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# Reversibility of Nucleoside Diphosphate Kinase Solubilization from the Surface of the Outer Mitochondrial Membrane

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**Abstract**—It was found that in medium with low ionic strength nucleoside diphosphate kinase (NDPK) solubilization from the outer membrane of liver mitochondria could be partially reversed by the addition of 3.3 mM $MgCl_2$. Complete rebinding of the enzyme after the addition of $MgCl_2$ was observed when the mitochondrial washing and storage medium contained leupeptin, an inhibitor of cathepsins. It was demonstrated that leupeptin and another inhibitor of cysteine proteinases, E-64, do not influence the rate of NDPK solubilization as well as its solubilized and membrane-associated activity. We conclude that NDPK becomes sensitive to proteolysis only after its solubilization; proteolysis does not affect the part of the enzyme molecule that is responsible for catalysis. After solubilization of NDPK in the absence of leupeptin, cathepsins damage sites of its binding on the membranes. The rate of the enzyme solubilization is dependent on the pH of the storage medium (pH 6.0-8.0); it decreases with increase in pH. It was shown that in the medium with high ionic strength, $MgCl_2$ does not reverse pH-dependent NDPK solubilization, but solubilization could be reversed by increase in medium pH in the presence of E-64 and BSA. The physiological importance of these results is discussed.

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*Key words*: nucleoside diphosphate kinase, mitochondria, outer compartment, solubilization, liver

Under physiological conditions nucleoside diphosphate kinase (NDPK; EC 2.7.4.6) catalyzes the synthesis of various nucleoside triphosphates (NTPs) from ATP and corresponding nucleoside diphosphates (NDPs). The resultant NTPs are involved in the main anabolic processes [1]. Besides, NDPK manifests activity of histidine proteinase [2] and 3′-5′-exonuclease [3, 4] and interacts with many proteins [5-8]. Owing to this, the enzyme reveals regulatory properties; it is involved in regulation of such processes as cell motility [5, 9], growth [6], development [10], malignant growth [5, 10-12], and apoptosis [6, 13, 14]. The catalytic and regulatory functions can exist independently [3, 9, 15, 16].

In humans, eight genes encoding eight types of homologous subunits constituting NDPK isoenzymes are recognized. NDPK isoenzymes differ by intracellular localization and tissue specificity [17-22]. All known eukaryotic NDPK isoenzymes are homohexamers [23, 24]. However, under certain conditions subunits of different isoenzymes can hybridize to form heterohexamers [24, 25]. 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 [17].

In hepatocytes, NDPK is localized within the cytoplasm; it is also associated with membranes [26, 27]. In mitochondria, the enzyme was found in the outer compartment and in the matrix [28, 29]. We have demonstrated that in rat liver mitochondria all NDPK activity of the outer compartment is associated with the outer surface of the outer mitochondrial membrane (omNDPK) [30, 31]. Whereas the physiological role of matrix NDPK is well understood [22, 28, 29], the specific functions of omNDPK remain unknown.

We have demonstrated that omNDPK of liver mitochondria is involved in functional coupling to the oxidative phosphorylation system; owing to this, a proportion of ADP formed during the NDPK-reaction is directly transferred into the mitochondrial matrix without mixing with ADP of the medium [32]. The existence of function-

*Abbreviations*: AP5A, $p^1p^5$-di(adenosine-5′)pentaphosphate; DMO, 5,5-dimethyl-2,4-oxazolidinedione; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanido)-butane; HK, hexokinase; leupeptin, acetyl-leucyl-leucyl-arginal; NDPK, nucleoside diphosphate kinase; omNDPK, outer mitochondrial membrane bound NDPK.

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al coupling facilitates ADP transfer from the liver cell cytoplasm (where its concentration is very low [33]) into the intermembrane space.

Earlier, functional coupling was demonstrated for hexokinase (HK) isoenzyme type I and glycerol kinase bound to the outer mitochondrial membrane [34-44]. Thus, functional coupling with oxidative phosphorylation appears to be a common feature of kinases bound to the outer mitochondrial membrane. However, in rat liver mitochondria HK and glycerol kinase activities are negligible compared with omNDPK activity. In rat liver mitochondria, HK activity is ~2.5 nmol/min per mg protein [45] and glycerol kinase activity is 0.08-0.2 nmol/min per mg protein [46], whereas omNDPK catalyzes conversion of 250-300 nmol CTP/min per mg protein [31, 32]. We have demonstrated 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; they are responsible for ~17% of the maximal rate of oxidative phosphorylation [31]. The role of other omNDPK molecules remains unknown.

Under experimental conditions, 95-97% of omNDPK activity can be solubilized from mitochondria [30, 31], but the enzyme molecules not involved in functional coupling are solubilized much faster compared with molecules participating in coupling [30, 31]. We assumed that the ability to be easily solubilized might be attributed to the function of this proportion of omNDPK molecules. We have already mentioned that NDPK isoforms participate in regulation of many intracellular processes; some of these processes (for example, malignant growth and apoptosis) involve enzymes associated with the outer mitochondrial membrane. The omNDPK might be one of the regulators of these processes. It is also possible that omNDPK regulates other processes proceeding on the outer surface of the outer membrane of mitochondria. It is obvious that solubilization of this enzyme should change its regulating properties. If the enzyme solubilization is physiologically important, it should be reversible.

The goal of present study was to elucidate whether solubilization of omNDPK molecules (not involved in functional coupling with oxidative phosphorylation) is reversible.

## MATERIALS AND METHODS

**Materials.** ATP, ADP, AP5A, and BSA (fatty acids free) were purchased from Sigma (USA); UDP and CDP were from Reanal (Hungary); leupeptin (acetyl-leucyl-leucyl-arginal) was from MP Biomedicals (USA), and E-64 (L-*trans*-epoxysuccinyl-leucylamide-(4-guanido)-butane) was from Biomol Research Labs (USA).

**Isolation of mitochondria.** Mitochondria were isolated from livers of albino rats (150-250 g) basically as described in our previous work [32]. The isolation medium contained 0.28 M mannitol and 2.1 mM Hepes, pH 7.4. The homogenate (10%) was centrifuged at 2000 rpm for 15 min in a JA-20 rotor of a Beckman J2-21 centrifuge (Austria). The supernatant was centrifuged at 8000 rpm for 10 min. The mitochondrial pellet (pellet 1) was suspended in one of the washing media and centrifuged at 10,300 rpm for 10 min. The mitochondrial pellet (pellet 2) was suspended in the corresponding washing medium or in the isolation medium. The resulting mitochondrial suspension was kept on ice.

In some experiments, supernatant was divided into several equal portions. After centrifugation, the same number of equal pellets 1 was obtained. The pellets were suspended in isolation medium or isolation medium containing leupeptin and used in experiments on the reversal of omNDPK solubilization.

**Reversal of omNDPK solubilization by the addition of $Mg^{2+}$.** All experiments were performed at 2°C. The suspension of pellet 2 in isolation medium (protein concentration ~60 mg/ml) was divided into three equal portions. Concentrated solution of $MgCl_2$ was added to the first and second portions (final concentration 3.33 mM) 30 and 60 min, respectively, after pellet 2 was obtained. After selected time intervals, aliquots of 30 µl were taken from each portion and added to the media of the respective composition. The total volume of samples was 2 ml. The samples were immediately centrifuged at 14,500 rpm for 1 min in a MiniSpin Plus centrifuge (Eppendorf, Germany). Pellets 3 were suspended in 35 µl of isolation medium and used for the polarographic determination of the remaining omNDPK activity. Periodically aliquots of pellet 2 suspensions were taken and placed into the cell of a polarograph for determination of total (solubilized plus bound) activity of the enzyme.

In other experiments, two pellets 1 were obtained. One of them was washed in 8 ml of isolation medium, and the other in 8 ml of isolation medium containing 50 µM leupeptin. The suspensions were centrifuged at 12,300 rpm for 10 min in a Metronex tabletop centrifuge (Poland). The pellets 2 were suspended in 0.4-3.0 ml of the respective washing media. After selected (indicated in figures) time intervals, concentrated solution of $MgCl_2$ was added to the suspension containing leupeptin up to the final concentration 3.33 mM. Periodically aliquots of 30-180 µl were taken from each suspension, diluted in respective media (final volumes were 2 ml), and centrifuged at 14,500 rpm for 1 min. The pellets 3 were suspended in 35 µl of isolation medium and used for the determination of the remaining omNDPK activity. Periodically aliquots of pellet 2 suspensions were taken for the determination of total enzyme activity.

During the study of location of the sites of leupeptin action, mitochondrial pellets 2 were obtained by washing

of pellets 1 in 8 ml of isolation medium or isolation medium with 50 μM leupeptin. The pellets were suspended in 3 ml of the respective media. Samples (500 μl) of each suspension were taken for the determination of total and bound omNDPK activity in these suspensions. Thirty minutes after pellets 2 were obtained, the remaining suspensions were centrifuged at 12,300 rpm for 10 min to obtain pellets 3. The resulting supernatants were quantitatively transferred to calibrated test tubes, and (1 h after pellets 2 were obtained) concentrated solutions of leupeptin and $MgCl_2$ were added to them. Then the second of these supernatants was added to the first pellet 3, and the first supernatant to the second pellet 3. The pellets were carefully resuspended. During the calculation of leupeptin and $MgCl_2$ additions, volume of the supernatants left in mitochondrial pellets 3 and leupeptin presence in one of them were taken into consideration. Owing to this, the final suspensions of pellets 3 had the same concentrations of leupeptin (50 μM) and $MgCl_2$ (3.33 mM). The total and membrane bound activity of omNDPK in the suspensions was determined as described earlier.

**Study of omNDPK solubilization at various pH values.** Mitochondrial pellets 1 were suspended in 1.8-2.0 ml of isolation medium or isolation medium containing 50 or 75 μM leupeptin. One set of washing media contained 1.8 ml of 10 mM Mes (pH 5.3-7.3) or 10 mM Hepes (pH 7.3-8.6). The ionic strength of these media was adjusted to total $I = 0.01$ by adding KCl, and total osmotic concentration of media was adjusted to 0.28 osM by adding mannitol. Another set of identical washing media contained 50 μM leupeptin. During the study of pH effect on omNDPK solubilization in high ionic strength media, all samples contained 75 μM leupeptin as well as 0.14 M KCl and 1 mM $MgCl_2$ instead of mannitol. Aliquots (0.2 ml) of pellet 1 suspensions in isolation medium or isolation medium with leupeptin were added to the washing media containing the same concentration of leupeptin.

The suspensions were placed into a Thermomixer Comfort (Eppendorf), incubated with periodic shaking at 2°C for 2 h, and centrifuged at 13,400 rpm for 10 min in the MiniSpin Plus centrifuge. The supernatants were used for determination of the pH values. The walls of the centrifuge tubes were carefully dried with filter paper, and mitochondrial pellets 2 were suspended in 100 μl of isolation medium. The activity of omNDPK remaining in the pellets was assayed by the polarographic method. Differences between pH values of the supernatants obtained after sedimentation of the pellets 2 and the initial buffers were usually less than 0.1 pH unit; only at pH > 8.0 the pH of the supernatant was lower than that of the initial buffer by 0.2-0.3 pH unit.

The ionic strength of the washing media was calculated using the equation $I = 1/2(\Sigma C_i Z_i^2)$, where $C_i$ represents concentration of each ion, and $Z_i$ represents the charge of the corresponding ion. The concentrations of ionized forms of buffer components were evaluated using the Henderson–Hasselbach equation.

**Reversal of omNDPK solubilization induced by change in storage medium pH.** Mitochondrial pellet 1 was suspended in 2 ml of isolation medium. Equal portions (0.2 ml) of pellet 1 suspension were added to test tubes containing 4.8 ml of 0.14 M KCl, 1 mM $MgCl_2$, and 10 mM Mes, pH 6.0, or 10 mM Hepes, pH 8.0. Some of the samples at pH 6.0 also contained 10 mg/ml BSA, 50 μM E-64, or both. Half of each suspension at pH 6.0 was used for study of omNDPK solubilization. Two hours after the suspension of pellet 1 was prepared, an amount of 1 M Tris increasing the buffer pH from 6.0 to 8.0 was added to the other half of the suspensions. After selected time intervals, portions in 750 μl were taken from each suspension and centrifuged at 13,400 rpm for 10 min. Pellets 2 were suspended in 35 μl of isolation medium, and the activity of omNDPK remaining in the pellets was assayed by the polarographic method. It was found that pH of the supernatants without added Tris was 6.1-6.2, and after Tris addition it was 8.4-8.7.

**Mitochondrial respiration.** The rate of mitochondrial oxygen consumption was determined at 22°C using 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 $MgCl_2$, and 5 mM potassium succinate. Other additions are given in the text and legends to the figures. Oxygen concentration in the main medium was assumed to be 290 μM at 22°C [47].

**Polarographic assay of omNDPK activity.** The main incubation medium also contained 300 μM ATP and 20 μM of the adenylate kinase inhibitor AP5A ($p^1p^5$-di(adenosine-5′)pentaphosphate). The activity of omNDPK was characterized as the ratio of phosphorylating respiration rate in the presence of 360 μM UDP to the phosphorylating respiration rate which 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 the addition of ADP and the rate after its phosphorylation (Fig. 1). The rate of phosphorylating respiration after the subsequent addition of UDP ($V^{UDP}$) was determined by the difference between the respiration rates after and before addition of UDP (Fig. 1a). We assumed that ADP/O ratio remained unchanged in one polarographic sample, and so the ratio $V^{UDP}/V^{ADP}$ 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. Where indicated, 600 μM CDP was used instead of UDP. ATP concentration in these samples was 1 mM.

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. 1), is a sensitive index of mitochondria functional capacities and their structural integrity [48-50]. In our experiments, RCR values during the entire experiment were at the level of 4.5-6.5.

Mitochondrial protein content was determined by the method of Gornall et al. [51] using BSA as a standard.

## RESULTS

We utilized a polarographic method to assay omNDPK activity [48, 49]. Figure 1 presents typical polarograms showing the effects of ADP, UDP, or CDP on the rate of mitochondrial respiration. ADP added to respiring mitochondria stimulated oxidative phosphorylation of this nucleotide. The affinity of the oxidative phosphorylation system for ADP is very high ($K_m$ ~ 20 μM) [48]. During oxidative phosphorylation an equimolar amount of ATP is formed. On a subsequent addition of UDP or CDP, the respiration was again stimulated as a result of ADP generation in the reaction catalyzed by omNDPK: UDP (CDP) + ATP → UTP (CTP) + ADP. It was demonstrated earlier that in the presence of ATP stimulation of mitochondrial respiration after the addition of non-adenine nucleoside diphosphates is only due to the activity of omNDPK, and the rate of phosphorylating respiration is proportional to the activity of this enzyme [32, 52]. Consequently, using the rate of mitochondrial respiration after UDP (CDP) addition, it is possible to calculate the rate of oxidative phosphorylation of ADP and the rate of UTP (CTP) production equal to it.

We characterized omNDPK activity as a $V^{UDP(CDP)}/V^{ADP}$ ratio. This ratio does not depend on the concentration of mitochondrial protein in the samples. The presentation of the results in the form of this ratio made it possible to compare data obtained in different experiments even if protein content in some mitochondrial suspensions was too low to be accurately measured. This approach was justified as we demonstrated in the previous study [31] that different conditions of mitochondria isolation and storage used in our study did not affect on the rate of oxidative phosphorylation. The omNDPK activity can be calculated from the rates of oxidative phosphorylation multiplied by the $V^{UDP(CDP)}/V^{ADP}$ ratio. In our experiments, the mean value of the rate of oxidative phosphorylation in the presence of 170 μM ADP was 298 ± 28 nmol ADP/min per mg ($n$ = 6).

**Reversal of omNDPK solubilization from the outer mitochondrial membrane by addition of $Mg^{2+}$.** In previous studies, we demonstrated that in the outer compartment of rat liver mitochondria the entire activity of omNDPK is associated with the outer surface of the outer membrane [30, 31]. We have also shown that the rate of omNDPK solubilization from the outer mitochondrial membrane (reflecting the tightness of its binding to the membranes) depended on conditions of storage of the mitochondrial preparation [31]. We suggested that various rates of the enzyme solubilization were due to the effects produced by mitochondrial storage media. These effects were produced only during storage of mitochondria, 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. If our suggestion is correct, then omNDPK solubilization could be possibly reversed by changes in the composition of storage medium. Earlier, we demonstrated [31] that omNDPK was fairly rapidly solubilized from mitochondria kept in medium with low ionic strength, but in the same medium containing additionally 3.33 mM $MgCl_2$ the enzyme remained associated with membranes during 5 h storage of the mitochondria on ice. We suggested that omNDPK solubilization in low ionic strength medium could be reversed by the addition of $MgCl_2$ to the mitochondrial suspension.

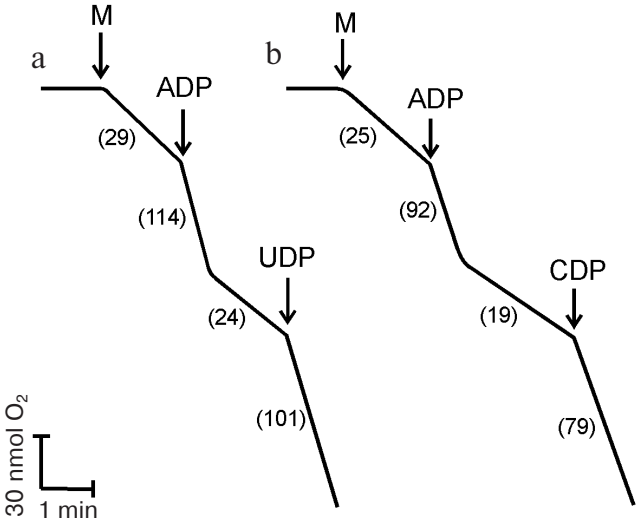


**Fig. 1.** Polarographic method for measuring omNDPK activity. The main incubation medium also contained 300 μM (a) or 1 mM (b) ATP. The reaction was initiated by the addition of 0.9 mg of mitochondrial protein (M). Where indicated, 170 μM ADP, 360 μM UDP, or 600 μM CDP were added to the closed reaction vessel (total volume 0.93 ml). Respiratory rates (in brackets) are expressed as nmol of oxygen consumed/min per mg mitochondrial protein.

Figure 2 shows that during storage of mitochondria in low ionic strength medium the proportion of the membrane-associated omNDPK activity declines (Fig. 2, curve *4*). Thirty minutes after the suspension of the pellet 2 was obtained, concentrated solution of $MgCl_2$ was added to this suspension up to its final concentration 3.33 mM. We observed rapid increase in the membrane-

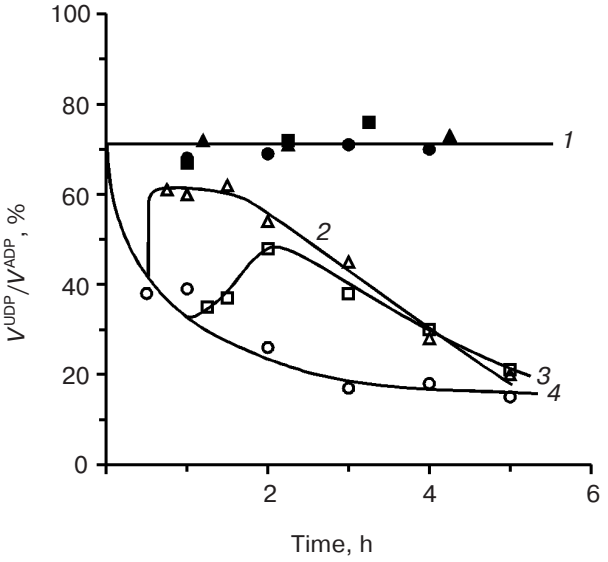


**Fig. 2.** Reversal of omNDPK solubilization by the addition of 3.33 mM $MgCl_2$. Circles, mitochondrial pellet 2 without additions; triangles and squares, $MgCl_2$ was added, respectively, 30 or 60 min after the suspension of pellet 2 was obtained. *1*) Relative total omNDPK activity; *2-4*) membrane-associated activity. Other details are given in "Materials and Methods" section.

associated enzyme activity; however, later omNDPK was solubilized again (Fig. 2, curve *2*). $MgCl_2$ added to the suspension after 60 min reversed solubilization at a slower rate, only slightly, and for a short time (Fig. 2, curve *3*).

It is well known that mitochondrial preparations are always contaminated with lysosomes. The latter are rich in proteolytic enzymes, cathepsins, which belong to the class of cysteine proteinases [53, 54]. We assumed that incomplete reversal of omNDPK solubilization was due to the activity of these enzymes damaging either omNDPK or sites of its binding (or both). To test this assumption, we added to the mitochondrial washing and storage media an inhibitor of cathepsins, leupeptin [53]. $MgCl_2$ added to the mitochondrial suspension after 60 min storage in a medium with low ionic strength in the presence of 50 μM leupeptin reversed omNDPK solubilization almost completely, and the extent of the reversal remained unchanged over time (Fig. 3, curve *2*).

To elucidate whether cathepsin action was directed at solubilized omNDPK, or at omNDPK-free sites of its binding, or both, the following experiments were performed (Fig. 4). In these experiments (Fig. 4a), after the addition of supernatants with leupeptin and $Mg^{2+}$, the suspensions of pellets 3(I) and 3(II) did not differ in composition, but they did differ in their history. In the first suspension, solubilized omNDPK present in the supernatant 3(II) was protected by leupeptin against the action of cathepsins from the very beginning, but the sites of its binding on the pellet 3(I) were accessible for proteolysis for 1 h. On the contrary, in the second suspension, in pellet 3(II) the sites of omNDPK binding on the outer membrane were protected against the action of cathepsins all the time; in the added supernatant 3(I) omNDPK was not protected against the action of cathepsins for 1 h (until leupeptin was added). If the action of cathepsins was mainly or only directed at solubilized omNDPK, then we would not observe reversal of solubilization in the second suspension, but if cathepsins only damaged sites of omNDPK binding, then reversal of the enzyme solubilization would be absent in the first suspension.

Figure 4b (curve *3*) shows that during 1 h in the absence of leupeptin cathepsins completely abrogated ability of solubilized omNDPK to reassociate with mitochondria kept in the presence of leupeptin (there is no augmentation of omNDPK activity in the mitochondrial pellet in the presence of $Mg^{2+}$). At the same time, adding of $Mg^{2+}$ to this suspension prevented further enzyme solubilization. Figure 4b (curve *2*) shows that if cathepsins acted on mitochondrial membranes, then in the presence of $Mg^{2+}$ insignificant rebinding of solubilized omNDPK from the extract kept in the presence of leupeptin was observed. However, this omNDPK was gradually solubilized again. As in the case of curve *3*, adding of $Mg^{2+}$ prevented further solubilization of omNDPK that was still associated with the outer membrane at the moment of cation addition (curves *2* and *3* finally meet). We concluded that cathepsins damaged both solubilized omNDPK and the sites of its binding, though the effect

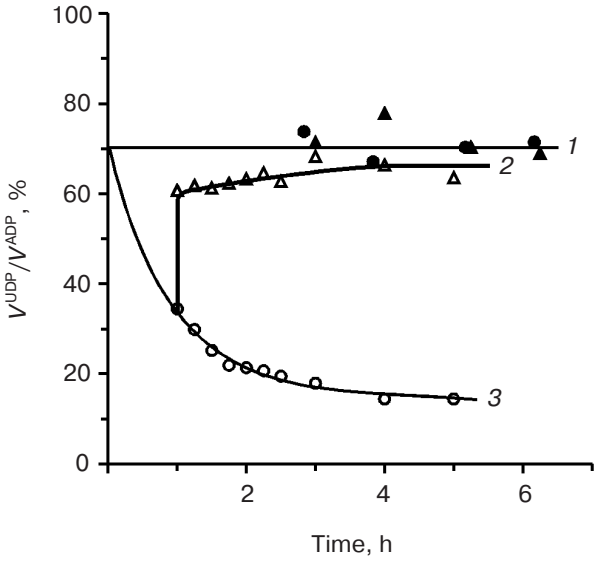


**Fig. 3.** Effect of leupeptin on the reversal of omNDPK solubilization by the addition of $Mg^{2+}$. Mitochondrial pellets 2 were suspended in 3 ml of isolation medium (circles) or isolation medium containing 50 μM leupeptin (triangles). One hour after the pellet 2 was obtained, 3.33 mM $MgCl_2$ was added to the suspension containing leupeptin. *1*) Relative total omNDPK activity; *2*, *3*) membrane-associated activity. Data represent one (of three) typical experiment. Other details are given in the "Materials and Methods" section.

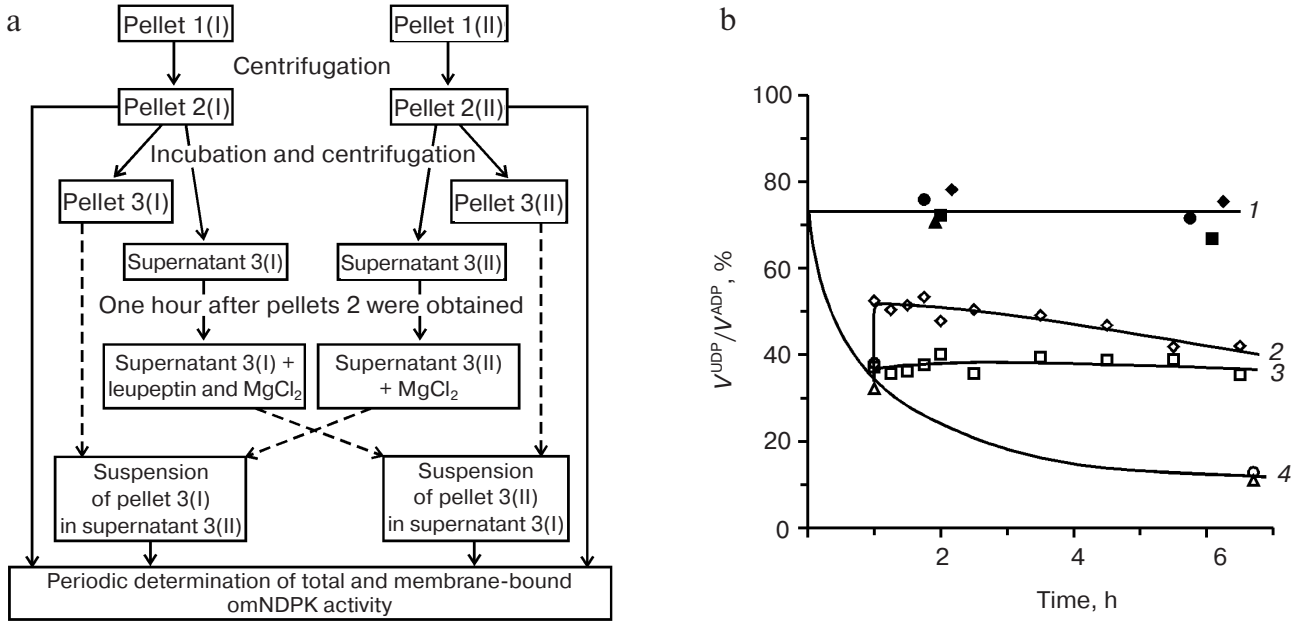


**Fig. 4.** a) Scheme of experiments on determination of localization of sites of cathepsin action. I and II, suspensions of mitochondrial pellets and supernatants contained, respectively, isolation medium or isolation medium with 50 μM leupeptin. b) Determination of localization of the sites of cathepsin action. *1*) Relative total omNDPK activity; *2-4*) membrane-associated activity. Circles, suspension of pellet 2(I); triangles, pellet 2(II); squares, suspension of pellet 3(II) in the supernatant 3(I); rhombs, pellet 3(I) in the supernatant 3(II). Designation of fractions as in Fig. 4a. Data represent results of one (of two) typical experiment. Other details are given in the "Materials and Methods" section.

on omNDPK was relatively more potent. Figure 4b also shows that presence of 50 μM leupeptin during mitochondria washing and storage in low ionic strength medium did not affect the rate of the enzyme solubilization (Fig. 4b, curve *4*) or its total activity (solubilized and membrane-associated) (Fig. 4b, curve *1*).

**Reversal of omNDPK solubilization induced by acidification of the storage medium.** We found that tightness of omNDPK binding to mitochondrial membranes depends on the pH of the storage medium and declines with medium acidification (Fig. 5a, curve *3*). It is known that cathepsins are most active at acidic pH values [54, 55]. However, inclusion of 50 μM leupeptin in mitochondrial suspensions kept at various pH values did not change the dependence of the enzyme solubilization on the pH of the storage medium (Fig. 5a, curve *2*).

The decrease in omNDPK activity in mitochondrial pellets kept in low pH medium was not caused by the enzyme inactivation at acidic pH (Fig. 5b). In this experiment, a concentrated suspension of mitochondrial pellet 2 was stored in buffer at pH 6.6. After various time intervals, we determined total omNDPK activity in the mitochondrial suspension (Fig. 5b, curve *1*) as well as the proportion of the enzyme activity associated with membranes at this time interval (Fig. 5b, curve *2*). Figure 5b shows that though a proportion of the membrane-associated activity decreased with the increase in mitochondria storage time, total omNDPK activity (membrane-bound and solubilized) remained unchanged.

As at pH 7.4 (Fig. 3), in the presence of leupeptin $Mg^{2+}$ reversed omNDPK solubilization caused by storage of mitochondria in low ionic strength medium at pH 6.6 (data not shown). However, as expected, 3.33 mM $MgCl_2$ did not reverse omNDPK solubilization at the same pH value in medium containing 0.14 M KCl (data not shown). During storage of mitochondria under conditions close to the physiological ones in respect to ionic strength as well as free $Mg^{2+}$ concentration [33], in the presence of leupeptin, the pH dependence of omNDPK solubilization shifted to the left, to more acidic pH values (Fig. 5a, curve *1*).

The solubilization of omNDPK at an acidic pH in high ionic strength medium was reversed after the increase in the pH of mitochondrial storage medium under conditions preventing cathepsin effects on the solubilized enzyme. In the experiment (Fig. 6), to inhibit cathepsins, we used irreversible cysteine proteinase inhibitor E-64 [53] bearing zero total charge on its molecule. BSA (10 mg/ml) was added to some samples as a nonspecific substrate for cathepsins. We assumed that in the presence of significant BSA excess over the omNDPK protein, the rate of omNDPK proteolysis would be decreased. Figure 6 (curve *5*) shows that neither E-64, nor BSA, nor a mixture of both affected the rate of omNDPK solubilization. After 2 h storage of mitochondria at pH 6.0, a concentrated Tris solution was added to all samples. The amount of Tris necessary to adjust pH of the storage medium to 8.0 was determined in a prelimi-

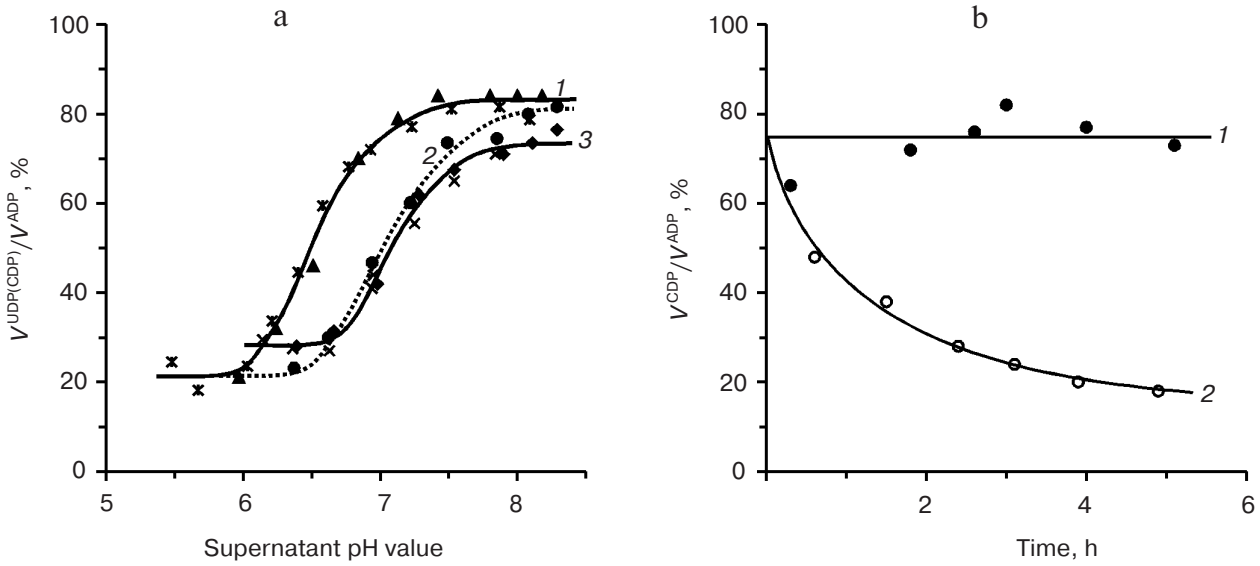


**Fig. 5.** a) Effect of pH of mitochondria washing and storage medium on omNDPK solubilization. *1*) High ionic strength medium; *2*, *3*) low ionic strength medium with or without leupeptin, respectively. *1*, *2*) Substrate UDP; *3*) substrate CDP. Various symbols on each curve show experimental points obtained in independent experiments. b) Dependence of omNDPK solubilization on the time of storage of mitochondria. Mitochondrial pellet 1 was washed (2.7 ml per gram of liver) in medium containing 1.8 mM KCl, 0.26 M mannitol, and 10 mM Mes, pH 6.6. Mitochondrial pellet 2 was kept in the same medium (protein concentration ~ 60 mg/ml). *1*) Relative omNDPK activity in the initial suspension of pellet 2; *2*) after re-sedimentation of mitochondria in the same medium. Other details are given in the "Materials and Methods" section.

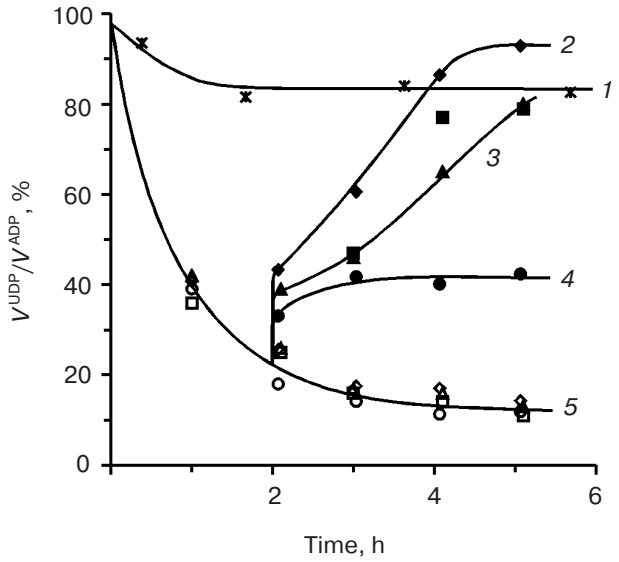


**Fig. 6.** Reversal of omNDPK solubilization induced by change in pH of the storage medium. Portions (0.2 ml) of the mitochondrial pellet 1 suspension were added to 4.8 ml of media containing 0.14 M KCl, 1 mM $MgCl_2$, 10 mM Mes, pH 6.0, as well as 10 mg/ml BSA (open triangles), 50 μM E-64 (open squares), BSA and E-64 (open rhombs), or instead of 10 mM Mes 10 mM Hepes, pH 8.0, was added (asterisks); without additions (open circles). Two hours after pellet 1 was obtained, Tris was added to some samples at pH 6.0 (closed symbols). *1*) Relative total omNDPK activity; *2-5*) membrane-associated activity. Other details are given in the "Materials and Methods" section.

nary experiment. Figure 6 (curve *4*) shows that in the absence of E-64 as well as BSA, increase in mitochondrial storage medium pH caused only insignificant reassociation of omNDPK but stopped its further solubilization. E-64 or BSA added separately reversed omNDPK solubilization more effectively and to the same extent (Fig. 6, curve *3*). Figure 6 (curve *2*) shows that E-64 and BSA added simultaneously reversed the enzyme solubilization up to the level of the initial omNDPK activity at pH 8.0 (curve *1*).

## DISCUSSION

Thus, it has been demonstrated that solubilization of omNDPK not involved in functional coupling to oxidative phosphorylation can be reversed. In our experiments, a compulsory condition of the complete reversal of solubilization was the presence of cathepsin inhibitors, leupeptin or E-64, in the mitochondrial washing and storage medium. Since leupeptin (Fig. 4b) and E-64 (Fig. 6) did not influence the rate of omNDPK solubilization, we concluded that the enzyme becomes sensitive to proteolysis only after its solubilization. In the absence of leupeptin, cathepsins also damaged not occupied by omNDPK sites of omNDPK binding on the membranes (Fig. 4b). Sensitivity of the sites of omNDPK binding to proteolysis serves as indirect evidence that on mitochon-

drial membranes omNDPK interacts with protein molecules. It was also demonstrated that total activity of solubilized and membrane-associated omNDPK does not depend on the presence of leupeptin (Figs. 3 and 4b) or E-64 (Fig. 6). It is reasonable to suggest that proteolysis does not damage a site of the enzyme molecule responsible for catalysis. Similar results were obtained during the study of cathepsin effects on the activity and solubilization of HK [54, 55].

It was found that the rate of omNDPK solubilization depends on the pH of the storage medium and increases with decrease in medium pH (Fig. 5a). The pH effect was not due to higher cathepsin activity in acidic medium [55] (Fig. 5a, curves *2* and *3*) or to omNDPK inactivation at acidic pH (Fig. 5b). Figure 5a shows that pH decrease did not cause the complete solubilization of the enzyme. Under physiological concentrations of $K^+$ and $Mg^{2+}$, 50% of subject to solubilization omNDPK activity was solubilized at pH 6.5 (Fig. 5a, curve *1*).

Earlier we showed that with increase in ionic strength of mitochondrial storage medium, the tightness of omNDPK association with the outer membrane increases [31]. We concluded that the enzyme binding to the membranes is mostly due to non-ionic interactions. How can we balance the idea about non-ionic interactions and the enzyme sensitivity to the pH of the storage medium? It can be speculated that a functional group exists in the region of omNDPK interaction with mitochondrial membranes. The extent of ionization of this group changes with pH in such a way that it is not charged at alkaline pH values, but it is ionized at acidic pH. The ionization of the group weakens hydrophobic interactions between omNDPK and the sites of its binding, thus generating the enzyme solubilization. The imidazole ring of histidine exhibits such properties. However, the pH effect might be indirect and caused by the influence of pH on the environment of the binding sites. Figure 5a (curve *1*) shows that in the high ionic strength medium the curve of omNDPK solubilization shifts to more acidic pH values. This effect might be caused by the increasing effect of ionic strength on the tightness of omNDPK association with the membranes.

Reversibility of omNDPK solubilization supports the suggestion that a similar process could also occur *in vivo*. In our experiments, $Mg^{2+}$ reversed solubilization that occurred in low ionic strength medium (Fig. 3), but it was ineffective in high ionic strength medium close to the ionic strength of cytoplasm. Therefore, $Mg^{2+}$ cannot be considered as a physiological regulator of omNDPK solubilization. At the same time, it is known that many physiological processes (for example, intensive muscle work, starvation, hypoxia, ischemia, diabetes) are accompanied with acidosis [57-62]. It is accepted that the pH of rat liver cell cytoplasm is 7.1-7.2 [56, 63-66]. Figure 5a (curve *1*) shows that under physiological concentrations of $K^+$ and $Mg^{2+}$, binding of omNDPK with mitochondrial membranes at the indicated pH values is close to the maximal magnitude. Owing to an activation of the physiological mechanisms regulating intracellular pH [67, 68], decrease in intracellular pH caused by physiological acidosis is relatively small, to pH 7.0-6.7 [58, 64, 66]. Decrease in intracellular pH to 6.8-6.7 could cause solubilization of only an insignificant proportion of omNDPK activity (Fig. 5a, curve *1*).

However, it should be taken into consideration that measurement of cytoplasm pH meets significant difficulties [56, 69]. Available data show that the pH of cytoplasm is much lower than the pH of the mitochondrial matrix [56, 70]. Meanwhile, compounds used for the determination of cytoplasmic pH (DMO (5,5-dimethyl-2,4-oxazolidinedione), $CO_2$) can penetrate mitochondria; therefore, the results obtained represent some mean values [56]. The same is true for the $^{31}$P-NMR method. Thus, for example, it was demonstrated that during diabetic ketoacidosis the pH of cytoplasm of rat liver cells located in the periportal region is 0.5 unit lower than the mean value determined by the $^{31}$P-NMR method [61]. In this connection, some authors [56] believe that the actual pH of the cell cytoplasm is lower than it is generally assumed to be. If that is the case, then it is possible that changes in intracellular pH could effectively change the proportion of omNDPK molecules associated with the outer membrane and this way affect processes in which this enzyme is involved. Besides, the influence of local acidification in the surrounding of the outer mitochondrial membrane cannot be excluded [71-73] if omNDPK is located close to enzymes catalyzing $H^+$-producing reactions.

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