompartment will be lower than K_{eq}^{app} (because in the mtCK active site the [ADP]/[ATP] ratio is lower than in the incubation medium) and the creatine phosphate synthesis in the microcompartment will be continued. But when the mass action ratio of the reaction components in the microcompartment becomes equal to the apparent equilibrium constant, this ratio determined by the substrate concentrations in the incubation medium will be higher than the K_{eq}^{app} because of the excess synthesis of creatine phosphate in the microcompartment. In fact, in experiments of DeFuria et al. [5] and afterwards of Saks et al. [6] and also of Sobol et al. [7, 8] the mass action ratio of the creatine kinase substrates determined in the incubation medium during oxidative phosphorylation by mitochondria [5, 7, 8] or by mitoplasts [6, 8] could become higher than the apparent equilibrium constant value determined under similar experimental conditions but with soluble creatine kinase instead of mitochondria [5, 7, 8] or on suppression of oxidative phosphorylation with carboxyatractyloside and oligomycin [6].

Those authors believe this finding confirms the difference between the concentrations of the creatine kinase reaction components in the region of the mtCK active site and in the incubation medium [5, 7, 8]. They also think the excess of Γ over the K_{eq}^{app} value proves the formation of the mtCK—ANT microcompartment during oxidative phosphorylation with its subsequent decomposition when oxidative phosphorylation is inhibited [6].

According to another viewpoint on the structural basis of the functional coupling between mtCK and ANT, the outer mitochondrial membrane has a limited permeability for charged molecules and, as a result, during oxidative phosphorylation local concentrations of adenine nucleotides become different from their concentrations in the medium [9]. The destruction of the outer membrane is associated with a disappearance of the functional coupling [10].

Gellerich et al. [11, 12] have shown that the mtCK activity during oxidative phosphorylation is associated with an increased concentration of ADP and a decreased concentration of ATP in the intermembrane space of mitochondria. According to their data, during the mtCK activity the concentration gradient of adenine nucleotides was maximal at a high value of the [ATP]/[ADP] ratio in the medium when the mtCK activity was high and the oxidative phosphorylation rate was low; this gradient was ~13 μM. The gradient value was comparable only to the physiological concentration of ADP but not of ATP and of creatine phosphate; therefore, the outer membrane seemed to be a permeability barrier only for ADP [12]. Gellerich et al. [13] suppose the physiological role of mtCK is an indirect transfer of ADP required for oxidative phosphorylation across the outer mitochondrial membrane. These authors called this concentration gradient of adenine nucleotides dynamic because it depended on the creatine kinase reaction rate and on the rate of oxidative phosphorylation. The termination of these reactions was associated with a disappearance of the dynamic concentration gradient of adenine nucleotides across the outer membrane [11, 12]. From this standpoint, when the creatine kinase reaction came to quasi-equilibrium, the concentration gradient of adenine nucleotides between the intermembrane space and extramitochondrial space should be close to zero and the mass action ratio of the creatine kinase reaction components should be equal to the apparent equilibrium constant of this reaction.

Thus, the thermodynamic test seemed to allow us to differentiate these two possibilities. However, any preparation of mitochondria exhibit ATPase activity due to the presence in it mainly of partially destroyed mitochondria and to a lesser degree of fragments of non-mitochondrial membranes [11]. The ATPase reaction is virtually irreversible and decreases the ATP concentration and increases the ADP concentration in the medium. The ATPase activity of fresh preparations of mitochondria is usually low compared to the rate of oxidative phosphorylation and the mtCK activity. However, when the creatine kinase reaction approaches quasi-equilibrium its rates in both directions approaches zero. A long-term incubation of mitochondria can decrease their ability to perform oxidative phosphorylation. As a result, the rate of ADP production by ATPase can be higher than the ability of mtCK and of the oxidative phosphorylation system to regenerate ATP. In this case the mass action ratio of the creatine kinase reaction components can be higher than the apparent equilibrium constant value, although not due to the excess synthesis of creatine phosphate but due to an increase in the ADP concentration and a decrease in the ATP concentration in the incubation medium produced by ATPase.

Heart mitochondria also contain adenylate kinase which can convert adenine nucleotides. However, the activity of adenylate kinase is low compared to the activities of mtCK and of the oxidative phosphorylation system and the reaction catalyzed by it is easily reversible; therefore, the activity of this enzyme cannot cause a devi-

ation of Γ from the K_{eq}^{app} and markedly affect the quasi-equilibrium concentrations of the mtCK substrates.

The information presented in the thermodynamic experiments described in the literature [5-8] does not allow us to differentiate these two possibilities. Having in mind the importance of the functional coupling problem for comprehension of the physiological role of mtCK and of general principles of metabolism, we performed experiments where changes in the concentrations of all components of the creatine kinase reaction were determined during its approach to the quasi-equilibrium during oxidative phosphorylation. We supposed that if an mtCKANT compartment was really formed during oxidative phosphorylation, an excess of the mass action ratio over the apparent equilibrium constant value should be obtained due to a persisting increase in the concentration of creatine phosphate (already after the mass action ratio became equal to the apparent equilibrium constant value) at constant [ADP]/[ATP] ratio in the medium. But if the functional coupling was structurally provided by a limited permeability of the outer membrane for ADP and the excess of the mass action ratio over the apparent equilibrium constant value was associated with the ATPase activity of mitochondria, the synthesis of creatine phosphate should be terminated when Γ of the creatine kinase reaction components determined by their concentrations in the medium would be equal to K_{eq}^{app}, and the subsequent excess of this ratio over the K_{eq}^{app} would be due to an increase in the [ADP]/[ATP] ratio in the medium. In our experiments the excess of the mass action ratio over the apparent equilibrium constant value was obtained at the cost of the excess synthesis of ADP as a result of a decrease in the rate of oxidative phosphorylation and of a relative increase in the ATPase activity. Thus, our findings contradict the concept on the structure–functional coupling between mtCK and ANT. We have also analyzed in detail the data presented in works [5-8] and have shown that in these works the excess of Γ over the K_{eq}^{app} value can also be due to the ATPase activity of mitochondria. We have concluded that the thermodynamic approach, which requires a long-term incubation of mitochondria at elevated temperatures, is not an adequate approach to solve the problem of the structural basis for the functional coupling between mtCK and ANT.

MATERIALS AND METHODS

Mitochondria were isolated from rat heart by a trypsin method of Jacobus and Saks [14]. In each experiment hearts of six rats with body weight of 250-300 g were used. Before the experiment the animals were fasted overnight but had free access to water. Mitochondria were finally suspended in 1 ml of the isolation medium which contained 0.3 M sucrose, 10 mM HEPES (pH 7.4), 0.2 mM EDTA, human serum albumin (HSA, 1 mg/ml) and kept on ice at 0°C. A spare solution of HSA was dialyzed for 3 days against the more than 100-fold volume of water which was changed every day. The dialyzed HSA solution was divided into portions and kept at −18°C.

Mitochondrial protein was assayed by the method of Gornall et al. [15].

Determination of the mass action ratio of the creatine kinase reaction components. *Incubation of samples.* The experimental conditions were similar to those used in [7, 8], only sucrose in the incubation medium was replaced with mannitol. The fundamental incubation medium (the sample volume was 1.2 ml, the vessel diameter was 18 mm) contained 0.25 M mannitol, 10 mM Tris (pH 7.2), 5 mM potassium phosphate, 0.2 mM EDTA, 20 mM KCl, 5 mM $MgCl_2$, 10 mM creatine, 2.5 mM glutamate, and 2 mM malate (medium A). In some experiments medium A was changed as indicated in legends to figures and tables.

The samples were supplemented with 1.2 mg mitochondrial protein and incubated to equalize the temperature at 37°C for 1 min with vigorous stirring by a magnetic stirrer. The reaction was initiated by addition of ATP to the final concentration of 1 mM and the samples were incubated for 1-20 min. To prevent an effect of the mitochondrial aging during the experiment on the results, in each experiment the samples were incubated in a random succession. After the incubation, 0.9 ml was taken from each sample and placed into tubes standing on ice and containing concentrated $HClO_4$ (final concentration 6%). The protein precipitate was centrifuged in the cold at 5000 rpm using a Metronex laboratory centrifuge (model 310, Poland). From the supernatants 0.9 ml was taken and neutralized with a known volume of 6 M K_2CO_3 in the presence of Bromothymol Blue until the indicator color changed from yellow to violet. The precipitate of $KClO_4$ was separated by centrifugation. The supernatants were frozen at −18°C and were used later for determination of the creatine kinase reaction substrates and products.

As the control for the ADP content in the mitochondrial matrix, samples without the ATP added were used and the reaction was stopped after the mitochondria had been incubated in medium A for 1 min. The resulting values of ADP concentration were subtracted from the values obtained for the experimental samples. Thus, the ADP concentrations presented in the tables and figures relate to its concentrations in the incubation medium. This approach was based on the finding described in the literature that the ADP content was virtually unchanged in the mitochondrial matrix after their transition from the rest to an active oxidative phosphorylation [7, 16]. For other substrates of mtCK the mitochondrial contribution was considered negligibly small and was not taken into account.

To obtain the K_{eq}^{app} value for our experimental conditions, the cytoplasmic isoenzyme of muscle creatine kinase (MM-CK, final concentration 55 μg/ml) was

added to the samples which contained medium A and 1 mM ATP and were incubated for 5 and 10 min to be sure that the creatine kinase reaction had come to equilibrium. The apparent equilibrium constant was also determined in the control mitochondria-containing samples in the presence of oligomycin (4 μg per mg protein) and rotenone to the final concentration of 1.7 μM in the incubation medium.

Contents of ATP and creatine phosphate were determined in the samples at 340 nm with a Hitachi 200-20 recording spectrophotometer (Japan) using coupled hexokinase and glucose-6-phosphate dehydrogenase enzymes as described in [17].

ADP content in the samples was assayed spectrophotometrically at 340 nm with a coupled system of pyruvate kinase and lactate dehydrogenase. In addition to the solution under study, the sample for spectrophotometry (total volume 0.7 ml) contained 86 mM HEPES (pH 7.4), 123 mM KCl, 13.3 mM $MgCl_2$, 0.14 mM NADH, 0.6 mM dithiothreitol, 2 mM phosphoenolpyruvate, and lactate dehydrogenase (2 units). The reaction was initiated by addition of pyruvate kinase (1 unit). The ADP content was determined using the molar absorption coefficient for NADH as $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ [18].

Creatine concentration in the samples was determined in the presence of diacetyl and α-naphthol [19].

Mass action ratio of the creatine kinase reaction was calculated by the formula:

$$\Gamma = [ADP] \cdot [CP] / [ATP] \cdot [Cr].$$

Determination of the optimal rate of sample agitation. Five positions were chosen for the rotation rate regulator of the magnetic stirrer. Positions 1 and 5 corresponded to the minimal and the maximal rate of stirring, respectively. At each of these rates the mitochondria samples were incubated in the medium A for 8 or 20 min after ATP had been added as described above. After the protein precipitation with $HClO_4$ and neutralization of the samples they were used to determine the contents of ADP and creatine phosphate.

The rate of mitochondrial respiration was assayed polarographically with a closed Clark-type electrode at 37°C and sometimes at room temperature. The latter case is specially pointed out in the text. The samples of 1 ml contained medium A and the mitochondrial protein (0.2-0.5 mg). Oxidative phosphorylation was initiated by addition to the samples of ADP to the final concentration of 0.3 mM. To determine the ATPase activity of the mitochondria preparation, the samples instead of ADP were supplemented with ATP to the final concentration of 1 mM.

The respiration rates were calculated assuming the oxygen concentration in the incubation medium to be 200 μM at 37°C and 250 μM at room temperature [20]. When calculating the rate of oxidative phosphorylation and the ATPase activity the respiration rate was found as the difference between the rate at state 3 and the respiration rate before the addition of ADP or ATP. The resulting difference was multiplied by the ADP/O value found for the specific preparation.

Studies on the effect of mitochondria incubation at 37°C on their functional characteristics. Mitochondria were incubated in medium A for 2 or 20 min after the addition of 1 mM ATP. To stop the reaction, the samples were placed into 40 ml of cold isolation medium and immediately centrifuged in the cold at 12,000*g* for 10 min using a Beckman centrifuge (model J2-21, Austria). The supernatants were carefully decanted, the vessel walls were dried with a filter paper, and the mitochondrial precipitate was suspended in 100 μl of the isolation medium without HSA. The resulting suspension was used for polarographic analysis.

RESULTS

Polarographic characteristics of the mitochondrial preparations are presented in Table 1. The mitochondrial preparations at 37°C exhibited a high values of respiratory control (about 6 in the presence of 5 mg $MgCl_2$) and a low ATPase activity which was ~12% of the oxidative phosphorylation rate. These values are comparable to data in the literature [11, 14].

In thermodynamic experiments described in the literature mitochondria were incubated for 20-60 min [7, 8]

Table 1. Some polarographic characteristics of rat heart mitochondria

Respiration rate in state 3, ng-atom O/min per mg protein	Respiratory control index	ADP/O	Rate of oxidative phosphorylation	ATPase activity
			nmol/min per mg protein	
888 ± 48	5.7 ± 0.5	3.2 ± 0.1	2313 ± 132	274 ± 36

Note: The results are presented as means SD (n = 5). The experimental conditions are described under "Materials and Methods".

and more [5]. During long-term experiments on oxidative phosphorylation it was necessary to provide conditions preventing anaerobiosis. Therefore, we incubated samples of a small volume in wide vessels and vigorously stirred them with a magnetic stirrer. But there was also another danger: a long-term incubation and an inappropriately high rate of the stirring could damage the mitochondria with a subsequent uncoupling between oxidation and phosphorylation.

Therefore, in the first experiments the optimal conditions for the sample stirring were chosen. In one experiment the samples were incubated for 8 min (by this time a constant concentration of creatine phosphate had been attained in the medium) and in another experiment the samples were incubated for 20 min at various rates of the agitation. After the incubation had been terminated, the contents of ADP and of creatine phosphate and also the CP/ADP ratio were determined. The ADP concentration in the samples depended on the ratio between the rates of three main reactions, those of creatine kinase and of ATPase which produce ADP and on the rate of oxidative phosphorylation which consumes ADP. We supposed that the optimal rate of the stirring should provide the minimal ADP concentration in the medium (this would suggest the minimal ATPase activity and the maximal rate of oxidative phosphorylation) and the maximal concentration of creatine phosphate that would suggest a high activity of mtCK. The results are presented in Table 2.

Table 2 shows that the ADP concentration in the samples did not depend on the rate of the stirring under widely varied conditions but increased at the maximal rate of the agitation, especially on incubation for 20 min. The minimal rate of the stirring was insufficient for manifestation of the maximal mtCK activity. At stirring rates 2-4 an increase in the stirring rate was not accompanied by an increase in the rate of creatine phosphate synthesis. This rate during the incubation for 20 min was lower than for the incubation for 8 min, especially at the maximal rate of stirring. As a result, for the 20-min incubation of the samples the CP/ADP ratio was lower than for the 8-min incubation. As for the 20-min incubation and for the maximal rate of the stirring the ADP concentration increased along with a decrease in the creatine phosphate concentration and with a sharp decrease in the CP/ADP ratio, we supposed that the increase in the ADP concentration during this period was associated not with an increase in the

Table 2. Effects of the incubation time and of the rate of the sample stirring on the ADP and creatine phosphate concentrations in the samples (the experimental conditions were described under "Materials and Methods")

Incubation time, min	Rate of stirring	Concentration, mM		CP/ADP
		ADP	CP	
8	1	0.017	2.52	148
	2	0.022	3.81	173
	3	0.016	3.97	248
	4	0.020	3.70	185
	5	0.027	3.58	133
20	1	0.021	2.21	105
	2	0.025	3.35	134
	3	0.026	3.38	130
	4	0.021	3.19	152
	5	0.054	2.12	39

Table 3. Determination of the apparent equilibrium constant (K_{eq}^{app}) of the creatine kinase reaction

Enzyme	Equilibrium concentration, mM				$K_{eq}^{app} \times 10^3$
	ATP	Cr	ADP	CP	
MM-CK (15)	0.653 ± 0.013	10.26 ± 0.23	0.246 ± 0.006	0.237 ± 0.006	8.82 ± 0.43
mtCK in mitochondria (8)	0.656 ± 0.044	9.85 ± 0.33	0.255 ± 0.021	0.251 ± 0.038	9.09 ± 0.44

Note: Experimental conditions are described under "Materials and Methods"; the results are presented as means SD. The number of determinations is given in parentheses.

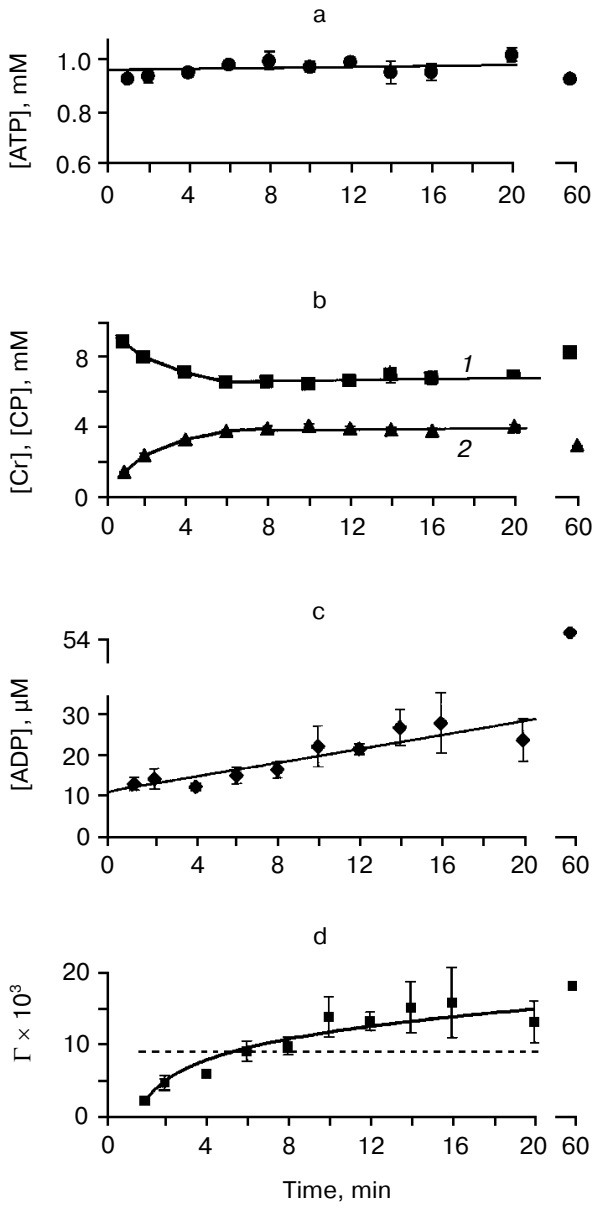

Fig. 1. Effect of incubation time on concentrations of the creatine kinase reaction components (a, b, c) and on the mass action ratio (Γ) of the reaction (d) (means SD, $n = 6$). The mitochondria were incubated in medium A for the time indicated. Other details of the experiments are given under "Materials and Methods". b) Creatine (Cr) (*1*), creatine phosphate (CP) (*2*). d) The position of K_{eq}^{app} is shown by the dotted line. For the 60-min incubation results of one experiment are presented.

mtCK activity but with a decrease in the rate of oxidative phosphorylation and/or with an increase in the ATPase activity of the mitochondria. We suggested that the maximal stirring rate and also the long-term incubations should be responsible for some damage to the mitochondria. With all this in mind, the medium stirring rate 3 and the incubation for no longer than 20 min were chosen.

In the next series of experiments we analyzed in detail time-dependent changes in the concentrations of the creatine kinase reaction components at the stirring rate chosen. From the resulting values the mass action ratios were calculated for this reaction. The experiment conditions were similar to those in the works of Sobol et al. [7, 8]. The data of experiments on mitochondria during oxidative phosphorylation are shown in Fig. 1, and the equilibrium concentrations of the substrates and products obtained in the experiments to determine the apparent equilibrium constant are presented in Table 3.

Thus, during the 20-min incubation the ATP concentration in the samples remained constant (Fig. 1a), concentration of creatine was decreasing during 6 min, and the creatine phosphate concentration was increasing (Fig. 1b). The creatine and creatine phosphate concentrations did not significantly change from 6 to 20 min. The ADP concentration was increasing during the whole period of incubation (Fig. 1c). The mass action ratio of the creatine kinase reaction (Fig. 1d) until 6 min was lower than the apparent equilibrium constant of this reaction determined under these experimental conditions (Table 3), from 6 to 8 min it was equal to K_{eq}^{app} and later it was higher. The excess of the mass action ratio of the creatine kinase reaction over the apparent equilibrium constant value was due to an increase in the concentration of ADP in the incubation medium because the concentrations of other reaction components were unchanged during this time (Fig. 1, a-d).

When the mass action ratio became equal to the apparent equilibrium constant value the creatine phosphate concentration in the incubation medium was ~3.8 mM (Fig. 1b). However, in the presence of cytoplasmic MM-CK the equilibrium creatine phosphate concentration was 0.24 mM (Table 3) that was ~6% of the creatine phosphate concentration attained in the medium under the experimental conditions presented at Fig. 1. The cause of this is clear from Fig. 1 and Table 3: when only MM-CK was active the equilibrium concentration of ADP was relatively high and equal to the equilibrium concentration of creatine phosphate. When mtCK and the oxidative phosphorylation system were active concurrently the latter removed ADP produced during the creatine kinase reaction, and as a result, the ADP concentration was maintained at the level of 13-27 μM (Fig. 1c). This promoted an additional synthesis of creatine phosphate by mtCK. As a result, its concentration became much higher than the concentration of ADP (Fig. 1, b and c). However, similarly to the case with MM-CK, the synthesis of creatine phosphate by the mitochondria stopped as soon as the mass action ratio of the reaction determined by the substrate and product concentrations in the reaction medium became equal to the apparent equilibrium constant value which was found in the experiments with MM-CK (Fig. 1 (b, d); Table 3). Obviously,

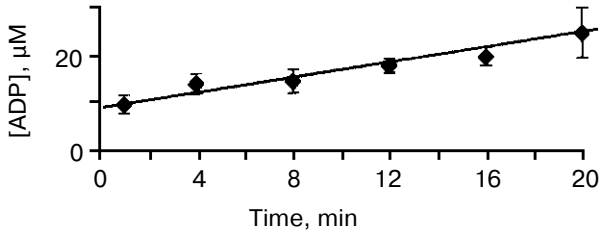

Fig. 2. ADP concentration in creatine-free samples depending on the incubation time (means SD, $n = 2$). The mitochondria were incubated for the time indicated in the creatine-free medium A with ATP. [P_i], 1 mM.

similar results could be obtained if a soluble ATP-regenerating system, e.g., that of pyruvate kinase, was used instead of oxidative phosphorylation [21]. In the experiments where oxidative phosphorylation was prevented by addition of oligomycin and rotenone into the medium the concentrations of creatine phosphate and ADP were the same as in the experiments with MM-CK, and Γ was also equal to K_{eq}^{app} (Table 3).

We supposed that a constant increase in the ADP concentration in the incubation medium (Fig. 1c) was not associated with the concurrent process of oxidative phosphorylation and the creatine kinase reaction but was due to a gradual decrease in the rate of oxidative phosphorylation and/or an increase in the ATPase activity of mitochondria during their incubation at 37°C. This should gradually increase the ADP concentration in the incubation medium. To check the hypothesis, mitochondria were incubated as usually but in the absence of creatine (Fig. 2). And, in spite of the absence of the creatine kinase reaction, the ADP concentration in the samples increased with an increase in the incubation time, and this increase was comparable to the increase observed in the creatine-containing samples (Fig. 1c).

Functional characteristics of mitochondria incubated for various times at 37°C in medium A are presented in Table 4. During the experiment the functional characteristics of the mitochondria which were kept on ice for 2.5-3.5 h changed insignificantly, although the respiratory control index was somewhat decreased. The same was observed on the mitochondria incubated for 2 min at 37°C. Thus, the procedure of mitochondria reprecipitation used by us insignificantly affected their functional characteristics. The 20-min incubation of mitochondria at 37°C sharply decreased the respiratory control value mainly due to a decrease in the respiration rate of mitochondria in state 3 (Table 4). The decrease in the respiration rate in state 3 could be caused by a decreased ability for oxidative phosphorylation because of the loss of Ca^{2+} by mitochondria [22] that could occur under the conditions of our experiments. Swelling of mitochondria, which also decreases their ability for oxidative phosphorylation, could also occur [23]. The reprecipitation was likely to more markedly affect the functional characteristics of the mitochondria incubated at 37°C for 20 min than of the mitochondria incubated for 2 min, i.e., the functional characteristics of these mitochondria were really less different than we had found in the experiments with the reprecipitation. However, these experiments clearly showed the direction of changes in the mitochondrial properties.

Based on the experiments presented in Fig. 2 and Table 4, we concluded that the increase in the ADP concentration in the samples during the incubation (Figs. 1c and 2) was caused by a decrease in the oxidative phosphorylation rate and by a relative increase in the ATPase activity of the mitochondria.

And a question arises: if the apparent equilibrium constant value of the creatine kinase reaction was exceed-

Table 4. Functional characteristics of mitochondria after incubation for different time at 37°C

Experiment number	Incubation time, min	Respiration rate, ng-atom O/min per mg protein		Respiratory control index
		state 3	state 4	
1	0	1009 (692)	150 (131)	6.7 (5.3)
	20	243	131	1.9
2	0	326 (310)	70 (85)	4.7 (3.6)
	2	261	73	3.6
	20	138	104	1.3

Note: Mitochondria were incubated for the time indicated in the table, the reaction was stopped by diluting the samples with 40 ml of cold isolation medium, and they were treated as described under "Materials and Methods". In the calculations a possible loss of an insignificant amount of mitochondrial protein during the reprecipitation was neglected. Polarographic experiments were performed at 37°C (1) or 22°C (2). Functional characteristics of the initial mitochondria (which were kept on ice) at the end of the experiment are shown in parentheses.

ed at the cost of an increase in the ADP concentration in the medium and there were actually no local concentrations of adenine nucleotides in the region of the mtCK active site, why these changes were not compensated by changes in the concentrations of all components of the creatine kinase reaction between 8 and 20 min? Figure 1c shows that the ADP concentration remained very low and was not higher than 30 μM, whereas the concentrations of other components of this reaction were on the level of 1-6 mM (Fig. 1, a and b). Under these conditions the mass action ratio value of the creatine kinase reaction could be decreased only due to a noticeable decrease in the ADP concentration, and the concentrations of all other substrates should be virtually unchanged. No decrease in the ADP concentration was observed because it was with an excess compensated by a concurrent ATPase reaction. However, the ADP concentration was increasing slowly because a part of ADP produced by ATPase was again converted to ATP during oxidative phosphorylation. Obviously, to provide noticeable changes in the concentrations of all components of the creatine kinase reaction, longer time intervals were required for the interaction of mtCK and ATPase which produced the substrate for creatine phosphate.

Indeed, Fig. 1 shows that during the incubation of mitochondria for 60 min the direction of the creatine kinase reaction was changed that was evidenced by a decrease in the concentration of creatine phosphate and by an increase in the creatine concentration (Fig. 1b). These changes were caused by the ATPase activity of mitochondria: in spite of the increase in the creatine concentration, the concentration of ATP decreased (Fig. 1a); the ADP concentration increased (Fig. 1c), in spite of the decrease in the concentration of creatine phosphate (Fig. 1b). Obviously, the rate of the ATPase reaction during this time was higher than the activities of mtCK and oxidative phosphorylation that were unable to fully compensate the changes in the concentrations of adenine nucleotides caused by ATPase. Nevertheless, the mass action ratio of the reaction was increased even more (Fig. 1d). No doubt, the excess of Γ over the apparent equilibrium constant value under these conditions was due not to the functional coupling of mtCK and ANT but due to the ATPase activity. Within 40 min (between 20 and 60 min) the creatine phosphate concentration decreased by 1.4 mM and the ATP concentration decreased by 0.1 mM (Fig. 1). Thus, within 40 min 1.5 mM ATP was hydrolyzed, and 1.4 mM of this amount was recovered at the cost of rephosphorylation with creatine phosphate of 1.4 mM ADP produced during the ATPase reaction. The diminution of creatine phosphate resulted in the same increase in the creatine concentration. The ATPase activity within this time was, on average, ~40 nmol/min per mg mitochondrial protein. The initial ATPase activity was 274 nmol/min per mg protein (Table 1). Thus, both the rate of oxidative phosphorylation and the ATPase activity of mitochondria decreased during the incubation of mitochondria. However, the ATPase activity decreased more slowly than the rate of oxidative phosphorylation.

An addition of creatine phosphate excess (1.5 mM) to mitochondria after 8 min of incubation, i.e., after its constant concentration had been attained, failed to significantly change the concentrations of creatine phosphate and ADP (Fig. 3). The ATP and creatine concentrations were also unchanged during the subsequent 10 min (not shown in the figure), although the mass action ratio of the creatine kinase reaction became significantly higher than the apparent equilibrium constant value (19.9 ± 0.7 and 8.72 ± 0.52, respectively). Obviously, in this case the apparently unchanged direction of the creatine kinase reaction was also due to the low ADP concentration in the medium.

DISCUSSION

Thus, creatine phosphate synthesis during oxidative phosphorylation was found to terminate when the mass action ratio of the reaction determined by the substrate concentrations in the incubation medium was equal to the apparent equilibrium constant of this reaction (Fig. 1, b and d). The subsequent excess of Γ over K_{eq}^{app} occurred at the cost of a further increase in the ADP concentration (Fig. 1c). This increase in the ADP concentration was not associated with the concurrent activities of mtCK and of the oxidative phosphorylation system; a comparable increase in the ADP concentration was also observed in the absence of creatine in the incubation medium (Fig. 2). The increase in the ADP concentration was associated with a decrease in the rate of oxidative phosphorylation and with a relative increase in the ATPase activity of mitochondria during the incubation (Table 4). The low ADP concentration in the incubation medium (<30 μM) (maintained by the concurrent activities of ATPase and of oxidative phosphorylation) and relatively high (1-6 mM) concentrations of other components of the creatine kinase reaction prevented the detection of the reverse creatine kinase reaction within 10 min after the mass action ratio exceeded the apparent equilibrium constant value, but the reverse reaction became evident on further incubation of the mitochondria. The reverse reaction was accompanied by a further increase in Γ (Fig. 1). The low ADP concentration in the medium was also responsible for the lack of an immediate rapid conversion of the excess creatine phosphate added, although $\Gamma > K_{eq}^{app}$ (Fig. 3, a and b).

Taken together, our findings contradict the concept on the structure-functional coupling between mtCK and ANT that considers the excess of the mass action ratio of the creatine kinase reaction over the apparent equilibrium constant value during oxidative phosphorylation to be

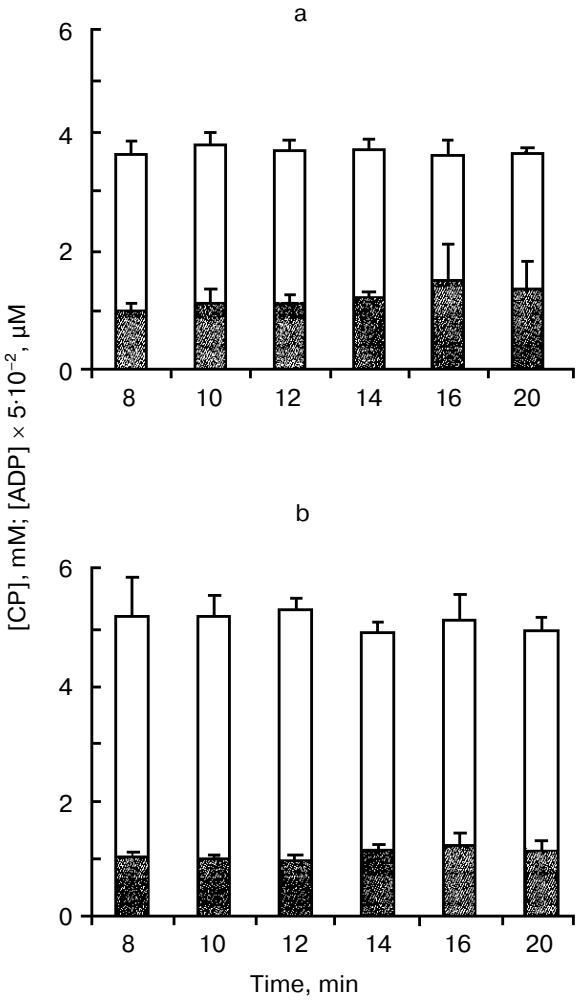

Fig. 3. Changes in concentrations of creatine phosphate (CP) and ADP in samples incubated without creatine phosphate (a) and after its addition (b). Creatine phosphate and ADP are shown by light and dark columns, respectively. Means SD are presented; $n = 2$. a) Mitochondria were incubated in the medium A for 8-20 min after the addition of 1 mM ATP; b) after 8 min of incubation the samples were supplemented with 1.5 mM creatine phosphate. The total incubation time of the samples after the addition of ATP is indicated. Other details are under "Materials and Methods", explanations are in the text.

associated with an excess synthesis of creatine phosphate due to generation in a mtCK–ANT microcompartment of local concentrations of adenine nucleotides different from their concentrations in the incubation medium [4]. In our experiments the excess of Γ over K_{eq}^{app} was obtained due to changes in adenine nucleotide concentrations in the medium caused by the ATPase activity and did not depend on the direction of the creatine kinase reaction (Fig. 1). Inhibition of the ATPase activity with oligomycin canceled the excess of the mass action ratio over the apparent equilibrium constant value (Table 3).

Our findings do not contradict the concept of dynamic compartmentation of adenine nucleotides in the intermembrane space provided by a relative impermeability of the outer membrane for ADP, because, according to this concept [11], under conditions of our experiments (after the attainment of the quasi-equilibrium of the creatine kinase reaction) the concentration gradient of ADP across the outer membrane should be close to zero. Consequently, the creatine phosphate synthesis should be terminated when the mass action ratio of the creatine kinase reaction determined by the substrate concentrations in the medium became equal to the apparent equilibrium constant value, and just this we observed in the experiment (Fig. 1).

In our experiments the incubation medium composition, temperature, and time of mitochondrial incubation were the same as in the experiments of Sobol et al. [7, 8]. Therefore, it was suggested that in the experiments of these authors the excess of the mass action ratio over the apparent equilibrium constant value should be also due to an increase in the concentration of ADP and not of creatine phosphate. The returning of Γ to the K_{eq}^{app} value on inhibition of oxidative phosphorylation with carboxyatractyloside, oligomycin, or under anaerobic conditions observed by these authors [7, 8] fails to prove the formation of mtCK–ANT microcompartment during oxidative phosphorylation because carboxyatractyloside and oligomycin are inhibitors of ATPase [23]. The removal of membrane potential under anaerobic conditions could also inhibit the mitochondrial ATPase because of its interaction with the protein inhibitor of ATPase under these conditions [24].

Sobol et al. also treated mitochondria hypotonically with 10 mM Tes for 5-30 min and then determined the ability of the resulting mitoplasts for the functional coupling of mtCK and ANT [8]. As in the case of untreated mitochondria, the authors observed the excess of the mass action ratio over the apparent equilibrium constant value, and this excess increased with an increase in the time of the hypotonic treatment. They think that these experiments have shown the unimportance of the outer membrane for the coupling (because it was destroyed during the hypotonic treatment), but that it is a permeability barrier for ATP and creatine; therefore, its more complete removal during the longer incubation in the hypotonic medium increased the rate of creatine phosphate synthesis and, consequently, the mass action ratio [8].

But we think that these findings can be explained otherwise and in a more likely way. High-amplitude swelling of mitochondria that should occur in the presence of 10 mM Tes is known to dramatically change their structure: it causes not only a rupture of the outer membrane but also the unfolding of cristae membranes and a several times increase in the matrix volume and its saturation with water [25, 26]. The mitochondria subjected to the high-amplitude swelling become loosely coupled

[25], and ATPase is stimulated in them [23]. The excess of the mass action ratio over the apparent equilibrium constant value in the experiments [8] was more likely caused by concurrent activities of mtCK and ATPase. The decrease in the mass action ratio observed in [8] on addition of KCl to the swelled mitochondria could be associated not with the microcompartment destruction due to mtCK dissociation from the membranes but with a reversion of the swelling by KCl and the resulting decrease in the ATPase activity of the mitoplasts. The increase in the ratio of mtCK activity to the activity of a matrix enzyme citrate synthase observed by Sobol et al. [8] after the hypotonic treatment suggested that the high-amplitude swelling could cause a rupture of the inner membrane and a partial loss of the matrix enzymes. Thus, without experiments characterizing the functional state of the hypotonically treated mitochondria the data presented in [8] are insufficient to prove the existence of a microcompartment between mtCK and ANT.

The works of DeFuria et al. [5] and of Saks et al. [6] also provoke some critical comments. On comparing the data presented in Fig. 1 and in Table 3 of the present work we have already paid attention to the different levels of the equilibrium concentrations of ADP and creatine phosphate attained in the absence and in the presence of oxidative phosphorylation: in the absence of oxidative phosphorylation [ADP] = [CP] (Table 3), whereas in the presence of oxidative phosphorylation [CP] >> [ADP] (Fig. 1) because the oxidative phosphorylation system maintains low ADP concentration in the medium. It could be also predicted that an addition of a new portion of creatine phosphate after the equilibrium was attained in the first case had to cause a rapid decrease in its concentration due to the reverse creatine kinase reaction and to the establishment of a new equilibrium state (because the medium contained a sufficient amount of ADP), whereas in the second case a rapid decrease in the creatine phosphate concentration would be prevented by an actual absence of the second substrate. Just this was shown in our experiments (Figs. 1 and 3). It was also shown that the different behavior of the two systems had no relation to the problem of the functional coupling. Meanwhile, in the works cited [5, 6] the predictable difference in the behavior of these two systems was still more enhanced by performing the experiments in the absence of oxidative phosphorylation at essentially higher concentrations of ATP, creatine, and creatine kinase than in its presence. This should promote a high rate of the reverse reaction on addition of excess creatine phosphate [6] and especially of creatine phosphate and ADP [5] after the attainment of the reaction equilibrium. Nevertheless, the differences found were considered to prove the structure-functional coupling between mtCK and ANT during oxidative phosphorylation [6].

In our experiments the creatine kinase reaction was initiated by addition of ATP and creatine into the medium and the moment of termination of the creatine phosphate synthesis was recorded. Such an approach allowed us to show that the creatine phosphate synthesis was terminated when the mass action ratio became equal to the apparent equilibrium constant value (Fig. 1). In works [5, 6] the authors added at once an excess of creatine phosphate in the medium; therefore, the equilibrium state was overcome not at the cost of excess creatine phosphate synthesis by mitochondria but at the cost of its excess added from the outside. And unlike us, these authors also observed the creatine phosphate synthesis somewhat afterwards [5, 6]. In their experiments the creatine kinase reaction changed its direction only on addition into the medium of inhibitors of oxidative phosphorylation, cyanide [5] or oligomycin [6]. In the experiments of DeFuria et al. the creatine phosphate concentration decreased by 1.1 mM (calculated by us from Fig. 12.2 of [5]) and in the experiments of Saks et al. it decreased by 0.6 mM within 3 min (calculated from Fig. 9A of [6]). In experiments on oxidative phosphorylation the total concentration of ATP and ADP was 0.4 and 0.17 mM in the work of the first and of the second group of authors, respectively; because of oxidative phosphorylation ATP was the main adenine nucleotide, whereas the ADP concentration should be still lower than in our experiments. To decrease the CP concentration by a value several times higher than both the ADP and ATP concentrations, in addition to a highly active mtCK a similarly active ATPase was required to supply ADP, the substrate for creatine phosphate. However, cyanide and oligomycin are inhibitors of the mitochondrial ATPase [23], and just a shutdown of oxidative phosphorylation under these conditions could not result in a noticeable degradation of CP because the ADP concentration in the medium was very low. The object studied seems also to be a poor choice. In the experiments of DeFuria et al. on rabbit heart mitochondria the activity of mtCK was ~50 nmol/min per mg protein that was ~5% of the oxidative phosphorylation rate in the presence of excess ADP (calculated based on Fig. 12.2 of [5]). Usually the activity of mtCK in mitochondria is comparable to the rate of oxidative phosphorylation at the ATP and creatine concentrations chosen by the authors. Rabbit heart mitochondria are not an exception [21, 27]. In our experiments the initial rate of the creatine phosphate synthesis was 1170 nmol/min per mg protein (Fig. 1b), or ~51% of the oxidative phosphorylation rate in state 3 (Table 1). The low activity of mtCK in the experiments of DeFuria et al. [5] suggests that the most part of it could be lost during the isolation of mitochondria, i.e., that the outer mitochondrial membrane was destroyed and the authors dealt with mitoplasts which were essentially depleted in mtCK. In the absence of conclusive evidence to the contrary, the ability of such preparations for oxidative phosphorylation during the incubation for 3 h at room temperature seems

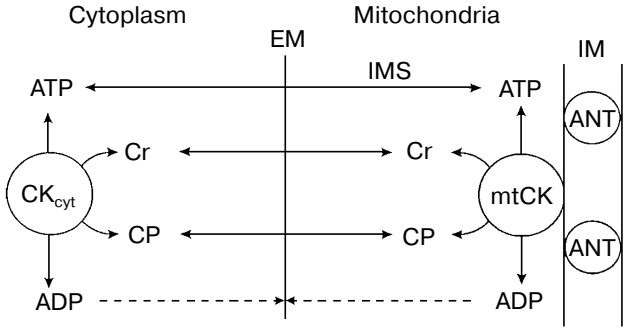

Fig. 4. Scheme of the mtCK role in elimination of the concentration gradient of ADP across the outer membrane (OM) of mitochondria: there is no direct exchange of nucleotides between mtCK and ANT; the concentrations of ATP, creatine (Cr), and creatine phosphate (CP) in the intermembrane space (IMS) are equal to their concentrations in the cytoplasm (shown by two-directional solid arrows intersecting the OM); the OM has a limited permeability for ADP (dotted lines not intersecting the OM); the creatine kinase reactions in the cytoplasm (CK_{cyt}) and in mitochondria are in a state close to equilibrium (the arrows show both directions of the reaction); as a result of the mtCK activity, the ADP concentration in the IMS is equal to its concentration in the cytoplasm. IM, the inner membrane (the figure is from [2]).

doubtful. Saks et al. deliberately choose mitoplasts to show that the outer membrane was unimportant for the functional coupling. From their data [6] on the rate of creatine phosphate conversion within the first minute of the incubation of the mitoplasts (Fig. 9A and Table 1 in [6]) the ATPase activity in their preparations was calculated to be 36% of the rate of oxidative phosphorylation (12% in our experiments). A high activity of ATPase is also shown by the polarogram (Fig. 2B from [6]). The reasons presented make us disagree with the conclusion of the authors [5-8] that their data have proved the existence of a compartment between mtCK and ANT. It should be also noted that for the above-mentioned reasons a thermodynamic approach to the problem of the functional coupling between mtCK and ANT was a poor choice. The presence in the system of the irreversible ATPase reaction is an obstacle for the mass action ratio of the creatine kinase reaction to attain a constant value. At present, it is impossible to eliminate the ATPase reaction because inhibitors of ATPase concurrently inhibit oxidative phosphorylation.

Other data which are considered in detail in [2] and also in [28] also contradict the concept on microcompartment formation between mtCK and ANT. We think it more likely that a compartment for adenine nucleotides in the intermembrane space of mitochondria is formed due to a limited permeability of the outer membrane. The concentration gradient produced across the outer membrane is of micromolar range [11, 12]; therefore, under physiological conditions it is essential for ADP which is present in the muscle cell cytoplasm in the concentration of 30-50 μM but not for the other substrates of mtCK [11, 12]. This means that in the cell concentrations of ATP, creatine, and creatine phosphate in the intermembrane space for any moment cannot be noticeably different from their concentrations in the cytoplasm. Under physiological conditions the muscle cell cytoplasm contains a highly active creatine kinase catalyzing the reaction which is near to equilibrium over a wide range of conditions. As concentrations of three from four substrates of creatine kinase are to be virtually the same in the cytoplasm and in the intermembrane space of mitochondria, under physiological conditions mtCK cannot provide in the intermembrane space the ADP concentration higher than that which is present in the cytoplasm for the moment. The nearer to equilibrium is the mtCK-catalyzed reaction, the less is the difference between the ADP concentrations in the intermembrane space and in the cytoplasm. The mtCK-catalyzed reaction close to equilibrium was shown by ^{31}P-NMR on transgenic mice lacking MM-CK [29]. We suppose that the physiological reason for appearance of a special creatine kinase isoenzyme in the intermembrane space and the physiological role of mtCK consist in a rapid equalizing of the ADP concentration (and, consequently, of the ATP/ADP ratio) in the intermembrane space of mitochondria with the ADP concentration in the cytoplasm for the moment [2]. The scheme of this concept is presented in Fig. 4. Due to the mtCK activity, the oxidative phosphorylation system is constantly provided with undistorted information about the phosphoryl potential in the cytoplasm and can adequately change its activity [2].

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