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In Rat Liver Mitochondria All Nucleoside Diphosphate Kinase of the Outer Compartment Is Associated with the Outer Surface of the Outer Membrane

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Abstract—Data on localization of nucleoside diphosphate kinase (NDPK) in the outer mitochondrial compartment are contradictory. We have demonstrated that repeated quintuple wash of a mitochondrial pellet (protein concentration is about 2 mg/ml) solubilized only 60% of total NDPK activity. Since no release of adenylate kinase, the marker enzyme of the intermembrane space, was observed, it was concluded that the solubilized NDPK activity was associated with the outer surface of the outer mitochondrial membrane. Treatment of mitochondria with digitonin solutions in low (sucrose, mannitol) or high (KCl) ionic strength media revealed that solubilization of remaining NDPK activity basically coincided with the solubilization curve of monoamine oxidase, the marker enzyme of the outer mitochondrial membrane, but differed from solubilization behavior of adenylate kinase and malate dehydrogenase. We concluded that the remaining NDPK activity was also associated with the outer mitochondrial membrane and electrostatic interactions were not essential for NDPK binding to mitochondrial membranes. Results of polarographic determination of remaining adenylate kinase and NDPK activities of mitochondria incubated in ice for different time intervals and subjected to subsequent centrifugation suggest that all NDPK activity of the outer compartment of rat liver mitochondria is associated with the outer surface of the outer mitochondrial membrane. We suggest the existence of at least three NDPK fractions. They represent 70, 15, and 15% of total NDPK activity of the outer compartment and differ by tightness of membrane binding.

Key words: nucleoside diphosphate kinase, mitochondria, localization, rat liver

In contrast to catabolism, many anabolic processes use nucleoside triphosphates (NTPs) other than ATP. These NTPs are formed in the reaction catalyzed by nucleoside diphosphate kinase (NDPK): ATP + NDP ↔ ADP + NTP [1]. This reaction proceeding via a ping-pong mechanism involves intermediate formation (phosphorylated histidine residue) at the active site of NDPK [2]. During the last decade it was demonstrated that NDPK plays not only a catalytic but also a regulatory role. The enzyme is involved in regulation of such important processes as proliferation, development, differentiation, malignant growth, and apoptosis (see for review [3-6]). In some cases it was demonstrated that the catalytic and regulatory functions of this enzyme exist independently [4, 6, 7].

In humans eight genes encoding eight types of homologous subunits constituting NDPK isoenzymes have been recognized. These isoenzymes are denominated by letters (from A to H) or by the names of corresponding genes, i.e., from Nm23-H1 to Nm23-H8 [3]. NDPK isoenzymes differ by intracellular localization and tissue specificity [3, 8-13]. All known eukaryotic NDPK isoenzymes are homohexamers [14, 15]. However, under certain conditions subunits of different isoenzymes may form heterohexamers [15, 16]. Such hybridization increases total number of NDPK isoforms. The existence of multiple forms of NDPK and their various intracellular localizations suggest that each isoform has a specific function and its intracellular localization is primarily important for this function [3].

Good evidence exists that in vertebrate mitochondria NDPK is localized in the outer and inner compartments [17, 18]. The former includes both surfaces of the outer mitochondrial membrane, intermembrane space and the outer surface of the inner mitochondrial membrane. The inner compartment is formed by the matrix surface of the inner mitochondrial membrane and matrix. In rat liver mitochondria 90% of NDPK activity is found

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in the outer compartment, whereas in pigeon liver mitochondria the outer compartment contains only 18% of the total mitochondrial NDPK activity; nevertheless, the specific NDPK activity of the outer compartment is identical in mitochondria of the two species [17].

The inner compartment NDPK has several known and putative functions [13, 17, 18]. Specific functions of the outer mitochondrial compartment NDPK remain unknown. Moreover, NDPK localization in this compartment is poorly characterized. In 1968 Schnaitman and Greenawalt [19] concluded that either NDPK is located in both outer mitochondrial membrane and intermembrane space or the enzyme is loosely bound to the outer membrane. Since in that study [19] the major proportion of NDPK activity was found in the soluble fraction, it became subsequently accepted that like adenylate kinase, NDPK is localized in the intermembrane space [18, 20, 21]. This viewpoint on the outer compartment NDPK localization in the intermembrane space of mitochondria was included into textbooks on biochemistry [1, 22].

Adams et al. [20] suggested that in the intermembrane space NDPK is localized in and out of contact sites. Later Brdiczka and Wallimann [21] positioned all NDPK of the intermembrane space into the contact site region.

Muhonen and Lambeth [17] suggest that in cardiac and liver mitochondria intermembrane space NDPK is associated with either the outer surface of the inner mitochondrial membrane or with the outer membrane. Data by Pedersen [23] suggest that a significant proportion of NDPK is associated with the outer surface of the outer mitochondrial membrane. However, this author only stated that NDPK is localized in the outer mitochondrial compartment [23].

Milon et al. [13] found specific mitochondrial NDPK isoenzyme, Nm23-H4 in the outer and inner membranes of mitochondria isolated from transformed human kidney embryonal cells. These authors suggest that NDPK is located in the contact sites of the mitochondrial membranes and it faces to the intermembrane space or matrix [13].

Thus, brief overview of the problem of mitochondrial NDPK localization clearly demonstrates that most authors believe that the outer mitochondrial compartment NDPK is located in the intermembrane space, particularly in the region of contact sites. However, certain evidence exists that NDPK is also associated with the outer surface of the outer mitochondrial membrane. The presence of NDPK in the region of contact sites also does not rule out its localization on the outer surface of the outer mitochondrial membrane (as demonstrated for hexokinase). However, function and modes of regulation of the enzyme located on the outer surface of the outer mitochondrial membrane and in the intermembrane space may differ.

In this study we employed rat liver mitochondria, because in these mitochondria almost all NDPK activity is associated with the outer compartment [17, 18].

Our study revealed that in rat liver mitochondria all NDPK activity of the outer compartment is associated with the outer surface of the outer mitochondrial membrane.

MATERIALS AND METHODS

Isolation of mitochondria. Male albino rats (150-170 g) starved for 16 h with free access to water were used in the experiments. Livers were quickly removed, washed free of blood, weighed, and squashed through a tissue press (pore diameter of 1 mm). The squashed liver was then homogenized in isolation medium containing 0.21 M mannitol, 0.07 M sucrose, 2.1 mM Hepes, pH 7.4, and human serum albumin (0.5 mg/ml) [18] for 1 min in a glass homogenizer using a Teflon pestle. The ratio of liver and the medium was 1 : 9 w/v. (The solution of human serum albumin was initially dialyzed for three days against a large volume of bidistilled water, which was changed every day. This treatment usually removes fatty acids bound to serum albumin. Aliquots of dialyzed human serum protein were stored at −18°C). The resultant 10% homogenate was initially centrifuged at 600*g* for 15 min in a Beckman J2-21 centrifuge (Austria) at 2°C to remove cell debris and nuclei. The supernatant was filtered through a nylon filter and centrifuged at 7000*g* for 10 min. After careful removal of supernatant, the mitochondrial pellet (P_1) was suspended in a measured volume of the isolation medium and transferred into a graduated tube (2 ml) for measurement of total volume of mitochondrial suspension and volume of P_1. An aliquot of P_1 was taken for protein determination and assay of enzyme activities. The supernatant centrifuged at 31,000*g* for 1 h was used for determination of soluble NDPK activity.

The remaining proportion of P_1 was then diluted with 40 ml of the isolation medium (protein concentration about 2 mg/ml) and centrifuged at 12,000*g* for 10 min. The resultant pellet (P_2) was suspended in the minimal volume of the isolation medium and (after taking an aliquot for determination of protein content and enzyme activities) diluted with 40 ml of the medium and immediately centrifuged as above. The same procedure was repeated up to preparation of P_6 pellet. Time of mitochondria sedimentation was fixed. The aliquots were kept at −18°C during 1-3 days until assay of enzyme activities.

In experiments shown in Fig. 3, where the method of aliquot collection was used, P_1 suspension was transferred into 16 ml of the isolation medium (protein concentration ~5 mg/ml) and mixed using a magnetic stirrer at 4°C. Aliquots of 2 ml periodically taken from this suspension were centrifuged using an Eppendorf MiniSpin plus (Germany) at 14,000*g* for 7 min. Supernatants were care-

fully removed and the resultant mitochondrial pellets were suspended in minimal volume of the isolation medium and placed into ice until assay of enzyme activities.

Calculation of contaminants of soluble NDPK activity in mitochondrial P_1 pellet. For calculation of water volume of mitochondrial intermembrane space and matrix in P_1, we determined protein content in P_1 suspension. The resultant value was multiplied by 4.67, because content of mitochondrial water was found to be 4.67 μl/mg [24]. Since the proportion of supernatant protein in P_1 suspension was less than 5%, we did not take it into consideration. Under our experimental conditions the volume of supernatant retained by P_1 pellet was very constant; it represented 60% of the volume of P_1.

Knowing the volume of supernatant contaminant in the initial P_1 suspension, we calculated its proportion in P_1 suspension used for assay of NDPK activity. During spectrophotometric assay of NDPK activity the resultant value was corrected for NDPK activity in supernatant. The latter activity was separately determined in supernatant obtained after centrifugation at 31,000*g* (see previous section). The NDPK activity of supernatant was subtracted from the NDPK activity determined in the sample containing P_1 suspension. During polarographic assay of NDPK activity the contribution of soluble NDPK to the total enzyme activity of P_1 was assessed as difference between NDPK activity in P_1 suspension and in the mixture of P_1 suspension and calculated supernatant volume. This difference was subtracted from NDPK activity found in P_1 suspension. The contamination of P_1 NDPK activity with supernatant NDPK activity did not exceed 11% of total NDPK activity found in P_1. The activity of supernatant NDPK in all subsequent mitochondrial pellets obtained during repeated sedimentation was too low to be taken into consideration. Supernatant soluble adenylate kinase activity was not detected in P_1 using the spectrophotometric assay.

Treatment of mitochondria with digitonin. The mitochondrial P_1 sediment was diluted with the isolation medium or with a medium containing 0.14 M KCl, 2.1 mM Hepes (pH 7.4), and 0.5 mg/ml human serum albumin (medium A) to the final protein concentration 20 mg/ml. Digitonin solution (20 mg/ml) was prepared just before experiment using heating in boiling bath for 30 min. The isolation medium without human serum albumin (medium B) or the medium containing 0.14 M KCl, 2.1 mM Hepes, pH 7.4, were used for preparation of digitonin solution. After cooling, stock digitonin solution was placed into ice. This solution was used for preparation of serial digitonin dilutions from 9.2 to 0.8 mg/ml, using the isolation medium or medium A, respectively.

Suspension of P_1 pellet in one of the media (0.25 ml) was mixed with an equal volume of one of the digitonin solutions and incubated in ice with stirring for 15 min. The incubation was stopped by adding 2.5 ml of the isolation medium (or medium A) and centrifuging at 12,500*g* for 6 min using a refrigerated Metronex centrifuge type 310 (Poland). Supernatants were carefully removed and pellets were suspended in 1.5 ml of the same medium and centrifuged again. The sediments were then suspended in 1 ml of the isolation media and kept at −18°C until assays of activities of NDPK and marker enzymes by spectrophotometric methods. Control samples underwent all these treatments but without digitonin.

Spectrophotometric assays of enzyme activities. *Assay of NDPK activity*. The reaction mixture (total volume of 1.1 ml) contained 3.3 mM glucose, 3.3 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.4, 0.3 mM $NADP^+$, 0.125 mM ADP, 0.3% Triton X-100 (for solubilization of mitochondrial membranes), 4 mM AMP (for inhibition of adenylate kinase), 10 mM NaN_3, 25 μM rotenone (for inhibition of mitochondrial electron transport chain), 0.85 U hexokinase, and 0.85 U glucose-6-phosphate dehydrogenase. After stabilization of absorbance at 340 nm, CTP was added to the final concentration of 2 mM. After recording of absorbance for several minutes, mitochondrial suspension (80-200 μg mitochondrial protein) was added and changes of absorbance were registered again. NDPK activity was determined as the difference between hexokinase activity after CTP addition and total activity of hexokinase and NDPK after addition of mitochondrial suspension. For evaluation of possible contribution of residual adenylate kinase activity to NDPK activity the same assay was carried out using a sample without CTP. For mitochondrial P_1 suspension we also determined contribution of soluble NDPK from supernatant contaminants (see above).

Assay of adenylate kinase activity. The composition of the assay medium was the similar to that used for determination of NDPK activity with several alterations: a) this medium did not contain AMP and CTP; b) ADP and $MgCl_2$ concentrations were 3 and 5 mM, respectively. The reaction was started by adding 10 μg of mitochondrial protein.

Assay of malate dehydrogenase activity. The reaction mixture (total volume 1.1 ml) contained 0.1 M Tris-HCl, pH 8.4, 0.15 mM NADH, 0.05% Triton X-100, and 20 μg of mitochondrial protein. The reaction was started by addition of oxaloacetate to the final concentration 20 μM. Molar absorbance coefficient for NADPH and NADH at 340 nm was assumed to be $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ [25].

Assay of monoamine oxidase activity. The reaction mixture (total volume 1.5 ml) contained 0.15 M potassium phosphate buffer, pH 7.6, 0.067% Triton X-100, and 200 μg mitochondrial protein. The reaction monitored by changes of absorbance at 250 nm was initiated by adding benzylamine to the final concentration of 0.67 mM. Molar absorbance coefficient of forming benzaldehyde was assumed to be $13.8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ [26].

For each preparation all spectrophotometric assays were repeated 2-3 times.

Polarographic assay of mitochondrial respiration. The rate of mitochondrial oxygen consumption was determined at 22°C using a closed Clark type platinum electrode and LP 7e polarograph (Czechoslovakia). The incubation medium (total volume 1 ml) contained 210 mM mannitol, 70 mM sucrose, 0.1 mM EGTA, 20 mM Tris-HCl, pH 7.4, 5 mM potassium phosphate, 3 mM $MgCl_2$, 5 mM potassium succinate [18], and (with exception of experiments with AMP) adenylate kinase inhibitor, P^1,P^5-di(adenosine-5′)pentaphosphate (AP5A). Other additions are given in corresponding legends to figures or mentioned in the text. Oxygen concentration in the incubation medium was assumed to be 250 μM [27]. The rate of oxidative phosphorylation was determined by multiplying the difference between State 3 and State 4 respiration rates by ADP/O value determined for a given sample $\{(V_{st3}^{ADP} - V_{st4}^{ADP}) \cdot ADP/O\}$ (see scheme in Fig. 3b).

Polarographic assay of adenylate kinase activity. The incubation medium was as described above but with addition of 300 μM ATP. The adenylate kinase reaction was initiated by adding 50 μM AMP. Adenylate kinase activity was calculated by multiplying the difference between respiration rates right after AMP addition and after its exhaustion by AMP/O value determined for a given sample $\{(V_{st3}^{AMP} - V_{st4}^{AMP}) \cdot AMP/O\}$ (see scheme in Fig. 3a).

Polarographic assay of NDPK activity. The activity was assayed in the presence of 600 μM CDP. If oxidative phosphorylation of ADP preceded CDP addition, NDPK activity was determined by multiplying the difference between respiration rates in the presence of CDP and in State 4 after ADP by the ADP/O value determined for a given sample $\{(V_{st3}^{CDP} - V_{st4}^{ADP}) \cdot ADP/O\}$ (see scheme in Fig. 3b).

If assay of adenylate kinase activity preceded assay of NDPK activity, we added AP5A (final concentration 20 μM) right after AMP exhaustion. In this case NDPK activity was calculated by multiplying the difference between the respiration rates after and before CDP addition by doubled AMP/O coefficient determined for this sample $\{(V_{st3}^{CDP} - V_{st4}^{AMP+AP5A}) \cdot 2 \cdot AMP/O\}$ (see Fig. 4). In our experiments the doubled AMP/O coefficient determined with AMP was equal to ADP/O coefficient determined in the experiments with ADP.

Mitochondrial protein content was determined by the method of Gornall et al. [28] using bovine serum albumin as standard.

RESULTS

Results of the study by Pedersen [23] suggested that a significant proportion of NDPK activity of the outer mitochondrial compartment in rat liver mitochondria is associated with the outer surface of the outer mitochondrial membrane. To determine which proportion of NDPK activity is associated with the outer surface of the outer mitochondrial membrane, we subjected a mitochondrial fraction to five sequential sedimentations followed by dilution in a large volume of the isolation medium (see "Materials and Methods" section for details).

Figure 1 shows that two mitochondrial washings resulted in a loss of 60% of NDPK activity present in P_1 sediment. Three subsequent washings (P_4-P_6 pellets) did not reduce NDPK activity in the sediment. (Mitochondrial NDPK activity remained unchanged during 1.5 h.) Specific activity of NDPK depended on the amount of mitochondrial protein used in the assay. For example, spectrophotometric assay of NDPK activity using 60-80 μg of protein gave the mean value of specific NDPK activity in P_1 of 52.9 ± 4.4 nmol CTP/min per mg protein (n = 4), whereas with 200 μg of mitochondrial protein NDPK activity in P_1 pellet was 25.8 ± 7.7 nmol CTP/min per mg protein (n = 5).

In these and subsequent experiments we assayed adenylate kinase activity to monitor permeability of the outer mitochondrial membrane. This enzyme is located in the intermembrane space [19]. Since its molecular mass of 30 kD is lower than that of NDPK (100 kD) [8] it is clear that lack of the outer membrane permeability for adenylate kinase means lack of permeability for NDPK provided that the latter is also located in the intermembrane space.

Figure 1 shows that adenylate kinase activity assayed in mitochondrial pellets after sequential washings

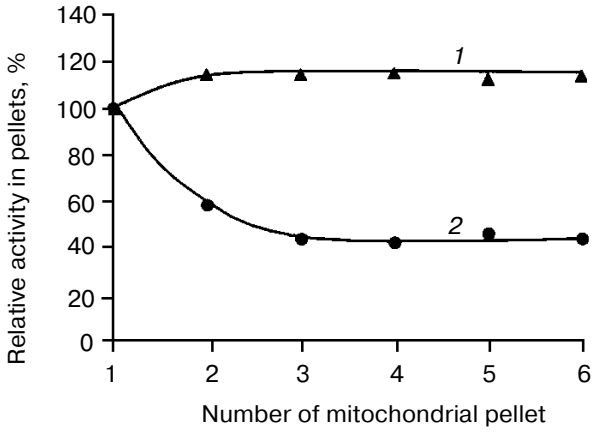

Fig. 1. Effect of repeated quintuple mitochondrial washings on solubilization of adenylate kinase (*1*) and NDPK (*2*) from rat liver mitochondria. Specific activity of these enzymes in P_1 pellet was defined as 100%: adenylate kinase (n = 5) and NDPK (at 80 μg mitochondrial protein in the spectrophotometric cuvette, n = 4) activities were 343 ± 62 and 52.9 ± 4.4 nmol ATP/min per mg protein, respectively. Other details are described in "Material and Methods".

remained at a constant level and, consequently, solubilization of NDPK activity observed in these experiments may be attributed to its release from the outer surface of the outer mitochondrial membrane.

To determine submitochondrial localization of the remaining 40% of NDPK activity, mitochondrial P_1 suspension was treated with different concentrations of digitonin in media with low (mannitol, sucrose) or high (KCl) ionic strength. After centrifugation of digitonin-treated mitochondria, we assayed remaining NDPK activity and also activity of the markers of the intermembrane space (adenylate kinase), the outer mitochondrial membrane (monoamine oxidase) [19], and mitochondrial matrix marker, malate dehydrogenase [30]. Since electrostatic forces determine malate dehydrogenase binding to the inner mitochondrial membrane, solubilization of this enzyme in low ionic strength media will reflect the extent of the inner mitochondrial membrane damage. Solubilization of malate dehydrogenase by digitonin solutions of high ionic strength would reflect solubilization of matrix enzymes.

Figure 2a shows that the curve of solubilization of NDPK activity by increasing digitonin concentrations basically coincided with the curve of monoamine oxidase solubilization. At digitonin concentration 0.2 mg/mg of mitochondrial protein all adenylate kinase activity was solubilized whereas about 80% NDPK and monoamine oxidase activity remained membrane bound at this concentration of digitonin. If all NDPK activity were freely located in the intermembrane space its solubilization would significantly exceed solubilization of monoamine oxidase. At digitonin concentration of 0.4 mg/mg of mitochondrial protein NDPK and monoamine oxidase activities were almost totally solubilized, whereas ~80% of malate dehydrogenase activity remained membrane-bound. So, we concluded that NDPK activity is associated with the outer mitochondrial membrane.

Figure 2b shows that adenylate kinase activity was totally solubilized at digitonin concentration of 0.2 mg/mg of mitochondrial protein. Behavior of malate dehydrogenase dramatically changed in medium A. At digitonin concentration of 0.4 mg/mg of mitochondrial protein malate dehydrogenase was totally solubilized. In this medium of high ionic strength the concentration-dependent curves of NDPK and monoamine oxidase solubilization shifted to higher digitonin concentrations. So, we concluded that ionic interactions are not essential for NDPK interaction with the outer mitochondrial membrane.

To test whether the NDPK activity remaining after two sequential mitochondrial sedimentations is also (maybe more tightly) bound to the outer surface of the outer mitochondrial membrane, we increased the number of mitochondrial washings and time intervals between repeated centrifugations. As in the case of experiments shown in Fig. 1, mitochondria were washed in media of

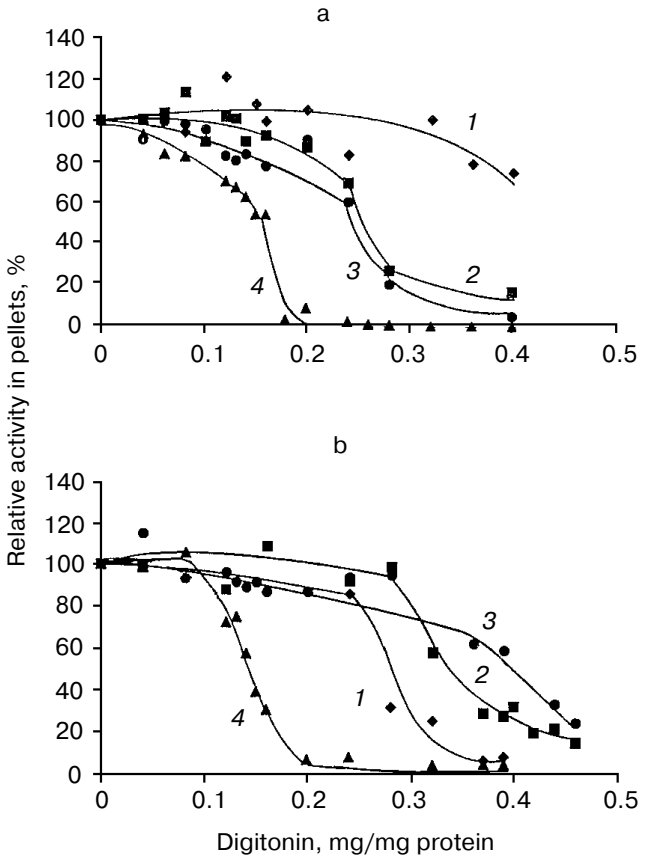

Fig. 2. Solubilization of mitochondrial enzymes by increasing concentrations of digitonin in isolation medium (a) and in salt medium A (b): *1*) malate dehydrogenase; *2*) monoamine oxidase; *3*) NDPK; *4*) adenylate kinase. Enzyme activities in control samples (in nmol/min per mg mitochondrial protein) were defined as 100%: a) adenylate kinase, 481 ± 62 (4); monoamine oxidase, 4.79 ± 0.43 (4); NDPK (at 200 μg in the spectrophotometric cuvette), 9.4 ± 1.7 (3); malate dehydrogenase, 310 ± 60 (2); b) adenylate kinase, 272; monoamine oxidase, 3.28; NDPK, 10.1; malate dehydrogenase, 356. Number of experiments is shown in parentheses. Other experimental details are given in "Material and Methods". See text for other explanations.

low ionic strength because increase of ionic strength tightened NDPK association with the membrane (Fig. 2). As in the case of experiments shown at Fig. 1, NDPK activity assayed in P_1 pellet 1 and 2.5 h after isolation of P_1 pellet remained at the level of 35-40% of initial activity of this enzyme in P_1. Subsequently, this activity gradually decreased reaching a new plateau during 7 h exposure after isolation of P_1 pellet. This new plateau corresponded to 15% of specific NDPK activity in initial P_1 pellet remained unchanged during 24 h exposure after P_1 isolation. Since increase in number of mitochondrial washings resulted in damage of the outer mitochondrial membrane and the loss of some portion of adenylate kinase activity,

we could not conclude that 25% of NDPK activity, which was solubilized before reaching the new plateau at the level of 15% of initial specific activity in P_1, might be attributed to NDPK association to the outer surface of the outer mitochondrial membrane.

Using spectrophotometric assay we determined total activity of mitochondrial NDPK, including that of the inner mitochondrial compartment. Consequently, it was possible that the remaining 15% NDPK activity originated from the inner compartment.

Taking into consideration such possibility, we used the polarographic method for assay of NDPK activity. Since the inner mitochondrial membrane is permeable only for ADP and ATP [31], but not for CDP [1], the polarographic method allows detection of only NDPK activity located in the outer mitochondrial compartment. Since repeated sequential mitochondrial washings dramatically impair polarographic parameters of mitochondria (the table, experiment 1) we used a single wash followed by long-term incubations of mitochondria in ice. Special experiments revealed that such storage in ice insignificantly influenced functional characteristics of the mitochondria (the table, experiment 2). Figure 3c shows results of an experiment when diluted suspension of P_1 was kept in ice and at certain time interval shown in this figure an aliquot was taken for subsequent centrifugation. Activities of NDPK and adenylate kinase in the pellet were determined by the polarographic method. The rates of mitochondrial respiration after addition of CDP and AMP did not exceed 70 and 90% of State 3 respiration, respectively. Thus, NDPK and adenylate kinase activities were not limited by capacity of the oxidative phosphorylation system. As shown in Fig. 1, the transition from P_1 to P_2 was accompanied by increase in adenylate kinase activity in the mitochondrial pellet and then it remained at a constant level for a long time. So, adenylate kinase activity of P_2 was defined as 100% (Fig. 3c). The mean value of P_1 NDPK activity measured by the polarographic method was 123 ± 4 nmol CDP/min per mg of mitochondrial protein ($n = 4$). Figure 3c shows that using the method of aliquot collection, adenylate kinase activity remained unchanged during storage for 8 h after sedimentation of the pellet P_1. The latter suggests integrity of the outer mitochondrial membrane over the whole period of this experiment. Use of this method also caused less pronounced decrease of NDPK activity in P_2 and P_3 pellets than in the case of sequential mitochondrial sedimentation. This explains why a plateau of NDPK activity observed from 1 to 2.5 h after P_1 isolation is not well defined in Fig. 3c: it is masked by the solubilization of tighter bound NDPK. The method of aliquot collection demonstrated heterogeneity of remaining NDPK activity. During storage for 5 and 6.5 h after P_1 isolation, the latter reached a plateau corresponding to 18% of specific NDPK activity in P_1 but after storage for 8 h NDPK activity further reduced to 3% of initial NDPK activity in P_1. A control experiment revealed that all activity solubilized from mitochondria was found in supernatant (not shown).

Figure 4 shows total solubilization of mitochondrial NDPK activity. In this experiment P_2 mitochondrial suspension was incubated in ice for 8 h. After this period two aliquots were taken. One was directly added to the polarographic cell (Fig. 4a) whereas the other one was initially centrifuged to remove NDPK activity solubilized during storage in ice (Fig. 4b). In each aliquot adenylate kinase activity was initially determined and then a specific inhibitor of this enzyme, AP5A was added and NDPK activity was determined in the same aliquot. Figure 4b shows that mitochondrial wash did not influence adenylate kinase activity but completely removed NDPK. A weak stimulation of mitochondrial respiration after addition of CDP (Fig. 4b) may be explained by NDPK solubilization rather than impairment of oxidative phosphorylation capacity because addition of ADP after CDP sharply stimulated mitochondrial respiration.

Effect of the method of mitochondrial pellet isolation on functional characteristics of the mitochondria

Number of experiment	Method of preparation of mitochondrial pellets	Fraction	Time before assay, h	State 3 respiration rate, ng-atoms O/min per mg protein	RCI	ADP/O	Rate of oxidative phosphorylation, nmol ADP/min per mg protein
1	Sequential sedimentation	P_1	11	138	5.6	1.6	181
		P_4	4	120	3.1	1.2	97
		P_6	4.5	95	1.7	1.1	43
2	Method of aliquot collection	P_1	3.5	197	5.0	1.5	237
		P_2	4.5	214	4.2	1.5	244
		P_2	12.5	262	4.1	1.4	277

Note: Time after the isolation of P_1 sediment is shown. State 3 respiration was initiated by adding 170 μM ADP. Other details are given in the "Material and Methods" section. RCI, respiratory control index.

Thus, results of experiments shown at Figs. 3c and 4 indicate that all NDPK activity associated with the outer mitochondrial membrane may be solubilized from mitochondria without damage to the outer membrane. So, we concluded that in rat liver mitochondria all NDPK activity of the outer compartment is associated with the outer surface of the outer mitochondrial membrane.

Since after 8 h of mitochondrial incubation in ice NDPK activity of the outer compartment was totally solubilized (see Figs. 3c and 4), we also concluded that 15% of the total NDPK activity that remained in mitochondrial suspension after 24 h incubation in ice represented NDPK activity of the inner mitochondrial compartment.

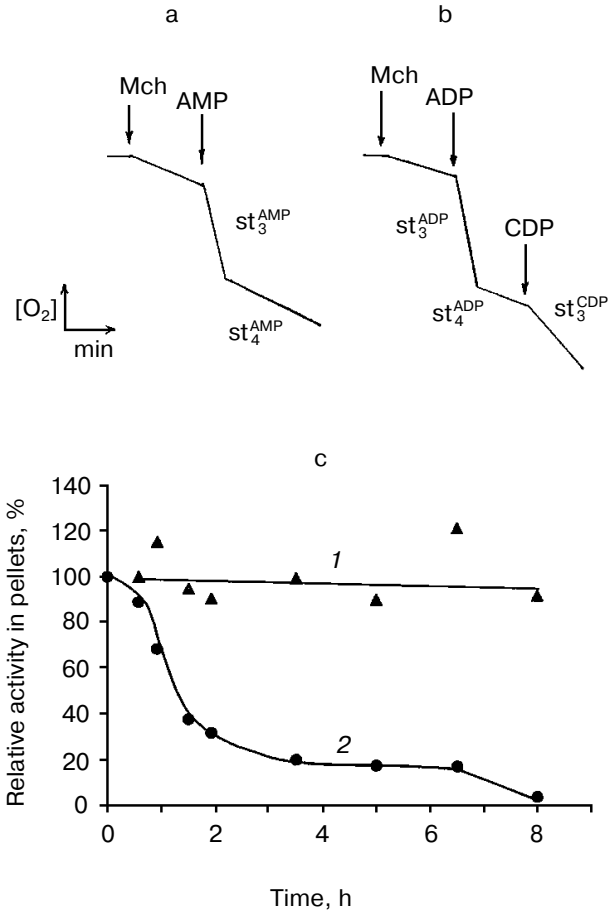

Fig. 3. Polarographic study of NDPK solubilization from rat liver mitochondria: a) the scheme of experiment demonstrating order of additions in the polarographic experiment of adenylate kinase assay; b) NDPK assay; c) results of typical experiment on solubilization of adenylate kinase (*1*) and NDPK (*2*). Abscissa shows time intervals after P_1 isolation. NDPK activity in P_1 pellet (125 nmol CDP/min per mg protein) and adenylate kinase activity in P_2 pellet (156 nmol AMP/min per mg protein) were defined as 100%. Results of NDPK activity assay were corrected for the presence of soluble enzyme in the mitochondrial pellet. Additions: mitochondria (Mch), 0.5-0.7 mg protein; AMP, 50 μM; ADP, 170 μM; CDP, 600 μM (final concentrations). Other details are given in the "Material and Methods" section. See the text for explanation.

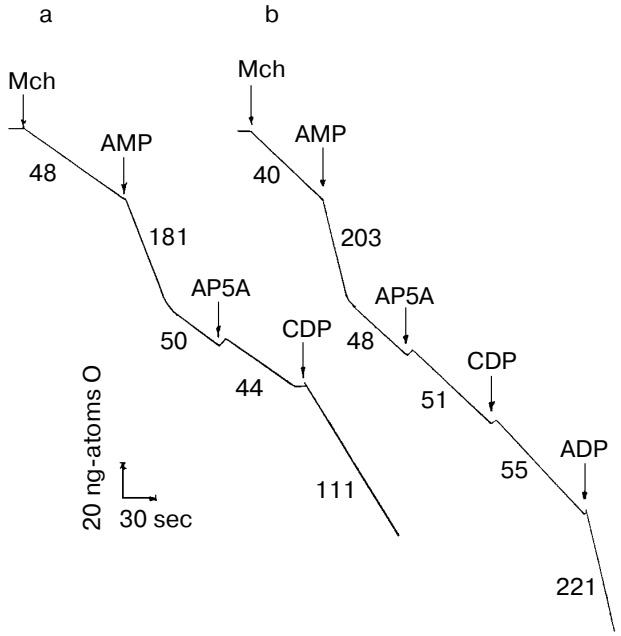

Fig. 4. Polarographic demonstration of solubilization of membrane-bound NDPK activity during storage of rat liver mitochondria in ice. a) Mitochondrial suspension P_2 (10 mg/ml) was kept in ice during 8 h after P_1 isolation. b) After this period 300 μl of P_2 suspension was centrifuged using the refrigerated centrifuge at 14,000*g* for 7 min. The sediment was resuspended in the volume of medium B that corresponded to the volume of the discarded supernatant. The incubation medium contained 300 μM ATP. Additions: mitochondria (Mch), 0.6 mg protein; AMP, 50 μM; AP5A, 20 μM; CDP, 600 μM; ADP, 170 μM (final concentrations). Numbers indicate the rate of mitochondrial respiration in ng-atoms O/min per mg protein. Other details are given in the "Material and Methods" section and in the text.

DISCUSSION

In the present study we have demonstrated for the first time that in rat liver mitochondria all NDPK activity of the outer compartment is associated with the cytoplasmic surface of the outer mitochondrial membrane. We did not find NDPK activity in the intermembrane space. It would be reasonable to suggest that lack of NDPK activity in the intermembrane space may be attributed to the loss of the enzyme from the intermembrane space during isolation of P_1. However, comparison of adenylate kinase and NDPK activities determined in the present study with literature data [17-19, 23] and high functional characteristics of P_1 (table) typical for mitochondria with intact outer membrane [19] argue against this suggestion.

In our experiments the enzyme was solubilized as at least three fractions. Release of these fractions of NDPK into solution was separated from each other by time intervals during which mitochondrial NDPK activity remained unchanged. The enzyme activity in sequentially prepared fractions represented ~70, 15, and 15% of NDPK activity in the outer mitochondrial compartment (or ~60, 12.5, and 12.5% of total NDPK activity of rat liver mitochondria). NDPK activity of the inner compartment represented 15% of total mitochondrial NDPK activity. This is consistent with the literature data on low NDPK activity in the inner compartment of rat liver mitochondria [17, 19]. The first readily solubilized fraction probably represents the proportion of NDPK activity that was released into solution in experiments by Schnaitman and Greenawalt [19] and Pedersen [23]. In experiments employing the method of sequential repeated mitochondrial sedimentation this fraction was time-separated from solubilization of two other fractions (Fig. 1). As in experiments by Pedersen [23], this separated fraction was not recognized using the method of sequential aliquot collection (Fig. 3). Two other fractions exhibited tighter association with mitochondria. Based on polarographic assays, we calculated that in rat liver NDPK activity of the outer mitochondrial compartment represents $13.2 \pm 1.4\%$ ($n = 6$) of total NDPK activity of the outer compartment and soluble fraction (31,000*g* supernatant). The content of mitochondria in hepatic tissue was assumed to be 30 mg/g wet weight [1].

The existence of three NDPK fractions in the outer mitochondrial compartment can be explained in various ways: heterogeneity of molecular composition of NDPK and/or heterogeneity of binding sites. Lambeth et al. found that readily solubilized NDPK from rat liver mitochondria represents a homolog of human NDPK B [3]. This enzyme (NDPK α) is the main form of NDPK found in cytoplasm of most rat tissues [32]. Using isoelectrofocusing, Cheng et al. found six fractions exhibiting NDPK activity in rat liver mitochondrial extract; their isoelectric points varied from 5.5 to 6.5 [33]. Kimura et al. suggest that the existence of multiple NDPK bands with various p*I* values may reflect independent phosphorylation at the active site of subunits of NDPK hexamer [34]. Thus, it is possible that the existence of three pools of NDPK activity found in the present study may be attributed to different phosphorylation degree at the active sites of NDPK molecules.

The other possibility includes the existence of several NDPK isoenzymes bound to mitochondrial membranes. Since these fractions are located on the outer surface of the outer mitochondrial membrane, the isoenzymes possessing cleavable N-terminal signal sequences for import into mitochondria should be taken out of consideration. NDPK isoenzymes Nm23-H3 and Nm23-H6 present in most tissues were found in cytoplasm and mitochondria [10, 11, 35]. At the N-terminus they have additional 17 and 7 (compared with NDPK A and B, respectively) amino acid residues [3, 36]. However, these sequences lack features typical for cleavable N-terminal sequences of proteins imported into mitochondria [36]. Dual localization of these isoenzymes and lack of signal sequences required for import into mitochondria suggest that these enzymes are located on the outer surface of the outer mitochondrial membrane. Homologs of these isoforms could be associated with the outer surface of the outer mitochondrial membrane in our study.

Distinct tightness of NDPK association with mitochondrial membranes may be also attributed to heterogeneity of binding sites rather than to heterogeneity of molecular forms of NDPK. It is possible that more tightly bound NDPK fractions interact with mitochondrial membranes in the region of the contact sites, whereas readily solubilized NDPK is located remote from these sites. Finally, heterogeneity of rat liver mitochondrial fraction is also possible. Heterogeneity of NDPK properties may mean that each fraction of NDPK may have a specific function in mitochondria. This requires further study.

It should be noted that there is no convincing evidence for the presence of NDPK in the intermembrane space of vertebrate mitochondria. The only known NDPK located in the intermembrane space was found in mitochondria of *Dictyostelium discoideum*. However, phylogenetically this enzyme is distinct from vertebrate NDPK [37]. So, we believe that previously described NDPK of the outer compartment of vertebrate mitochondria [17-20] is associated with the outer surface of the outer mitochondrial membrane.

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