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Transducin-mediated, isoform-specific interaction of recombinant rat nucleoside diphosphate kinases with bleached bovine retinal rod outer segment membranes

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Abstract The properties of the binding of recombinant rat nucleoside diphosphate (NDP) kinase isoforms α and β (NDP kinase α and β, respectively) to bleached bovine retinal rod outer segment (ROS) membranes were investigated. It was found that: (1) both NDP kinase isoforms interacted with ROS membranes in a pH-, cation- and GTPγS-dependent manner; (2) the retinal G-protein transducin was an obligatory factor for the interaction; (3) the apparent affinity of NDP kinase α for ROS membranes was about 100-fold higher than that of NDP kinase β; and (4) an α-isoform-specific peptide, corresponding to the sequence of the N-terminal third (variable region), had the ability to displace bovine NDP kinase from ROS membranes. The results suggest the possible involvement of NDP kinases in cellular regulation via interaction with G-proteins and provide a structural basis for the possible differential roles of mammalian NDP kinase isoforms in the cell.

Key words: Nucleoside diphosphate kinase; Isoform; Retinal rod outer segment membrane; G-protein; Transducin

1. Introduction

Nucleoside diphosphate (NDP) kinase exchanges γ-phosphates between nucleoside tri- and diphosphates in cells [1,2] and is found in a wide range of life forms from lower to higher organisms. In contrast to lower organisms, mammals contain two NDP kinase isoforms, which are encoded by independent genes but show remarkable similarity in their primary structure and catalytic properties [2–6]. The physiological meaning of the presence of the NDP kinase isoforms in mammalian cells remains unclear and is currently under intensive investigation in many laboratories.

Transcripts and proteins for both isoforms are expressed ubiquitously but in a tissue-dependent manner [2,5]. The distribution of the isoforms in cells was noted to be different [7]. These data suggest that mammalian NDP kinase isoforms may play distinctive roles in cells. Meanwhile, NDP kinases have been identified as a tumor metastasis suppressor (nm23)[8], a morphological regulator (Awd)[9], a transcription factor (PuF)[10] and a differentiation inhibitor (I-factor)[11], leading to the notion that they are rather multifunctional proteins involved in different levels of cellular regulation [12–14]. One potential mechanism of regulation performed by NDP kinases[12–15] may be ascribed to the interaction of the enzyme with heterotrimeric GTP-binding proteins (G-proteins) [16,17] which mediate the information flow from activated membrane receptor to the target proteins in signal transduction systems. Recently, we showed that bovine retinal rod outer segments (ROS) preparations contain soluble NDP kinase (bovine ROS NDP kinase) which exhibits equilibrium binding to the photoreceptor membranes (ROS membranes) containing components of the visual transduction system; bleached light receptor rhodopsin (R*) and the G-protein transducin (G_t)[18–20]. The finding that the equilibrium binding was under the control of guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) at submicromolar concentration leads to the suggestion that G_t may be involved as a constituent of the NDP kinase binding sites in ROS membranes.

In our present work, attempts were made to search for possible differences in the binding properties of the mammalian NDP kinase isoforms. For this purpose, taking advantage of the structural conservation of each NDP kinase isoform among mammalian species, we used recombinant rat NDP kinase α and β (NDP kinase α and β, respectively) [5] and examined their ability to interact with two kinds of bleached bovine ROS membranes, which differed in G_t content. Such an approach confirmed the obligatory role of G_t in the interaction and further revealed that, whereas NDP kinase α bound tightly to the G_t-containing ROS membranes, as was seen with the retinal enzyme, the apparent affinity of NDP kinase β to the membranes was much weaker. Moreover, the interaction between NDP kinase and ROS membranes was suggested to occur through an α-isoform-specific amino acid sequence corresponding to the N-terminal third variable region (V1 region) of the enzyme. The results thus demonstrate that NDP kinase may interact with the ROS membranes through G-protein in an isoform-specific manner.

2. Materials and methods

2.1. Materials

Unless otherwise mentioned, all of the reagents and enzymes used were as described previously [5].

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2.3. NDP kinase assays

NDP kinase activity of the samples (2–20 μl) was measured at 30°C in 140 mM Tris-acetate buffer, containing 36 mM KCl and 14 mM $MgCl_2$ (total volume 400 μl) photometrically at 340 nm by a coupling enzyme method via estimation of the rate of ADP formation from ATP (2.9 mM) during the phosphorylation of TDP (0.4 mM) by NDP kinase [1,21] and expressed as the amount of ADP formed/min. Buffers, salts and peptides used in this study did not interfere with the NDP kinase activity measurements. Nucleotides used (GTPγS, guanosine 5′-(β,γ-imino)triphosphate (GppNHp) or adenosine 5′-(β,γ-imino)triphosphate (AppNHp)) had no influence on the activity at their final concentrations below 5–10 μM. Assays were performed in triplicate and agreed within 7%. NDP kinase protein content was determined by SDS-PAGE followed by Western blotting with an affinity-purified polyclonal anti-rat NDP kinase antibody NK2 which reacted with the bovine enzyme as well [21].

2.4. Preparations of ROS suspensions and ROS membranes

Bovine ROS ($A_{280/500}$=2.7–3.0) were prepared by two-step flotation in 40% (w/v) sucrose-isotonic buffer solution [22] and stored in the dark at −80°C. SDS-PAGE revealed that about 60% of the protein in the preparations was constituted by rhodopsin protein (M_r approx. 40 000 [16,17]). The preparations contained typical amounts of G_t (6–8 G_t copies (M_r about 80 000[16,17]) per 100 rhodopsin molecules [2]), as determined by SDS-PAGE followed by Western blotting with an antibody against G-protein α-subunits (DuPont/NEN). The NDP kinase content (about 2 copies of NDP kinase as a hexamer with M_r=100 000 [1] per 1000 rhodopsin molecules) and enzyme activity (1.1–1.4 μmol ADP/min per mg of the total ROS proteins) in the ROS preparations were similar to those previously obtained [20].

NDP kinase-depleted G_t-containing ROS membranes (N-membranes) were prepared at 2°C under dim red light by repeated washings (100 000×*g* for 10 min) of the ROS preparations in neutral buffer N (10 mM Tris-HCl,pH 7.5, 0.25 mM $MgCl_2$), which had been partially bleached (R*/rhodopsin =0.2–0.3) with orange light (λ > 540 nm) for 1 min on ice. As determined by SDS-PAGE, 75–80% of the protein in these membranes was constituted by rhodopsin protein. The contents of G_t and NDP kinase in the membranes were 80–90% and 7–10% of those in the ROS preparations, respectively. NDP kinase- and G_t-depleted ROS membranes (W-membranes) were prepared under dim red light by successive treatments of the ROS preparations with GTP, EDTA and urea-containing media [23,24]. SDS-PAGE revealed that >90% of the protein in these membranes was constituted by rhodopsin protein. The content of G_t in the membranes was lower than the sensitivity of the method used (< 5% of the G_t content in the ROS preparations). The NDP kinase content of the membranes was 5–7% of the ROS preparations. N- and W-membranes were stored in acidic buffer A (10 mM MES-NaOH, pH 5.5, 0.25 mM $MgCl_2$) at −80°C in the dark. ROS and membrane preparations were totally bleached with orange light for 10 min at 0°C before use.

Three independently obtained preparations of ROS, N- and W-membranes were used in this study with similar results.

2.5. Preparation of G_t and NDP kinases α and β

G_t prepared by the method described previously [23] was further dialyzed to remove free nucleotides against buffer (2 mM MES-NaOH, pH 6.0, 0.25 mM $MgCl_2$). The preparation was 95% pure as determined by SDS-PAGE and was essentially free of NDP kinase activity (< 0.1 μmol ADP/min per mg G_t).

Hexameric NDP kinases α and β (M_r approx. 100 000) were prepared as described [5] and stored on ice in buffer (2 mM Tris-HCl, pH 7.0, 0.25 mM $MgCl_2$) as a concentrated stock solution (30 mg/ml).

2.6. Peptide preparation

Peptides α1 (AMKFLRASEEHLKQ) and β1 (GLKFIQASEDLLKE), corresponding to residues 37–50 of NDPK kinase α and β, respectively, peptides α2 (DYKSCAHDWVYE) and β2 (DYKSCAQNWIYE), corresponding to residues 141–152 of enzymes α and β, respectively, a common peptide α/β (TGRVMLGETNPADSKPGTI) corresponding to residues 86–104 of both NDP kinases and an unrelated peptide X (IPLDPVAGYKEPA) were synthesized using an automated peptide synthesizer (430-A or 432-A, Applied Byosystem Inc., USA) as described [25] and stored in buffer A at concentrations of 10–40 mg/ml.

2.7. Binding of NDP kinases to the ROS membranes

The N- and W-membranes were incubated with NDP kinase α or β in a final volume of 50–100 μl on ice for 5 min under various conditions. Final concentrations of R* and of NDP kinase α or β in the experiments were typically 0.6–3 μM and 10–20 nM, respectively, unless otherwise mentioned. The samples were centrifuged (TL-100 ultracentrifuge, Beckman, USA) at 100 000×*g* for 10 min at 2°C. The supernatants obtained were stored on ice and used for measurements of the activity of free NDP kinase. The bound NDP kinase was usually calculated by subtracting the free NDP kinase activity from the total enzyme activity added to the membranes, based on the fact that the sum of the free NDP kinase activity and bound NDP kinase activity measured after extraction with buffer N containing 100 mM NaCl was shown to be equal to the enzyme activity added. The binding of NDP kinases to the membranes was completed within 5 min of incubation under the conditions employed. Without membranes NDP kinases neither sedimented at 100 000×*g* for 30 min nor changed their activities irreversibly under the conditions examined. The separation of free and bound NDP kinases in the ROS preparations was carried out similarly by centrifugation as described above. The distribution of the NDP kinase activity between supernatants and pellets paralleled that for NDP kinase protein.

The endogenous NDP kinase activity present in the N-membranes was minimally released by buffer N or A containing either GTPγS or salts, and its amount was very low (< 0.02 μmol ADP/min per mg rhodopsin) and never exceeded a few percent of those of the recombinant NDP kinases added during the binding experiments. W-membranes did not release any measurable NDP kinase activity during these treatments. The contribution from the trace amount of NDP kinase present in the G_t preparations was less than 1% of the total enzyme activity used.

Each binding experiment described was repeated at least 3 times with similar results, and representative data from one experiment are shown.

2.8. Other methods

The rhodopsin concentration was determined as described [22]. Protein concentration was measured using BCA Protein Assay Reagent (Pierce, USA) using bovine serum albumin as a standard.

3. Results

3.1. Interaction of NDP kinases α and β with ROS membranes

It was shown previously [18–20] that soluble bovine ROS NDP kinase exhibits equilibrium binding to ROS membranes. Acidic pH and low ionic strength were favorable for the membrane-bound form (half-maximal binding at pH 7), whereas inclusion of either salts (100 mM NaCl or KCl, 10 mM $CaCl_2$ or $MgCl_2$) or 5 μM GTPγS prevented the binding.

In this study we investigated the interaction of NDP kinase α and β with N-membranes under different conditions. Whereas NDP kinase α did not sediment with the membranes at pH 8.0, most of the enzyme bound to the membranes at pH 5.5 (Fig. 1A). Binding was half-maximal at pH 6.2–6.4 (not shown). Acid-induced binding was greatly decreased in the presence of salts or GTPγS (Fig. 1A). Repeated washing of the membranes with GTPγS released the rest of the bound enzyme (not shown). Binding of NDP kinase α was dependent on the concentration of N-membranes (Fig. 1B): half-maximal binding occurred at R* concentration as low as 50–150 nM, being comparable to the value (60 nM) obtained for the bovine enzyme in ROS suspensions [20]. These data indicated that rat NDP kinase α and bovine ROS NDP kinase are very similar in binding properties.

NDP kinase β bound to N-membranes with similar proper-

ties in terms of pH, cation and GTPγS dependences (data not shown) to those observed for NDP kinase α, except that the interaction of NDP kinase β required greater membrane concentrations: half-maximal binding was observed at 10 μM R* (Fig. 1B). Thus, NDP kinase β seems to interact with the ROS membranes with an about 100-fold lower apparent affinity than does NDP kinase α.

3.2. Binding of NDP kinases to the ROS membranes is mediated by G_t

We next investigated whether G_t participates in the interaction of NDP kinase to the ROS membranes under these conditions. The results obtained with NDP kinase α showed (Fig. 2) that: (1) the effectiveness of GTP analogs at inhibiting the binding of NDP kinase was reminiscent of those for G_t-mediated reactions [26,27]; (2) GTPγS releasing most of the G_t from ROS membranes [27–30] resulted in a dramatic decrease in the apparent affinity of N-membranes for NDP kinase; (3) W-membranes depleted of G_t provided only low-affinity binding of NDP kinase and this binding was insensitive to GTPγS; and (4) addition of purified G_t to W-membranes restored the binding of NDP kinase, which was suppressed by the presence of GTPγS.

Taken together, these data clearly show that G_t is an important participant in the interaction between NDP kinase and ROS membranes. The binding sites seem to be complexes between R* and G_t, that are formed in bleached ROS membranes over a wide range of pH and ionic conditions [16,17,23,28,29]. In turn, the release of NDP kinases in the presence of GTPγS would appear to be due to disruption of the complexes [16,17,23,27–30].

3.3. A domain of NDP kinase involved in the interaction with ROS membranes

The differential binding properties of recombinant NDP kinase isoforms revealed with ROS membranes prompted us to conduct a search of the site(s) of the enzymes involved in the interaction as the possible structural origin of the difference. NDP kinases α and β are known to be highly homologous. They differ in 16 out of 152 amino acids; 7 and 3 of the 16 amino acid substitutions take place in the two major variable regions, one near the N-terminal third (V1 region) and the other at the C-terminal end (V2 region), respectively. Therefore, if the two isoforms demonstrate different properties in a certain experimental system, the difference would be ascribed to one of these variable regions of NDP kinases. To test this possibility, peptides α1 and β1, derived from the V1 region of NDP kinases α and β, respectively, peptides α2 and β2 from the V2 region of the enzymes, and a common peptide α/β derived from other conservative parts of the enzymes were synthesized and used to examine whether these peptides are capable of interfering with the interaction of bovine ROS NDP kinase with the ROS membranes. The results clearly demonstrated that peptide α1 was able to inhibit the interaction, whereas other peptides including β1 had no appreciable effects (Fig. 3).

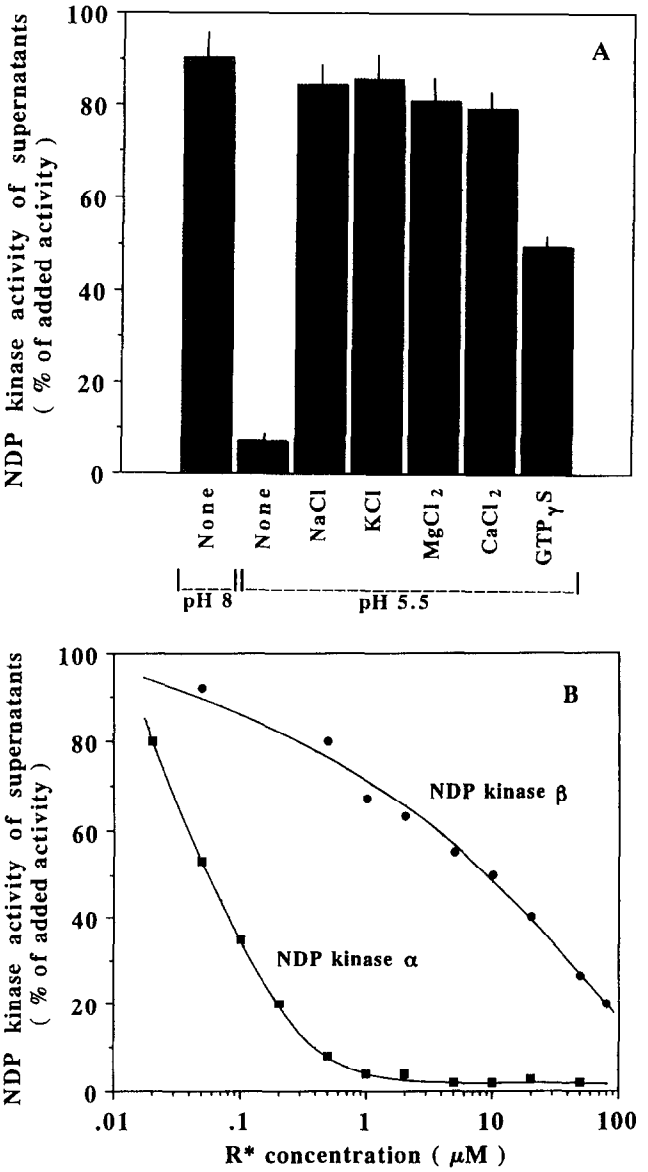


Fig. 1. Interaction of NDP kinase α and β with N-membranes. (A) Effects of pH, salts and GTPγS on the binding of NDP kinase α. N-membranes at a final R* concentration of 3 μM were mixed with NDP kinase α (final activity, 8.3 nmol ADP/min per 2 μl). Mixing was performed in either hypotonic neutral buffer N (pH 8.0) or hypotonic acidic buffer A (pH 5.5) in the absence or presence of either 100 mM NaCl, 100 mM KCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$ or 5 μM GTPγS as indicated. The samples were centrifuged and the activity of the supernatants was measured. The results are expressed as percent of the total activity added to the suspensions. Each column represents the means ± S.E.M. (bars) of triplicate samples. (B) Different apparent affinities of NDP kinase α and β for N-membranes. Different amounts of N-membranes at the final R* concentrations indicated were mixed in buffer A with NDP kinase α or β (final activities, 7.5 and 6 nmol ADP/min per 2 μl, respectively). The samples were centrifuged and the activity of the supernatants was measured. The results are expressed as percent of the total activity added to the suspensions.

4. Discussion

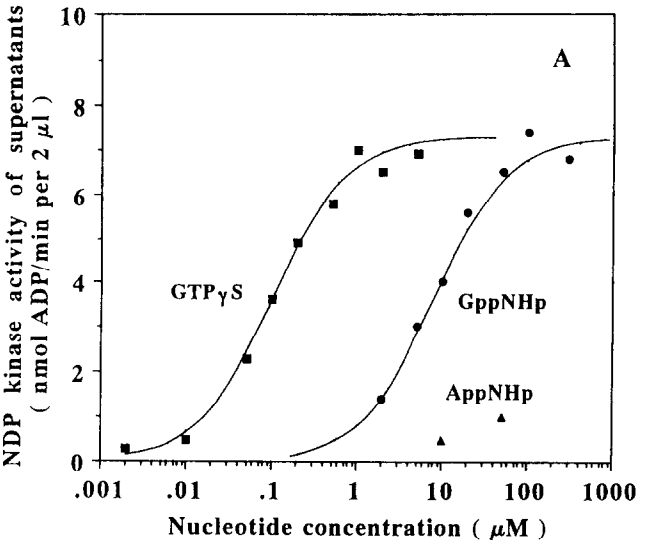
NDP kinases α and β exhibited pH-, cation- and GTPγS-sensitive binding to ROS membranes as did bovine ROS NDP kinase. Although differences in the binding properties of the rat isoforms were noted, the findings suggest that the interaction may reflect a common feature of members of the NDP kinase family.

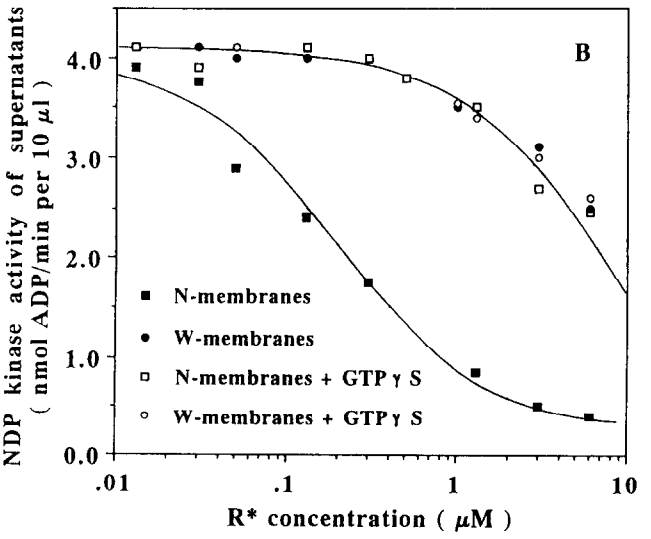
According to our data, G_t is an obligatory factor for the

high-affinity interaction of NDP kinases with bleached ROS membranes. Such results may support the suggestion [12–15] that NDP kinases play a role in cellular regulation such as signal transduction via the interaction with G-proteins. The possible physiological meaning of the interaction between NDP kinase, G_t and R* has been discussed in our previous work [20].

There are some reports describing possible distinctive roles of NDP kinase isoforms [31,32]. Nevertheless, because of the extreme structural similarity between them, their respective roles have not been clearly presented as yet. This study has revealed that NDP kinases α and β are dramatically different in their affinity for the candidate binding site, the complex between R* and G_t present in ROS membranes. Furthermore, the finding that the binding of the endogenous bovine NDP kinase to ROS membranes was interrupted by α1 peptide demonstrates the involvement of the V1 region of the α isoform in the binding. It could be extrapolated from these results that the two isoforms may exert their functions via interacting with their own target proteins through the V1 region. The crystallographic observation [33] that the V1 region encompasses the region from the β2 strand to the α_A helix of the molecule and extends to the outer surface of the hexameric structure may support this view.

In this work we have described a simple system to investigate the physical interaction of NDP kinases with G-proteins in the presence of activated receptor. Moreover, this system allows one to distinguish the NDP kinase isoforms on the basis of their binding properties. We believe that this work should provide a molecular basis for future studies on the mode of action of NDP kinase in cellular regulation as well as the possible diversity of the function of NDP kinase in cells.





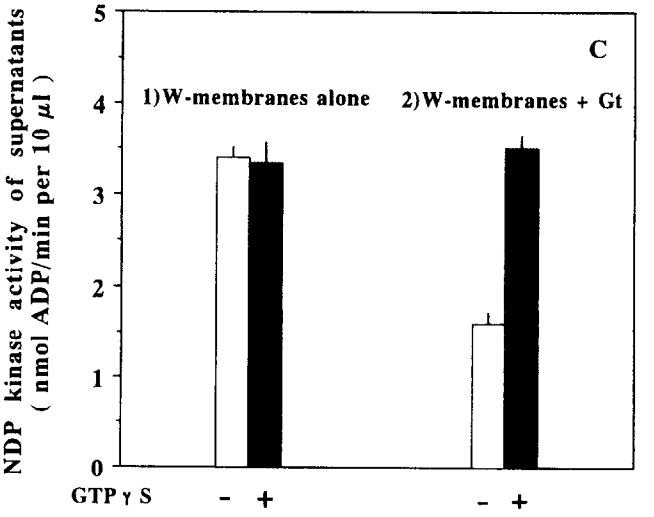


Fig. 2. Involvement of G_t in the binding of NDP kinase α to ROS membranes. (A) Guanine-nucleotide-sensitive binding. N-membranes at a final R* concentration of 2 μM were mixed with NDP kinase α (final activity, 12.3 nmol ADP/min per 2 μl) in buffer A in the presence of indicated concentrations of either GTPγS, GppNHp or AppNHp, were centrifuged and the activity of the supernatants was measured. The curves are theoretical plots computed according to the Hill equation as previously described [20] with a Hill coefficient of 1 and half-saturating concentrations of GTPγS and GppNHp of 0.1 and 8 μM, respectively. (B) Loss of high-affinity NDP kinase binding in G_t-depleted ROS membranes. Different amounts of N-membranes (squares) or W-membranes (circles) at the final R* concentrations indicated were mixed with NDP kinase α at a final concentration of 1.5 μM (final activity, 4.3 nmol ADP/min per 10 μl) in buffer A in the absence (closed symbols) or presence of 5 μM GTPγS (open symbols), centrifuged and the activity of supernatants was measured. (C) G_t-induced restoration of the GTPγS-sensitive interaction of NDP kinase α with W-membranes. (1) W-membranes alone: W-membranes at a final R* concentration of 0.6 μM were mixed with NDP kinase α at a final concentration of 1.5 μM (final activity of 4.3 nmol ADP/min per 10 μl) in buffer A in the absence or presence of 5 μM GTPγS as indicated and the activity of the supernatants was measured. (2) W-membranes plus G_t: The experiment was carried out as described above except that G_t (final concentration of 80 nM) was added prior to the incubation with NDP kinase α. Each column represents the mean ± S.E.M. (bars) of triplicate samples.

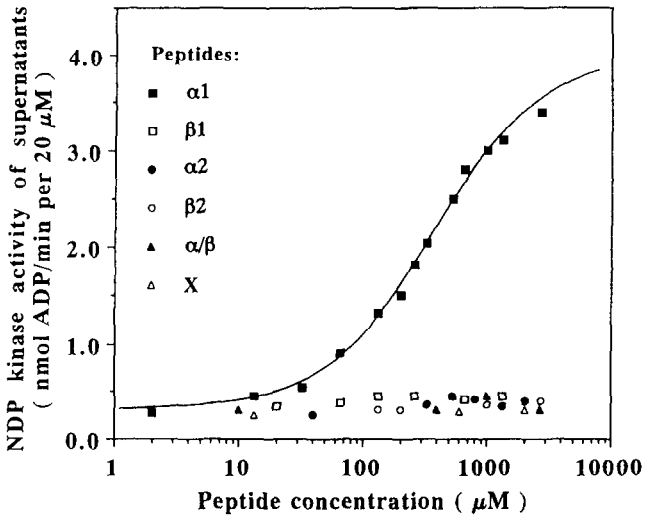


Fig. 3. Influence of synthetic peptides on the binding properties of bovine ROS NDP kinase. Either one of the peptides, α1, β1, α2, β2, α/β or X, was added to the bleached ROS suspensions in buffer A at the final concentrations indicated. Final NDP kinase activity and ROS protein concentration present in the ROS preparation used were 4.4 nmol/min per 20 μl and 170 μg/ml, respectively. The samples were incubated, centrifuged and the NDP kinase activity of the supernatants was measured. The curve was fitted with the Hill equation as mentioned above with a Hill coefficient of 1 and half-saturating α1 concentration of 360 μM.

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