Interaction of recombinant rat nucleoside diphosphate kinase with bleached bovine retinal rod outer segment membranes: A possible mode of pH and salt effects

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INTERACTION OF RECOMBINANT RAT NUCLEOSIDE DIPHOSPHATE KINASE α WITH BLEACHED BOVINE RETINAL ROD OUTER SEGMENT MEMBRANES: A POSSIBLE MODE OF pH AND SALT EFFECTS.

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Summary
An attempt was made to reveal the mode of action of protons and salts on the recently discovered GTPγS-dependent interaction of bovine retinal rod outer segments (ROS) nucleoside diphosphate kinase (NDP kinase) with the complex between bleached visual receptor rhodopsin and retinal G-protein transducin in bovine ROS membranes. The properties of recombinant rat NDP kinase α, that is immunologically similar to the soluble NDP kinase from bovine ROS preparation, have been studied in solution by means of protein fluorescence at different pH and salt concentrations and results were compared with pH and salt effects on the binding of NDP kinase α to bleached bovine ROS membranes. The results suggest that NDP kinase α itself may serve as a target for protons and salts and mediates their effects on the interaction between the enzyme and ROS membranes.

Keywords: nucleoside diphosphate kinase, retinal rod outer segment membranes, G-proteins, transducin, intrinsic protein fluorescence

Introduction
Nucleoside diphosphate kinase (NDP kinase) catalyses the transfer of γ-phosphate from nucleoside triphosphates to nucleoside diphosphates in cells (1). The vertebrate enzymes are typically hexamers of small (M, of ca. 17,000) subunits, organized as a double trimer (2). Some recent findings (see for the review (3-5)) lead to the notion that NDP kinases are rather multifunctional proteins and some of their functions would operate via the interaction with G-proteins (6).

1 Abbreviations: ROS, retinal rod outer segments; NDP kinase, nucleoside diphosphate kinase; ROS NDP kinase, soluble NDP kinase from bovine ROS preparation; Gt, transducin; R*, bleached rhodopsin; R*-Gt, complex between R* and Gt; N-membranes, NDP kinase-depleted Gt-containing ROS membranes.
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Vertebrate retinal rod outer segments (ROS) are highly enriched with soluble G-protein transducin (Gt) that interacts with bleached rhodopsin, R* (7,8). The complex between R* and Gt (R*-Gt) in ROS membranes is very stable under a wide range of pH and ionic conditions, but dissociates in the presence of GTPγS (9,10). Recently, we used these remarkable features of these components of ROS phototransduction system to search for an interaction of NDP kinase with G-proteins and revealed that the soluble NDP kinase from bovine ROS preparation (ROS NDP kinase) is able to bind to the R*-Gt and releases after dissociation of R*-Gt under GTPγS added (11,12). We suggested (12) that such an interaction would involve in the process, that provides extremely high rate of Gt activation in living ROS (7,8,13).

It was also shown, that the equilibrium binding of NDP kinase to the ROS membranes strongly depended on the pH and salt concentration (11,12). In the present work the attempt was made to clear up the mechanism of the pH and salt effects on the interaction between R*-Gt and NDP kinase. For this purpose, we used the recombinant rat NDP kinase α (NDP kinase α) that strongly interacts with ROS membranes (14). During the first step of this work it was shown that the pH and salt effects on the binding of NDP kinase α to the bovine R*-Gt containing ROS membranes practically coincide with those measured earlier for the ROS NDP kinase (11,12). Further fluorometric study revealed that the pronounced changes of the quantum yield of fluorescence of isolated NDP kinase α occur in the same ranges of pH and salt concentrations as binding did. Such as coincidence allowed to hypothesize that the pH and salts induce the changes in the NDP kinase properties, reflected in its fluorescence behavior, which may serve as the cause of their effects on the enzyme-to- R*-Gt binding in ROS membranes.

Materials and methods

Materials. Unless otherwise mentioned, all of the reagents were as described previously (15).

Preparation of NDP kinase α. NDP kinase α was prepared and stored as described earlier (14,16). Molar extinction coefficients of hexameric NDP kinase α (Mₐ of ca. 100,000) at 280 and 296.7 nm were estimated to be of 130,000 and ca. 40,000 M⁻¹cm⁻¹, respectively.

NDP kinase assay. NDP kinase activity was measured photometrically by the coupling enzyme method via estimation of the rate of ADP formation from ATP at phosphorylation of TDP by NDP kinase (14,16) and expressed as amount of formed ADP/min. Buffers and salts used did not interfere with the NDP kinase activity measurements. Assays were done
in triplicate and agree within 7%. NDP kinase protein content was determined by SDS-PAGE (17) and Western blotting with affinity-purified polyclonal anti-rat-NDP-kinase antibody NK2 which reacted with bovine enzyme as well (16).

Preparations of NDP kinase-depleted Gt-containing ROS membranes (N-membranes). N-membranes were prepared by repeated washing of bovine ROS preparations (18) in neutral buffer N (10 mM Tris-HCl, pH 7.5, 0.25 mM MgCl₂) as described earlier (14). SDS-PAGE has revealed that 75-80% of the protein in the N-membranes was constituted by rhodopsin. NDP kinase content (about 2 copies of NDP kinase as a hexamer per 10,000 rhodopsin molecules) and its specific enzyme activity (0.1-0.15 μmol ADP/min per mg of the total protein) were 7-10% of those shown in the ROS preparations (12,14). The preparations contained 6-7 Gt copies per 100 rhodopsin molecules (ca. 80-90% of those in ROS preparations (9)), as determined by SDS-PAGE followed by Western blotting with an antibody against G-protein α-subunits (DuPont/NEN). The membranes were dark stored in acidic buffer A (10 mM MES-NaOH, pH 5.5, 0.25 mM MgCl₂) at -80°C and totally bleached with orange light for 10 min at 0°C before use.

Binding of NDP ldnase α to the N-membranes. The binding experiments were carried out as described early (14). Briefly, N-membranes were mixed under appropriate conditions with NDP kinase α up to final R* and enzyme concentrations of about 1 μM and 15-20 nM, respectively. The samples were centrifuged (TL-100 ultracentrifuge, Beckman, USA, 100,000 x g for 10 min, 2°C) and the supernatants and pellets obtained were used for measurements of the activity of free and bound NDP kinase α, respectively. 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 α added to the membranes, only the activity of supernatants was usually measured. In accordance with our previous work (14) the distribution of the NDP kinase activity between supernatants and pellets paralleled with that for NDP kinase protein.

NDP kinase activity of N-membranes was as low as 0.2-0.3% of that of NDP kinase α added to the suspensions. NDP kinase α alone neither sedimented at 100,000 x g for 30 min, nor irreversibly changed its activity in the presence of any buffer and salt used.

Fluorescence measurements. The protein fluorescence spectra were measured with the lab-made registering spectrofluorimeter with monochromatic excitation and light collecting from the front face of standard silica cell [19,20] or in the Shimazu RF 5000 spectrofluorimeter. Spectra were excited at 296.7 nm, where only tryptophan absorbs in proteins. The spectral resolution was not worse than 2 nm. All emission spectra were corrected for the instrument spectral sensitivity (21) and for the Raman scattering band. Intensities in the corrected spectra were proportional to the number of photons emitted per a unit wavelength interval in a time unit. NDP kinase α concentrations in samples (2-3 ml) was of 0.3-1 μM. The absorbance of the protein solutions never exceeded 0.04 at 296.7 nm. The measurements were done at 22-28°C.

The fluorescence quantum yield of the NDP kinase α at pH 8.0 (10 mM HEPES-NaOH) was taken for 100%. The relative quantum yield values (qₐ) for a sample at another condition were estimated by comparing areas under given fluorescence spectra with that of NDP kinase α solution at pH 8 with the same absorbance at 296.7 nm. An accuracy of qₐ determination was 5-7%.

Other methods. The pH values were measured with an accuracy of 0.05 using pH meters pH-340 (USSR), pHM-82 (Radiometer, Denmark) or Centron 2001 (Centron, Netherlands). Titration with salts was carried out by the progressive addition of aliquots of 4 M NaCl, 3 M KCl, 2 M MgCl₂ or 2 M CaCl₂. The measured emission intensities under titration were corrected for dilution. Rhodopsin and protein concentrations were
determined as described earlier (14,18). Absorption spectra were recorded with Specord UV-VIS (Karl Zeiss, Jena) or Beckman DU 650 (Beckman, USA) spectrophotometers.

The salt and pH effect data were analyzed using Hill equation:

$$A = A_o + \frac{A_{max} - A_o}{1 + \left(\frac{K_{1/2}}{L}\right)^n},$$

where $A$, $A_o$, and $A_{max}$ are either fluorescence intensity at 350 nm or the supernatant NDP kinase activity values at, respectively, the current, zero and saturating $H^+$ or salt concentrations; $L$ is the current $H^+$ or salt concentration; $K_{1/2}$ is the half-saturating $H^+$ or salt concentration, related with equilibrium dissociation and association constants as $K_{1/2} = K_d^{-1/n} = K_u^{1/n}$, that is $pK_u = -n\log(K_{1/2})$; $n$ is the Hill coefficient. The fitting accuracy of $K_{1/2}$ and $n$ estimation was not worse than 10%. Each of the experiments described was repeated at least three times with the very similar results. The $K_{1/2}$ and $n$ values evaluated in independent experiments agreed within 20%.

Results

Interaction of NDP kinase $\alpha$ with N-membranes. Influence of pH and salts. At low ionic strength, the binding of NDP kinase $\alpha$ to the N-membranes is induced by lowering pH value (Fig. 1A), the half-maximal effect being at pH ca. 6.3 (Table 1). The binding was reversible: the bound NDP kinase $\alpha$ released into supernatant during extraction of the membranes by neutral buffer N (not shown). According to our previous work (14), the binding is prevented by submicromolar concentration of GTP$\gamma$S.

The binding at low pH effectively decreased in the presence of salts studied (see Fig. 1 and Table 1). The effects of NaCl and KCl were identical, whereas the effects of CaCl$_2$ and MgCl$_2$, being close one another, were observed at some lower concentrations. The salt effects were cooperative, with Hill coefficient of about 3 (Table 1).

These results clearly demonstrate that the NDP kinase $\alpha$ is able to the pH- and salt-dependent equilibrium binding to R*-G$_t$ complexes in ROS membranes and, in this aspect, it is quite similar to the intrinsic soluble ROS NDP kinase (Table 1). Therefore, in order to check, if the properties of NDP kinase itself can determine the pH- and salt-dependent behavior of this associating system, we studied parameters of intrinsic fluorescence of isolated NDP kinase $\alpha$ in solutions at various pH and salt concentrations.

Fluorometric pH- and salt-titration of NDP kinase $\alpha$. Fluorescence spectra of NDP kinase $\alpha$ at pH 8.0 display an emission maximum ($\lambda_{max}$) at 340.9±0.3 nm. Its relative quantum yield $q_r$ at such conditions was maximal and taken for 100% (see "Materials and Methods"). The acid titration to pH 4.0-5.5 resulted in a 4-nm shift of $\lambda_{max}$ to shorter wavelengths (22) and decrease in $q_r$ by ca. 40% (Fig. 2A). The decrease in $q_r$ was half-maximal at pH ca. 6.4 (Table 1). These fluorescence changes were reversible: the back titration up to pH 8.0-8.7
Fig. 1. Interaction of NDP kinase α with N-membranes. Influence of pH (A) and salt concentration (B-E). N-membranes were mixed with NDP kinase α (final activity of 9 nmol ADP/min per 2 µl) in either hypotonic buffers (10 mM MES-NaOH or HEPES-NaOH, 0.25 mM MgCl₂) at pH indicated (A) or an acidic buffer A containing indicated salt concentrations (B-E). The samples were centrifuged and the activity of the supernatants was measured. Theoretical curves were fitted by the Hill equation (1) with the parameters shown in Table 1.
Table 1. Parameters of Equation (1) for pH- and salt-effects on the interaction of NDP kinase α with N-membranes (Binding) and the NDP kinase α fluorescence yield (Fluorescence). The results of a typical experiment are shown. For comparison the parameters of Equation (1) for pH- and salt-effects on the interaction of ROS NDP kinase with ROS membranes (11,12) are shown in parentheses.

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practically restored the $q_r$ and $\lambda_{max}$ values (22). These results suggest that structural and/or physical state of the enzyme molecules may differ at pH 8.0-8.7 and pH 4.0-5.5 and the states exist in an pH-dependent equilibrium.

At pH around 8.0, addition of 0.5-1 M KCl did not practically change the NDP kinase fluorescence. However, at pH 4.0-5.5 0.5-1 M KCl caused an additional decrease in $q_r$ by ca. 15% (Fig.2A) without any change in $\lambda_{max}$. The presence of KCl added did not change the mid-point position (and, thus, of association constant) of the pH-dependent quantum yield changes (see Fig.2A and Table 1). The effects of other salts studied (0.5 M NaCl, 20 mM CaCl₂ or MgCl₂) were similar to those of KCl (not shown). These results suggest that, whereas salts studied did not practically influence the enzyme state at pH 8.0-8.7, at pH 4.0-5.5 they significantly affected molecular properties of NDP kinase. The more detailed analysis of pH-induced molecular events, which lead to the changes of fluorescence properties of rat NDP kinases, will be done elsewhere (22).
Since in the acidic pH range addition of NaCl, KCl, CaCl₂ or MgCl₂ decreased $q_r$ without any alteration in the shape and position of fluorescence spectra (22), the changes of emission intensity at 350 nm can be used as a measure of the $q_r$ changes. Therefore we used it to record the salt concentration dependencies. As it is seen in Fig.2 and Table 1, the effects of NaCl and KCl are essentially similar. The effects of CaCl₂ and MgCl₂ were also close to one another, but shifted to some lower concentration range, comparing with those of monovalent cation salts. The fluorometric effects of all the salts studied were non-cooperative (the Hill coefficient $n \approx 1$).

**Discussion**

Recently, we succeeded to show that the ROS NDP kinase exhibits equilibrium binding with $R^*-G_1$ complexes in the ROS membranes (11,12). The equilibrium was demonstrated to be pH- and salt-dependent, but mechanism of these effects remained unclear. Since $R^*-G_1$ are known to be stable under a wide range of pH and ionic conditions (9,10), in this work an attempt is made to investigate another simplest possibility, that the NDP kinase itself may be a target for the effects of pH and salts, mediating then their influence on the enzyme-membrane interaction.

To check this possibility we studied the properties and behavior of isolated NDP kinase α in solution using intrinsic protein fluorescence techniques. The binding properties of the ROS NDP kinase and NDP kinase α in the experimental system used was shown to be remarkable similar (Table 1). It assumed that NDP kinase α can substitute ROS NDP kinase in the fluorescence study as well.

The fluorescence study of isolated NDP kinase α in solution revealed that under the almost physiological conditions, there exist at least three states of the protein, differing in their fluorescence properties and, possibly, in their structural states. These states exist in an equilibrium which depends upon pH and salt concentration (Fig. 3). One of them, refereed as NDPK-I, is stable at pH 8.0-8.7. NDP kinase α has in this state $\lambda_{max}$ at ca. 341 nm and maximal quantum yield. The protein fluorescence in this state is insensitive to salt addition. Acidification to pH 4.0-5.5 results in a reversible transition of the protein into another state, refereed as NDPK-II, with $\lambda_{max}$ at about 337 nm and $q_r$ of ca. 60%. Increase in the salt concentration at pH 4.0-5.5 induces another transition of the protein, from NDPK-II state into the third state, NDPK-III, with the same $\lambda_{max}$ at 337 nm, but with lowest observed $q_r$ value (45-50% of that at pH ca. 8).
Fig. 2. Fluorimetric titration of NDP kinase α. Theoretical curves were fitted by the Hill equation with the parameters shown in Table 1. (A) pH-dependencies of relative quantum yield $q_r$ of the enzyme (final protein concentration of 30 µg/ml) in 10 mM HEPES-NaOH in the absence or presence of either 0.5 M or 1 M KCl, measured at 21°C. (B-E) Salt titration of NDP kinase α (protein concentration of about 100 µg/ml) by salts under acidic conditions (2.5 mM MES-NaOH, pH 5.5). Fluorescence intensity decrease values (a.u.) at 350 nm were measured at 25°C. In the absence of salts, the absolute intensities were 410 a.u.(B,C) and 500 a.u.(D,E).
Fig. 3. A possible scheme of the influence of pH and salt concentration on the interaction of NDP kinase α with R*-G₁ complexes in ROS membranes (see text for the explanation).

The comparison of the data obtained under studies of NDP kinase α using two different experimental approaches (Table 1) indicates that the transitions between the states, revealed in fluorescence experiments, might be related to the switching by pH and ionic strength changes of the ability of the enzyme to interact with membrane R*-G₁ complexes. The NDPK-II state, stable under acidic pH and low ionic strength, is a most suitable candidate to be directly involved in the interaction with the membranes. The NDPK-I and NDPK-III states evidently have a significantly lowered affinity to the bleached ROS membranes.

However, the salt effect revealed by fluorescence changes is non cooperative, whereas the effect on the NDP kinase binding is highly cooperative (n=2-3). It suggests that the enzyme subunits in solution are independent of each another and contribute equally in the emission signal measured, but the binding of the enzyme to R*-G₁ complex either may induce an interaction between subunits through an allosteric-like mechanism or needs that 2-3 ion-binding sites would be vacant simultaneously. For example, the dissociation of NDP kinase from the complex may demand the simultaneous occupancy of ion-binding sites on the three subunits in a trimer of the hexameric quaternary structure of the enzyme (2). On the other hand, it cannot be exclude that the ion binding in three sites has a strong positive feedback. In any case, it is much probable that the cooperativity of the salt effect appears only when NDP kinase is bound to the R*-G₁ complex. In other words, we can hypothesize that the binding to R*-G₁ complex induces in NDP kinase α a transition into a new structural and/or physical state, designated in the Fig. 3 as NDPK-II*.

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