ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 8, pp. 835-842. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 8, pp. 1027-1036.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-031, May 27, 2007.

# Investigation of Chimerical and Tagged Forms of Recombinant Rat Nucleoside Diphosphate Kinases α and β. Interaction with Rhodopsin–Transducin Complex and Thermal Stability

**N. Ya. Orlov[1,2]*, Y. Ishijima[1], D. N. Orlov[2], T. G. Orlova[1,2], E. A. Burstein[2], and N. Kimura[1]**

[1]*Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan; fax: (8133)579-4776; E-mail: nkimura@tmig.or.jp*
[2]*Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia; fax: (496)733-0553; E-mail: orlov@iteb.ru*

Received February 5, 2007
Revision received April 19, 2007

**Abstract**—To elucidate the physicochemical basis of differences between the isoforms of mammalian multifunctional nucleoside diphosphate kinase (NDP), we investigated the recombinant rat homohexameric NDP kinases α and β, consisting of highly homologous α or β subunits of 152 residues each and differing only in variable regions V1 and V2, and their chimerical forms (NDP kinase $\alpha^{1\text{-}130}\beta^{131\text{-}152}$ and NDP kinase $\beta^{1\text{-}130}\alpha^{131\text{-}152}$) and tagged derivatives (NDP kinase HA-$\alpha^{1\text{-}130}\beta^{131\text{-}152}$, NDP kinase HA-$\beta^{1\text{-}130}\alpha^{131\text{-}152}$, and NDP kinase HA-β). The thermal stability of these proteins and the ability of some of them to interact with the rhodopsin–transducin (R*Gt) complex have been studied. It was found that NDP kinase α, NDP kinase $\alpha^{1\text{-}130}\beta^{131\text{-}152}$, and NDP kinase HA-$\alpha^{1\text{-}130}\beta^{131\text{-}152}$ were similar in their thermal stability ($T_{1/2}$ = 61-63°C). NDP kinase β, NDP kinase $\beta^{1\text{-}130}\alpha^{131\text{-}152}$, NDP kinase HA-$\beta^{1\text{-}130}\alpha^{131\text{-}152}$, and NDP kinase HA-β were inactivated at a lower temperature ($T_{1/2}$ = 51-54°C). NDP kinase HA-$\alpha^{1\text{-}130}\beta^{131\text{-}152}$ interacted with the R*Gt complex in the same manner as NDP kinase α, whereas the interaction of NDP kinase HA-$\beta^{1\text{-}130}\alpha^{131\text{-}152}$ and NDP kinase β with the photoreceptor membranes under the same conditions was very weak. It is suggested that the variability of the region V1 is a structural basis for the multifunctionality of NDP kinase hexamers in the cell.

**DOI**: 10.1134/S0006297907080044

*Key words*: nucleoside diphosphate kinase, G protein transducin, thermal stability of proteins

Until recently, it was accepted that the only function of nucleoside diphosphate kinase (NDP kinase, EC 2.7.4.6) is the transfer of γ-phosphate from nucleoside triphosphates to nucleoside diphosphates [1]. However, during the last decade, NDP kinase has been identified as a tumor metastasis suppressor (nm23), a morphological regulator (Awd), a transcription factor (PuF), and a differentiation inhibitor (I-factor) (for review, see [2]). These results and some other data (for review, see [2-10]) led to the notion that NDP kinases are most likely multifunctional proteins and may be involved in different cellular regulatory pathways.

NDP kinases are typically hexamers of small subunits with a molecular mass of about 17 kD. In contrast to the enzymes of lower organisms, the NDP kinase hexamers of mammals contain two sorts of randomly associated subunits of 152 residues each and, thus, exist as a set of NDP kinase isoforms [11, 12]. The subunits are encoded by two independent genes but show remarkable similarity (90%) in their primary structure, all differences being due to the variable regions V1 (residues 37-50, seven substitutions) and V2 (residues 131-150, six substitutions). Moreover, the mammalian NDP kinase isoforms are remarkably similar in their catalytic properties, mode of hexamer organization, nucleoside binding site composi-

*Abbreviations*: Gt) transducin; GTPγS) guanosine 5′-O-(3-thio)triphosphate; NDP kinase) nucleoside diphosphate kinase; NDP kinase α and β) α and β isoforms of recombinant rat NDP kinase; NDP kinase $\alpha^{1\text{-}130}\beta^{131\text{-}152}$) a chimerical form consisting of the N-terminal part (residues 1-130) of NDP kinase α and the C-terminal part (residues 131-152) of NDP kinase β; NDP kinase $\beta^{1\text{-}130}\alpha^{131\text{-}152}$) a chimerical form consisting of the N-terminal part (residues 1-130) of NDP kinase β and the C-terminal part (residues 131-152) of NDP kinase α; NDP kinase HA-β, NDP kinase HA-$\alpha^{1\text{-}130}\beta^{131\text{-}152}$, and NDP kinase HA-$\beta^{1\text{-}130}\alpha^{131\text{-}152}$) tagged proteins containing the hemagglutinin (HA) epitope (YPYDVPDYA) between Ala2 and Asp3; N-membranes) NDP kinase-depleted Gt-containing retinal ROS photoreceptor membranes; R) rhodopsin; R*) photoactivated rhodopsin; R*Gt complex) a complex between R* and Gt in retinal ROS photoreceptor membranes; ROS) rod outer segments.

* To whom correspondence should be addressed.

tion, and three-dimensional structures (for review, see [13]). The physiological significance of existence in the mammalian cells of NDP kinase isoforms with similar catalytic and structural properties is as yet not completely understood in spite of the efforts of many laboratories.

To explain the physiological meaning of the existence of NDP kinases as isoforms in mammalian cells as well as the multifunctional role of these enzymes, it has been suggested [3-7, 14, 15] that targets for the regulatory action of NDP kinases might be different GTP-binding proteins including heterotrimeric GTP-binding proteins (G proteins), which mediate the information flow in signal transduction systems [16, 17]. In accord with this suggestion, experiments carried out in the past 15 years using different methods and approaches showed that: 1) β-subunits of the vertebrate photoreceptor G protein transducin (Gt) and of G proteins of different membranes are phosphorylated on one of their histidine residues (His266 of Gt β-subunit) [18-26] by an endogenous enzyme whose role is played by NDP kinase, which works here as histidine kinase [21, 25, 26]; 2) βγ-subunits of Gt and human NDP kinase B form a complex [24]; 3) there is indication that phosphate is transferred from the phosphorylated β-subunit to GDP in the active site of the G protein α-subunit [20, 27-31]; 4) human recombinant NDP kinase NM23-H1 interacts with small GTPases (Ras, Ral, Gem) and phosphorylates GDP covalently attached to their active sites by UV-induced cross-linking [32].

We examined the suggestion about a possible coupling between NDP kinase and G protein using vertebrate retinal rod outer segments (ROS) [14, 33]. ROS are very suitable for such kind of investigation. In fact, this highly specialized part of the retinal rod contains the membrane light receptor rhodopsin (R, 37 kD) and the G-protein transducin (80 kD) at extremely high concentrations (~5 and ~0.5 mM, respectively). These two components of the phototransduction system constitute most ROS protein (~80%). Moreover, R-containing photoreceptor membranes may be used as a sorbent that interacts specifically with some components of the phototransduction system (Gt, arrestin, rhodopsin kinase, etc.) [34]. Specifically, photoactivated rhodopsin (R*) forms a functional membrane complex with Gt (R*Gt complex), which in the presence of micromolar concentrations of GTP and relative compounds, dissociates under certain conditions into R* localized in membranes and soluble Gt [34]. These properties allows one to easily obtain highly purified Gt, to regulate the concentration of R*Gt complexes in photoreceptor membranes, and are widely used in studying molecular mechanisms of phototransduction. Also, ROS contain NDP kinase [35], whose concentration is relatively high (~10 μM) [14]. An essential function of NDP kinase in ROS is phosphorylation of GDP to GTP, which is crucial for providing the functional cycle of transducin independently of its activation mechanism, and for the synthesis of the mediator cGMP by ROS guanylate cyclase [36]. The donor in this reaction is ATP that is produced in the retinal rod inner segment by mitochondria.

The use of bovine retinal ROS preparations showed [14] that water-soluble endogenous NDP kinase does not bind to Gt until the functional membrane R*Gt complex is formed, and it is released when this complex dissociates in the presence of GTP or its analogs. Transducin is an obligatory participant of the interaction process: removal of Gt from photoreceptor membranes blocks the binding, whereas addition of Gt restores it [14]. The interaction is highly specific—the $K_d$ value for the interaction between NDP kinase and the R*Gt complex was estimated to be about 5 nM [14]. Thus, our results support the suggestion that there is a functionally important interaction between NDP kinase and Gt within the R*Gt complex.

In our further work, we used bovine ROS photoreceptor membranes free of endogenous NDP kinase to study the interaction between the R*Gt complex and recombinant homohexameric rat NDP kinases α and β (NDP kinases α and β), each containing only one sort of subunits (α or β). It was shown that these α- and β-isoforms are quite different in their interaction with the R*Gt complex [33]: the interaction of NDP kinase α with the R*Gt complex was as high as that of the endogenous ROS NDP kinase ($K_d$ ~ 5 nM), whereas the affinity of NDP kinase β for the R*Gt complex was about 100 times lower. In our parallel investigations carried out by using intrinsic protein fluorescence, it was shown too that isolated NDP kinases α and β even though they had the same fluorophore positions (Trp78, -133, and -149, Tyr52, -67, -147, and -151), were quite different in intrinsic protein fluorescence characteristics (quantum yield, $q$; spectral maximum position, $\lambda_{max}$; shape and multicomponent character of spectrum, and availability of fluorophores for quenchers) in the solution at pH 8.0 [37]. Moreover, NDP kinases α and β were shown to be very different in their conformational mobility: unlike NDP kinase β, NDP kinase α exists in solution in two different structural states, the equilibrium between these states being under the control of several factors, pH in particular [37, 38]. Also, it was shown that one of these states has a high affinity ($K_d$ ~ 5 nM) for the R*Gt complex [33, 38]. Thus, our studies helped us to find experimental approaches to reveal differences not known earlier in the behavior of NDP kinase isoforms.

The approaches described above were used to investigate the physicochemical basis of differences between NDP kinase isoforms. The detailed studies of the chimerical forms of recombinant rat NDP kinases (NDP kinase $\alpha^{1\text{-}130}\beta^{131\text{-}152}$ and NDP kinase $\beta^{1\text{-}130}\alpha^{131\text{-}152}$) and their tag-derivatives containing hemagglutinin (HA) epitope (YPYDVPDYA) between Ala2 and Asp3 (NDP kinase HA-$\alpha^{1\text{-}130}\beta^{131\text{-}152}$ and NDP kinase HA-$\beta^{1\text{-}130}\alpha^{131\text{-}152}$) by intrinsic protein fluorescence allowed us to suggest that the dif-

ferences in the fluorescence properties and structural mobility of NDP kinases α and β in solution may be due to differences in their variable region V1 [39].

For further analysis of the physicochemical basis of the differences between the isoforms, we studied in this work the thermal stability of recombinant rat NDP kinases α and β, the chimerical enzymes NDP kinase $\alpha^{1\text{-}130}\beta^{131\text{-}152}$ and NDP kinase $\beta^{1\text{-}130}\alpha^{131\text{-}152}$, and the tagged enzymes NDP kinase HA-β, NDP kinase HA-$\alpha^{1\text{-}130}\beta^{131\text{-}152}$, and NDP kinase HA-$\beta^{1\text{-}130}\alpha^{131\text{-}152}$ obtained as described earlier [40]. The interaction of NDP kinase HA-$\alpha^{1\text{-}130}\beta^{131\text{-}152}$ and NDP kinase HA-$\beta^{1\text{-}130}\alpha^{131\text{-}152}$ with the R*Gt complex in the bovine retinal ROS photoreceptor membranes was also investigated. The binding properties of NDP kinase HA-$\alpha^{1\text{-}130}\beta^{131\text{-}152}$ were shown to be similar to those described for NDP kinase α, whereas the interaction of NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ with the R*Gt complex under the same conditions (pH 5.5, low ionic strength) was very weak, as previously observed for NDP kinase β [33]. It was found that NDP kinase α, NDP kinase $\alpha^{1-130}\beta^{131-152}$, and NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ were similar in their thermal stability. Half-inactivation of these enzymes was observed at 61-63°C. NDP kinase β, NDP kinase $\beta^{1-130}\alpha^{131-152}$, NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$, and NDP kinase HA-β were inactivated at lower temperatures (51-54°C). The results point to different roles of the variable regions V1 and V2 of NDP kinase in the interaction with the R*Gt complex and in the maintenance of thermal stability, and, in general, support the suggestion about the principal importance of the region V1 for realization of the multifunctional role of this enzyme in the cell.

Preliminary reports of this study were presented at the 5th International Congress of the Genetics, Biochemistry and Physiology of NDP Kinase/NM23/AWD (Lexington, KY, USA, October 13-16, 2003), at the Symposium "Biological Motility" dedicated to the memory of academician G. M. Frank (1904-1976) (Pushchino, May 23-June 1, 2004), and at the III Congress of Biophysicists of Russia (Voronezh, June 24-29, 2004).

## MATERIALS AND METHODS

**Preparation and purification of recombinant rat NDP kinases α and β and their derivatives.** Recombinant rat NDP kinases α and β and their chimerical and tagged forms were expressed in *E. coli* BL21(D3) pLysS competent cells as previously described [40]. The recombinant NDP kinases were recovered from a high-speed supernatant fraction obtained after lysis of transfected cells [21] and purified by one-step affinity chromatography using an ATP-Sepharose (Sigma, USA) column [40, 41]. All proteins were stored at −80°C in concentrations of 1-2 mg/ml. All preparations were almost homogeneous, which was confirmed by SDS-PAGE [42].

**Estimation of NDP kinase activity and content.** NDP kinase activity was measured photometrically at 30°C by the coupling enzyme method via estimation of the rate of ADP formation from ATP in the phosphorylation of TDP by NDP kinase [33] and expressed as the amount of formed ADP per minute. Buffers and salts used did not interfere with the NDP kinase activity measurements. Assays were done in duplicate and agreed within 4%.

NDP kinase protein content was determined by SDS-PAGE [42] and Western blotting with affinity-purified polyclonal anti-rat-NDP-kinase antibodies NK2, which reacted with bovine enzyme as well [12, 33]. Since NDP kinase activity and NDP kinase protein amount were shown to parallel each other, the enzyme activity was monitored in most experiments.

**Preparations of Gt-containing, NDP kinase-depleted bovine retinal ROS photoreceptor membranes (N-membranes).** N-Membranes were prepared by repeated washing of partially bleached (R*/R = 0.2-0.3) bovine retinal ROS preparations [43] in neutral buffer N (10 mM Tris-HCl, pH 7.5, 0.25 mM $MgCl_2$) as described earlier [33]. SDS-PAGE revealed that 75-80% of the protein in the N-membranes was constituted by rhodopsin. The content of NDP kinase (about two copies of NDP kinase per 10,000 molecules of R) and its specific enzyme activity (0.1-0.15 μmol ADP/min per mg of total protein) were 7-10% of the values shown for the ROS preparations [14, 33]. The preparations contained six-seven Gt copies per 100 molecules of R (ca. 80-90% of those in ROS preparations [34]), as determined by SDS-PAGE followed by Western blotting with an antibody against G-protein α-subunits (DuPont/NEN, USA). The membranes ([R] ≈ 5 mg/ml) were stored in the dark in neutral buffer N at −80°C. The membranes were totally bleached with orange light for 10 min at 0°C before use.

**Binding of recombinant derivatives of rat NDP kinases with N-membranes.** The binding of recombinant rat NDP kinases with the R*Gt complex of bovine retinal ROS was carried out as described earlier [33], using high speed centrifugation for separation of free and bound forms of the enzyme. Most experiments were carried out at a standard low photoreceptor membrane concentration ([R*] ≈ 1 μM). Briefly, N-membranes were mixed with NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ or NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ (final enzyme concentrations were 15-20 nM) at pH 5.5-8.0 in appropriate buffers (5 mM Hepes-NaOH or Mes-NaOH, 0.25 mM $MgCl_2$) and incubated for 5 min at 0°C. In some experiments, the samples contained 1-300 mM of salts (NaCl, KCl, $MgCl_2$, or $CaCl_2$) or 0.01-5 μM GTPγS (guanosine 5′-O-(3-thio)triphosphate). The samples were centrifuged at 100,000*g* for 10 min (TL-100 ultracentrifuge; Beckman, USA) at 2°C and the supernatants and pellets obtained were used for measurements of the activity of free and bound NDP kinase, respectively. In some special experiments, the membrane concentration was varied over a

wide range ([R*] ≈ 0.005-10 μM); in these cases, the enzymes were added at a concentration of about 2 nM. Since the sum of activities of free and bound forms of the enzyme was shown to be equal to the activity of the NDP kinase added to the membranes, only the activity of supernatants was usually measured. In accordance with our previous data [33], the distribution of the NDP kinase activity between supernatants and pellets paralleled that for NDP kinase protein.

NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ alone neither sedimented on centrifugation at 100,000*g* for 30 min, nor irreversibly changed its activity in the presence of any buffer and salt used. The NDP kinase activity of N-membranes was as low as 0.2-0.3% of the activity of recombinant rat NDP kinases added to the suspensions.

**Measurements of thermal stability of recombinant NDP kinases α and β and their derivatives.** The thermal stability of rat recombinant NDP kinases was determined, as previously described [43], by measuring the remaining enzyme activity of samples after their incubation at given temperatures for 15 min. The measurements were carried out at an enzyme concentration of about 5 μg/ml in 5 mM HEPES-NaOH, pH 8.1, containing 0.5 mM $MgCl_2$ and 1 mg/ml BSA. The samples (20-50 μl) were incubated in glass tubes (total volume 0.1 ml) placed in a liquid thermostat. The desired temperature was reached in about 15 sec. The accuracy of maintenance of temperature was not worse than 0.3°C. Immediately after incubation, the samples were cooled for 15 sec at 0°C and used for enzyme activity measurements. The enzyme activity was constant during at least 1-2 h after the end of incubation.

**Other methods.** The pH values were measured with pH meters pH-340 (USSR), pHM-82 (Radiometer, Denmark), or Centron 2001 (Centron, The Netherlands). Rhodopsin and protein concentrations were determined as described earlier [33, 43]. Absorption spectra were recorded with Specord UV-VIS (Karl Zeiss, Germany) or Beckman DU 650 (Beckman, USA) spectrophotometers.

The NDP kinase binding data obtained at different pH values were analyzed by using a modified Hill equation [37, 38]:

$$A = A_{max}/\{1+[((10^{(-pKa)})/(10^{(-pH)}))^h]\}, \quad (1)$$

where $A_{max}$ and $A$ are the NDP kinase activities of supernatants obtained at pH 8.0 and pH < 8.0, respectively; p*K*a is the pH values at which the half-maximal NDP kinase binding occurred; and $h$ is the Hill coefficient.

The NDP kinase binding data obtained in the presence of salts and GTPγS were analyzed using the equation:

$$A = A_0 + A_{max}/[1+(K_{1/2}/L)^h], \quad (2)$$

where $A$, $A_0$, and $A_{max}$ are the NDP kinase activities of supernatants obtained for the ligand concentration $L$, in the absence of ligands, and at the saturation concentration of ligand, respectively; $K_{1/2}$ is the half-saturation concentration of ligand; and $h$ is the Hill coefficient.

The NDP kinase binding data obtained for the different concentrations of N-membranes were analyzed using the equation:

$$A = A_{max} - A_{max}/[1+(R_{1/2}/R)], \quad (3)$$

where $A_{max}$ and $A$ are the NDP kinase activities of supernatants obtained in the absence of the N-membranes and at the N-membrane concentration $R$, respectively; $R_{1/2}$ is the half-saturation concentration of N-membranes.

Thermal inactivation data were analyzed using the equation:

$$A = A_{max}/[1+(T_{1/2}/t)^h], \quad (4)$$

where $A_{max}$ and $A$ are the NDP kinase activities of supernatants obtained in the temperature ranges 20-25 and 25-80°C, respectively; $t$ is the temperature of preincubation; $T_{1/2}$ is the temperature at which the half-maximal NDP kinase inactivation occurred; and $h$ is the Hill coefficient for the thermal inactivation process. The fitting accuracy was not worse than 10%. Each of the experiments described was repeated at least three times with very similar results.

## RESULTS

**Binding of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ to the R*Gt complex in bovine ROS photoreceptor membranes.** We showed earlier [33] that at a low ionic strength and pH 8.0 the recombinant rat NDP kinase α did not interact with bovine ROS photoreceptor membranes, whereas at pH 5.5 the enzyme was totally bound to the R*Gt complex in the membranes. Unlike NDP kinase α, the recombinant rat NDP kinase β did not interact with the R*Gt complex under acidic conditions.

In the present work, the binding of chimeric proteins NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ with the R*Gt complex in N-membrane preparations was studied. The chimeric NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ had its V1 region which was similar to that of NDP kinase α, but in contrast to NDP kinase α, it contained the C-terminal V2 site of NDP kinase β and an additional HA-sequence in the N-terminal part of the molecule. The chimeric NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ contained the V1 region of NDP kinase β, the V2 region of NDP kinase α, and the N-terminal HA-peptide. The results demonstrated (Fig. 1a) that under typical conditions (low ionic strength, [R*] ≈ 1 μM) and at pH 8.0, the

NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$, like NDP kinase α, did not bind to the membranes and was present in the supernatant, whereas at pH 5.5 the most part of the enzyme sedimented with the membranes and addition of GTPγS released the bound enzyme. NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$, much like NDP kinase β itself, existed in the free state at pH 8.0 and showed only weak binding at pH 5.5 (Fig. 1b).

The characteristics of binding of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ with N-membranes were studied in more detail. The pH dependence of binding of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ with N-membranes in the pH range 5.5-8.0 was shown to be half-maximal at pH 6.1 (Fig. 1c). Essentially the same value (pH 6.2-6.3) was obtained previously for NDP kinase α [33]. In this pH range, the binding of NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$, like the binding of NDP kinase β [33], was negligible (Fig. 1c).

Recently it has been shown that the binding of bovine ROS NDP kinase and recombinant rat NDP kinase α to N-membranes depends on membrane concentration [14, 33]. The binding was half-maximal at very low membrane concentrations ([R*] ≈ 50 nM). Under such conditions the concentration of R*Gt complexes which play the role of binding sites for NDP kinases was 4-5 nM (the molar ratio R/Gt in ROS membranes was

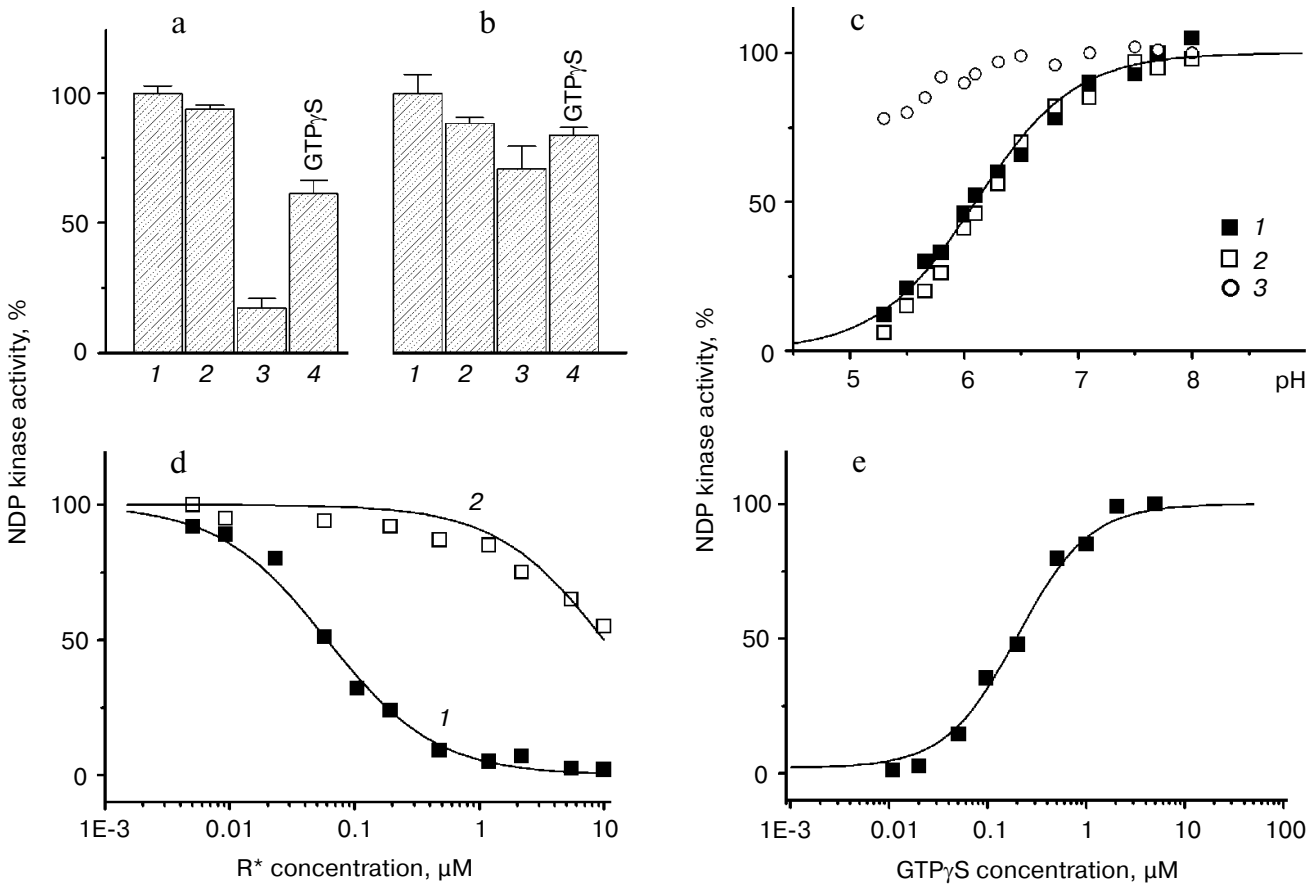


**Fig. 1.** Characteristics of interaction of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ with bovine retinal ROS photoreceptor membranes. a, b) Interaction of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ (a) and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ (b) with N-membranes. Effects of pH and GTPγS. N-Membranes ([R*] ≈ 1 μM) were mixed with the enzymes under conditions mentioned below and centrifuged, and supernatant activity was measured. *1*) NDP kinase activity of the suspension at pH 8.0 before centrifugation (100%); *2*, *3*) NDP kinase activity of supernatants obtained at pH 8.0 and pH 5.5, respectively; *4*) NDP kinase activity of the supernatants obtained at pH 5.5 in the presence of 5 μM GTPγS. Values obtained are the averages of three independent experiments. c) The pH dependence of binding of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ (*1*, *2*) (the results of two independent experiments) and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ (*3*) with N-membranes. N-Membranes ([R*] ≈ 1 μM) were mixed with the enzymes at different pH values, centrifuged, and supernatant activity was measured. The solid line is the theoretical curve computed according to a modified Hill equation (1). d) Binding of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ (*1*) and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ (*2*) with N-membranes. Dependence on membrane concentration. N-Membranes at different concentrations were incubated at pH 5.5 with the enzymes (final enzyme concentration was about 2 nM), centrifuged, and supernatant activity was measured. The solid lines are theoretical curves computed according to the modified Hill equation (3). e) Binding of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ with N-membranes. Dependence on GTPγS concentration. N-Membranes ([R*] ≈ 1 μM) were mixed with NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ at pH 5.5 and different GTPγS concentrations, centrifuged, and supernatant activity was measured. The solid line is the theoretical curve computed according to the modified Hill equation (2).

estimated to be about 10-12 [34]). This means that the $K_d$ value for the interaction between bovine ROS NDP kinase or recombinant rat NDP kinase α is about 4-5 nM. In the present work, the binding of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ to N-membranes was half-maximal at R* concentrations of about 50-60 nM (Fig. 1d). Thus, NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ and NDP kinase α have the same affinity for the R*Gt complex.

Previously it was shown that the affinity of NDP kinase β for photoreceptor membranes was only 1/100 of that for NDP kinase α: the half-maximal binding of NDP kinase β was observed at a very high ROS membrane concentration ([R*] ≈ 10 μM) [33]. The same value was obtained in this work in the experiments carried out using NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ (Fig. 1d).

Thus, the experimental approaches used showed that the binding properties of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ are very similar to the properties of NDP kinases α and β, respectively. These findings confirm the assumption that the interaction of NDP kinase isoforms with the rhodopsin–transducin complex in photoreceptor membranes seems to be determined by the variable region V1 and does not depend on the V2 variable region and the presence of the additional N-terminal HA-peptide.

Further experiments were carried out to study the influence of GTPγS and salts on the interaction of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ with photoreceptor membranes. The half-maximal elution of bound NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ was found at 200 nM GTPγS (Fig. 1e). The

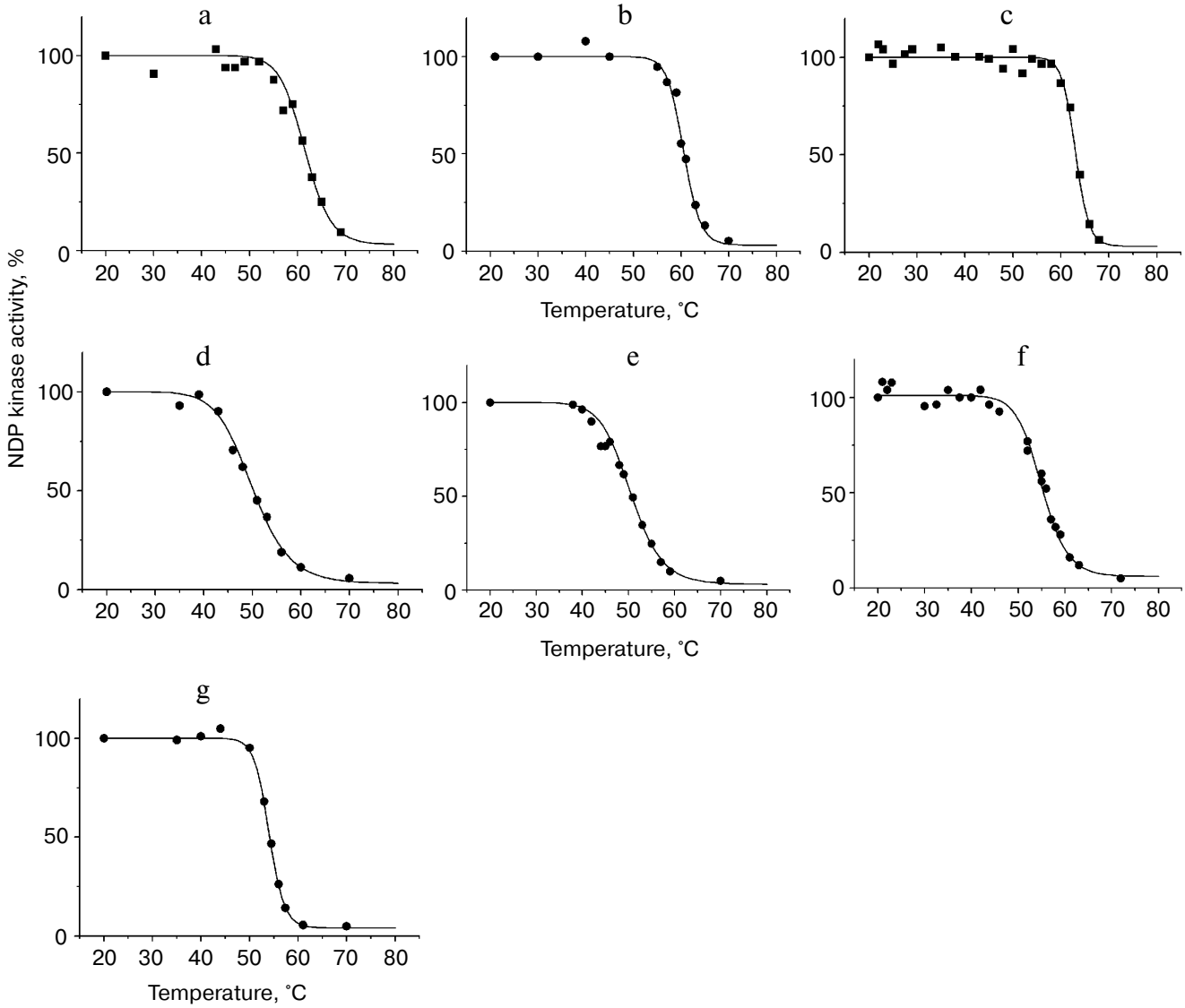


**Fig. 2.** Thermal inactivation of NDP kinase α (a), NDP kinase $\alpha^{1-130}\beta^{131-152}$ (b), NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ (c), NDP kinase β (d), NDP kinase $\beta^{1-130}\alpha^{131-152}$ (e), NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ (f), and NDP kinase HA-β (g). The solid lines are theoretical curves computed according to the modified Hill equation (4).

same GTPγS concentration was needed for the half-maximal elution of bound NDP kinase α [33]. This means that the binding sites in ROS photoreceptor membranes for NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ as well as for NDP kinase α are R*Gt complexes [14, 33]. It was shown previously that salts (NaCl, KCl, $MgCl_2$, and $CaCl_2$) interacted with NDP kinase α itself and shifted the equilibrium between the bound and free forms of the enzyme towards the free form [39]. In accord with those results, in the present work it was shown that the acid-induced binding of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ was prevented by addition of NaCl or KCl. Moreover, the dependences of the binding of this enzyme on salt concentration were similar to those obtained earlier for NDP kinase α [38]: for both enzymes the half-maximal elution was observed at 35 mM NaCl or KCl with the Hill coefficient of about 3 (data not shown). Thus, in the experiments described the behavior of NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ did not differ from that for NDP kinase α.

**Thermal inactivation of recombinant rat NDP kinases α and β and their derivatives.** Recombinant rat NDP kinases α and β were half inactivated at different temperatures: 61.5 and 50°C, respectively (Fig. 2 and table). These values are very close to the half-inactivation temperatures of recombinant human NDP kinases B and A, respectively [44, 45]. It is well known that rat NDP kinases α and β are highly homologous (98-99%) to the human NDP kinases B and A, respectively [46].

The half-inactivation temperature for NDP kinase $\alpha^{1-130}\beta^{131-152}$ that contained the V2 region of NDP kinase β instead of the V2 variable region of NDP kinase α, was similar to that for NDP kinase α, whereas the cooperativity of thermal inactivation process for NDP kinase $\alpha^{1-130}\beta^{131-152}$ was markedly higher than that for NDP kinase α (Fig. 2 and table). Insertion of the HA-sequence into the N-terminal part of NDP kinase $\alpha^{1-130}\beta^{131-152}$ (NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$) resulted in a small increase in half-inactivation temperature and further increase in cooperativity (Fig. 2 and table). A similar tendency was revealed in the study of thermal inactivation processes of NDP kinase β and its derivatives (Fig. 2 and table). The thermal inactivation parameters for NDP kinase $\beta^{1-130}\alpha^{131-152}$ were very similar to those for the original NDP kinase β. Insertion of the HA-sequence into both proteins (NDP kinase HA-β and NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$) slightly increased the thermal stability and cooperativity of the inactivation process (Fig. 2 and table).

## DISCUSSION

Our previous study of recombinant rat NDP kinases α and β, each containing only one sort of subunits (α or β), revealed that they are quite different in their acid-induced GTP- and salt-dependent interaction with the R*Gt complex in bovine ROS photoreceptor membranes [33]. Moreover, it was shown that an artificial peptide α1 simulating the V1 region of NDP kinase α was able to inhibit the interaction of the enzyme with the R*Gt complex, whereas the peptide β1 derived from the V1 region of NDP kinase β as well as all other model peptides for the V2 region of NDP kinases α and β had no marked effects on the binding [33]. These results imply that it is the V1 region of NDP kinase α that is involved in the interaction of the enzyme with the R*Gt complex.

In the present work, it was shown that the chimerical form of NDP kinase α (NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$) in which the V1 region of NDP kinase α and the V2 region of the β isoform was included was very similar to NDP kinase α in its interaction with the R*Gt complex. In contrast, the chimerical form of NDP kinase β (NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$) that contained the V1 region of NDP kinase β and the V2 region of the α isoform did not interact, like NDP kinase β itself, with the R*Gt complex. These results are in agreement with the conclusion about the importance of the V1 region of NDP kinase α for the binding of the isoform α with the R*Gt complex.

The investigation of thermal inactivation of NDP kinases α and β and their derivatives clearly demonstrated that the difference in thermal stability of recombinant rat NDP kinases α and β is almost entirely due to their differences in the variable V1 regions, whereas the contribution of the C-terminal variable region V2 and/or the HA-sequence to the thermal inactivation processes of the enzymes seems to be negligible.

Our previous investigations of NDP kinases α and β and their derivatives by intrinsic protein fluorescence [37-39] showed that the unusual fluorescence properties of NDP kinase α and its ability to exist in two different con-

Parameters of thermal inactivation of NDP kinase α (a), NDP kinase $\alpha^{1-130}\beta^{131-152}$ (b), NDP kinase HA-$\alpha^{1-130}\beta^{131-152}$ (c), NDP kinase β (d), NDP kinase $\beta^{1-130}\alpha^{131-152}$ (e), NDP kinase HA-$\beta^{1-130}\alpha^{131-152}$ (f), and NDP kinase HA-β (g). The values of half-inactivation temperature ($T_{1/2}$) and inactivation cooperativity ($h$) were obtained by treatment of experimental data according to a modified Hill equation (4)

| | a | b | c | d | e | f | g |
|---|---|---|---|---|---|---|---|
| $T_{½}$, °C | 61.5 | 60.5 | 63 | 50 | 50.5 | 54 | 53 |
| $h$ | 23 | 35 | 45 | 13 | 15 | 20 | 35 |

formational states, in one of which it is able to strongly interact with the R*Gt complex, are determined by the V1 variable region. Taken together with the results of the present work, this means that the differences in the V1 variable regions in NDP kinases α and β seems to serve as the main physicochemical basis of their differences in structural lability, fluorescence properties, interaction with the R*Gt complex, thermal stability and, as a consequence, is the main source of the diversity in the physiological roles of hexameric isoforms of NDP kinase in the cell.

We are grateful to Ms. N. Shimada for running SDS-PAGE and Western blotting and Drs. T. Toda and N. Ishikawa for fruitful discussions.

This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture, the Human Frontier Science Program Organization, and the Russian Foundation for Basic Research (No. 00-04-48127).

## REFERENCES

1. Park, R. E., Jr., and Agarwal, R. E. (1973) in *The Enzymes* (Boyer, P. D., ed.) Vol. 8, Academic Press, New York, pp. 307-333.
2. Kimura, N., Shimada, N., Ishijima, Y., Fukuda, M., Takagi, Y., and Ishikawa, N. (2003) *J. Bioenerg. Biomembr.*, **35**, 41-47.
3. Otero, A. S. (1990) *Biochem. Pharmacol.*, **39**, 1399-1404.
4. Lacombe, M. L., and Jakobs, K. H (1992) *Trends Pharmacol. Sci.*, **13**, 46-48.
5. Kimura, N. (1993) in *GTPases in Biology. Handbook of Experimental Pharmacology* (Dickey, B., and Birnbaumer, L., eds.) Vol. 108/II, Springer, Berlin, pp. 485-498.
6. Otero, A. S. (2000) *J. Bioenerg. Biomembr.*, **32**, 269-275.
7. Kimura, N., Shimada, N., Fukuda, M., Ishijima, Y., Miyazaki, H., Ishii, A., Takagi, Y., and Ishikawa, N. (2000) *J. Bioenerg. Biomembr.*, **32**, 309-315.
8. Timmons, L., and Shearn, A. (2000) *J. Bioenerg. Biomembr.*, **32**, 293-300.
9. Postel, E. H., Berberich, S. J., and Rooney, J. W. (2000) *J. Bioenerg. Biomembr.*, **32**, 277-284.
10. De La Rosa, A., Williams, R. L., and Steeg, P. S. (1995) *Bioassays*, **17**, 53-62.
11. Gilles, A., Presecan, F., Vonika, A., and Lascu, I. (1991) *J. Biol. Chem.*, **266**, 8784-8789.
12. Fukuchi, T., Shimada, N., Hanai, N., Ishikawa, N., Watanabe, K., and Kimura, N. (1994) *Biochim. Biophys. Acta*, **1205**, 113-122.
13. Janin, J., Dumas, C., Morera, S., Xu, Y., Meyer, P., Chiadmi, M., and Cherfils, J. (2000) *J. Bioenerg. Biomembr.*, **32**, 215-225.
14. Orlov, N. Ya., and Kimura, N. (1998) *Biochemistry (Moscow)*, **63**, 171-179.
15. Orlov, N. Ya., Freidin, A. A., Orlov, D. N., and Kimura, N. (2003) *Biol. Membr. (Moscow)*, **20**, 46-52.
16. Gilman, A. G. (1987) *Ann. Rev. Biochem.*, **56**, 615-649.
17. Stryer, L. (1986) *Ann. Rev. Neurosci.*, **9**, 87-119.
18. Wieland, T., Ulibarri, I., Gierschik, P., and Jakobs, K. H. (1991) *Eur. J. Biochem.*, **196**, 707-716.
19. Wieland, T., Ronzani, M., and Jakobs, K. H. (1992) *J. Biol. Chem.*, **267**, 20791-20797.
20. Wieland, T., Nurnberg, B., Ulibarri, I., Kaldenberg-Stasch, S., Schultz, G., and Jakobs, K. H. (1993) *J. Biol. Chem.*, **268**, 18111-18118.
21. Nurnberg, B., Harhammer, R., Exner, T., Schulze, R. A., and Wieland, T. (1996) *Biochem. J.*, **318**, 717-722.
22. Hohenegger, M., Mitterauer, T., Voss, T., Nanoff, C., and Freissmuth, M. (1996) *Mol. Pharmacol.*, **49**, 73-80.
23. Kowluru, A., Seavey, S. E., Rhodes, C. J., and Metz, S. A. (1996) *Biochem. J.*, **313**, 97-108.
24. Cuello, F., Schulze, R. A., Heemeyer, F., Meyer, H. E., Lutz, S., Jakobs, K. H., Niroomand, F., and Wieland, T. (2003) *J. Biol. Chem.*, **278**, 7220-7226.
25. Kowluru, A., and Metz, S. A. (1994) *Biochemistry*, **33**, 12495-12503.
26. Kowluru, A. (2002) *Biochem. Pharmacol.*, **63**, 2091-2100.
27. Seifert, R., Rosental, W., Scultz, G., Wieland, T., Gierschick, P., and Jakobs, K. H. (1988) *Eur. J. Biochem.*, **175**, 51-55.
28. Piacentini, L., and Niroomand, F. (1996) *Mol. Cell Biochem.*, **157**, 59-63.
29. Niroomand, F., Mura, R., Jakobs, K. H., Rauch, B., and Kubler, W. (1997) *J. Mol. Cell. Cardiol.*, **29**, 1479-1486.
30. Hippe, H. J., Lutz, S., Cuello, F., Knorr, K., Vogt, A., Jakobs, K. H., Wieland, T., and Niroomand, F. (2003) *J. Biol. Chem.*, **278**, 7227-7233.
31. Lutz, S., Hippe, H. J., Niroomand, F., and Wieland, T. (2004) *Meth. Enzymol.*, **390**, 403-418.
32. Zhu, J., Tseng, Yu-H., Kantor, J. D., Rhodes, C. J., Zetter, B. R., Moyers, J. S., and Kahn, C. R. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 14991-14918.
33. Orlov, N. Ya., Orlova, T. G., Nomura, K., Hanai, N., and Kimura, N. (1996) *FEBS Lett.*, **389**, 186-190.
34. Kuhn, H. (1981) in *Current Topics in Membranes and Transport* (Miller, W. H., ed.) Vol. 15, Academic Press, New York, pp. 171-201.
35. Hall, S. W., and Kuhn, H. (1986) *Eur. J. Biochem.*, **161**, 551-556.
36. Dizhoor, A. (2000) *Cell. Signal.*, **12**, 711-719.
37. Orlov, N. Ya., Orlova, T. G., Reshetnyak, Ya. K., Burstein, E. A., and Kimura, N. (1999) *J. Biomol. Struct. Dynam.*, **16**, 955-968.
38. Orlov, N. Ya., Orlova, T. G., Reshetnyak, Ya. K., Burstein, E. A., and Kimura, N. (1997) *Biochem. Mol. Biol. Int.*, **41**, 189-198.
39. Orlov, N. Ya., Reshetnyak, Ya. K., Orlova, T. G., Orlov, D. N., Burstein, E. A., Ishijima, Y., and Kimura, N. (2003) *Biol. Membr. (Moscow)*, **20**, 52-58.
40. Ishijima, Y., Shimada, N., Fukuda, M., Miyazaki, H., Orlov, N. Ya., Orlova, T. G., Yamada, T., and Kimura, N. (1999) *FEBS Lett.*, **445**, 155-159.
41. Kim, S. Y., Chang, K. H., Doh, H. J., Jung, J. A., Kim, E., Sim, C. J., and Lee, K. J. (1997) *Mol. Cells*, **7**, 630-634.
42. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
43. Orlov, N. Ya., Kalinin, E. V., Orlova, T. G., and Freidin, A. A. (1988) *Biochim. Biophys. Acta*, **954**, 325-335.
44. Giartosio, A., Erent, M., Cervoni, L., Morera, S., Janin, J., Konrad, M., and Lascu, I. (1996) *J. Biol. Chem.*, **271**, 17845-17851.
45. Milon, L., Meyer, P., Chiadmi, M., Munier, A., Johansson, M., Karlsson, A., Lascu, I., Capeau, J., Janin, J., and Lacombe, M. L. (2000) *J. Biol. Chem.*, **275**, 14264-14272.
46. Lascu, I., Giartosio, A., Ransac, S., and Erent, M. (2000) *J. Bioenerg. Biomembr.*, **32**, 227-236.