ISSN 0006-2979, Biochemistry (Moscow), 2012, Vol. 77, No. 6, pp. 593-602. © Pleiades Publishing, Ltd., 2012.
Original Russian Text © T. Yu. Lipskaya, V. V. Voinova, 2012, published in Biokhimiya, 2012, Vol. 77, No. 6, pp. 731-741.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM12-021, April 8, 2012.

Activity of Nucleoside Diphosphate Kinase α (NDPK α) Capable of Binding to Outer Mitochondrial Membrane Accounts for Less than 10% of Total NDPK Activity Present in Cytoplasm of Liver Cells

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

Department of Biochemistry, Faculty of Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-3955; E-mail: tlipskaya@yandex.ru

Received February 6, 2012
Revision received February 28, 2012

Abstract—During incubation of a constant volume of rat liver cytosol with an increasing quantity of mitochondrial protein in the presence of 3.3 mM $MgCl_2$, the binding of nucleoside diphosphate kinase (NDPK) from the cytosol to mitochondrial membranes is described by a saturation curve. The highest bound NDPK activity accounts for less than 9% of the added activity. Analysis of the results suggests that only one NDPK isozyme is bound to the membranes. Western blotting showed it to be NDPK α, a homolog of human NDPK-B. Substrates of NDPK, hexokinase, and glycerol kinase, as well as N,N′-dicyclohexylcarbodiimide and palmitate, did not influence the association of NDPK with mitochondrial membranes. We conclude that the sites of NDPK binding to the outer mitochondrial membrane are not identical to those of hexokinase and glycerol kinase.

DOI: 10.1134/S0006297912060053

Key words: nucleoside diphosphate kinase, mitochondria, outer compartment, liver, isozymes, binding

Under physiological conditions, nucleoside diphosphate kinase (EC 2.7.4.6, NDPK) catalyzes the reactions of nucleoside triphosphate (NTP) synthesis from ATP and the respective nucleoside diphosphates. These nucleoside triphosphates are involved in the main anabolic processes [1]. Ten genes encoding the ten types of homologous subunits used for construction of NDPK isoenzymes were found in human tissues. NDPK shows protein kinase and 3′-5′-exonuclease activities and interacts with many proteins, thereby participating in the regulation of cell mobility, growth and development, malignant growth, and apoptosis. The catalytic and regulatory functions can be realized independently from each other. The abundance and different intracellular localizations of NDPK isoforms suggest that each of them has a specific function in a cell, and its intracellular localization is crucially important for performance of this function (see, e.g. the reviews [2-5]).

In liver cells, NDPK is localized in the cytoplasm and bound to membranes [6, 7]. In mitochondria, this enzyme was found in the outer compartment and in the matrix [8, 9]. We have shown that all NDPK activity of the outer compartment in rat liver mitochondria is localized at the external surface of the outer membrane (omNDPK) [10, 11]. Specific functions of NDPK from the outer compartment have not been studied to date.

We have shown that the omNDPK of liver mitochondria is involved in functional coupling with the oxidative phosphorylation system; hence, some part of ADP formed during the NDPK reaction is directly transferred into the mitochondrial matrix, bypassing the stage of mixing with ADP of the medium [12].

We have shown for the first time that functional coupling involves only a small fraction of the omNDPK molecules, which are most tightly bound to mitochondria and are responsible for 22-24% of the total enzyme activity [11].

Abbreviations: AP5A, p^1p^5-di(adenosine) pentaphosphate; DCCD, N,N′-dicyclohexylcarbodiimide; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)-butane; leupeptin, acetyl-leucyl-leucyl-argininal; NDPK, nucleoside diphosphate kinase; omNDPK, NDPK associated with the outer mitochondrial membrane.

* To whom correspondence should be addressed.

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

Mitochondria washing medium	Preincubation of mitochondria	Oxidative phosphorylation rate, nmol ADP/min per mg protein	NDPK activity, nmol CDP/min per mg protein	V^{CDP}/V^{ADP}, %	RCR
IM with $MgCl_2$ (4)	no	335 ± 30	287 ± 35	85 ± 5.0	4.0 ± 0.2
With high ionic strength (4)	no	345 ± 58	264 ± 18	79 ± 13	4.8 ± 0.5
IM (3)	no	315 ± 22	169 ± 38	54 ± 12	4.8 ± 0.4
IM	yes	482 ± 75 (4)	64 ± 10 (3)	13 ± 0.3 (3)	6.1 ± 0.6 (4)

Note: In experiments with pre-incubation, the suspension of mitochondrial precipitate 1 was stirred in the cold for 2 h. RCR is respiratory control ratio. The number of experiments is given in parentheses. Other details are described in "Materials and Methods". Explanations are given in the text.

Hexokinase and glycerol kinase are also capable of functional coupling with the oxidative phosphorylation system [13-17]. Both enzymes were shown to bind to the same region of porin [18, 19]. To date, it is unknown whether there is an antagonist partner of omNDPK at the outer membrane of mitochondria.

In this work we continued the study of the properties of omNDPK. Previously, it was shown that omNDPK activity is only ~13% of the total enzyme activity in the outer mitochondrial compartment and in the soluble fraction [10]. Such highly excessive activity of cytosol NDPK compared to the mitochondria-bound enzyme suggests that the enzyme binding to the mitochondria is limited by the number of specific binding regions at the external surface of the outer membrane. On the other hand, there are several NDPK isoforms in the cytoplasm of liver cells [6]; the enzyme easily forms complexes with other proteins [3] and relatively low-molecular-weight compounds, e.g. monosaccharides [20]. Therefore, it is likely that only a small proportion of total NDPK activity present in the cytoplasm of liver cells can bind to the external surface of the outer mitochondrial membrane.

The goal of this work was to determine whether the cytoplasm of liver cells contains a specific fraction of NDPK molecules that are capable of binding to the outer mitochondrial membrane; to identify the NDPK isoform associated with the outer membrane; and to compare the effects of substances influencing the association of hexokinase and glycerol kinase with mitochondrial membranes to their effects on omNDPK association with the membranes.

MATERIALS AND METHODS

Materials. The following materials were used: ATP, ADP, CTP, CDP, GTP, GDP, p^1p^5-di(adenosine) pentaphosphate (AP5A), and BSA (defatted) (Sigma, USA), UDP (Reanal, Hungary), leupeptin (MP Biomedicals, Inc, USA), L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)-butane (E-64) (BIOMOL Research Labs, Inc, USA), and primary mouse monoclonal antibodies against NME2 (ab60602) (Abcam, USA). The secondary rabbit polyclonal antibodies against mouse IgG conjugated with horseradish peroxidase were kindly provided by Prof. A. G. Katrukha, Department of Biochemistry of the Biological Faculty of Moscow State University.

Binding of NDPK from the cytosol with mitochondrial membranes. *Isolation of mitochondria and solubilization of omNDPK.* Mitochondria were isolated from the liver of white rats (180-220 g) mainly as described in work [12]. The isolation medium (IM) contained 0.28 M mannitol and 2.1 mM Hepes, pH 7.4. The 10% homogenate was centrifuged for 15 min in a J2-21 centrifuge (Beckman, Austria) at 2000 rpm in a JA-20 rotor. The supernatant was centrifuged for 10 min at 8000 rpm, the volume of resulting supernatant (S) was measured, and 10-15 ml of the supernatant was taken, followed by leupeptin addition up to final concentration of 50 μM, and centrifuged for 1 h in a L-90K centrifuge (Beckman) at 30,000 rpm in a SW 55Ti bucket rotor. The resulting supernatant containing all soluble proteins of the cytoplasm including NDPK will be hereinafter called "cytosol". Mitochondrial precipitate after centrifugation at 8000 rpm (precipitate 1) was suspended in the IM with leupeptin (2.7 ml per 1 g tissue). The suspension of precipitate 1 was stirred on a magnetic stirrer at 2°C for 2 h and centrifuged for 10 min in the JA-20 rotor at 10,300 rpm. The mitochondrial precipitate (precipitate 2) devoid of most of the omNDPK activity was suspended in the IM with leupeptin to final concentration of about 60 mg/ml. The total volume of suspension (V_1) was measured, and the total volume of precipitate 2 (V_2) was calculated by the difference between the total volume and the volume of the added IM with leupeptin. The results were used for further calculations, and the suspension was immediately used in the

experiments on the cytosol NDPK binding to mitochondrial membranes.

In some experiments presented in Table 1, mitochondrial precipitate 1 was suspended in IM or in one of the following wash media: 3.33 mM $MgCl_2$, 0.27 M mannitol, 2.1 mM Hepes, pH 7.4 (the medium with Mg^{2+}) or 0.14 M KCl, 2.1 mM Hepes, pH 7.4 (the medium with high ionic strength). Mitochondrial precipitate 2 was suspended in the respective wash medium to a concentration of about 60 mg of mitochondrial protein in 1 ml. The suspensions were stored on ice and used in polarographic experiments.

Experiments on cytosol NDPK binding to mitochondrial membranes. A number of test tubes contained the calculated volume of IM with leupeptin, 1.15 ml cytosol, and $MgCl_2$ to the final concentration of 3.33 mM. The reaction was started by adding 40-400 µl of the suspension of precipitate 2 containing 2.5 to 25 mg protein. Control samples (for residual omNDPK activity in precipitate 2) contained 100-400 µl of the suspension of precipitate 2 and did not contain the cytosol; some samples with the cytosol contained no $MgCl_2$. The total volume of each sample was 1.9 ml.

All samples were simultaneously shaken for 5 min at 2°C in a Thermomixer Comfort (Eppendorf) and centrifuged for 1 min at 14,500 rpm in a MiniSpin plus centrifuge (Eppendorf). The supernatant was discharged and preserved; mitochondrial precipitates (precipitates 3) were rinsed by 0.5 ml of IM with leupeptin and $MgCl_2$; then the test tube walls were thoroughly dried and the precipitates were suspended in IM (35 µl per each 30 µl of the taken suspension of precipitate 2). NDPK activity was determined by the polarographic method.

Mitochondrial respiration. The rate of oxygen consumption by mitochondria at 22°C was measured with a closed Clark-type oxygen electrode and a LP 7e polarograph (Laboratorni Pristroje Praha, Czechoslovakia). The basic 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. Additional components are indicated in the text. Dissolved oxygen concentration in the basic incubation medium at 22°C was considered to be equal to 290 µM [21].

Determination of omNDPK activity by the polarographic method. The basic incubation medium additionally contained 1 mM ATP. The suspension of one of the mitochondrial precipitates (20 µl) was placed in a 0.93-ml polarographic cell. In 1.5-2 min ADP was added at final concentration of 170 µM and then, after respiration entered into state 4 according to Chance [22], 600 µM CDP was added (Fig. 1).

The initial NDPK activity in the cytosol and residual activity in the supernatants after obtaining precipitates 3 were measured in the incubation medium additionally containing 20 µM AP5A (the inhibitor of adenylate kinase). The suspension of precipitate 2 (18-20 µl) and the cytosol or the respective supernatant (0-12 µl) were introduced into the polarographic cell. Then ADP and CDP were successively added as described above.

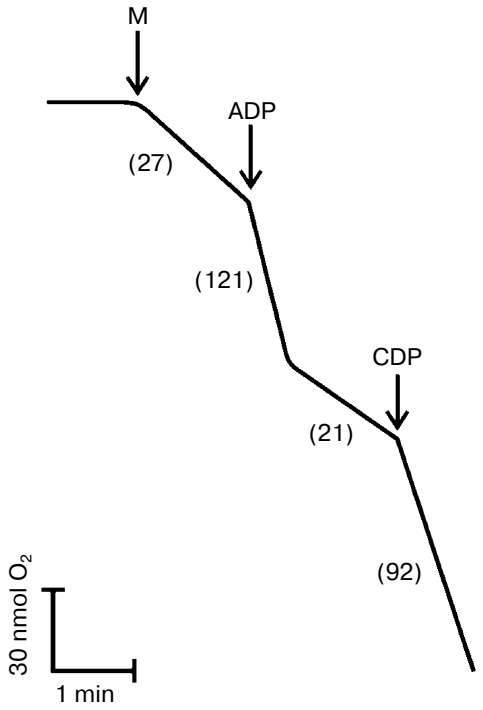

Fig. 1. Polarographic method for measuring omNDPK activity. The reaction was started by adding 20 µl of mitochondrial (M) suspension containing 0.64 mg of protein. ADP (170 µM) and CDP (600 µM) were added to the samples where indicated. Respiration rates (in parentheses) are expressed in nmol O_2/min per mg protein. Other details are described in "Materials and Methods". Explanations are given in the text.

Phosphorylating respiration rate after addition of ADP (V^{ADP}) was determined as a difference in respiration rates immediately after ADP addition and after phosphorylation of all ADP (Fig. 1). The phosphorylating respiration rate after subsequent addition of CDP (V^{CDP}) was found as a difference of respiration rates before and after CDP addition (Fig. 1). For calculation of oxidative phosphorylation rate and NDPK activity, the respective values of phosphorylating respiration rates (in ng-atom O/min per mg of protein) were multiplied by the ADP/O ratio, being equal to 2 during succinate oxidation. The ADP/O ratio was considered invariable in the frame of a single polarographic test; therefore, V^{CDP}/V^{ADP} is a ratio between omNDPK activity and ADP oxidative phosphorylation rate in this test.

Calculation of NDPK activity in fractions. The results of polarographic analysis were used for calculation of the total NDPK activity present in the suspensions of precipitates 3. The volume of the suspensions of precipitate 3 was calculated taking into account not only the volume of

IM added to each precipitate 3, but also the volume of the precipitate itself. This volume was considered to be directly proportional to the volume of the respective suspension of precipitate 2 that yielded precipitate 3. The volume of each precipitate 3 was found by the formula: $V_3 = C \cdot V_1/V_2$ (ml), where C was the volume of suspension of precipitate 2 that yielded the respective precipitate 3. NDPK activity bound to the mitochondrial membranes from the cytosol was found as a difference between the total NDPK activity in the suspensions of precipitate 3 obtained after the incubation of precipitate 2 with the cytosol and the control samples incubated without the cytosol.

The initial activities of NDPK from the cytosol and NDPK not bound to mitochondria were determined with preliminary plotting of the diagrams of enzyme activity dependence on the cytosol volume added into the polarographic cell. The linear sections of the diagrams were used for calculations. The volume of the fraction of NDPK activity from the cytosol not bound to the mitochondria was determined by subtracting the volume taken by the mitochondria proper in precipitate 3 from the total volume of the fraction (1.9 ml). It was considered to be 40% of the total volume of the precipitate [10].

Creating Scatchard plots. The equation used was analogous to the modified Scatchard equation: $K_{bind} = A_{bound}/A_{free} \cdot (mP - A_{bound})$, where K_{bind} was the equilibrium constant of NDPK binding to mitochondrial membranes: A_{bound} was the total mitochondria-bound NDPK activity in each precipitate 3 or NDPK activity bound to the given mitochondrial suspension from the cytosol (nmol CDP/min); A_{free} was NDPK activity not bound to the given precipitate 3 (nmol CDP/min); m was the maximum specific NDPK activity in the given preparation of mitochondria (nmol CDP/min per mg of protein); P was mitochondrial protein (mg). Thus, the product of $m \cdot P$ (nmol CDP/min) reflects the maximum NDPK activity (total or from the cytosol), which in principle the given precipitate 3 can bind. The following dependence curves were plotted: A_{bound}/A_{free}·mg (1/mg) of A_{bound}/mg (nmol CDP/min per mg protein).

Western blotting. Mitochondrial precipitate 1 after centrifugation at 8000 rpm was suspended in the IM with leupeptin (2.7 ml per gram tissue) and centrifuged for 10 min at 10,300 rpm. Mitochondrial precipitate 2 was suspended in the same medium to a concentration of about 60 mg mitochondrial protein in 1 ml. Portions of suspension (500 µl) were poured into test tubes and shaken in a thermomixer at 2°C. After 4 h, the suspension was centrifuged at 14,500 rpm in a MiniSpin plus centrifuge for 10 min. The precipitate was removed, and the supernatants were centrifuged two more times under the same conditions to remove mitochondrial residuals.

The supernatants were combined, followed by addition of a denaturing buffer containing: 0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% sucrose, 20% β-mercaptoethanol, 0.0165% bromophenol blue (3 : 1) and heated on a water thermostat at 95°C for 10 min. For separating large particles, the samples were filtered through an Ultracel YM-100 membrane (Microcon Centrifugal Filter Device; Millipore, USA) for 40 min at 13.9g in a MiniSpin plus centrifuge at 4°C. Filtrate proteins were separated in 12.5% SDS-polyacrylamide gel in the Laemmli buffer system [23]. (The cell for electrophoresis was a Mini-Protean 3; Bio-Rad, USA). The treated extract was applied at 30 µl to each gel lane.

After electrophoresis, the gel was washed free from SDS for 15 min in electrotransfer buffer containing 25 mM Tris, 192 mM glycine, and 20% ethanol. Electrotransfer of proteins to the nitrocellulose membrane (Bio-Rad) was performed in a Mini Trans-Blot (Bio-Rad) for 1 h at 100 V and 4°C on a magnetic stirrer. After the electrotransfer was completed, the membrane was placed for 15 min into 5% BSA solution in TBST buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1% Tween-20) with shaking in Sky Line S-4 (ELMI, Latvia). The membrane was washed free from BSA by a single rinsing in TBST and incubated for 1 h at room temperature in a solution of the primary mouse monoclonal antibodies against NME2 in TBST (dilution 1 : 278). Before 60-min incubation with the rabbit anti-mouse antibodies against IgG conjugated with horseradish peroxidase (dilution 1 : 1000), the membrane was washed two times for 4.5 min in TBST. After subsequent washing of the membrane in TBST (3 times for 5 min), the proteins were stained in a solution containing 50 mM Tris-HCl, pH 7.6, 0.3% 3,3′-diaminobenzidine, and 0.03% H_2O_2. The control samples for nonspecific protein staining were incubated only with the secondary antibodies.

Study of effects of metabolites on omNDPK binding to mitochondrial membranes. The supernatant after centrifugation of liver homogenate at 2000 rpm was divided into 8-10 equal portions and centrifuged with cooling for 10 min at 9700 rpm in a desktop centrifuge model 310 (Metronex, Poland). Mitochondrial precipitates 1 were suspended in 1.3-2 ml of media containing the metabolites under study (medium compositions are given in the text); the suspensions were incubated under periodic shaking in a thermomixer for 1-2 h at 2°C and centrifuged for 10 min in the Metronex centrifuge at 12,000 rpm. The resulting precipitates were suspended in a small volume of IM, followed by the measurement of residual omNDPK activity and calculation of V^{NDPK}/V^{ADP} as described above.

To study the effects of NDPK substrates, mitochondria were shaken for 1 h in medium containing, in addition to the substrates, 2.1 mM Hepes, pH 7.4, 50 µM leupeptin, and 1 mM EDTA; ionic strength was brought to 0.08 by adding KCl, and osmotic concentration was brought to 0.28 OsM by adding mannitol. In the polarographic tests of these experiments, the basic incubation medium additionally contained 300 µM ATP, 170 µM ADP, and 360 µM UDP.

Ionic strength of the media was determined by the equation $I = 1/2\,(\Sigma Ci \cdot Zi^2)$, where *Ci* was the concentration of each ion and *Zi* was the charge of this ion. When calculating the ionic strength of the buffers, the concentrations of ionic forms were determined using the Henderson–Hasselbach equation.

Study of the effect of N,N′-dicyclohexylcarbodiimide (DCCD) on NDPK binding to mitochondrial membranes. Mitochondrial precipitate 1 after centrifugation at 8000 rpm was suspended in 10 ml of IM containing 50 μM leupeptin and 25 μM E-64 (IM with cathepsin inhibitors); the suspension was incubated for 1 h with stirring at 2°C and centrifuged for 10 min at 10,300 rpm in the JA-20 rotor. The resulting supernatant 2 containing solubilized omNDPK was stored in ice until the beginning of the experiment on repeated binding of omNDPK with the mitochondrial membrane. Mitochondrial precipitate 2 was suspended in 3 ml of IM with the inhibitors.

During the incubation of mitochondria with DCCD, the 500-μl samples contained 375 μl of IM with the inhibitors and 5 μl of DCCD solution in ethanol up to a final concentration of 0.02 or 0.1 mM. Control samples contained 5 μl of ethanol. The reaction was started by adding 120 μl of the suspension of precipitate 2 (1.9 mg protein). The samples were shaken for 15 min at 22°C in the thermomixer; then each was layered onto 11 ml of IM with the inhibitors containing 1 mg/ml of defatted BSA in 15-ml test tubes of the JA-20.1 rotor of an Avanti J-E centrifuge (Beckman) and centrifuged under cooling for 15 min at 30,000*g*. Mitochondrial precipitates 3 were rinsed with 1 ml of IM with the inhibitors; then the liquid was thoroughly removed and the precipitates were suspended in 1.2 ml of the same medium. For the repeated binding of omNDPK solubilized from mitochondria, the suspensions of mitochondrial precipitates 3 were quantitatively transferred into 2-ml test tubes, with addition of 0.6 ml of supernatant 2 and a concentrated $MgCl_2$ solution to the final concentration of 3.3 mM. The samples were shaken in the thermomixer for 1 h at 2°C and centrifuged for 10 min at 14,500 rpm in the MiniSpin plus centrifuge. The resulting supernatants 4 and residuals of supernatant 2 were stored at −18°C until determination of omNDPK activity by spectrophotometry.

Spectrophotometric detection of NDPK activity. The samples with total volume of 1 ml contained 3.3 mM glucose, 10 mM $MgCl_2$, 150 mM Hepes, pH 7.4, 0.6 mM EDTA, 0.125 mM ADP, 0.3 mM NADP, 0.1 unit of glucose-6-phosphate dehydrogenase, 0.7 unit of hexokinase, 20 μM AP5A, and 0-200 μl of one of supernatants 2 or 4. The reaction was started by adding 3 mM CTP, and the rate of absorption increase was measured at 340 nm. Residual NDPK activity in the supernatants was determined as a difference between the absorptions of supernatant-containing samples and the sample without the supernatants. The bound NDPK activity was determined as a difference between the added activity from supernatant 2 and the activity remaining in supernatants 4.

Investigation of effect of palmitic acid on omNDPK binding to mitochondrial membranes. Mitochondrial precipitate 1 after centrifugation at 8000 rpm was suspended in 20 ml of the medium containing 0.26 M mannitol, 10 mM KCl, and 2.1 mM Hepes, pH 7.4. The suspension was divided into six equal portions and centrifuged for 10 min at 12,300 rpm in the Metronex centrifuge. One milliliter of the above medium containing 0-100 μM of palmitic acid was added to mitochondrial precipitates 2. Palmitic acid solutions in ethanol (5 μl) were added to the suspension media immediately before their use. Suspensions containing 13 mg mitochondrial protein each were incubated at 2°C for 30 min, followed by the addition of 7 ml of the medium without palmitic acid, and centrifuged again. Mitochondrial precipitates 3 were suspended in a small volume of IM containing 5% defatted BSA, and their residual NDPK activity was measured by the polarographic method. The medium of the polarographic experiment additionally contained 1% BSA, 300 μM ATP, 170 μM ADP, and 360 μM UDP.

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

Mitochondrial protein was assayed by the method of Gornall et al. [24] with BSA as a standard.

RESULTS

In most of our experiments, NDPK activity was measured by the polarographic method (Fig. 1). The advantages of this method have been described previously [11, 12, 25].

In the experiments with DCCD, which influences the functional characteristics of mitochondria, NDPK activity was measured by spectrophotometry.

Mitochondrial respiration is stimulated as a result of NDPK functioning, irrespective of whether the enzyme is bound to mitochondrial membranes or is in solution. Our previous studies have shown that the total activity of bound and solubilized omNDPK did not depend on the degree of enzyme solubilization at saturating substrate concentrations (1 mM ATP and 600 μM CDP or 300 μM ATP and 360 μM UDP) [25].

Binding of NDPK from cytosol to mitochondrial membranes. To determine whether the binding of NDPK from the cytoplasm of liver cells with the outer mitochondrial membrane is limited by the number of specific binding sites or by the number of specific NDPK molecules, a fixed volume of rat liver cytosol was incubated with increasing quantity of mitochondria. The cytosol volume added to the samples was equal to the volume of supernatant S per ~2 mg of the protein from precipitate 1. In our experiments, the content of mitochondria in experi-

mental samples varied 10-fold (from 2.5 to 25 mg). According to the calculations, the added NDPK activity exceeded 3.0-3.5-fold the expected activity of the bound NDPK in the sample with the maximum content of organelles; hence, we supposed that if the activity was limited only by the number of binding sites of the enzyme, upon increase in the quantity of mitochondria we would observe linear dependence of the bound enzyme activity on the content of mitochondria in a sample. If only some portion of NDPK activity from the cytosol can specifically bind to the outer membrane, the increase in the quantity of mitochondrial protein will give a saturation curve. Mitochondria contain different NDPK activities depending on isolation conditions (Table 1). We have used mitochondria with most of the omNDPK solubilized (Table 1). The conditions necessary for repeated NDPK binding to mitochondrial membranes were selected previously [25].

Experimental results are shown in Fig. 2. It can be seen that, while the activity of omNDPK remaining in mitochondria linearly increased along with the increase in mitochondrial protein (curve *1*), the activity of NDPK bound from the cytosol reached a certain limit (curve *2*), though this activity was less than 9% of NDPK activity in the cytosol added to the suspensions of precipitate 2 (23,323 ± 478 nmol CDP/min). Verification has shown that all NDPK activity from the cytosol not bound to mitochondria is found in the supernatants of precipitates 3 (data not shown). In the absence of Mg^{2+}, the maximum binding of NDPK from the cytosol with mitochondria was 2.5 times less than in its presence (data not shown). In one of the experiments, a protease and phosphatase inhibitor cocktail (50 μM leupeptin, 10 μM E-64, 0.25 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 1 mM dithiothreitol) was used instead of 50 μM leupeptin; however, it did not result in enhanced binding of NDPK from the cytosol (data not shown).

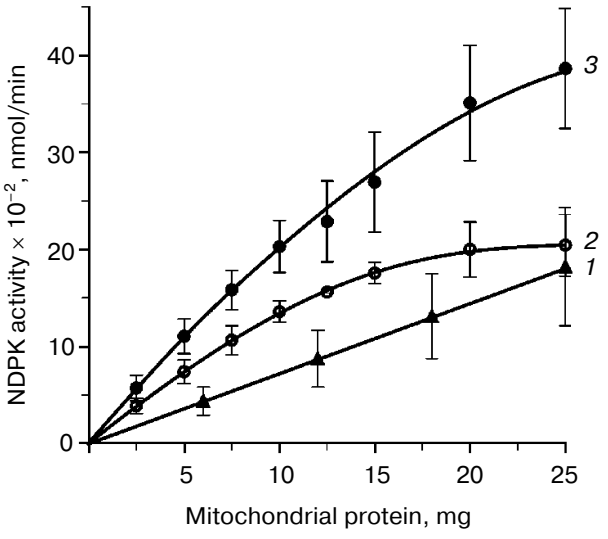

Fig. 2. Binding of NDPK from cytosol to mitochondrial membranes: *1*) omNDPK activity in control samples incubated without cytosol; *2*) mitochondria-bound NDPK activity from cytosol; *3*) total bound NDPK activity. Results are averages of three experiments. NDPK activity in the added cytosol is 23,323 ± 478 nmol CDP/min. Other details are described in "Materials and Methods".

The binding of NDPK to mitochondrial membranes was quantitatively characterized by an equation analogous to the Scatchard equation. The calculations were made using the average results of experiments presented in Fig. 2. The A_{bound} values were obtained on the basis of curves *3* or *2*. The A_{free} values are a difference between the maximum bound NDPK activity from the cytosol (2000 nmol CDP/min (Fig. 2, curve *2*)) and NDPK activity from the cytosol bound to one of precipitates 3 (Fig. 2, curve *2*).

If the free and bound NDPK are in the state of equilibrium, then the dependence plots for A_{bound}/A_{free} per mg of the protein (1/mg) on A_{bound}/mg (nmol CDP/min per mg of protein) must be in the form of straight lines, which cross the abscissa axis giving value m; intersection of the ordinate axis gives a $K_{bind} \cdot m$ product, from where the K_{bind} value can be found.

When using the total bound NDPK activity in the calculations, i.e. the NDPK activity remaining in precipitates 2 plus the activity bound from the cytosol (according to the data of curve *3*, Fig. 2), we obtained curve *1* in Fig. 3, where all points fell on a straight line. We arrived at the conclusion that NDPK from the cytosol was bound to regions at the outer mitochondrial membrane previously occupied with omNDPK and with the same affinity. This conclusion was confirmed by plotting curve *2*, where only NDPK activity bound from the cytosol was taken into consideration. The calculations were based on the data of the ascending part of curve *2*, Fig. 2. Figure 3 shows that curve *2* is parallel to curve *1*. The total maximum specific activity of NDPK m in the used mitochondrial preparations was 238 nmol CDP/min per mg protein on average (Fig. 3, curve *1*), while the specific activity of NDPK bound from the cytosol was 160 nmol CDP/min per mg protein (Fig. 3, curve *2*). The difference between these values (78 nmol CDP/min per mg protein) is omNDPK activity not solubilized from mitochondria in the course of obtaining precipitates 2 and 3. According to the data of Fig. 2 (curve *1*), this value was 73 ± 24 nmol CDP/min per mg protein on average. The constants of NDPK binding with mitochondrial membranes were the same for both curves: 0.0096 min/nmol CDP.

We concluded that only a small part (<9%) of free NDPK present in the cytoplasm of rat liver cells can be bound to the outer mitochondrial membrane, and it is in a state of equilibrium with the bound enzyme.

Identification of NDPK isoform bound to outer mitochondrial membrane. The two main NDPK isoforms identified in rat liver, α and β, are homologous to human

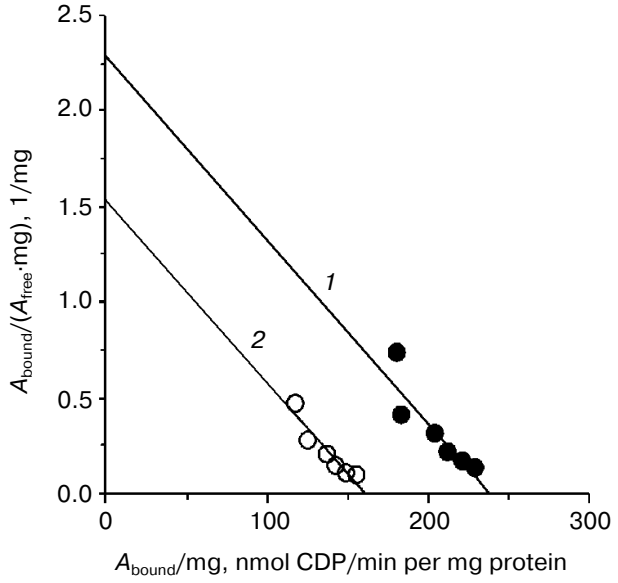

Fig. 3. Scatchard plots based on data of Fig. 2. Details are described in "Materials and Methods". Explanations are given in the text.

NDPK-B and -A by 98 and 95%, respectively [6]. While NDPK-A was found in the cytoplasm only, NDPK-B and NDPK α were found both in the cytoplasm and in the membrane fraction [7, 26].

The results of analysis of the extract from the mitochondrial outer membrane surface for the presence of NDPK α are shown in Fig. 4. Monoclonal antibodies against the recombinant fragment of NDPK-B corresponding to amino acid residues 51-153 and containing the maximum number of amino acid substitutions compared to NDPK-A was used in this work. Figure 4 shows that NDPK α is actually present in the extract from the surface of liver mitochondria. The mean molecular mass of the NDPK α isozyme was 19.7 ± 0.2 kDa (three experiments).

Effects of metabolites on omNDPK binding to mitochondrial membranes. In search of the omNDPK partners (omNDPK antagonists) at the mitochondrial surface, the effects of substances influencing upon the binding of hexokinase and glycerol kinase with the outer mitochondrial membrane were investigated. Previously we have shown that the increased ionic strength of the storage medium for mitochondria makes the association between omNDPK and the membranes tighter [11]. The maximum and half-maximum effects were observed at an ionic strength of ≥0.03 and ~0.01, respectively [11]. At an ionic strength of 0.01 (Table 2, experiments 1 and 2), it was possible to observe both association-intensifying and solubilizing effects of glucose and glucose-6-phosphate (supposing they took place). Because of the difficulties with exact calculation of the ionic strength of salt solutions under study, we increased the ionic strength of the medium in experiment 3 (Table 2) so that the final result was not influenced by possible ambiguity in the calculations of ionic strength. The results in Table 2 show that the tested substances had no effect on the association of omNDPK with the membranes of liver mitochondria.

The effects of sodium salts of ATP, CTP, GTP, ADP, CDP, GDP, IDP, and UDP at a final concentration of 1 mM were investigated. None of these substances had any effect on the association of omNDPK with the membranes (data not shown). It is known that the true NDPK substrates are not free nucleotides but their complexes with bivalent metals. In our experiments, Mg-complexes of these substances increased the binding of omNDPK to the membranes, but their effects were comparable with the effect of free Mg^{2+} (data not shown).

Effects of DCCD and palmitic acid on omNDPK binding to mitochondrial membranes. There are two types of hexokinase binding sites on the outer mitochondrial membrane: *A* and *B*. These sites are located in domains of different phospholipid composition [27]. Glucose-6-phosphate, DCCD, and palmitic acid inhibit hexokinase binding to the *A* regions [27-30]. Only solubilization from the *A* regions is reversible [28]. In one of our three experiments with DCCD, which gave analogous results, the activity of newly bound NDPK in the control sample (with two repeats) was 17.3 ± 5.2 nmol CTP/min per mg protein, at a DCCD concentration of 0.02 mM (5.3 nmol/mg protein), 19.0 ± 0.0 and 14.7 ± 2.6 at a DCCD concentration of 0.10 mM (26 nmol/mg protein).

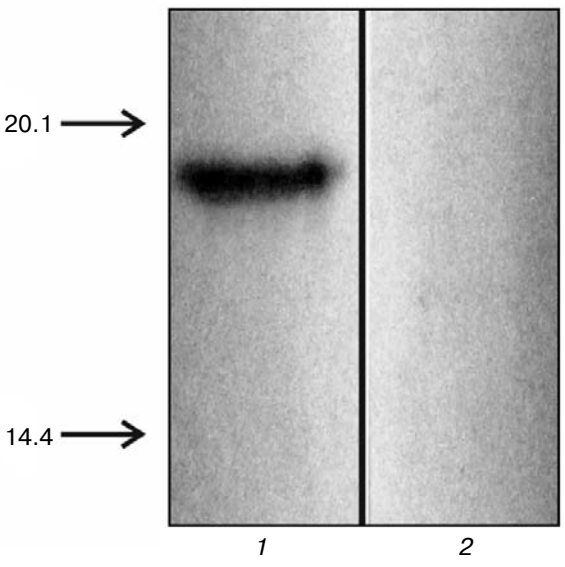

Fig. 4. Identification of NDPK isozyme bound to the outer mitochondrial membrane. Results of immunoblotting. The nitrocellulose membrane was incubated in the presence of: *1*) primary and secondary antibodies; *2*) secondary antibodies only. The arrows show the position of standard proteins: the inhibitor of trypsin (20.1 kDa) and α-lactalbumin (14.4 kDa). Other details are described in "Materials and Methods".

Table 2. Effects of some metabolites on omNDPK association with membranes of liver mitochondria

Experiment No.	Ionic strength	Metabolite	V^{CDP}/V^{ADP}, %
1	0.01	KCl	49.5 ± 10.2 (4)
		glucose, 20 mM	44.1 ± 8.7 (4)
2	0.01	KCl	37.8 ± 3.1 (3)
		gl-6-P, 1 mM	37.6 ± 0.3 (2)
3	0.033	KCl	77 ± 2.0 (2)
		glucose, 20 mM	73.5 ± 2.5 (2)
		gl-6-P, 1 mM	78.5 ± 2.5 (2)
		gl-6-P, 10 mM	82.5 ± 0.5 (2)
		α-glyc-P, 1 mM	77.5 ± 3.5 (2)
		α-glyc-P, 10 mM	79.0 ± 4.0 (2)

Note: In addition to the specified substances, the washing media for mitochondria contained Hepes, pH 7.4, at a concentration of 2.1 mM (Experiments No. 1, 3) or 10 mM (Experiment No. 2). Total ionic strength of the media was brought to the above values by adding KCl and osmotic concentration was brought to 0.28 OsM by adding mannitol. The samples were shaken in the thermomixer for 2 h (Experiments No. 1, 2) or 1 h (Experiment No. 3). The medium for the polarographic experiment additionally contained 1 mM ATP, 170 μM ADP, and 600 μM CDP. Gl-6-P is glucose-6-phospate; α-glyc-P is α-glycerol phosphate. The number of measurements is given in parentheses. Other details are described in "Materials and Methods"; explanations are given in the text.

The specific activity of added omNDPK was 36.2 nmol CTP/min per mg protein. In one of the three similar experiments with palmitic acid, V^{UDP}/V^{ADP} was 54% in the control and remained at the same level on variation of the concentration of the acid from 0.01 to 100 μM.

The presented results show that neither substance had an effect on the association between omNDPK and mitochondrial membranes.

DISCUSSION

Comparison of the data from Table 1 and Fig. 3 shows that the maximum specific NDPK activity obtained by extrapolation of curve *1* (Fig. 3) (238 nmol CDP/min per mg protein) exceeds the value obtained for the mitochondria isolated in IM (169 ± 38) and unreliably differs from the activity found in mitochondria isolated in the medium of high ionic strength or in the presence of $MgCl_2$ (264 ± 18 and 287 ± 35 nmol CDP/min per mg protein, respectively). Consequently, the mitochondria isolated in IM lose some part of omNDPK activity during isolation, while specific omNDPK activity in the mitochondria isolated in the two other media reflects the initial content of this enzyme in the mitochondria of liver cells. Table 1 shows that omNDPK can provide 80-90% of the maximum oxidative phosphorylation rate at a saturating CDP concentration.

On curve *1*, Fig. 3, all points fell on a straight line, though the ratio of the omNDPK activity remaining in mitochondria to the bound activity was not constant and varied from 0.29 when using 2.5 mg mitochondrial protein to 0.89 when using 25 mg. We have come to the conclusion that NDPK from the cytosol was bound to the regions on the outer mitochondrial membrane previously occupied by omNDPK, and with the same affinity. The shape of curves *1* and *2* on the Scatchard plots (Fig. 3) suggests that only one NDPK isozyme was bound to the outer mitochondrial membrane in our experiments. Enzyme immunoassay showed it to be NDPK α. The results correlate with the literature data that only NDPK α is found in the membrane fraction [7]. The found molecular mass of NDPK α subunit coincides with the value for NDPK-B from human erythrocytes [31].

We have ascertained that <9% of NDPK activity present in the cytoplasm of liver cells can be bound to the outer mitochondrial membrane. At the same time, it is known that NDPK α is the most expressed form in the cytoplasm of liver cells [6]. It seems that NDPK α bound to the outer mitochondrial membrane possesses some specific properties that distinguish it from other molecules of this isoenzyme in the cytoplasm. To date we know nothing about the nature of these differences.

Previously we have shown that omNDPK of mitochondria isolated in IM is incapable of the functional coupling with oxidative phosphorylation [11]. Thus, all omNDPK molecules in the mitochondria isolated and stored in IM show identical properties both under repeated binding with the mitochondrial membranes and during the study of functional coupling. At the same time, in the mitochondria isolated in the medium of high ionic strength or in the presence of Mg^{2+}, 22-24% of omNDPK activity is capable of functional coupling [11], i.e. under these conditions the enzyme shows heterogeneity of its properties. It may be supposed that in these media, where omNDPK is more tightly bound to the outer membrane (Table 1), heterogeneity of the enzyme properties could be caused by the differences in its surroundings on the membrane that do not manifest themselves under shallower binding of the enzyme.

One more assumption, namely that a part of the bound omNDPK could be replaced by some other fraction of NDPK α from the cytosol, capable of functional coupling, during isolation of mitochondria in the medium of high ionic strength or in the presence of Mg^{2+}, seems to be unlikely, because the first mitochondrial precipitate in all cases was obtained in IM, and different media were used only for washing this precipitate, i.e. after removal of the cytosol fraction. In addition, Mg^{2+} was present in the experiments on the binding of NDPK from the cytosol to the mitochondrial membrane; there-

fore, if the cytosol contained two fractions of NDPK α capable of binding to the outer membrane, the Scatchard plots would show a twist on the curves.

Hexokinase and glycerol kinase are also capable of functional coupling with oxidative phosphorylation in liver mitochondria [13-17]. Model experiments on the binding of hexokinase isolated from rat brain to liver mitochondria showed that the functional coupling provided by this bound hexokinase [17, 32], in terms of percentage of the maximum oxidative phosphorylation rate, was very close to the values obtained in our experiments with omNDPK [12]. These data suggest that hexokinase and NDPK are bound in the same regions at the surface of the outer mitochondrial membrane, especially as in the experiments of Laterveer et al. [32] mitochondria were isolated in medium resulting in omNDPK removal from many binding sites [11].

This assumption seemed even more likely, because evidence was obtained that hexokinase and glycerol kinase are bound to the same regions on the porin. It was shown that reaction products solubilized their own enzyme and increased the binding of the other enzyme [19, 33]. In rat liver mitochondria, omNDPK activity is incomparably higher than hexokinase and glycerol kinase activities [12]; therefore, the presence of the binding site in common with omNDPK on the outer mitochondrial membrane could be of great importance for regulation of activities of these enzymes.

We have studied the influence of the substrates and products of these reactions, as well as the inhibitors of hexokinase binding (DCCD and palmitic acid), on the association of omNDPK with the outer mitochondrial membrane and demonstrated that none of the tested substances had any effect on the association between omNDPK and the membranes. In our experiments, free nucleoside di- and triphosphates had no effect on the association of omNDPK with mitochondrial membranes, whereas free nucleoside triphosphates solubilized hexokinase [34].

There are other differences as well. It is known that hexokinase is bound to porin mainly via electrostatic interactions, and high ionic strength solubilizes the enzyme [35]. DCCD and palmitic acid are inhibitors of hexokinase binding with the membranes, disturbing their electrostatic interactions with the binding sites [29, 30]. At the same time, high ionic strength tightens the association between omNDPK and the membranes, i.e. the enzyme is bound to the membranes mainly via hydrophobic interactions [11]. The association of hexokinase and omNDPK with the mitochondrial membranes becomes tighter at acid [35] and alkaline [25] pH values, respectively. Finally, it was shown that dextran increased the functional coupling of hexokinase with oxidative phosphorylation [32] but had no effect on the functional coupling of omNDPK [11] though, as had been described earlier [32], increased the functional coupling of adenylate kinase [11].

The results lead to the conclusion that omNDPK binding sites on the outer mitochondrial membrane are not identical to hexokinase and glycerol kinase binding sites.

REFERENCES

1. Lehninger, A. (1974) *Biochemistry* [Russian translation], Mir, Moscow.
2. Kimura, N., Shimada, N., Fukuda, M., Ishijima, Y., Miyazaki, H., Ishii, A., Takagi, Y., and Ishikawa, N. (2000) *J. Bioenerg. Biomembr.*, **32**, 309-315.
3. Roymans, D., Willems, R., van Blockstaele, D. R., and Slegers, H. (2002) *Clin. Exper. Metastasis*, **19**, 465-476.
4. Kim, H. D., Youn, B. H., Kim, T. S., Kim, S. H., Shin, H. S., and Kim, J. (2009) *Mol. Cell. Biochem.*, **329**, 167-173.
5. Boissan, M., Dabernat, S., Peuchant, E., Schlattner, U., Lascu, I., and Lacombe, M.-L. (2009) *Mol. Cell. Biochem.*, **329**, 51-62.
6. Shimada, N., Ishikawa, N., Munakata, Y., Toda, T., Watanabe, K., and Kimura, N. (1993) *J. Biol. Chem.*, **268**, 2583-2589.
7. Kimura, N., and Shimada, N. (1988) *J. Biol. Chem.*, **263**, 4647-4653.
8. Muchonen, W. W., and Lambeth, D. O. (1995) *Comp. Biochem. Physiol.*, **110B**, 211-223.
9. Jacobus, W. E., and Evans, J. J. (1977) *J. Biol. Chem.*, **252**, 4232-4241.
10. Lipskaya, T. Yu., and Plakida, K. N. (2003) *Biochemistry (Moscow)*, **68**, 1136-1144.
11. Lipskaya, T. Yu., and Voinova, V. V. (2008) *Biochemistry (Moscow)*, **73**, 321-331.
12. Lipskaya, T. Yu., and Voinova, V. V. (2005) *Biochemistry (Moscow)*, **70**, 1354-1362.
13. Viitanen, P. V., Geiger, P. J., Erickson-Viitanen, S., and Bessman, S. P. (1984) *J. Biol. Chem.*, **259**, 9679-9686.
14. Lipskaya, T. Yu., Geiger, P. J., and Bessman, S. P. (1995) *Biochem. Mol. Med.*, **55**, 81-89.
15. Ostlund, A. K., Gohring, U., Krause, J., and Brdiczka, D. (1983) *Biochem. Med.*, **30**, 231-245.
16. Adams, V., Griffin, L., Towbin, J., Gelb, B., Worley, K., and McCabe, E. R. (1991) *Biochem. Med. Metab. Biol.*, **45**, 271-291.
17. Laterveer, F. D., Gellerich, F. N., and Nicolay, K. (1995) *Eur. J. Biochem.*, **232**, 569-577.
18. Adams, V., Griffin, L., Towbin, J., Gelb, B., Worley, K., and McCabe, E. R. (1991) *Biochem. Med. Metab. Biol.*, **45**, 271-291.
19. Kaneko, M., Kurokawa, M., and Ishibashi, S. (1985) *Arch. Biochem. Biophys.*, **237**, 135-141.
20. Abdulaev, N., Karaschuk, G. N., Ladner, J. E., Kakuev, D. L., Yakhyaev, A. V., Tordova, M., Gaidarov, I. O., Popov, V. I., Fujiwara, J. H., Chinchilla, D., Eisenstein, E., Gilliland, G. L., and Ridge, K. D. (1998) *Biochemistry*, **37**, 13958-13967.
21. Goronovskii, I. T., Nazarenko, Yu. P., and Nekryach, E. F. (1962) *Quick Reference Guide in Chemistry* [in Russian], USSR Academy of Sciences Publishers, Kiev.
22. Chance, B., and Williams, G. R. (1955) *J. Biol. Chem.*, **217**, 383-393.
23. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.

24. Gornall, A. G., Bardawill, C. L., and David, M. M. (1949) *J. Biol. Chem.*, **177**, 751-766.
25. Lipskaya, T. Yu., and Voinova, V. V. (2009) *Biochemistry (Moscow)*, **74**, 578-587.
26. Kowluru, A., Tannous, M., and Chen, H. Q. (2002) *Arch. Biochem. Biophys.*, **398**, 160-169.
27. Hutny, J., and Wilson, J. E. (2000) *Acta Biochim. Polon.*, **47**, 1045-1060.
28. Golestani, A., Ramshini, H., and Nemat-Gorgani, M. (2007) *BMC Biochem.*, **8**, 20 (doi:10.1186/1471-2091-8-20).
29. Nakashima, R. A., Mangan, P. S., Colombini, M., and Pedersen, P. L. (1986) *Biochemistry*, **25**, 1015-1021.
30. Klug, G. A., Krause, J., Ostlund, A.-K., Knoll, G., and Brdiczka, D. (1984) *Biochim. Biophys. Acta*, **764**, 272-282.
31. Gilles, A.-M., Presecan, E., Vonica, A., and Lascu, I. (1991) *J. Biol. Chem.*, **266**, 8784-8789.
32. Laterveer, F. D., Nicolay, K., and Gellerich, F. N. (1997) *Mol. Cell. Biochem.*, **174**, 43-51.
33. Muller, G., Kourndorfer, A., Kornak, U., and Molaisse, W. J. (1994) *Arch. Biochem. Biophys.*, **308**, 8-23.
34. Rose, I. A., and Warms, J. V. B. (1967) *J. Biol. Chem.*, **242**, 1635-1645.
35. Felgner, P. L., and Wilson, J. E. (1977) *Arch. Biochem. Biophys.*, **182**, 282-294.