Autophosphorylation of nucleoside diphosphate kinase on non-histidine residues

Anthony A. Bominaar, Annemiek D. Tepper, Michel Véron*

Unité de Biochimie Cellulaire, CNRS-URA 1129, Institut Pasteur, 75724 Paris Cedex 15, France

Abstract Recently, several reports appeared which described auto-phosphorylation of NDP kinase on residues different from the active-site histidine. Based on these findings conclusions were drawn with respect to a regulation of enzyme activity and to a possible role as a metastasis suppressor. In this paper we show that although non-histidine autophosphorylation occurs on NDP kinases from mammals, lower eukaryotes and bacteria, less than 0.2% of the subunits are phosphorylated. Using site-directed mutagenesis, we show that the active site histidine is essential for non-histidine autophosphorylation. The low stoichiometry of phosphate incorporation excludes a role of autophosphorylation in regulating overall enzyme activity.

Key words: NDP kinase; nm23; Phosphorylation

1. Introduction

Nucleoside diphosphate kinase (NDP kinase; EC 2.7.4.6) is the enzyme responsible for the equilibration of all non-adenine nucleoside triphosphates and nucleoside diphosphates. NDP kinase also appears to be involved in processes related to differentiation, cell-proliferation and metastasis [1–4]. These functions are not obviously related to its enzymatic function. Recently, autophosphorylation on residues different from the active site histidine (notably serine) was reported. In human NDP kinase A, encoded by the gene *nm23-H1*, the presence of phospho-serine was related to metastasis suppression [5]. Although no data on stoichiometry were provided, the authors suggest that phosphate incorporation is substantial. The residue phosphorylated was mapped to be serine-45 and it was suggested that 'in vivo' phosphate turnover on this residue might result in a significant ATPase activity. For the NDP kinase from *Myxococcus xanthus* [6] a 1:1 ratio of serine to histidine phosphate has been described. Given the known importance of phosphorylation as a regulatory mechanism in many different signal transduction pathways, auto-phosphorylation of NDP kinase has tentatively been related to its 'second function(s)'.

In this paper we have examined the autophosphorylation of NDP kinase in more detail. We used *Dictyostelium* NDP kinase for which the structure of the active site is known from X-ray studies [7,8], as well as the enzymes from *Myxococcus* for which the structure has also been solved [9] and from human. We show that although autophosphorylation on non-histidine residues can be demonstrated, non of the three NDP kinases investigated showed a stoichiometry of phosphorylation above 0.2% of the subunits. The implications with respect to biological function are discussed.

2. Materials and methods

2.1. Materials

[γ-³²P]ATP (> 3000 Ci/mmol) was obtained from Amersham plc.,

ATP, ADP, EDTA and HEPES were from Sigma (St. Louis), pre-stained SDS-PAGE molecular weight markers were from BDL. Nitrocellulose filters were from Whatman.

2.2. Proteins

Recombinant *Dictyostelium discoideum* NDP kinase was purified from *E. coli* [10], pure *Myxococcus xanthus* NDP kinase was a kind gift of Dr. R. Williams (MRC, Cambridge, UK), pure recombinant Human NDP kinase-A was a kind gift of Dr. I. Lascu (Université Bordeaux 2, France). Mutant of *Dictyostelium* NDP Kinase were made by site directed mutagenesis according to Kunkel et al. [11], and the mutations were verified by standard DNA-sequencing techniques. The synthetic oligo nucleotides were:

H122C: 5'-GAAACATCATCTGCGGTTCTGATTC-3'
S124C: 5'-CATCCACGGTTGCGATTCAGTTG-3'
S124A: 5'-CATCCACGGTGCGGATTCAGTTG-3'
S124G: 5'-CATCCACGGTGGGGATTCAGTTG-3'

Altered bases as compared to the wild-type sequence are underlined. Mutant proteins were purified to homogeneity following the same procedure as for wild type enzyme.

2.3. Phosphorylation

NDP kinase (1 μg) was incubated with 100 μM ATP (2000 cpm/pmol) in a buffer containing 20 mM HEPES/NaOH pH 7.4, 50 mM NaCl and 1 mM MgCl₂ in a total volume of 10 μl. For histidine phosphorylation, reactions were quenched by adding SDS-gel sample buffer and applied to gel immediately. For non-histidine phosphorylation, the phosphate bound to histidine was chased by the addition of a ten fold excess of ADP. After 1 min the reactions were terminated by the addition of SDS-gel sample buffer and boiling of the samples for 3 min. Samples were applied to 12.5% SDS-PA gels to separate the proteins from ATP and P$_i$. Kodak X-OMAT AR films were exposed to the dried gels at −80°C for the time indicated in the figure legends. The radioactivity incorporated was quantified by excission of the protein bands and counting in a liquid scintillation counter.

2.4. Instability of histidine phosphate in NDP kinase

NDP kinase (1 μg) was incubated for 15 min at 20°C together with 1 μCi ATP (> 3000 Ci/mmol) in a buffer composed of 40 mM HEPES/NaOH pH 7.4, 1 mg/ml BSA, 1 mM EDTA, in a final volume of 10 μl, denatured by the addition of 2 μl of 5 × concentrated SDS-PAGE gel sample buffer and spotted on nitrocellulose filters. The filters were washed 3 times in a buffer containing 50 mM Tris, 40 mM glycine pH 8.4 and 20% methanol and twice in PBS. Dephosphorylation was determined by incubating the filters in either 1.5 ml of 100 mM HCl pH 1.0 or in 1.5 ml of 100 mM HEPES/NaOH pH 7.4 at 22°C or 45°C. At the indicated time points the filters were removed from the bathing solution, rinsed once with 1 ml of H₂O and counted. The incubation solution and the H₂O used for rinsing were combined and counted as well.

*Corresponding author: Fax: (33) (1) 4568 8399.

Abbreviations: NDP, nucleoside diphosphate; SDS-PAGE, SDS-poly-acrylamine gel electrophoresis.

Dephosphorylation was determined from the fraction of total radioactivity found in the liquid phase.

3. Results and discussion

3.1. Release of histidine-bound phosphate

The major problem in the study of protein phosphorylation of NDP kinase is that the enzymatic reaction catalyzed by the protein proceeds via a phospho-histidine (His-122) intermediate. Combined with the fact that ATP is a natural substrate this implies that any (auto)phosphorylation event will be masked by a high level of histidine phosphate. In previous studies the ratio between acid labile and alkaline labile phosphate was used to determine the presence of non-histidine phosphate. This method, however, is less straightforward than generally assumed. Although phospho-histidine is acid labile and alkali stable, it is often forgotten that phospho-histidine is also thermo-labile. As is shown in Fig. 1, release of phosphate at 22°C at pH 1.0 has a half-life of 78 min which is 5-fold shorter than that for pH 7.4, however increase of the temperature to 45°C decreased the half-life at neutral pH 11-fold to 38 min. These data clearly show that the loss of phosphate upon incubation at different pH is not a solid means to examine the presence of non-histidine phosphate on protein known to contain histidine phosphate. Thermal instability of histidine can be an important factor as well. Therefore a more reliable method is required to remove all of the histidine bound phosphate before examining the presence of alkali labile phosphate.

3.2. Non-histidine autophosphorylation

To circumvent the acid treatment we made use of the enzymic properties of the protein, which readily releases its histidine bound phosphate as soon as an appropriate acceptor is present. With a k_{cat} <2000 s^{-1} the time needed to release the phosphate is extremely short, allowing rapid further processing of the protein. A second advantage of using a high ratio of acceptor over donor is that the acceptor acts as a competitive inhibitor preventing the rephosphorylation of the protein [12]. Followed by a 3-min heat treatment this method should release all histidine phosphate. Fig. 2 demonstrates the dramatic difference between the levels of histidine phosphate and non-histidine phosphate. In panel A, NDP kinase from Dictyostelium, Myxococcus, and human (1 µg, 1 µg and 0.6 µg, respectively) was phosphorylated and separated without further treatment; in panel B, identical samples were first treated as described to remove histidine bound phosphate and than separated. Subsequently films were exposed to the two gels, 2 h for panel A, 120 h for panel B. The non-histidine phosphorylation for both Myxococcus and Dictyostelium NDP kinases is extremely low (Fig. 2B, lanes 1 and 2). No major differences in the levels of phosphorylation between the two enzymes were observed. The human enzyme NDP kinase A is more strongly labeled under these conditions (Fig. 2B, lane 3). However, when the large difference in time of development of the autoradiographs in Fig. 2A and B is taken into account (respectively of 2 h and 120 h), we estimated the maximal level of non-histidine phosphorylation as less than 1/50 of the histidine-phosphate.

In Fig. 3A the amount of phosphate remaining after chasing with ADP, is plotted as a function of the incubation time with ATP. A time dependency comparable to the histidine phosphorylation would be expected if the remaining phosphate was
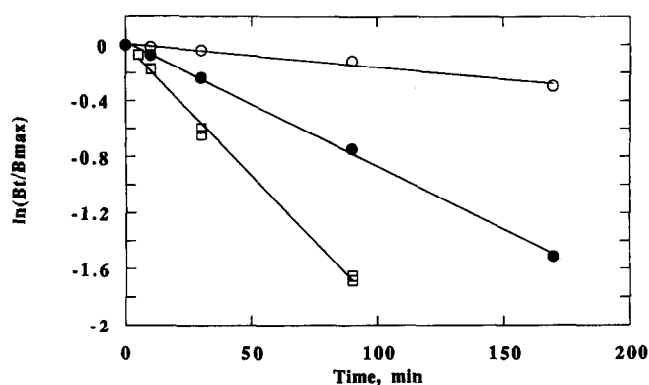

Fig. 1. Dissociation kinetics of histidine bound phosphate. NDP kinase was phosphorylated, SDS-denatured and spotted onto nitrocellulose. Filters were incubated at pH 1.0 (●) or pH 7.4 (○) at 22°C or at pH 7.4 at 45°C (□)

bound to histidine. However this latter reaction reaches plateau levels within milliseconds, whereas the phosphorylation observed here reaches a plateau in 5 to 10 min. This clearly illustrates that the phosphorylation observed here is due to a different process than the normal enzymic histidine phosphorylation. Data from Fig. 3B lead to a computation of a K_m for ATP of 100 µM and the maximal stoichiometry 0.043 ± 0.006% of the total protein. Although the human enzyme reaches a higher level of autophosphorylation (see also Fig. 2B), the maximal stoichiometry of autophosphorylation of the human enzyme is 0.14 ± 0.02%, which is only three fold higher than the enzyme from Dictyostelium, and still very low. The time needed to reach these levels is slightly longer (20 min as compared to 5–10 min in the experiment shown in Fig. 3 using Dictyostelium NDP kinase). The K_m is comparable to what we observed for the Dictyostelium enzyme, approx. 100 µM.

Our results are contradictory to those reported by Muñoz-Dorado for the myxococcal protein: analogous levels for histidine- and serine-phosphorylation were reported, and thus the authors concluded a high serine-phosphate content. The most likely cause of the discrepancy observed lies in the fact that
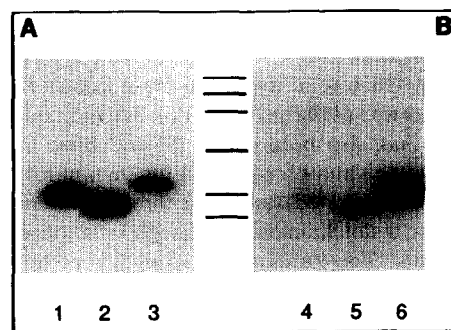

Fig. 2. Comparison of the extent of histidine to non-histidine phosphorylation. One µg NDP kinase from Dictyostelium (lanes 1 and 4), Myxococcus (lanes 2 and 5) and 0.6 µg Human NDP kinase-A (lanes 3 and 6) were autophosphorylated in the presence of 100 µM ATP (2000 cpm/pmol) and either processed to retain histidine phosphate (panel A) or histidine phosphate was removed (panel B). Subsequently samples were autoradiographed after SDS-PAGE separation. Exposure times were panel A: 2 h, panel B: 120 h. The markers indicated are 105, 70, 43, 28, 18, 15.4 kDa.
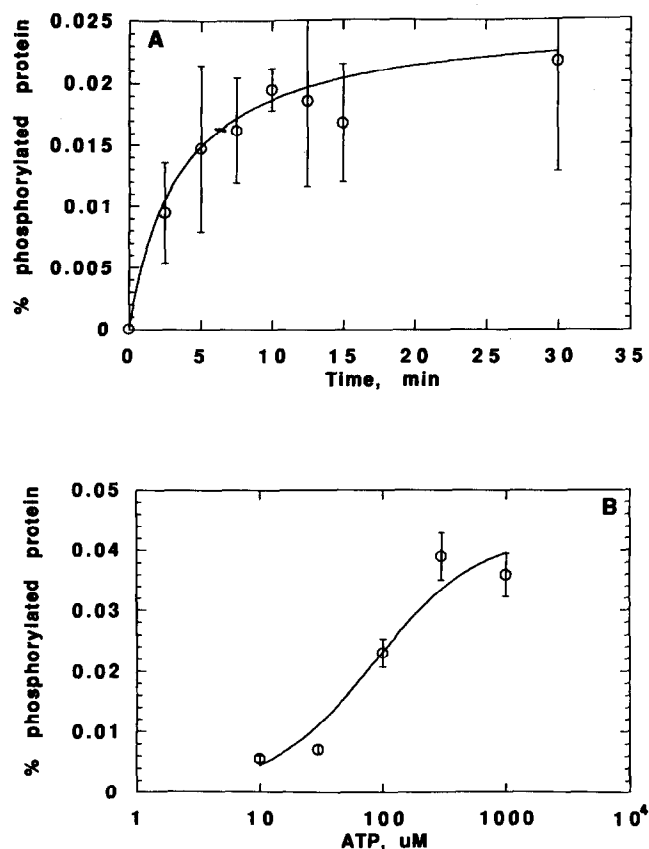
Fig. 3. Characterization of non-histidine auto-phosphorylation. *Dictyostelium* NDP kinase was incubated in the presence of 100 μM ATP (2000 cpm/pmol) (panel A) or at different concentrations of ATP (2×10^5 cpm/sample) (panel B). At the indicated time points (15 min for panel B) the reaction was terminated and incorporation of phosphate was determined. Data are expressed as mean ± S.E.M. from three independent experiments, each in duplicate.

Muñoz-Dorado et al. [6] used alkaline hydrolysis at 45°C, which will also release phosphate from histidine thus giving an overestimation of the alkali labile fraction. The fact that the ATP concentration used was such that the stoichiometry of histidine phosphorylation will have been relatively low, again resulted in an overestimation of the stoichiometry of serine phosphorylation. As far as the results of McDonald et al. [5] are concerned, here the discrepancy is only apparent and is merely the result of the conclusions drawn by the authors regarding the biological significance of their results. Although no stoichiometric data are provided, the computing of a maximal stoichiometry from their data, indeed indicate a very low level of phosphorylation, which is in no way contradictory to the levels described here.

3.3. Residues involved in non-histidine autophosphorylation

Mutant H122C is a completely inactive mutant, lacking the active site histidine [7]. Using this mutant we are able to show that non-histidine phosphorylation requires the active site histidine (Fig. 4). Again these results are in apparant contrast with what was found by McDonald et al. using mutant H118G (H118 is the nm23 homolog of H122 in *Dictyostelium* NDP kinase), retaining autophosphorylation. However their H118G mutant still had a residual activity of 2.5%. This makes a con-

tamination of their protein with *E. coli* NDP kinase very likely since the catalytic mechanism of the enzyme implies that replacement of this histidine should lead to a totally inactive protein. The *E. coli* NDP kinase might then also account for the phosphorylation observed.

Considering the fact that the stoichiometry of *Dictyostelium* NDP kinase phosphorylation was less than 0.1%, microsequencing of a tryptic digest to determine the phosphorylated residue was not possible. However, since serine 124 (Ser-124) is in close proximity to the active site histidine, not only in the sequence [10] but also in the 3D structure [7], we used site-directed mutagenesis to test whether Ser-124 could be the phosphorylated residue. Three different substitutions were made. S124A and S124G in which the serine side chain is removed and S124C a 'neutral' replacement since cysteine can be considered as sterically similar to serine and also has a nucleophile group. While H122C showed a total lack of non-histidine phosphorylation, (Fig. 4, lane 2) a very low but reproducible phosphorylation was observed with mutant S124A NDP kinase (Fig. 4, lane 3). With the S124G mutant (Fig. 4, lane 4) auto-phosphorylation was irreproducible and was only observed on fresh preparations. It should be noted that S124G NDP kinase mutant is a very unstable protein. (A.D. Tepper, unpublished results). Finally, the S124C mutation resulted in a much stronger autophosphorylation (Fig. 4, lane 5), which however still corresponded to a stoichiometry of less than 1% of the subunits. This increased phosphorylation could result from an effect of the mutation on the enzyme conformation or could directly be due to the fact that a thiol is a more potent nucleophile than the hydroxyl of serine, supporting the hypothesis of direct phosphorylation of Ser-124.

In conclusion, we demonstrate that the low non-histidine phosphorylation is dependent on the presence of the active site histidine, and that while S124 might be phosphorylated, additional residues can be phosphorylated at a low level as well.

Serine-45 in human NDP kinase (corresponding to threonine 48 in *Dictyostelium* NDP kinase) was reported to be phosphorylated [5]. Ser-45 is very unlikely to receive a phosphate from the active site histidine given its position in the structure [7,8]. Microsequencing of digests of proteins phosphorylated with a stoichiometry as low as described here can easily result in the assignment of phosphate to the wrong
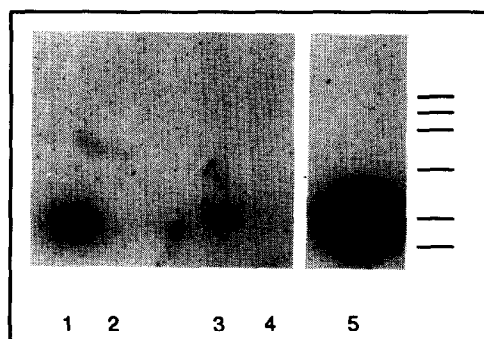

Fig. 4. Non-histidine autophosphorylation of *Dictyostelium* NDP kinase mutants. NDP kinase was phosphorylated and non-histidine autophosphorylation was detected by autoradiography. Lane 1, wild type *Dictyostelium* NDP kinase; lane 2, H122C; lane 3, S124A; lane 4, S124G; lane 5, S124C. Autoradiograms were exposed for 120 h. Markers are as in Fig. 2.

peptide because separation of a very small phosphorylated fraction from the rest of the peptides will almost certainly result in contamination with another peptide. An alternative possibility is that the phosphotransfer would occur between two different molecules, NDP kinase acting as a histidine protein kinase. If NDP kinase is acting as a protein kinase, its 'second function' might well be this kinase activity on other proteins and not so much its auto-phosphorylating activity. However, this hypothesis is unlikely because the phosphate-bound to histidine is not in a position favorable to be transferred to another protein.

3.4. Biological significance of non-histidine autophosphorylation

The biological significance of the observed phosphorylation has to be questioned. Considering the enzyme activity of the protein it will be obvious that a phosphorylation in the order of magnitude of 0.1% with relatively slow kinetics can have no effect on total protein activity unless it would result in a dramatic activation. Preincubation of *Dictyostelium* NDP kinase with ATP during 15 min had no effect on activity (data not shown) thereby excluding a significant activation. The single possibility which cannot be excluded is that the phosphorylated protein has another function besides catalysis. The existence of a second function for NDP kinase has been suggested by several authors. NDP kinase B (encoded by *nm23-H2*) has been shown to bind to and activate the transcription of the *c-myc* promoter in vitro [13]. These functions are conserved in an inactive mutant of NDP kinase B lacking the active site histidine [14], indicating that autophosphorylation plays no role in the DNA binding activity of NDP kinase. Non histidine autophosphorylation might simply be the result of an erroneous transfer of phosphate from the active site histidine to an accepting amino acid in its vicinity. Under natural conditions it probably does not occur at all because in vivo the lifetime of a phospho-histidine is extremely short.

References

[1] Hailat, N., Keim, D.R., Melhem, R.F., Zhu, X., Eckersckorn, C., Brodeur, G.M., Reynols, C.P., Seeger, R.C., Lottspeich, F., Strahler, J.R. and Hanash, S.M. (1991) J. Clin. Invest. 88, 341–345.
[2] Keim, D., Hailat, N., Melhem, R., Zhu, X.X., Lascu, I., Veron, M., Strahler, J. and Hanash, S.M. (1992) J. Clinic. Invest. 89, 919–924.
[3] Rosengard, A.M., Krutzsch, H.C., Shearn, A., Biggs, J.R., Barker, E., Margulies, I.M.K., King, C.R., Liotta, L.A. and Steeg, P.S. (1989) Nature. 342, 177–180.
[4] Steeg, P.S., Bevilacqua, G., Pozzatti, R., Liotta, L.A. and Sobel, M.E. (1988) Cancer Res. 48, 6550–6554.
[5] Mac Donald, N.J., De La Rosa, A., Benedict, M.A., Freije, J.M.P., Krutsch, H. and Steeg, P.S. (1993) J. Biol. Chem. 268, 25780–25789.
[6] Munoz-Dorado, J., Almaula, N., Inouye, S. and Inouye, M. (1993) J. Bacteriol. 175, 1176–1181.
[7] Dumas, C., Lascu, I., Moréra, S., Glaser, P., Fourme, R., Wallet, V., Lacombe, M.-L., Véron, M. and Janin, J. (1992) EMBO J. 11, 3203–3208.
[8] Moréra, S., Lascu, I., Dumas, C., LeBras, G., Briozzo, P., Véron, M. and Janin, J. (1994) Biochemistry 33, 459–467.
[9] Williams, R.L., Oren, D.A., Munoz-Dorado, J., Inouye, S., Inouye, M. and Arnold, E. (1993) J. Mol. Biol. 234, 1230–1247.
[10] Lacombe, M.L., Wallet, V., Troll, H. and Veron, M. (1990) J. Biol. Chem. 265, 10012–10018.
[11] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367–382.
[12] Parks, R.E.J. and Agarwal, R.P. (1973) The Enzymes. 8, 307–334.
[13] Postel, E.H., Berberich, S.J., Flint, S.J. and Ferrone, C.A. (1993) Science 261, 478–480.
[14] Postel, E.H. and Ferrone, C.A. (1994) J. Biol. Chem. 269, 8627–8630.