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Functional Coupling between Nucleoside Diphosphate Kinase of the Outer Mitochondrial Compartment and Oxidative Phosphorylation

T. Yu. Lipskaya* and V. V. Voinova

Department of Biochemistry, Faculty of Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (7-095) 939-3955; E-mail: TLipskaya@yandex.ru

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Abstract—In rat liver mitochondria all nucleoside diphosphate kinase of the outer compartment is associated with the outer surface of the outer membrane (Lipskaya, T. Yu., and Plakida, K. N. (2003) *Biochemistry* (Moscow), **68**, 1136-1144). In the present study, three systems operating as ADP donors for oxidative phosphorylation have been investigated. The outer membrane bound nucleoside diphosphate kinase was the first system tested. Two others employed yeast hexokinase and yeast nucleoside diphosphate kinase. The two enzymes exhibited the same activity but could not bind to mitochondrial membranes. In all three systems, muscle creatine phosphokinase was the external agent competing with the oxidative phosphorylation system for ADP. Determination of mitochondrial respiration rate in the presence of increasing quantities of creatine phosphokinase revealed that at large excess of creatine phosphokinase activity over other kinase activities (of the three systems tested) and oxidative phosphorylation the creatine phosphokinase reaction reached a quasi-equilibrium state. Under these conditions equilibrium concentrations of all creatine phosphokinase substrates were determined and K_{eq}^{app} of this reaction was calculated for the system with yeast hexokinase. In samples containing active mitochondrial nucleoside diphosphate kinase the concentrations of ATP, creatine, and phosphocreatine were determined and the quasi-equilibrium concentration of ADP was calculated using the K_{eq}^{app} value. At balance of quasi-equilibrium concentrations of ADP and ATP/ADP ratio the mitochondrial respiration rate in the system containing nucleoside diphosphate kinase was 21% of the respiration rate assayed in the absence of creatine phosphokinase; in the system containing yeast hexokinase this parameter was only 7% of the respiration rate assayed in the absence of creatine phosphokinase. Substitution of mitochondrial nucleoside diphosphate kinase with yeast nucleoside diphosphate kinase abolished this difference. It is concluded that oxidative phosphorylation is accompanied by appearance of functional coupling between mitochondrial nucleoside diphosphate kinase and the oxidative phosphorylation system. Possible mechanisms of this coupling are discussed.

Key words: nucleoside diphosphate kinase, liver mitochondria, oxidative phosphorylation, functional coupling

Under physiological conditions nucleoside diphosphate kinase (NDPK; EC 2.7.4.6) catalyzes reactions of synthesis of various nucleoside triphosphates (NTPs) from ATP and corresponding nucleoside diphosphates (NDPs). The resultant NTPs are involved in the main anabolic processes [1].

In hepatocytes, NDPK is localized within the cytoplasm; it is also associated with membranes [2, 3]. In mitochondria, this enzyme was found in the outer compartment and also in the matrix [4, 5]. Recently we have demonstrated that in rat liver mitochondria all NDPK activity of the outer mitochondrial compartment is associated with the outer surface of the outer mitochondrial membrane [6]. In mitochondria NDPK of the outer mitochondrial compartment catalyzes the following reaction: ATP + NDP → ADP + NTP.

ADP formed in this reaction is further consumed during oxidative phosphorylation, and this is accompanied by the stimulation of respiration [5-7]. Although mitochondrial NDPK represents only 13% of NDPK present in soluble fraction of hepatic homogenate [6], its activity may account for nearly the maximal rate of oxidative phosphorylation [5-7].

The functions of matrix NDPK are well understood [4, 5, 8], whereas a physiological role of NDPK in the outer mitochondrial compartment remains unknown.

Abbreviations: ANT) adenine nucleotide translocase; AP5A) P^1,P^5-di(adenosine-5′)pentaphosphate; CPK) muscle creatine phosphokinase; Cr) creatine; HK) hexokinase; yHK) yeast hexokinase; NDPK) nucleoside diphosphate kinase; mNDPK) mitochondrial nucleoside diphosphate kinase; yNDPK) yeast nucleoside diphosphate kinase; PC) phosphocreatine.

* To whom correspondence should be addressed.

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Studies of other kinases localized on the external surface of the outer mitochondrial membrane, hexokinase (HK) and glycerol kinase, revealed the existence of functional coupling between these kinases and oxidative phosphorylation. The most numerous data have been obtained for isozyme I of HK.

Bessman et al. demonstrated that oxidative phosphorylation is accompanied by a decrease in apparent K_m of mitochondrial HK for ATP synthesized in mitochondria compared with external medium ATP [9, 10]. Later the decrease in HK K_m for ATP and the increase in catalytic efficacy of HK during oxidative phosphorylation have also been observed by other researchers [11-13]. Similar results have also been obtained for glycerol kinase [14, 15]. It was suggested that functional coupling between HK (or glycerol kinase) and the oxidative phosphorylation system might involve formation of a kinase microcompartment limiting adenine nucleotide diffusion during oxidative phosphorylation. This results in generation of higher local concentration of ATP in this micro-compartment than in the external environment because ATP formed during oxidative phosphorylation is more available to kinases than ATP of external medium [10, 12, 16-20]. The adenine nucleotide pool of this micro-compartment is not totally autonomous and prolonged incubation of mitochondria results in convergence of K_m values of HK for external ATP and ATP generated by mitochondria [9, 10]. The degree of ATP compartmentation in the active site of HK also decreased with the increase in ATP concentration in the external solution [17].

There is convincing evidence that mitochondrial contact sites containing these kinases, porin, and adenine nucleotide translocase (ANT) represent the structural basis of the functional compartment of these kinases [21, 22]. *In vitro* experiments directly demonstrated interaction of HK and glycerol kinase with porin [20, 23] and porin interaction with ANT [24]. This implies formation of a kinase–porin–ANT multienzyme complex [24]. Formation of the multienzyme complex is accompanied by conformational changes in each individual component [13, 18, 25, 26].

In model experiments employing rat liver mitochondria with brain mitochondrial HK isoenzyme I, Laterveer et al. demonstrated [27, 28] that during oxidative phosphorylation some proportion of ADP formed in the hexokinase reaction was inaccessible to exogenously added pyruvate kinase system though its activity significantly exceeded the rate of oxidative phosphorylation. These authors concluded that the outer mitochondrial membrane represents a permeability barrier for ADP and during catalysis by mitochondrial HK some proportion of ADP is directly tunneled into the mitochondrial matrix without mixing with external ADP [27, 28]. It is suggested that kinetic advantages of membrane bound HK are not significant at physiological concentrations of ATP and mainly ADP tunneling into the matrix has the only physiological importance [27]. It is also suggested that mitochondrial kinases may be crucial for maintenance of intramitochondrial ADP level and ATP/ADP ratio favorable for oxidative phosphorylation without generation of high ADP concentration in cytoplasm [28].

We have suggested that liver NDPK localized on the outer surface of the outer mitochondrial membrane (mNDPK) may exhibit the same properties because in liver mitochondria at least part of the mNDPK activity is localized within the contact site region [18]. There is one difficulty of experiments with mNDPK. This is that pyruvate kinase usually employed as the external ADP-consuming system in polarographic experiments and also for determination of ADP concentration in the experimental samples (when the reaction is stopped) is not specific with respect to ADP and may also use other NDPs, the NDPK substrates present in the medium. These problems could be excluded by using the creatine phosphokinase system as an ADP-consuming system. Since liver mitochondria (in contrast to cardiac or skeletal muscle mitochondria) lack this enzyme [29], use of muscle creatine phosphokinase (CPK) is methodologically correct. The other reason for use of CPK is the reversibility of the creatine phosphokinase reaction (the pyruvate kinase reaction is practically irreversible).

We have analyzed three systems shown in Fig. 1. In the first system (Fig. 1a), mNDPK bound to the outer surface of the outer mitochondrial membrane acted as the ADP donor. In the second system (Fig. 1b), membrane-unbound yeast HK (yHK) was the ADP donor, whereas in the third system membrane-unbound yeast NDPK (yNDPK) served as the ADP donor. In all three systems, CPK was the external agent competing with the oxidative phosphorylation system for ADP.

In all these systems, we have determined mitochondrial respiration rate in the presence of increasing quantities of CPK. In the presence of a large excess of CPK over activity of mNDPK, yHK, and oxidative phosphorylation system, the creatine phosphokinase reaction is in a quasi-equilibrium state. So in the samples incubated in the presence of yHK we determined equilibrium concentrations of all components of the creatine phosphokinase reaction and calculated K_{eq}^{app} of this reaction under our experimental conditions. In samples containing active mNDPK we also determined concentrations of ATP, creatine, and phosphocreatine, and using the value of K_{eq}^{app} we calculated the quasi-equilibrium concentration of ADP.

We found that in the presence of a large excess of CPK activity quasi-equilibrium concentrations of ADP and also ATP/ADP ratios in the systems with yHK and mNDPK were the same, but the rate of mitochondrial respiration with mNDPK was higher than the corresponding value determined in the system with yHK. This difference disappeared when mNDPK was replaced by yNDPK.

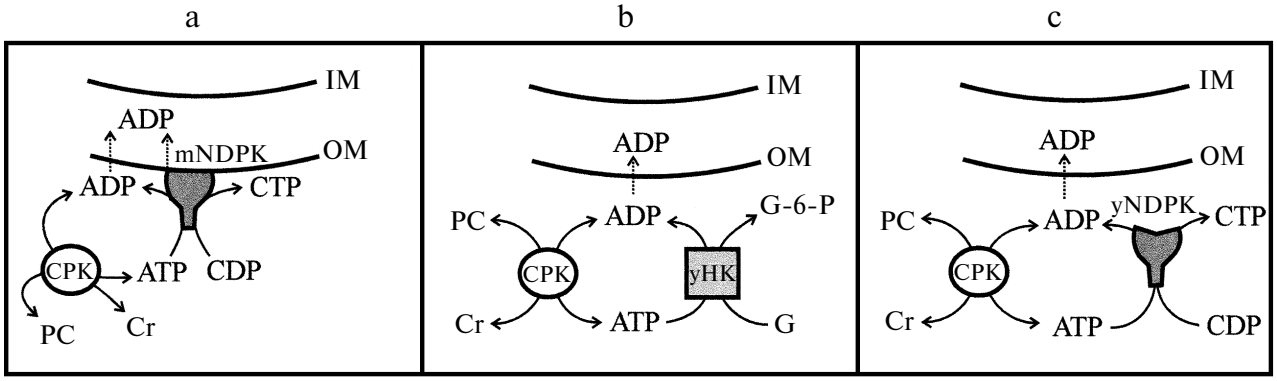

Fig. 1. Three experimental models used in this study. OM and IM are the outer and the inner mitochondrial membranes, respectively; G and G-6-P are glucose and glucose-6-phosphate, respectively. Other comments are given in the text.

MATERIALS AND METHODS

Materials. ATP, ADP, AP5A, phosphocreatine, carboxyatractyloside, and yNDPK were purchased from Sigma (USA), creatine was from Eastman Kodak Co (USA), yHK was from Fluka (Switzerland), and CPK was produced by Reanal (Hungary).

Isolation of mitochondria. Mitochondria were isolated from livers of albino rats (180-200 g) starved for 16 h with free access to water. All operations were carried out at 2-4°C. Livers were quickly removed, washed free from blood in 0.25 M sucrose, weighed, and squashed through a tissue press (pore diameter of 1 mm). The squashed liver was then homogenized in 40 ml of an isolation medium containing 0.28 M mannitol, 2.1 mM Hepes, pH 7.4, for 1 min in a glass homogenizer using a Teflon pestle. After homogenization, we added the isolation medium to obtain 10% homogenate. The resultant 10% homogenate was initially centrifuged at 2000 rpm for 15 min in a Beckman J2-21 centrifuge (Austria) to remove cell debris and nuclei. The supernatant (S_1) was filtered through a nylon filter and centrifuged at 8000 rpm for 10 min. After careful removal of the supernatant, the mitochondrial pellet (P_1) was suspended in 16 ml of washing medium containing 3.33 mM $MgCl_2$, 0.27 M mannitol, 2.1 mM Hepes, pH 7.4. The suspension of pellet P_1 was centrifuged at 12,000 rpm for 10 min. The supernatant was carefully removed and the mitochondrial pellet (P_2) was suspended in the washing medium (protein concentration ~60 mg/ml). The resultant mitochondrial suspension was used in polarographic experiments.

For preparation of mitochondria with low activity of mNDPK the mitochondrial pellet P_1 was suspended in 16 ml of the isolation medium; the suspension was incubated in the cold for 8 h under constant stirring and after the incubation it was centrifuged at 12,000 rpm for 10 min. The mitochondrial pellet P_2 was suspended in an isolation medium as described above. During preparation of mitochondria with high and low mNDPK content using the same rat liver the supernatant S_1 was subdivided into two parts and two identical P_1 pellets were obtained. Each pellet was suspended in a half volume of washing or isolation medium and treated as above.

Polarographic assay of mitochondrial respiration. The rate of mitochondrial oxygen consumption was determined at 22°C using a covered Clark type platinum electrode and LP 7e polarograph (Czechoslovakia). The main incubation medium contained 85 mM KCl, 110 mM mannitol, 0.1 mM EGTA, 20 mM Tris-HCl, pH 7.4, 5 mM potassium phosphate, 3 mM $MgCl_2$, and 5 mM potassium succinate. Other additions are given in the text or corresponding legends to the figures and tables. Oxygen concentration in the incubation medium was assumed to be 290 μM at 22°C [30].

Polarographic experiments in the presence of increasing concentrations of CPK. Increasing concentrations of freshly prepared solution of rabbit muscle CPK (0-34.1 U/ml) were added to the main incubation medium containing the following additions: 1 mM ATP, 6 mM phosphocreatine, and 0.02 mM adenylate kinase inhibitor AP5A (incubation medium-1), and also 5 mM glucose (incubation medium-2). The reaction was initiated by adding mitochondrial suspension P_2. Using incubation medium-1, we added 600 μM CDP 1.5 min after addition of the mitochondrial suspension, whereas in the case of incubation medium-2 we added the predetermined amount of yHK that caused the same stimulation of mitochondria respiration in the absence of CPK as 600 μM CDP did.

To exclude a possible influence of mitochondrial storage on the data obtained, samples prepared in incubation media-1 and -2 and containing different amounts of CPK were used in a random succession.

Subsequent treatment of samples. One minute after the last addition, the reaction was stopped by placing the samples into concentrated solution of $HClO_4$ containing

bromothymol blue (final concentration of $HClO_4$ was 0.6 M). Samples were incubated on ice for 1 h. Pilot experiments revealed that such incubation period caused total inactivation of CPK even in samples where CPK concentration was especially high. The samples were centrifuged at 13,400 rpm for 5 min using a MiniSpin plus centrifuge (Eppendorf, Germany). The supernatant was neutralized with sufficient amount of 5 M K_2CO_3 until the indicator color changed (from yellow to violet) and after 30 min it was centrifuged at 5000 rpm for 5 min using a Metronex bench centrifuge (Poland). The resultant supernatant was stored at −18°C before determination of concentration of creatine phosphokinase reaction components and also glucose-6-phosphate and CTP.

For evaluation of the contribution of matrix ADP to the total pool of ADP, the mitochondrial suspension P_2 was added to the main incubation medium, and after 1 min the enzymes were inactivated by adding $HClO_4$. This approach employed the finding described in the literature that ADP content in mitochondrial matrix remained basically unchanged during mitochondria transition from a resting state to active oxidative phosphorylation [31, 32]. We found that storage of samples treated with 0.6 M $HClO_4$ for 1 h on ice was accompanied by negligible hydrolysis of ATP and phosphocreatine. However, because of ADP concentration below 10 μM in samples with high CPK activity we had to take into consideration of contribution of ADP derived from ATP hydrolysis. For this purpose, three parallel samples containing incubation medium-2 were incubated with $HClO_4$ for 60 min and then treated as above. For determination of initial contaminations of ADP in the ATP preparation, the other samples were initially treated with K_2CO_3 and then with $HClO_4$.

Concentrations of ADP, glucose-6-phosphate, ATP, CTP, and phosphocreatine were determined spectrophotometrically using well-known coupling enzyme systems using a Hitachi 200-20 recording spectrophotometer (Japan) at 340 nm. ADP concentration in the samples was determined as described in [33].

The reaction mixture for assay of glucose-6-phosphate (final volume 1 ml) contained 20 μl of an experimental sample, 20 mM Hepes, pH 7.4, 0.4 mM EDTA, 10 mM $MgCl_2$, 3 mM glucose, and 0.3 mM $NADP^+$. The reaction initiated by adding 0.8 U glucose-6-phosphate dehydrogenase was monitored by the increase in absorbance at 340 nm. After termination of this reaction, ATP concentration in the same sample was determined by adding 0.75 U yHK. A special set of experiments revealed that under our experimental conditions the yHK did not use CTP as a substrate. For determination of CTP concentration, we added 0.3 mM ADP to the same sample and after stabilization of the absorbance 2.5 U yNDPK was added.

Phosphocreatine concentration was determined in 10 μl of samples, which was added to the same medium used for CTP determination (but lacking yNDPK); the reaction was initiated by adding 12 U CPK. Molar absorbance coefficient for NADH and NAD(P)H was assumed to be $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 340 nm [34].

Creatine concentration was determined using diacetyl and α-naphthol [35]. The results were corrected for contribution of sample components to the development of color.

Creatine phosphokinase activity assay using CDP as substrate. After addition of 34.1 U/ml CPK to incubation medium-2, the samples were incubated at 22°C for 1.5 min with periodic shaking using a Thermomixer comfort (Eppendorf) and then 600 μM CDP and the amount of yHK equivalent to that used in polarographic experiments were simultaneously added to the reaction mixture. The reaction was stopped 1 min after by adding $HClO_4$, and glucose-6-phosphate and CTP were assayed. These experiments imitated ADP and CDP concentrations that were present in polarographic experiments with mNDPK and CPK; these experiments allowed the maximal amount of CDP utilized by CPK to be determined. Under these experimental conditions, the maximal amount of CDP utilization was 10-12% of the initial concentration of this compound. Such decrease in CDP concentration did not influence mNDPK activity.

Study of mNDPK solubilization during mitochondria storage. The concentrated suspension of P_2 sediment was stored on ice in the washing medium; after certain time intervals aliquots of 30 μl (~2 mg of protein) were taken and added to 1970 μl of the same medium, mixed, and immediately centrifuged in the cold at 14,500 rpm for 1 min in the MiniSpin plus centrifuge. For evaluation of possible additional solubilization of mNDPK from mitochondria during a polarographic experiment the aliquots of 30 μl of P_2 suspension were added to incubation medium-1 and incubated at 22°C with periodical shaking in the Thermomixer comfort for 1.5 min; after that 600 μM CDP was added. Total volume of samples was 2 ml. After the incubation for 1 min, samples were centrifuged as above. Supernatants were carefully removed, and the pellets were suspended in 35 μl of the washing medium and used for the polarographic determination of remaining mNDPK activity using the main incubation medium containing 1 mM ATP. The proportion of remaining mNDPK was characterized by the ratio of the phosphorylating respiration after CDP addition to the rate of phosphorylating respiration determined earlier in the same polarographic sample after addition of 170 μM ADP. The rate of phosphorylating respiration after ADP addition (V^{ADP}) was determined as the difference between the respiration rate registered immediately after ADP addition and the rate after its exhaustion. The rate of phosphorylating respiration after subsequent CDP addition (V^{CDP}) was determined as the difference in the respiration rate after and before CDP addition. The ratio V^{CDP}/V^{ADP} in the initial P_2 suspension was determined in a similar way.

In the presence of CPK, the rate of phosphorylating respiration during catalytic activity of NDPK or yHK was determined by deducing the respiration rate after mitochondria addition from the respiration rate after addition of CDP or yHK, respectively.

Mitochondrial protein content was determined by the method of Gornall et al. [36].

RESULTS

Earlier we demonstrated that mNDPK is readily solubilized during storage of mitochondria [6]. The goals of the present study required tight enzyme binding to mitochondrial membranes for at least 8 h of storage on ice. After testing several washing media, we selected medium containing 3.33 mM $MgCl_2$, 0.27 M mannitol, 2.1 mM Hepes, pH 7.4 (the washing medium). Figure 2 shows results of a typical experiment.

Figure 2 shows that mNDPK was basically bound to mitochondria during 8 h and enzyme solubilization did not occur (curves *1* and *2*). In incubation medium-1, there was insignificant solubilization of mNDPK (curve *3*), but about 80% of the catalytic activity was still associated with the mitochondria.

Functional characteristics of mitochondrial preparations used in this study are summarized in Table 1. The table shows that under our experimental conditions for isolation and storage the resultant mitochondrial preparations were well coupled and their functional characteristics remained almost unchanged over the whole experiment; there was a small decrease in respiratory control ratio due to a small increase in the respiration rate observed after ADP exhaustion. This may be attributed to some stimulation of mitochondrial ATPase activity.

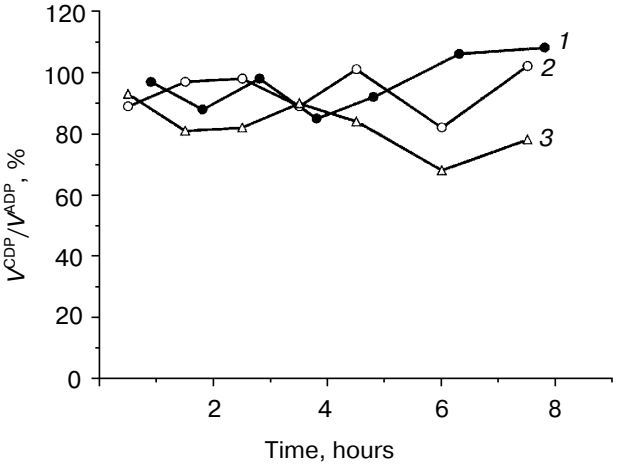

Fig. 2. Solubilization of mNDPK during mitochondria storage in the washing medium: *1*) initial P_2 suspension; *2*) after re-sedimentation of the mitochondria in the washing medium; *3*) after incubation of the mitochondria in incubation medium-1 and re-sedimentation.

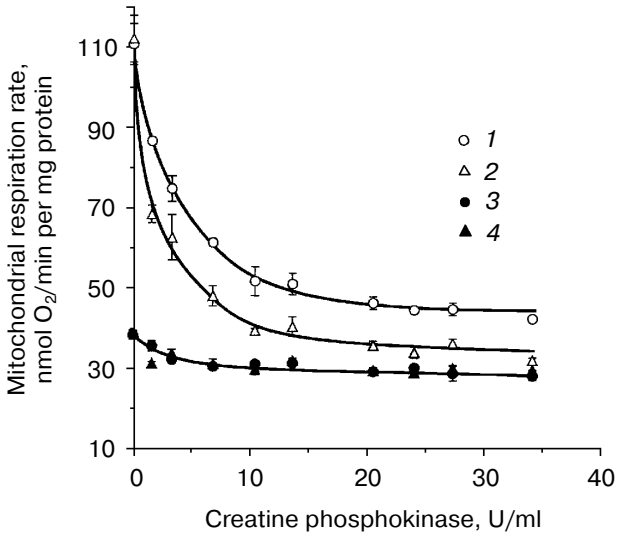

Fig. 3. Dependence of mitochondrial respiration rate on the activity of creatine phosphokinase added: *1*, *3*) mitochondria incubated in incubation medium-1; *2*, *4*) mitochondria incubated in incubation medium-2; *1*) respiration rate after CDP addition; *2*) respiration rate after yHK addition; *3*, *4*) mitochondrial respiration rates in incubation media 1 and 2 before addition of CDP or yHK, respectively. Data represent mean ± SEM of three independent experiments.

Figure 3 shows the effect of increasing concentrations of CPK on the rate of mitochondrial respiration studied in two systems: with mNDPK and in the presence of yHK (Figs. 1a and 1b). It is seen that in the absence of added CDP and yHK (Fig. 3, curves *3* and *4*, respectively) the respiration rates were identical in both systems irrespective of the amount of CPK added. In the absence of CPK addition of yHK caused the same stimulation of mitochondrial respiration as the addition of 600 μM CDP, i.e., activities of yHK and mNDPK in our experimental systems were equal. During operation of these systems State 3 respiration represented about 90% of the respiration rate assayed in the presence of 170 μM ADP (Table 1). Equality of yHK and mNDPK activities was demonstrated by spectrophotometric measurements of the reaction products; the activity of yHK was 293 ± 4 nmol glucose-6-phosphate/min per mg mitochondrial protein, and the activity of mNDPK was 292 ± 2 nmol CTP/min per mg protein (n = 3). However, during the increase in CPK concentration added the respiration rate decreased more slowly in the case of mNDPK activity compared with the samples with yHK (Fig. 3, curves *1* and *2*).

Figure 3 shows, that in the presence of 20.5 U CPK/ml the mitochondrial respiration rates reached the minimal values in both systems and did not change further. Under these conditions the CPK activity exceeded the rate of oxidative phosphorylation by 60-100 times.

Table 1. Functional characteristics of mitochondrial preparations

Mitochondria storage in ice, hours	Respiration rate, nmol O_2/min per mg protein			RCR
	st_4^S	st_3^{ADP}	st_4^{ADP}	
0.5-2.1	27.6 ± 1.2	117 ± 7	26.2 ± 0.8	4.4 ± 0.1
7.4-8.8	30.2 ± 1.6	129 ± 5	32.5 ± 1.8	4.0 ± 0.1

Note: The main incubation medium contained 20 μM AP5A. Mitochondrial protein was 0.9-1.1 mg/ml. The rates of mitochondrial respiration: st_4^S) after addition of mitochondria; st_3^{ADP}) after addition of 170 μM ADP; st_4^{ADP}) after ADP exhaustion. The results are the mean ± SEM of three independent experiments. Here and in Table 3, RCR is respiratory control ratio.

Table 2. Some parameters characterizing the quasi-equilibrium state of the creatine phosphokinase reaction in the systems containing yHK and mNDPK

Active enzyme	Γ = [ADP]·[PC]/[ATP]·[Cr]	[ADP], μM	ATP/ADP	Phosphorylating respiration rate			
				−CPK		+CPK	
				nmol O_2/min per mg protein	%	nmol O_2/min per mg protein	%
yHK	0.0259 ± 0.002 (10)	3.22 ± 0.49 (11)	288 ± 36 (11)	73.3 ± 5.6 (3)	100	5.3 ± 0.5 (12)	7
mNDPK	—	3.16 ± 0.25 (10)	311 ± 27 (10)	72.4 ± 5.4 (3)	100	15.0 ± 0.8 (12)	21

Note: The calculation was made using data of the three experiments shown in Fig. 3. In the presence of CPK, the mean values for samples containing 20.5-34.1 U/ml of CPK were used. Data represent the mean ± SEM. Numbers in the brackets indicate numbers of observations.

Figure 4 shows the dependence of the mass action ratio of the components of the creatine phosphokinase reaction (Γ = [ADP]·[PC]/[ATP]·[Cr]) in the samples with yHK on the activity of CPK added. Figure 4 shows that at CPK concentration equal to or exceeding 20.5 U/ml, the Γ values became constant. This means that in these samples the reaction reached the quasi-equilibrium state, i.e., $\Gamma = K_{eq}^{app}$. The mean of the calculated K_{eq}^{app} value was 0.0259 ± 0.002 (Table 2).

As we said above the activities of mNDPK and yHK were equal in these experiments. Consequently, in samples with active mNDPK the creatine phosphokinase reaction had to reach the quasi-equilibrium state at the CPK concentration of 20.5 U/ml and above. Using the value of K_{eq}^{app} found in the experiments with yHK we calculated the quasi-equilibrium ADP concentration in samples containing active mNDPK for these CPK concentrations. Table 2 summarizes the results of these calculations. It is known that in the presence of excess substrate and P_i the submaximal rate of mitochondrial respiration is determined by the ratio of concentrations of ATP and ADP in the incubation medium [37]. The calculated values of the ATP/ADP ratios are also given in Table 2.

Table 2 shows that the quasi-equilibrium concentrations of ADP, ATP/ADP ratios, and initial rates of mitochondrial respiration in the absence of CPK did not differ in these two systems. However, under conditions of quasi-equilibrium of the creatine phosphokinase reaction the rate of phosphorylating respiration in the system with yHK was significantly lower than with active mNDPK. The proportion of phosphorylating respiration remaining

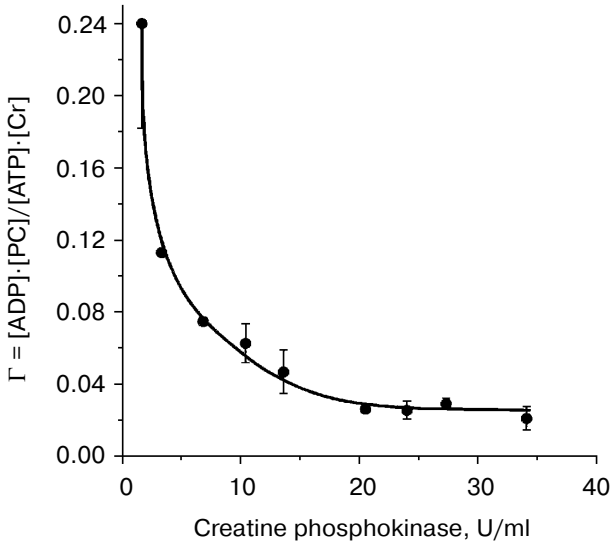

Fig. 4. Dependence of the mass action ratio (Γ) of the creatine phosphokinase reaction components on CPK activity of samples containing yHK. The concentrations of ATP, ADP, creatine, and phosphocreatine were determined as described in the "Materials and Methods" section.

Table 3. Effect of excess of CPK activity on the rate of mitochondrial respiration during catalytic activity of mNDPK and yNDPK

Suspension number	Active enzyme	RCR	V^{CDP}/V^{ADP}, % (mNDPK)	Phosphorylating respiration rate			
				−CPK		+CPK	
				nmol O_2/min per mg protein	%	nmol O_2/min per mg protein	%
1	mNDPK	4.8	91.0	112	100	25.5 ± 1.6 (3)	23
2	yNDPK	4.9	7.0	120	100	3.4 ± 1.5 (2)	3

Note: Mitochondria with high and low mNDPK content (suspensions 1 and 2, respectively) were isolated from the same liver as described in the "Materials and Methods" section. Polarographic assay was started after suspension 2 was prepared. Mitochondrial protein content was 1.4 mg/ml. In the case of suspension 2, yNDPK was added in amounts sufficient to maintain equal phosphorylating respiration after CDP addition in the absence of CPK in both systems. Where indicated 20.5, 24.0, and 27.4 units of CPK/ml were added to suspension 1. In the case of liver mitochondria in the presence of yNDPK, 20.5 and 27.3 units of CPK/ml were added. The results represent mean ± SEM, the number of observations being given in parentheses.

in the presence of excess of CPK activity was 21% in the system with mNDPK, whereas in the system with yHK this parameter was only 7%.

Completely different results were obtained when yNDPK was added to liver mitochondria that had mNDPK removed (Table 3). Table 3 shows that mitochondria in suspensions 1 and 2 did not differ in the respiratory control ratio; this means that both preparations were well coupled. The mitochondrial preparations sharply differed in mNDPK activity (V^{CDP}/V^{ADP} = 91 and 7%, respectively). However, the rates of phosphorylating respiration differed insignificantly after yNDPK addition to suspension 2 (112 and 120 nmol O_2/min per mg protein). This table also shows that in the presence of the added yNDPK the excess of CPK activity almost removed the stimulation of respiration after CDP addition (remaining activity was 3% of initial). In mitochondria with high mNDPK content mitochondria retained about 23% of the initial phosphorylating respiration in the presence of the same CPK activity. Addition of 10 μM carboxyatractyloside after CDP reduced the respiration rate in both systems to the same level, which was lower than before CDP addition (not shown in Table 3).

DISCUSSION

In the present study, we have demonstrated that in the presence of an excess of an external ADP-consuming system NDPK bound to mitochondrial membrane provides higher mitochondrial respiration rate than yNDPK and yHK not bound to mitochondria. Under our experimental conditions, the substrate (succinate) and P_i were in excess; under these conditions, the mitochondrial respiration rate depended on concentrations of ADP and ATP in the medium [37]. In the presence of soluble enzymes, the rate of mitochondrial respiration provided by quasi-equilibrium ADP concentration in the medium was 3-7% of the mitochondrial respiration rate determined in the absence of CPK (Tables 2 and 3). During mNDPK functioning the remaining mitochondrial respiration rate was 21-23% of the initial rate (Tables 2 and 3), though external concentration of ADP and ATP/ADP ratio insignificantly differed in these systems (Table 2). This difference in the mitochondrial respiration rates disappeared after addition of carboxyatractyloside, the adenine nucleotide translocase inhibitor; this means that this difference was determined by differences in oxidative phosphorylation rates.

The data suggest appearance of functional coupling between mNDPK and the oxidative phosphorylation system during their active functioning. We have not found such data for mNDPK in the literature, and so these observations on the mitochondrial enzyme have been obtained for the first time. Earlier functional coupling was demonstrated for HK isoenzyme type I [9-13] and glycerol kinase [14, 15] bound to the outer surface of the outer mitochondrial membrane. The value of mitochondrial respiration insensitive to inhibition by excess of CPK (21-23% of the mitochondrial respiration rate in the absence of CPK) found in the present study is comparable to the value (19%) obtained by Laterveer et al. [27] in medium without dextran and in the presence of excess pyruvate kinase and using liver mitochondria bound HK isoenzyme type I from brain mitochondria. Thus, functional coupling with the oxidative phosphorylation system appears to be a common feature of kinases bound to the outer mitochondrial membrane. However, it should be noted that in rat liver mitochondria HK and glycerol kinase activities are very low compared with mNDPK activity. According to literature data in rat liver mitochondria HK activity is ~2.5 nmol/min per mg protein [21] whereas glycerol kinase activity varies from 0.08

to 0.2 nmol/min per mg protein [38]; nevertheless, mitochondria contain many potential binding sites for these enzymes [21, 27, 28, 38]. Under our experimental conditions mNDPK catalyzed conversion of ~290 nmol CDP/min per mg protein (assuming that ADP/O = 2, see Table 2). This corresponds to ~80% of the maximal rate of oxidative phosphorylation. This comparison shows that in rat liver mitochondria mNDPK molecules obviously make a major contribution to the functional coupling between these kinases and oxidative phosphorylation.

What are the possible mechanisms underlying the functional coupling between mNDPK and the oxidative phosphorylation system? We may suggest that all (or at least some proportion of) mNDPK molecules of the outer mitochondrial compartment are localized near porin pores. The membrane surfaces are characterized by the existence of an unstirred water layer. At close positioning of active site of mNDPK and the porin pore some proportion of ADP molecules formed in the nucleoside diphosphate kinase reaction may reach the pore before leaving the layer of unstirred water, which is crucial for ADP availability to CPK. The existence of the unstirred aqueous layer on the surface of the outer mitochondrial membrane may create a microcompartment for ADP where ADP concentration is higher than in the external medium. This would result in the increase in ADP concentration in the intermembrane space and the increase in mitochondrial respiration rate. Another possibility is the formation of a multienzyme complex mNDPK–porin–ANT at the contact sites between outer and inner mitochondrial membranes during oxidative phosphorylation; the formation of such a complex has already been demonstrated for HK–porin–ANT [24]. Appearance of the multienzyme complex may cause conformational changes in the ANT molecule accompanied by changes in its kinetic properties with respect to ADP and ATP, which result in increased affinity to ADP. In the presence of CPK excess and at the same low steady state ADP concentration and equal ATP/ADP ratios the mitochondrial respiration rate will be higher in the case of active functioning mNDPK than yHK and yNDPK. Complex formation might also alter permeability of the porin pores for adenine nucleotides. It is possible that the effects observed in the present study can be attributed to a combination of these possible mechanisms.

Earlier we have shown that 2-3 fractions of mNDPK are associated with the outer mitochondrial membrane; these fractions exhibit different tightness of association with the outer mitochondrial membrane [6]. It is possible that only one of these fractions accounts for the mNDPK effects described here. Further studies are required to elucidate which possibility is actually the case in functional coupling between mNDPK and oxidative phosphorylation.

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REFERENCES

1. Lehninger, A. (1974) *Biochemistry* [Russian translation], Mir, Moscow.
2. Shimada, N., Ishikawa, N., Munakata, Y., Toda, T., Watanabe, K., and Kimura, N. (1993) *J. Biol. Chem.*, **268**, 2583-2589.
3. Kimura, N., Shimada, N., Nomura, K., and Watanabe, K. (1990) *J. Biol. Chem.*, **265**, 15744-15749.
4. Muchonen, W. W., and Lambeth, D. O. (1995) *Comp. Biochem. Physiol.*, **110B**, 211-223.
5. Jacobus, W. E., and Evans, J. J. (1977) *J. Biol. Chem.*, **252**, 4232-4241.
6. Lipskaya, T. Yu., and Plakida, K. N. (2003) *Biochemistry (Moscow)*, **68**, 1136-1144.
7. Pedersen, P. L. (1973) *J. Biol. Chem.*, **248**, 3956-3962.
8. Milon, L., Meyer, P., Chiadmi, M., Munier, A., Johansson, M., Karlsson, A., Lascu, I., Capeau, J., Janin, J., and Lacombe, M.-L. (2000) *J. Biol. Chem.*, **275**, 14264-14272.
9. Gots, R. E., Gorin, F. A., and Bessman, S. P. (1972) *Biochem. Biophys. Res. Commun.*, **49**, 1249-1255.
10. Gots, R. E., and Bessman, S. P. (1974) *Arch. Biochem. Biophys.*, **163**, 7-14.
11. Lipskaya, T. Yu., Geiger, P. J., and Bessman, S. P. (1995) *Biochem. Molec. Med.*, **55**, 81-89.
12. Laterveer, F. D., van der Heijden, R., Toonen, M., and Nicolay, K. (1994) *Biochim. Biophys. Acta*, **1188**, 251-259.
13. Wicker, U., Bucheler, K., Gellerich, F. N., Wagner, M., Kapischke, M., and Brdiczka, D. (1993) *Biochim. Biophys. Acta*, **1142**, 228-239.
14. Seltzer, W. K., and McCabe, E. R. (1984) *Mol. Cell. Biochem.*, **64**, 51-61.
15. Ostlund, A. K., Gohring, U., Krause, J., and Brdiczka, D. (1983) *Biochem. Med.*, **30**, 231-245.
16. Bessman, S. P., Borrebaek, B., Geiger, P. J., and Ben-Or, S. (1978) in *Microenvironments and Metabolic Compartmentation* (Srere, P. A., and Estabrook, R. W., eds.) Academic Press, New York, pp. 111-128.
17. Viitanen, P. V., Geiger, P. J., Erickson-Viitanen, S., and Bessman, S. P. (1984) *J. Biol. Chem.*, **259**, 9679-9686.
18. Brdiczka, D. (1991) *Biochim. Biophys. Acta*, **1071**, 291-312.
19. Seltzer, W. K., Firminger, H., Klein, J., Pike, A., Fennessey, P., and McCabe, E. R. (1985) *Biochem. Med.*, **33**, 189-199.
20. Adams, V., Griffin, L., Towbin, J., Gelb, B., Worley, K., and McCabe, E. R. (1991) *Biochem. Med. Metab. Biol.*, **45**, 271-291.
21. Weiler, U., Riesinger, I., Knoll, G., and Brdiczka, D. (1985) *Biochem. Med.*, **33**, 223-235.
22. McCabe, E. R. (1994) *J. Bioenerg. Biomembr.*, **26**, 317-325.
23. Fiek, C., Benz, R., Roos, N., and Brdiczka, D. (1982) *Biochim. Biophys. Acta*, **688**, 429-440.
24. Vyssokikch, M. Y., and Brdiczka, D. (2003) *Acta Biochim. Pol.*, **50**, 389-404.
25. Hashimoto, M., and Wilson, J. E. (2000) *Arch. Biochem. Biophys.*, **884**, 163-173.
26. Beutner, G., Ruck, A., Riede, B., and Brdiczka, D. (1998) *Biochim. Biophys. Acta*, **1368**, 7-18.

27. Laterveer, F. D., Gellerich, F. N., and Nicolay, K. (1995) *Eur. J. Biochem.*, **232**, 569-577.
28. Laterveer, F. D., Nicolay, K., and Gellerich, F. N. (1997) *Molec. Cell. Biochem.*, **174**, 43-51.
29. Jacobus, W. E., and Lehninger, A. L. (1973) *J. Biol. Chem.*, **248**, 4803-4810.
30. Goronovsky, I. T., Nazarenko, Yu. P., and Nekryach, E. F. (1962) *Short Reference Book on Chemistry* [in Russian], Ukrainian Academy Press, Kiev.
31. Sobol, S., Conrad, A., Keller, M., and Hebisch, S. (1992) *Biochim. Biophys. Acta*, **1100**, 27-32.
32. Davis, E. J., and Lumeng, L. (1975) *J. Biol. Chem.*, **250**, 2275-2282.
33. Lipskaya, T. Yu., and Savchenko, M. S. (2003) *Biochemistry (Moscow)*, **68**, 68-79.
34. Sober, H. A. (ed.) (1970) *Handbook of Biochemistry. Selected Data for Molecular Biology*, CRC Press, Cleveland, Ohio.
35. Eggleton, P., Elsden, S. R., and Gough, N. (1943) *Biochem. J.*, **37**, 526-529.
36. Gornall, A. G., Bardawill, C. L., and David, M. M. (1949) *J. Biol. Chem.*, **177**, 751-766.
37. Kunz, W., Bochnensack, R., Bohme, G., Kuster, U., Letko, G., and Schonfeld, P. (1981) *Arch. Biochem. Biophys.*, **209**, 219-229.
38. Kaneko, M., Kurokawa, M., and Ishibashi, S. (1985) *Arch. Biochem. Biophys.*, **237**, 135-141.