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Mitochondrial Nucleoside Diphosphate Kinase: Mode of Interaction with the Outer Mitochondrial Membrane and Proportion of Catalytic Activity Functionally Coupled to Oxidative Phosphorylation

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Abstract—In the present study, we found that ionic interactions are not essential for the binding of nucleoside diphosphate kinase of liver mitochondria outer compartment to outer mitochondrial membrane and that the proportion of the enzyme activity involved in functional coupling with oxidative phosphorylation (we demonstrated the existence of functional coupling earlier) is only 17%. Additional evidence was obtained that functionally coupled activity of nucleoside diphosphate kinase is associated with the outer surface of mitochondria. Dextran (10%) did not increase functional coupling. The physiological importance of these effects is discussed.

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Key words: nucleoside diphosphate kinase, mitochondria, outer compartment, localization, oxidative phosphorylation, functional coupling

Mechanisms providing ADP transport across the outer mitochondrial membrane remain unclear. Since ADP concentration in the cell cytoplasm is very low (~30 μM [1]), and the outer mitochondrial membrane represents a diffusion barrier for charged molecules [2], potentially the outer membrane may limit the rate of intracellular energy transport. The problem of the outer membrane permeability for ADP is solved as a result of functional coupling between kinases of the outer mitochondrial compartment and the oxidative phosphorylation system.

According to a current concept, a microenvironment in the region of interacting proteins or near the intracellular surfaces can form so-called metabolic or functional compartments [3] that may have no physical boundaries, but maintain complete or partial kinetic isolation [4]. The constituents of the compartment are functionally coupled, i.e. they prefer to use substrates generated inside the compartment [5]. Formation of a functional compartment is a characteristic feature that distinguishes functional coupling from usual coupling between activities of two enzymes with a common substrate in solution. *In vivo* regulation of metabolism occurs at the microenvironmental and compartmental level [3].

Studies have revealed the existence of functional coupling between activities of kinases localized on the external surface of the outer mitochondrial membrane, hexokinase (HK) and glycerol kinase, and oxidative phosphorylation [6-15]. Mitochondrial contact sites containing HK or glycerol kinase, porin, and adenine nucleotide translocase represent the structural basis of the functional compartment of these kinases [16, 17]. *In vitro* experiments demonstrated the formation of a kinase–porin–adenine nucleotide translocase multienzyme complex [18]. Formation of the complex is accompanied by conformational changes in each individual component [8, 12, 19, 20].

Abbreviations: AP5A) p^1p^5-di(adenosine-5′)pentaphosphate; CPK) muscle creatine phosphokinase; FCCP) carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HK) hexokinase; mAK) mitochondrial adenylate kinase; NDPK) nucleoside diphosphate kinase; omNDPK) outer mitochondrial membrane bound NDPK; RCR) respiratory control ratio; yHK) yeast HK.

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We study functional coupling between activity of nucleoside diphosphate kinase (NDPK) (EC 2.7.4.6) in liver mitochondria and oxidative phosphorylation.

Under physiological conditions, NDPK catalyzes synthesis reactions of various nucleoside triphosphates (NTPs) from ATP and the corresponding nucleoside diphosphates (NDPs). NTPs are involved in anabolic processes [21]. The enzyme participates in the regulation of proliferation, development, differentiation, malignant growth, and apoptosis [22-25]. The catalytic and regulatory functions of this enzyme may exist independently [23, 25, 26].

In hepatocytes, NDPK is localized within the cytoplasm, in mitochondria, and is associated with membranes [27-32]. In rat liver mitochondria, 90% of NDPK activity is found in the outer compartment [29, 32]. Earlier, we demonstrated that all NDPK of the outer compartment is associated with the outer surface of the outer mitochondrial membrane (omNDPK) [32].

Pedersen [31] suggested the existence of functional coupling between activity of NDPK of the outer mitochondrial compartment and oxidative phosphorylation but did not produce any evidence of this.

We demonstrated for the first time that omNDPK localized on the outer surface of the outer mitochondrial membrane [32] is indeed functionally coupled to oxidative phosphorylation [33]. In the present study, we demonstrated that functional coupling involves only a small proportion of this enzyme activity. Additional evidence was also obtained that this functionally coupled omNDPK activity is associated with the outer surface of the outer mitochondrial membrane. A preliminary report of this work has been presented as an abstract [34].

MATERIALS AND METHODS

Materials. ATP, ADP, AP5A (p^1p^5-di(adenosine-5′)pentaphosphate), phosphocreatine, cytochrome *c*, FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone), and yeast NDPK (nucleoside diphosphate kinase) were purchased from Sigma Chemical Co (USA); creatine was from Eastman Kodak Co (USA); yHK (yeast hexokinase) was from Fluka AG (Switzerland); CDP, AMP, and CPK (muscle creatine phosphokinase) were produced by Reanal (Hungary); dextran (Mw 15,000-20,000) was from ICN (USA).

Isolation of mitochondria. Mitochondria were isolated from livers of albino rats (180-270 g) basically as described in work [33]. The isolation medium contained 0.28 M mannitol, 2.1 mM Hepes, pH 7.4. A 10% homogenate was centrifuged at 2000 rpm for 15 min in a JA-20 rotor of a Beckman J2-21 centrifuge (Austria). The supernatant (S_1) was centrifuged at 8000 rpm for 10 min. The mitochondrial pellet (P_1) was suspended (2.7 ml per gram of liver) in isolation medium (low salt medium) or in one of the following media: 3.33 mM $MgCl_2$, 0.27 M mannitol, 2.1 mM Hepes, pH 7.4 (Mg^{2+} washing medium); 0.14 M KCl, 2.1 mM Hepes, pH 7.4 (high salt washing medium). The suspension of pellet P_1 was centrifuged at 10,300 rpm for 10 min. The mitochondrial pellet (P_2) was suspended in the same medium (protein concentration ~60 mg/ml). The resulting mitochondrial suspension was kept on ice.

In some experiments liver was homogenized in a medium containing 250 mM mannitol, 0.5 mM EGTA, 5 mM Hepes, pH 7.4, 0.1% (w/v) BSA [35]. The homogenate was divided into two equal portions. From one portion, mitochondria were isolated by the method described above ("short method"), and from another one they were isolated by the method of Hovius et al. [35], which includes a Percoll gradient centrifugation of mitochondrial suspension. Finally, mitochondria were suspended in a low salt medium and used in polarographic experiments.

Mitochondrial respiration. The rate of mitochondrial oxygen consumption was determined at 22°C in a reaction vessel equipped with a covered Clark type oxygen electrode and LP 7e polarograph (Laboratorni Pristroje Praha, 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 $MgCl_2$, 5 mM potassium succinate. Other additions are given in the text or legends to the figures and tables. Oxygen concentration in the incubation medium was assumed to be 290 μM at 22°C [36].

Polarographic assay of omNDPK (outer mitochondrial membrane bound NDPK) activity. The main incubation medium also contained 1 mM ATP and 20 μM adenylate kinase inhibitor AP5A. The activity of omNDPK was characterized as the percent ratio of phosphorylating respiration rate in the presence of 600 μM CDP to the phosphorylating respiration rate that was initially determined in the same polarographic sample after addition of 170 μM ADP. The rate of phosphorylating respiration after addition of ADP (V^{ADP}) was determined by measuring the difference between the respiration rate initiated by addition of ADP and the rate after its exhaustion (ADP phosphorylation) (Fig. 1a). The rate of phosphorylating respiration after the subsequent addition of CDP (V^{CDP}) was determined by the difference between the respiration rates after and before addition of CDP (Fig. 1a). We assumed that ADP/O ratio remained unchanged in one polarographic sample and so the ratio V^{CDP}/V^{ADP} reflects the ratio of omNDPK activity to the rate of oxidative phosphorylation (of ADP) in the same sample. ADP (170 μM) provides close to maximal rate of oxidative phosphorylation.

Polarographic experiments in the presence of creatine kinase (CPK). Freshly prepared solution of rabbit muscle CPK (0-37.5 U/ml final concentration) was added to the main incubation medium containing the following addi-

tions: 1 mM ATP, 6-7 mM phosphocreatine, and 20 μM AP5A (incubation medium-1), and also 5 mM glucose (incubation medium-2). In experiments with active mitochondrial adenylate kinase (mAK), the main incubation medium also contained 1 mM ATP, 6.2-7.7 mM phosphocreatine, and 7.4-9.6 μM AP5A (incubation medium-3). The reaction was initiated by adding a suspension of mitochondrial pellet P_2 (0.9-1.2 mg of protein/ml). Using incubation medium-1, we added 600 μM CDP 1.5 min after the addition of the mitochondrial suspension, whereas in the case of incubation medium-3, we added 250-400 μM AMP. Concentrations of AMP and AP5A in samples containing active mAK were selected to maintain the respiration rate in the absence of CPK at a level of 80-90% of the respiration rate in the presence of 170 μM ADP. Using 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 or 250-400 μM AMP (in the presence of AP5A) did. Mitochondrial oxygen consumption was recorded for 1 min. In experiments performed in the presence of CPK, the rates of phosphorylating respiration in the presence of active omNDPK, yHK, or mAK in incubation media -1, -2, and -3, respectively, were determined by the difference in the mitochondrial respiration rates after and before additions of CDP, yHK, or AMP.

Removal of cytochrome *c* from mitochondria. The method of Jacobs and Sanady [37] was used. Rat liver mitochondria were isolated and washed in 0.3 M sucrose as described above. Mitochondrial P_2 pellet was carefully drained of excess sucrose solution, suspended in 17 ml of 0.015 M KCl, and stirred on ice for 10 min (hypotonic treatment). The mitochondria were then sedimented at 10,300 rpm for 10 min, resuspended in 17 ml of 0.15 M KCl (cytochrome *c* solubilizing medium), stirred for 10 min, and again centrifuged. The resulting mitochondrial pellet was suspended in a small volume of 0.3 M sucrose and used in polarographic experiments.

Study of omNDPK solubilization during mitochondria storage. The concentrated suspension of P_2 sediment was stored on ice in one of the washing media; 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 a MiniSpin plus centrifuge (Eppendorf, Germany). In some experiments, for evaluation of the possible additional solubilization of omNDPK from mitochondria during a polarographic experiment, aliquots of 30 μl of P_2 suspension were added to incubation medium-1 and incubated at 22°C with periodic shaking in a Thermomixer comfort (Eppendorf) for 1.5 min; afterwards, 600 μM CDP was added. The total volume of samples was 2 ml. After incubation for 1 min, samples were centrifuged as above. Supernatants were carefully removed, and the pellets were suspended in 35 μl of the same washing medium and used for the polarographic determination of the remaining omNDPK activity.

During study of NDPK localization in the outer mitochondrial compartment, suspension of P_1 sediment in low salt medium was stirred on ice for 10 h. After certain time intervals, aliquots of 0.5 ml were taken and centrifuged at 13,400 rpm for 10 min. Pellets P_2 were rinsed with a small volume of low salt medium, carefully drained of excess medium, and suspended in 0.5 ml of 0.15 M KCl. The samples were incubated at 2°C with periodic shaking in the thermomixer. After incubation for 10 min, the samples were centrifuged as above. Supernatants were carefully removed, and the pellets were suspended in 100 μl of low salt medium and used for the determination of the remaining omNDPK and mAK activities, as well as the effect of cytochrome *c* on mitochondria respiration.

Solubilization of omNDPK induced by various substances. Aliquots (7-8 ml) of the supernatant S_1 were centrifuged at 9700 rpm for 10 min in the cold using a bench top Metronex centrifuge (Poland). Each P_1 pellet was suspended in 1.8 ml of washing medium, and placed into the thermomixer at 2°C. (Composition of the washing media is given in legends to the corresponding figures and tables.) After incubation for 1-5 h with periodic shaking, the samples were centrifuged at 13,400 rpm for 10 min in the MiniSpin plus centrifuge. The resulting pellets, P_2, were suspended in a small volume of low salt medium (protein concentration of ~60 mg/ml). The remaining omNDPK activity was assayed by the polarographic method and the V^{CDP}/V^{ADP} ratio was calculated as above. The ionic strength of the extracting solutions was calculated using the following equation: $I = 1/2\ (\Sigma C_i Z_i^2)$, where C_i represents concentration of every ion, and Z_i represents a charge of this ion. The concentrations of ionized forms of buffer components were evaluated using the Henderson–Hasselbach equation.

Statistical evaluation of the results is shown as means ± SEM. The number of measurements is indicated in the legends to figures and tables.

Mitochondrial protein content was determined by the method of Gornall et al. [38] (using BSA as standard).

RESULTS

To study the functional coupling between omNDPK activity and oxidative phosphorylation, we utilized the polarographic method [39, 40]. The magnitude of the respiratory control ratio (RCR) defined as a ratio of the rate of mitochondrial respiration immediately after ADP addition to that after all ADP was phosphorylated (Fig. 1a), is a sensitive index of mitochondria functional capacities and their structural integrity [39-41].

Figure 1a represents a typical polarogram showing the effects of ADP and CDP on the rate of mitochondrial respiration. ADP produced during the reaction cat-

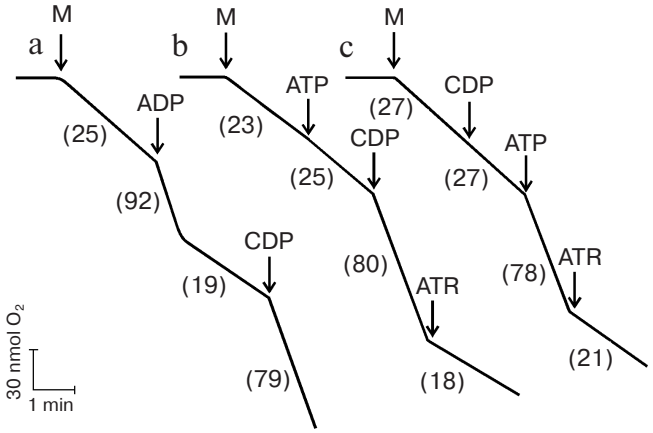

Fig. 1. Polarographic method for measuring omNDPK activity. Mitochondria were washed and kept in high salt medium. The main incubation medium also contained 0.02 mM AP5A (a-c) and 1 mM ATP (a). The reaction was initiated by the addition of 0.9 mg of mitochondrial protein (M). Where indicated, 170 μM ADP, 600 μM CDP, 1 mM ATP, and 10 μM atractyloside (ATR) were added to the closed reaction vessel. Respiratory rates (in brackets) are expressed as nmol O_2/min per mg mitochondrial protein.

alyzed by omNDPK (CDP + ATP → CTP + ADP) stimulates respiration. The affinity of the oxidative phosphorylation system for ADP is very high (K_m ~ 20 μM) [39]. Addition of only ATP or CDP does not stimulate respiration (Fig. 1, b and c; see also [31]). Atractyloside, an adenine nucleotide translocase inhibitor, abolishes mitochondrial respiration in the presence of ATP and CDP (Fig. 1, b and c). Using the rate of mitochondrial respiration after addition of CDP, it is possible to calculate the rate of oxidative phosphorylation of ADP and the rate of CTP production, equal to it. The rate of oxidative phosphorylation of ADP is calculated as the rate of phosphorylating respiration in ng-atom O/min per mg of protein multiplied by ADP/O ratio, which equals 2 for succinate oxidation.

We demonstrated [33] that omNDPK activity measured by the rate of mitochondrial phosphorylating respiration after the addition of CDP coincides with the enzyme activity calculated on the basis of the amount of CTP measured spectrophotometrically at 340 nm in a coupled enzyme system containing HK, glucose-6-phosphate dehydrogenase, and yeast NDPK. Pedersen [31] found that stimulation of respiration by different nucleoside diphosphates is associated with nucleoside triphosphates formation. Consequently, in the presence of ATP, stimulation of mitochondrial respiration caused by CDP addition is only due to the activity of omNDPK, and the rate of phosphorylating respiration is proportional to the activity of this enzyme.

We characterized omNDPK activity as a V^{CDP}/V^{ADP} ratio. The presentation of the results in the form of this ratio is more informative (in relation to omNDPK function) than just activity of omNDPK. It makes possible comparison of data obtained in different experiments and during activity of different kinases. At the same time, omNDPK activity can be calculated from the rates of oxidative phosphorylation in the presence of 170 μM ADP (mean values are given in the Table 1) multiplied by V^{CDP}/V^{ADP} ratio.

Comparison of two methods of mitochondria isolation. We compared the "short" method of mitochondria isolation and the method of Hovius et al., which includes Percoll gradient purification of mitochondria [35]. RCR magnitudes of mitochondria isolated by the "short"

Table 1. Functional characteristics of mitochondrial preparations stored in different washing media

Washing medium	*n*	Time of storage, h	Respiration rate, nmol O_2/min per mg				Rate of oxidative phosphorylation, nmol ADP/min per mg	omNDPK activity, nmol CTP/min per mg	V^{CDP}/V^{ADP}, %	RCR
			st_4^S	st_3^{ADP}	st_4^{ADP}	st_3^{CDP}				
Low salt	6	0.5-3.0	26 ± 3	95 ± 10	21 ± 3	61 ± 10	298 ± 28	161 ± 34	55 ± 13	4.7 ± 0.3
	6	5.0-8.0	32 ± 4	107 ± 14	25 ± 5	69 ± 11	331 ± 38	177 ± 35	55 ± 14	4.4 ± 0.4
High salt	9	0.5-3.0	31 ± 5	120 ± 27	25 ± 4	100 ± 21	352 ± 76	273 ± 32	81 ± 12	4.8 ± 0.5
	10	5.0-8.0	31 ± 3	102 ± 13	26 ± 2	87 ± 11	306 ± 51	247 ± 43	78 ± 8	4.1 ± 0.5
Mg^{2+}	16	0.5-3.0	33 ± 5	108 ± 15	29 ± 5	102 ± 13	316 ± 48	265 ± 46	87 ± 6	3.8 ± 0.3
	9	5.0-8.0	38 ± 10	113 ± 18	31 ± 4	100 ± 11	328 ± 60	277 ± 34	87 ± 9	3.6 ± 0.3

Note: Mitochondria were washed and kept in one of the indicated media. The main incubation medium also contained 1 mM ATP and 20 μM AP5A. Time after isolation of P_2 pellet is shown. 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; st_3^{CDP}, after addition of 600 μM CDP (see Fig. 1a). The rates of oxidative phosphorylation are calculated with the use of the ADP/O ratio of 2 for respiration on succinate. *n*, number of observations.

method were slightly higher (especially in the presence of 1 mM ATP) than those of mitochondria isolated using a Percoll gradient [35] (6.2-6.3 and 5.3-5.9 in the presence of ATP, respectively). RCR magnitude measured in the presence of ATP represents a test for the attendance of any membrane fragments in mitochondria, since all of them exhibit ATPase activity, decreasing the magnitude of RCR. In mitochondria isolated by the "short" method, omNDPK activity was higher than that in mitochondria isolated by the method of Hovius et al. (V^{CDP}/V^{ADP} was 72-81 and 50-53%, respectively). This was not due to the contamination of mitochondrial preparation with cytoplasmic NDPK [32] or NDPK of microsomes, as the latter do not contain the enzyme [30]. Lower NDPK content in preparation obtained by the method of Hovius et al. is possibly caused by NDPK solubilization during multiple and prolonged washing of mitochondria. Taking into consideration all these observations, in subsequent experiments we used the "short" method of mitochondria isolation.

Nature of forces involved in the interaction of omNDPK with mitochondrial membranes; the role of Mg^{2+}. Figure 2 shows the dependence of the proportion of omNDPK activity associated with the mitochondrial pellet on KCl concentration in the washing medium. These data demonstrate that during an increase of KCl concentration from 0 to 50 mM, tightness of omNDPK binding to membranes increased and reaches its maximum at 40-50 mM KCl. These observations corroborate our suggestion [32] about an important role of nonionic interactions in enzyme binding to membranes.

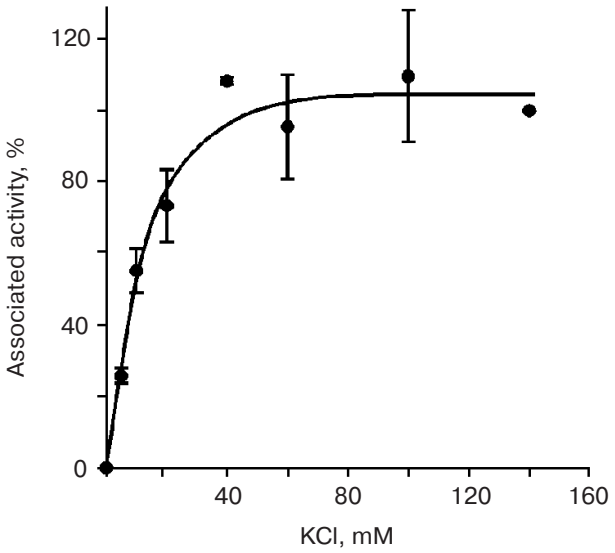

Fig. 2. Effect of KCl concentration on omNDPK binding to mitochondrial membranes. Rat liver mitochondrial pellets, P_1, were suspended in washing media containing 2.1 mM Hepes, pH 7.4, indicated KCl concentrations, and mannitol up to a total osmotic concentration of 0.28 osM. Samples were shaken in the thermomixer for 1 h. The ratio V^{CDP}/V^{ADP} obtained for mitochondria suspended in the high salt washing medium was defined as 100% (in these experiments, this ratio was 69.0 ± 0.9%); the V^{CDP}/V^{ADP} ratio obtained for mitochondria suspended in low salt medium was defined as 0% (in these experiments, this ratio was 34.5 ± 0.2%). The results of two independent experiments are presented.

Figure 2 shows that half-maximal binding of omNDPK was observed at KCl concentration of ~10 mM (I = 0.01). Substitution of 10 mM KCl by 10 mM NaCl did not affect omNDPK binding to mitochondrial membranes (data not shown). This suggests that the effects of K^+ and Na^+ may be attributed to a nonspecific influence of ionic strength on the enzyme binding. $MgCl_2$ increased the tightness of omNDPK binding to membranes. The effect of 3.33 mM $MgCl_2$ (I = 0.01) was two times higher than the effect of 10 mM KCl (data not shown). It was reasonable to suggest that Mg^{2+} ions are directly involved in the enzyme binding to mitochondrial membranes. However, washing of mitochondria in low salt medium at pH 8.0 in the presence of 1 mM EDTA did not cause additional solubilization of omNDPK (data not shown).

Solubilization of omNDPK from mitochondria stored in various media. We investigated the effects of washing and storage of mitochondria in various media on the rate of oxidative phosphorylation and on the tightness of omNDPK binding to mitochondrial membranes.

Table 1 shows that mitochondria isolated under different conditions were tightly coupled. Their main functional characteristics basically did not differ and did not change during the whole experiment. This means that different conditions of mitochondria isolation and storage did not produce any indirect action on the respiration machinery. However, mitochondria kept in Mg^{2+} washing medium revealed a small decrease in the magnitude of RCR, perhaps, due to some stimulation of mitochondrial ATPase activity in the presence of Mg^{2+}. Table 1 shows that omNDPK activity in mitochondria washed in Mg^{2+} and high salt media was higher than that in mitochondria isolated in low salt medium.

In these experiments, all the effects on the omNDPK activity were possibly produced during storage of the mitochondria in different media, since during the measurement of the enzyme activity by the polarographic method the components of the mitochondria storage medium were diluted at least 50 times and could not affect omNDPK activity. Besides, during omNDPK activity measurements, the incubation medium contained saturating concentrations of the enzyme substrates (1-1.17 mM ATP, 0.6 mM CDP) and Mg^{2+} (3 mM).

Figure 3 (curves *1* and *2*) shows that the tightness of omNDPK association with membranes significantly varied in mitochondria kept in various media. The omNDPK was easily solubilized from mitochondria washed and kept in low salt medium (Fig. 3c, curve *2*). The solubilization of omNDPK from mitochondria washed and kept in the washing medium with high ionic strength occurred at a slower rate (Fig. 3a, curve *2*). In

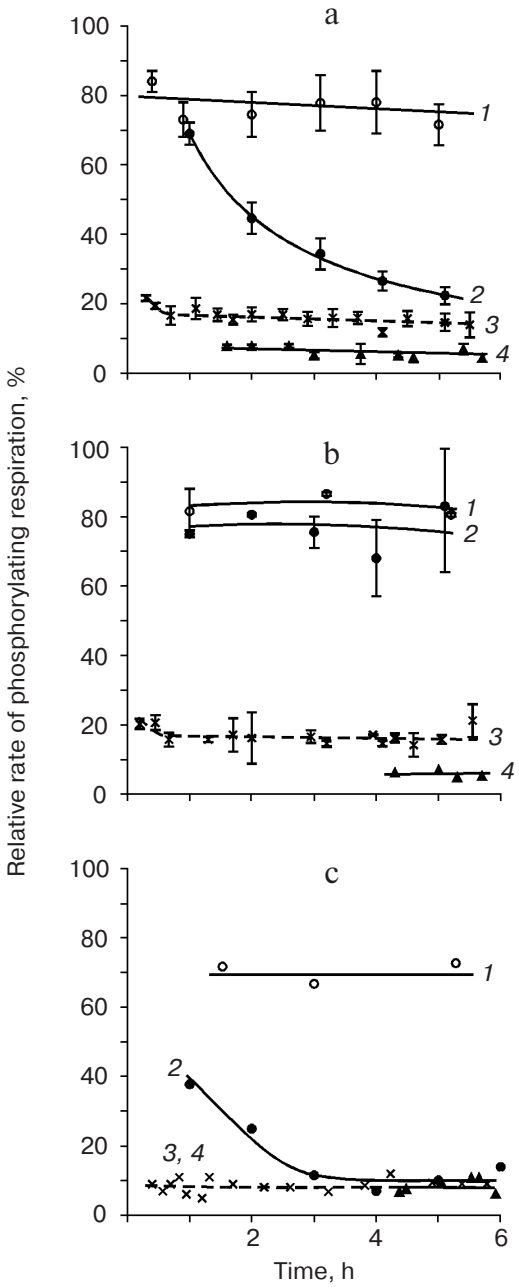

Fig. 3. Proportion of omNDPK activity involved in functional coupling with oxidative phosphorylation. Mitochondria were washed and stored in high salt washing medium (a), Mg^{2+} washing medium (b), or low salt medium (c). Mitochondrial respiration was measured in the main incubation medium also containing 1 mM ATP and 20 μM AP5A (curves *1* and *2*); in incubation medium-1 or -2 containing 22.5-36.0 U/ml CPK (curves *3* and *4*, respectively). *1*, *3*, *4*) Initial suspension of P_2 pellet; *2*) after resedimentation in the corresponding washing medium. Mean values of V^{ADP} obtained in the absence of phosphocreatine and CPK for the nearest time intervals during studies of suspension of P_2 pellets were used for calculation of V^{CDP}/V^{ADP} or V^{HK}/V^{ADP} for curves *3* and *4*, respectively. Initial V^{ADP} values were 85.3 ± 10.0, 89.5 ± 0.5, 87.2 nmol O_2/min per mg protein (a-c, respectively). a) Curves *1-3*, number of observations, $n = 6$; curve *4*, $n = 3$; b) curves *1-3*, $n = 2$; curve *4* and panel (c) are results of one experiment.

mitochondria washed and kept in the washing medium containing $MgCl_2$, the enzyme remained associated with membranes during 5 h storage of mitochondria on ice (Fig. 3b, curve *2*). Pilot experiments revealed all solubilized omNDPK activity in the supernatant after sedimentation of the mitochondria (data not shown).

Figure 3 also shows that in all cases, total activity of solubilized and membrane-associated omNDPK remained basically unchanged during storage (curves *1*). Under conditions of the polarographic experiment, we basically did not observe additional solubilization of omNDPK from mitochondria (data not shown). These results were used to elucidate the proportion of omNDPK activity involved in functional coupling to oxidative phosphorylation.

Proportion of omNDPK activity involved in functional coupling to oxidative phosphorylation. Previously [33], we developed methodological approaches required for studies of functional coupling between omNDPK and oxidative phosphorylation. We analyzed two systems. In one system, omNDPK played the role of ADP donor for oxidative phosphorylation, and in the other system, yeast HK or yeast NDPK (unbound to mitochondrial membranes and exhibiting equal activity) were the ADP donors. Both systems employed muscle CPK as the external agent competing for ADP with oxidative phosphorylation. CPK activity exceeded the oxidative phosphorylation rate by 60-100 times; in both systems, quasi-equilibrium concentrations of ADP and ATP/ADP ratio were the same [33]. Under these conditions, the rate of mitochondrial phosphorylating respiration in the system containing active omNDPK represented 21% of the phosphorylating respiration rate determined in the absence of CPK. In the system containing yHK, this parameter represented 7%, and in the system containing yeast NDPK it was only 3%. Thus, functional coupling between active omNDPK and the oxidative phosphorylation system can be characterized by the parameter of phosphorylating respiration rate maintained in the presence of excess of CPK activity.

Using mitochondria isolated and kept in different media, we have investigated whether all molecules of omNDPK are involved in functional coupling to oxidative phosphorylation. Figure 3a (curve *2*) shows that storage of a mitochondrial suspension in a high salt washing medium for 5 h was accompanied by the decrease of membrane-associated omNDPK activity from 78 to 22% of the rate of oxidative phosphorylation; in mitochondria kept in the Mg^{2+} washing medium, a proportion of membrane-associated omNDPK activity remained basically unchanged and represented 70-80% of the rate of oxidative phosphorylation (Fig. 3b, curve *2*). During this period in both suspensions, the respiration rate insensitive to the presence of excess of CPK activity remained unchanged; it represented ~17% of the rate of oxidative phosphorylation (Fig. 3, a and b, curves *3*). In the pres-

ence of active yHK, the proportion of phosphorylating respiration insensitive to the presence of excess of CPK activity represented only ~6% (Fig. 3, a and b, curves *4*). We concluded that in both types of experiments with omNDPK, only some (and the same) proportion of membrane-bound omNDPK molecules ensured that CPK insensitive respiration was involved in functional coupling to oxidative phosphorylation. The activity of these molecules accounts for ~20% of total omNDPK activity.

Figure 3c shows that in mitochondria washed and kept in the low salt medium, the rate of phosphorylating respiration in the presence of CDP and an excess of CPK activity was comparable to the respiration rate in the presence of yHK even during the first two hours of mitochondria storage (when omNDPK was only partially solubilized) (Fig. 3c, curves *3* and *4*). We concluded that in these mitochondria, functional coupling between omNDPK activity and oxidative phosphorylation is absent.

Effect of 10% dextran on functional coupling of omNDPK and mAK to oxidative phosphorylation. To determine whether the proportion of omNDPK molecules involved in functional coupling to oxidative phosphorylation can be increased, we added 10% dextran to the polarographic incubation medium. Pilot experiments revealed that 10% dextran did not solubilize omNDPK from mitochondrial membranes (data not shown). It is well documented that dextran increases the number of contact sites between the outer and inner mitochondrial membranes and reduces the volume of the intermembrane space by mimicking the oncotic pressure of cytoplasmic proteins [15]. In the presence of 10% dextran, functional coupling between HK-I or an enzyme from the mitochondrial intermembrane space, mAK, and oxidative phosphorylation increases [2, 15].

In parallel, we carried out experiments with omNDPK and mAK. Figure 4 shows the results of separate experiments and Table 2 summarizes all data. Figure 4 (a and b) shows that omNDPK and mAK exhibited functional coupling to oxidative phosphorylation in mitochondria washed and kept in the Mg^{2+} washing medium. Indeed, catalytically active omNDPK and mAK provided a higher rate of phosphorylating respiration in the presence of excess of CPK activity compared with the rate of phosphorylating respiration determined in the presence of yHK exhibiting equal activity in the absence of CPK. However, in these mitochondria, dextran did not increase functional coupling to oxidative phosphorylation (Fig. 4, c and d). Figure 4 shows that the presence of 10% dextran did not influence functional coupling between omNDPK and oxidative phosphorylation in all systems studied (Fig. 4, c, e, and g). However, in mitochondria washed and kept in Mg^{2+} free media, dextran did increase functional coupling between oxidative phosphorylation and mAK (Fig. 4, f and h).

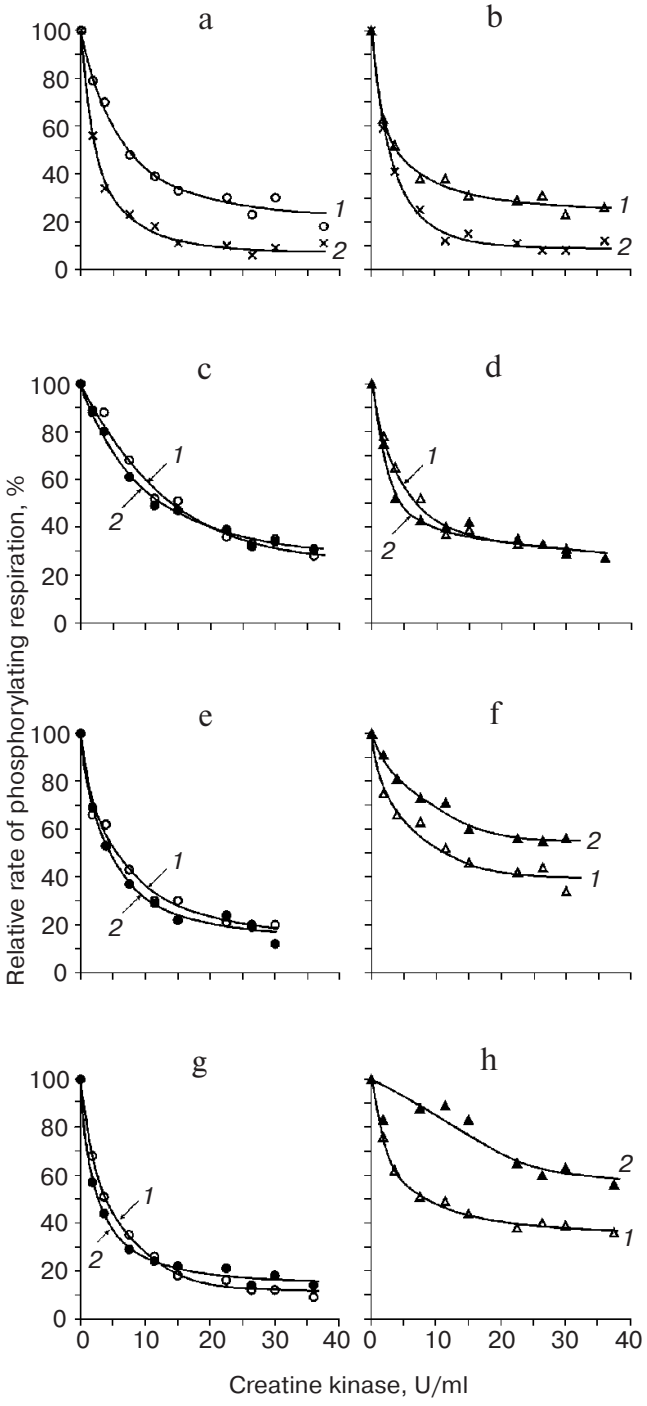

Fig. 4. Effect of 10% dextran on functional coupling of omNDPK (a, c, e, g) or mAK (b, d, f, h) with oxidative phosphorylation. Mitochondria were washed and kept in Mg^{2+} washing medium (a-d), in high salt washing medium (e, f), or in low salt medium (g, h). Mitochondria were incubated in incubation medium-1 (a, curve *1*; c, e, g), in incubation medium-2 (a and b, curves *2*), or in incubation medium-3 (b, curve *1*; d, f, h). Dark circles and triangles show that the corresponding incubation media also contained 10% dextran (w/v). The rates of phosphorylating respiration in the presence of substrates for corresponding kinases in the corresponding incubation media, but in the absence of CPK, were defined as 100%. Data represent results of one of 2-3 typical experiment.

Table 2. Effect of 10% dextran on mitochondrial respiration in the presence of excess of CPK activity

Mitochondria washing medium	Active enzyme	Rate of phosphorylating respiration, %	
		– dextran	+ dextran
Mg^{2+}	omNDPK	23.9 ± 2.6 (12)	21.2 ± 4.9 (8)
	mAK	29.1 ± 1.3 (8)	31.0 ± 1.8 (4)
High salt	omNDPK	22.7 ± 1.9 (7)	24.0 ± 2.7 (7)
	mAK	36.4 ± 1.8 (7)	52.6 ± 1.6 (7)
Low salt	omNDPK	12.2 ± 2.5 (4)	16.8 ± 2.9 (4)
	mAK	38.2 ± 1.1 (10)	55.4 ± 1.9 (10)

Note: In each experiment, the relative rates of mitochondrial phosphorylating respiration in the presence of substrates for corresponding kinase and in the absence of CPK were defined as 100%. Results given in the table were obtained in the presence of CPK (22.5-37.5 U/ml). The number of measurements is given in brackets.

Study of omNDPK localization in the outer mitochondrial compartment. Earlier, we demonstrated that in rat liver mitochondria, all NDPK activity of the outer compartment is associated with the cytoplasmic surface of the outer mitochondrial membrane [32].

In our present experiments, we found that only a small proportion of omNDPK molecules took part in the functional coupling to oxidative phosphorylation. It could be supposed that these molecules are located in the intermembrane space.

To verify this suggestion, we carried out an additional study of omNDPK localization in the outer mitochondrial compartment. It is known that a component of the mitochondrial respiratory chain—cytochrome *c*—is bound to the outer surface of the inner membrane by electrostatic forces [37]; it is easily solubilized from mitochondria with damaged outer membrane in a salt medium [37]. In rat liver mitochondria, there is no molar excess of cytochrome *c* compared with other components of mitochondrial respiratory chain [42]. It participates in all reactions of electron transport [37]. Cytochrome *c* solubilization results in a reduced rate of respiration, which could be restored after addition of its low concentration to the medium [37]. At present, analysis of the effects of added cytochrome *c* on mitochondria respiration is the most sensitive and reliable test for the outer mitochondrial membrane integrity.

Figure 5 shows the results of the experiment. In this experiment, suspension of mitochondrial pellet, P_1, was incubated on ice for 10 h. At certain time intervals, two aliquots were taken. One of them was directly added to the polarographic cell, whereas the other one was initially centrifuged (to remove omNDPK and mAK activities solubilized during storage on ice) and then washed in 0.15 M KCl to remove cytochrome *c* from mitochondria with damaged outer membrane. The figure shows that washing of mitochondria did not affect mAK activity (Fig. 5a, curves *1* and *2*), but almost completely removed activity of omNDPK after 10 h of storage of mitochondria on ice (Fig. 5a, curves *3* and *4*). After 10 h storage, cytochrome *c* did not increase the rate of mitochondrial respiration, which was stimulated by FCCP addition

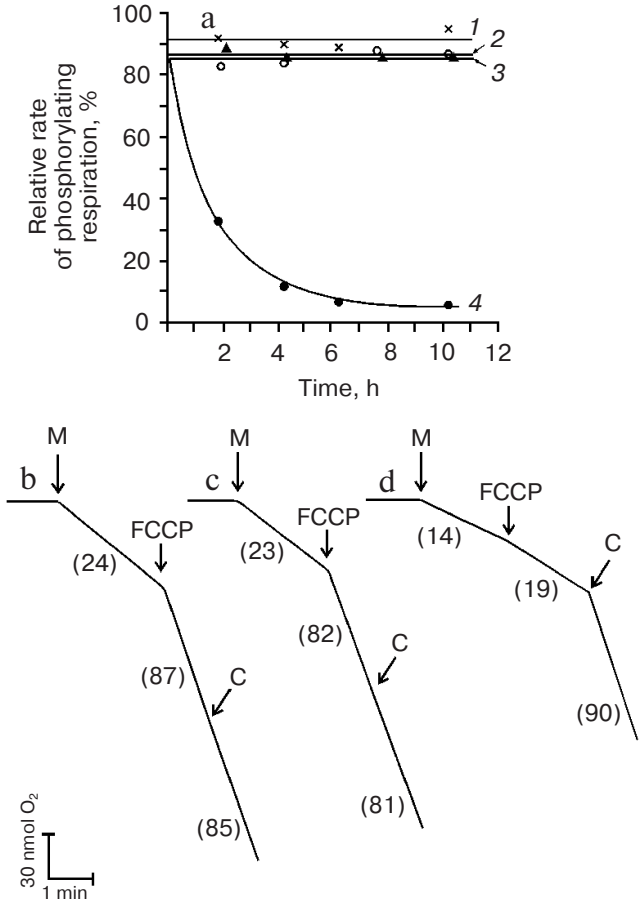

Fig. 5. Solubilization of omNDPK, mAK, and cytochrome *c* during storage of mitochondria. a) Main incubation medium also contained 0.3 mM ATP and 40 μM AMP (curves *1* and *2*), 1 mM ATP and 20 μM AP5A (curves *3* and *4*). *2*, *3*) Initial suspension of P_1 pellet; *1*, *4*) P_1 suspension after treatment with 0.15 M KCl. b) Initial suspension of P_1 after 10 h storage of mitochondria on ice; c) P_1 suspension after 10 h storage and treatment with 0.15 M KCl; d) mitochondria after hypotonic treatment, incubation with 0.15 M KCl and re-sedimentation. Where indicated, 0.5 μM FCCP and 10 μg of cytochrome *c* were added. Respiratory rates (in brackets) are expressed as nmol O_2/min per mg protein in the initial mitochondrial suspensions. Data are representatives of one of two experiments with similar results.

(Fig. 5, b and c), but it stimulated respiration of mitochondria with damaged outer membrane (Fig. 5d). Thus, like mAK test, the cytochrome *c* test also shows that in our experiments, washing and storage of mitochondria did not affect the integrity of the outer membrane (Fig. 5, b and c). We concluded that in our experiments, all solubilized omNDPK activity was associated with the outer surface of the outer mitochondrial membrane.

DISCUSSION

Table 1 shows that during our experiments the main functional characteristics of mitochondria basically did not change, and that the mitochondria were rather pure and tightly coupled.

The increase in the ionic strength of the storage medium increased the tightness of omNDPK association with membranes (Figs. 2, 3a, and 3c). The solubilization of omNDPK from mitochondria was slow (Fig. 3); solubilization time for this process was within the scale of hours. These results support our hypothesis [32] that ionic interactions are not essential for omNDPK binding. The presence of Mg^{2+} in the storage medium significantly increased the enzyme association with mitochondrial membranes (Fig. 3b). Addition of a chelating agent, EDTA, into the washing medium did not increase the enzyme solubilization. We concluded that Mg^{2+} is not directly involved in omNDPK binding to the outer mitochondrial membrane. It is possible that omNDPK solubilization is caused by differences existing between mitochondria storage media in our experiments and the environment of mitochondria in liver cells with respect to pH and metabolite composition. As a result, the system could establish new association/dissociation equilibrium. Indeed, we found that solubilization of omNDPK in low salt medium is reversible (unpublished data).

It is possible that association with mitochondrial membranes alters the kinetic properties of omNDPK and its substrate specificity. Since we used saturating concentrations of omNDPK substrates, a possible difference in kinetic parameters (perhaps, in K_m's) between membrane-associated and solubilized omNDPK did not affect the interpretation of our results. Indeed, it can be seen (Figs. 3 and 5a) that total activity of the enzyme (solubilized plus bound) was constant during the experiment.

We previously demonstrated [33] (and have confirmed in this study) that omNDPK may be functionally coupled to oxidative phosphorylation (Fig. 4a). Thus, functional coupling is the common feature of all studied kinases that can bind to the outer mitochondrial membrane. However, in rat liver mitochondria, activities of HK and glycerol kinase are negligibly small compared with the activity of omNDPK [16, 43].

We demonstrated here for the first time that functional coupling involves only a small proportion of omNDPK molecules exhibiting the tightest binding to mitochondrial membranes. These molecules represent 22-24% of the total activity of this enzyme (Table 2); they are responsible for ~17% of the maximal rate of oxidative phosphorylation (Fig. 3). Functional coupling of these molecules to oxidative phosphorylation depends on conditions of isolation of the mitochondria (Fig. 3).

We suppose that functional coupling involves those omNDPK molecules that are localized at the contact sites of mitochondrial membranes and are associated with porin or its neighboring environment. This is consistent with data by Adams et al. [44] demonstrating that in rat liver mitochondria subjected to multiple washing in medium of low ionic strength, the remaining activity of NDPK was detected at the contact sites.

What are the possible mechanisms underlying the functional coupling between omNDPK and the oxidative phosphorylation system? Firstly, the unstirred aqueous layer on the surface of the outer mitochondrial membrane may create a microcompartment for ADP, where its concentration is higher than that in the external medium; secondly, the formation of a multienzyme complex omNDPK–porin–adenine nucleotide translocase may result in increased translocase affinity to ADP or may alter permeability of the porin pores for adenine nucleotides. A combination of some of these supposed mechanisms is also possible [33].

Dextran (10%) did not influence functional coupling of omNDPK to oxidative phosphorylation (Fig. 4, c, e, and g). However, dextran increased functional coupling between mAK and oxidative phosphorylation in mitochondria kept in the washing media without Mg^{2+} (Fig. 4, f and h). It is relevant to suggest that at least in these mitochondria, dextran not only reduced the volume of the intermembrane space (a precondition required for increase of mAK functional coupling to oxidative phosphorylation [45]) but also increased the number of contact sites [8]. In this case, dextran inefficiency observed in experiments with omNDPK may be attributed to the small proportion of omNDPK molecules, which may bind to contact sites of liver mitochondrial membranes and participate in functional coupling with oxidative phosphorylation.

At the same time, functional coupling might be limited by a number of functional compartments that can be formed in the contact sites. It is known that mitochondria contain several isoforms of porin and an adenylate carrier [18, 46], but only some of them may exhibit a tight interaction; these isoforms found in the contact sites represent the structural basis of the functional compartments [18].

Since in our experiments only 22-24% of omNDPK activity (provided by the most tightly bound enzyme molecules) took part in the functional coupling to oxidative phosphorylation, it could be supposed that these molecules are localized not on the surface of the outer mito-

chondrial membrane, but in the intermembrane space. However, our data (Fig. 5) show that ~95% of omNDPK activity is solubilized from mitochondria without loss of the outer membrane integrity. The same results (97%) were obtained in a previous work [32]. Table 1 also shows that mitochondria, which were kept in high salt medium, do not differ in the rates of respiration after ADP addition from mitochondria isolated in low salt medium. We concluded that in rat liver mitochondria, all omNDPK molecules of the outer compartment, including the molecules participating in the functional coupling to oxidative phosphorylation, are associated with the outer surface of the outer membrane. Data of Fig. 4 also confirm our conclusion. Figure 4h shows that activity of an enzyme from the mitochondrial intermembrane space, mAK, is functionally coupled to oxidative phosphorylation in mitochondria isolated in low salt medium. At the same time, in these mitochondria, activity of omNDPK is not coupled to oxidative phosphorylation (Fig. 4g). It may be well explained by omNDPK localization on the outer surface of the outer membrane, since in mitochondria isolated in low salt medium the enzyme is not tightly bound to the membrane (Fig. 3c). On the other hand, if omNDPK was located in the intermembrane space, it should be coupled to oxidative phosphorylation under conditions of mAK coupling.

Our conclusions contradict an idea that in mitochondria NDPK of the outer compartment is localized in the intermembrane space. The opinion about NDPK localization in the intermembrane space of rat liver mitochondria was accepted in the literature since the work by Schnaitman and Greenawalt [47]. Using digitonin treatment of mitochondria, they found NDPK activity in the outer membrane and soluble fractions. The last observation was considered by others [12, 30, 44] as evidence of the enzyme localization in the intermembrane space, though Schnaitman and Greenawalt did not exclude the possibility that the enzyme was solubilized from the outer membrane [47]. Digitonin treatment does not allow for the identification of the surface of the outer membrane that binds NDPK. Later, Adams et al. [44] provided evidence that in rat liver mitochondria, some proportion of NDPK activity is associated with contact site regions. In the contact sites, the enzyme may be associated with the outer surface of the outer membrane (like HK), or located in the intermembrane space (like creatine kinase in heart mitochondria), or associated with the matrix surface of the inner mitochondrial membrane.

In humans, Milon et al. identified a specific mitochondrial form of NDPK, Nm23-H4 [27]. The enzyme is associated with the outer and inner membranes; it also possesses the cleavable *N*-terminal extension characteristic of mitochondrial targeting [27]. The authors concluded that the enzyme is specifically associated only with contact site components and may be oriented toward intermembrane space or matrix sides [27]. They also observed a NDPK activity in mitochondrial soluble fraction [27]. It can be seen from this analysis, that there is no direct evidence in the literature that in vertebrates NDPK is localized in the intermembrane space of mitochondria. At the same time, some data give support to the suggestion that Nm23-H4 is oriented toward the matrix side of the inner membrane. Indeed, the Nm23-H4 *N*-terminal presequence [27] resembles presequences of matrix proteins rather than proteins from the intermembrane space [48, 49]. The sequence of Nm23-H4 has several features that are different from cytoplasmic isoforms but are present in the sequence of NDPK isolated from the matrix space of pigeon liver mitochondria [50]. A significant amount of Nm23-H4 from pancreatic β-cells [51], as well as NDPK from the matrix space of rabbit liver mitochondria [52] was found in a complex with the matrix enzyme succinyl-CoA synthetase. Finally, our data revealed that all NDPK activity from the outer compartment of rat liver mitochondria can be solubilized without loss of the outer membrane integrity (Fig. 5 and [32]).

We still do not know the total number of NDPK isoforms associated with the outer surface of the outer mitochondrial membrane, as well as the isoform participating in the functional coupling to oxidative phosphorylation. Solution of this intricate problem needs further studies. However, the main conclusions of our present and previous works [32, 33] (that omNDPK is associated with the outer surface of the outer mitochondrial membrane, that only some proportion of omNDPK molecules takes part in the functional coupling to oxidative phosphorylation, about the mode of the enzyme interaction with the outer mitochondrial membrane) do not require knowledge of the nature of isoforms and number of them.

Physiological conditions (150 mM (K^+ + Na^+), ~1 mM free Mg^{2+} [53]) favor tight binding of omNDPK to mitochondrial membranes. The activity of the enzyme molecules involved in functional coupling with oxidative phosphorylation facilitates ADP transfer from the liver cell cytoplasm into the intermembrane space. This is a new, previously unknown, function of NDPK. In spite of limited permeability of the outer mitochondrial membrane for ADP (and its low concentration in cytoplasm), concerted action of omNDPK and mAK may contribute to more than 60% of the maximal rate of oxidative phosphorylation in liver mitochondria.

It remains unclear whether omNDPK molecules are involved in the regulation of mitochondrial permeability transition pore required for triggering apoptosis, as it has been demonstrated for HK-I [18, 20]. Taking into consideration the key role of NDPK in energy supply of anabolic processes and its regulatory functions [22-25], involvement of this enzyme in such processes seems to be quite reasonable. The main role of other omNDPK molecules probably involves the energy supply of anabolic processes associated with the outer mitochondrial compartment (e.g. phospholipid synthesis [12]) or processes

proceeding in the cell close to the surface of the outer mitochondrial membrane.

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