Upsala J Med Sci 87: 243–250, 1982

Effect of Chemical Modification of a Histidine and a Lysine Residue of Pea Seed Nucleoside Diphosphate Kinase

Bror Edlund*, Carl Henrik Heldin and Lorentz Engström

From the Institute of Medical and Physiological Chemistry, Biomedical Centre, University of Uppsala, Sweden

**Present address: Department of Clinical Chemistry, University Hospital, University of Uppsala, Sweden*

ABSTRACT

Chemical modification of a histidine and lysine residue inactivates pea seed nucleoside diphosphate kinase (NDP kinase). Thus there seems to be a reactive lysine residue, at the active site of pea seed NDP kinase, in addition to the histidine residue phosphorylated by the substrate ATP as a consequence of the enzyme reaction. The presence of a reactive lysine at the active site of the enzyme could explain why a small amount of N-ε-phospholysine, as well as 1-phosphohistidine and 3-phosphohistidine, is formed on alkaline hydrolysis of the enzyme.

INTRODUCTION

Earlier studies have indicated that NDP kinase (nucleoside diphosphate: ATP transphosporylase EC 2.7.4.6) from pea seed is a tetrameric protein with a molecular weight of 70 000, with four identical subunits, each containing an active site (1,2). Evidence has also been obtained that the enzyme is intermediately phosphorylated by its substrate ATP on a histidine residue at the active site, presumably as 1-phosphohistidine, the phosphoamino acid dominating in an alkaline hydrolysate of phosphorylated NDP kinase from baker's yeast (3,4).

The amino acid sequences of the dominating phosphopeptides from the active site of the pea seed enzyme inactivated in two different ways, i.e. with alkali and acid, and degraded with two different proteases, trypsin and pepsin respectively, are overlapping (5,6) indicating that the phosphoryl group is bound to the same histidine residue in the enzyme irrespective of the inactivation method used.

Two tryptic phosphopeptides with the same amino acid sequence but differing in stability to acid of the phosphoryl bond are obtained, probably by a phosphoryl group migration from 1-phosphohistidine to 3-phosphohistidine (7).

Alkaline hydrolysis of phosphorylated pea seed NDP kinase gives mainly phosphopeptides, and in addition small amounts of phosphohistidine and N-ε-

phospholysine (8,9). The present investigation was therefore focused on the possibility that there might exist, in addition to the reactive histidine residue, a lysine residue, essential for enzyme activity, in the active site of pea seed NDP kinase. If so, these amino acid residues should be exposed in the active site and more chemically reactive than the rest of the amino acid residues of the enzyme.

It was considered that support for this view would be obtained if chemical modification of one lysine and one histidine residue were to inactivate the enzyme and if substrate and product protected the enzyme from inactivation. The enzyme was therefore treated with 1-fluoro-2,4-dinitrobenzene (FDNB) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) which are known to react preferentially with lysine residues (10,11,12) and diethylpyrocarbonate (DPC) which reacts with histidine residues (13,14). The activity of the treated enzyme was studied in relation to the degree of chemical modification obtained, also in the presence of the substrate ATP and product ADP.

MATERIALS AND METHODS

The enzyme was prepared as previously described (8). An absorbance value of $E_{280}^{1\%} = 14.1$ was used for the purified enzyme (8). The enzyme activity was determined by a coupled assay according to Mourad and Parks, using dGDP as nucleoside diphosphate (15). FDNB was obtained from Pierce, TNBS from Sigma, DPC from Eastman and sodium dodecylsulfate (SDS) from Pierce. N-tris-(hydroxymethyl)-methyl-2-amino-ethanol sulfonic acid (TES) was purchased from British Drughouse Ltd. All chemicals were of highest quality available. Spectroscopic measurements were made with a PMQ II Zeiss spectrophotometer, using a recorder for registration.

Reaction of NDP kinase with FDNB. To 20 ml of NDP kinase (15 µmoles/l) in TES buffer (0.2 moles/l, pH 8.5) 1.6 ml of a solution of FDNB in ethanol (0.1 moles/l) were added. The solution was kept at room temperature (23°C). Aliquots (1 ml) were taken at fixed intervals and chromatographed at 23°C on a Sephadex G-50 column (1.4x16 cm) equilibrated and eluted with sodium phosphate buffer (0.05 moles/l, pH 6.5). The absorbance at 280 nm of the fractions eluted with the void volume were measured and the molar amount of incorporated dinitrophenyl groups was calculated. A molar absorbance of 16 000 cm^{-1} at 365 nm was used for the dinitrophenyl groups (10) and the number of modified lysine residues per subunit was calculated. The enzyme activities were determined and compared with that of a sample chromatographed without previous addition of FDNB.

Treatment of NDP kinase with DPC. The number of histidyl residues reacting with DPC were determined by difference spectroscopy at 240 nm, using a molar absorbance of 3200 cm^{-1} (13). The carbetoxylation of the enzyme was

carried out in sodium phosphate buffer (0.05 moles/l, pH 6.5) or in triethanolamine-acetic acid buffer (0.05 moles/l, pH 7.4). Solutions of DPC in ethanol were prepared ex tempore. The final concentration of DPC was 1 mmole/l and that of the enzyme 15 μmoles/l. The extent of inactivation of the enzyme was calculated in relation to that of the enzyme treated in the same way but without addition of DPC.

Trinitrophenylation of lysine residues. To 1 ml of NDP kinase (30 μmoles/) in sodium hydrogen carbonate (0.25 moles/l), 1 ml of TNBS (1.7 mmoles/l) were added at zero time. A blank solution without NDP kinase was also prepared. The reaction mixtures were kept at 40°C. Aliquots were taken for enzyme assay at fixed intervals. At the same time 100 μl of sample and blank solutions were diluted with 100 μl of 10% (w/v) SDS in water followed immediately by 50 μl of HCl (1 mole/l) and 3 ml of HCl (0.01 moles/l) (12). The difference in absorbance between sample and blank solutions at 344 nm was determined and the number of trinitrophenyl groups incorporated was calculated from the molar absorbance of 11 000 cm^{-1} at 344 nm given for a trinitrophenyl group in a protein (12). The extent of inhibition was calculated as per cent of enzyme activity remaining after the experimental period.

Inactivation of NDP kinase in the presence of ATP and ADP. In all experiments the final NDP kinase concentration was about 0.2 μmoles/l. The enzyme solutions contained magnesium acetate (1.5 mmoles/l) in order to obtain the nucleotides in the magnesium form. Samples containing ATP (0.8 mmoles/l) or ADP (0.8 mmoles/l) were incubated at 30°C with DPC (1 mmole/l) in triethanolamineacetic acid buffer (40 mmoles/l, pH 7.4) for 10 min, TNBS (2 mmoles/l) in sodium hydrogen carbonate (10 mmoles/l) for 30 min or FDNB (20 mmoles/l) in sodium dihydrogen carbonate (40 mmoles/l) for 30 min. The residual enzyme activity was compared with that of a control solution in which the modifying agent was omitted. In the experiments with DPC and FDNB, where the reagent was dissolved in ethanol, the same amount of ethanol was included in the control experiment (5% to 10% (v/v)). The enzyme activity was not affected by these ethanol concentrations.

RESULTS AND DISCUSSION

The reaction of pea seed NDP kinase with FDNB indicates that there was one lysine residue per subunit which reacted faster than the other nine (2). All enzyme activity was lost when this residue had reacted, but the enzyme activity seemed to decrease more rapidly than the dinitrophenylation of the lysine residue, indicating that another kind of amino acid residue was also reacting. Imidazole-dinitrophenyl-histidine does not show an absorbance peak at 365 nm and would therefore not have been detected by the method used for measuring the degree of dinitrophenylation of the enzyme in the present work

(16). It is therefore possible that a histidine residue was also blocked by FDNB, leading to inactivation of the enzyme (Fig. 1).

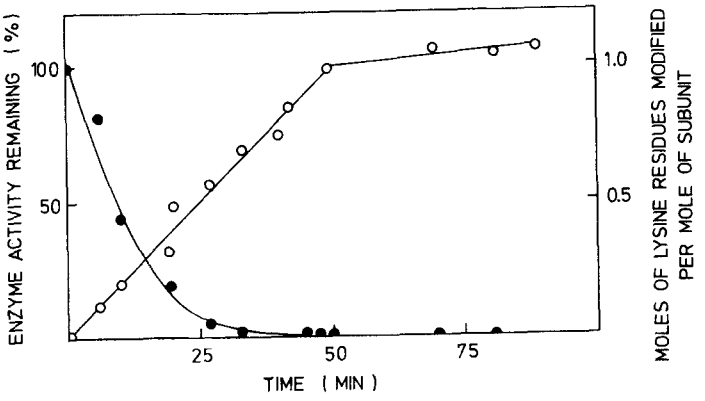

Fig. 1. Inhibition of pea seed NDP kinase by FDNB at pH 8.5. Filled circles represent enzyme activity remaining and open circles represent DNP groups incorporated per mole of subunit.

The carbetoxylation at pH 7.4 of one histidine residue per subunit of the enzyme out of three (2) leads to inactivation of enzyme (Fig. 2).

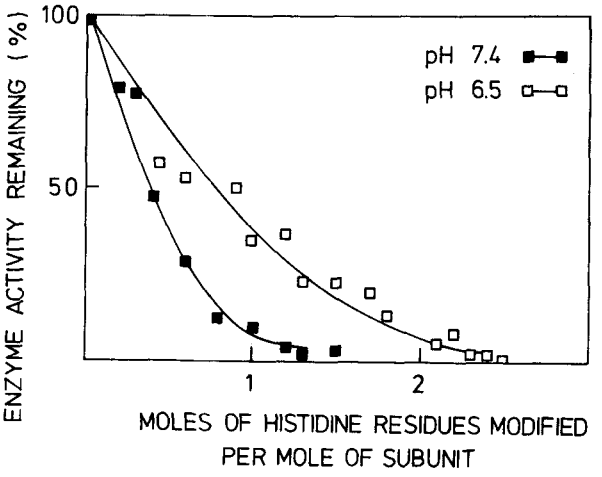

Fig. 2. Modification of histidine residues of pea seed NDP kinase by carbetoxylation with DPC and its effect on enzyme activity. Filled squares represent enzyme activity remaining at pH 7.4 and open squares represent enzyme activity remaining at pH 6.5 after modification of the indicated number of histidine residues per subunit.

At pH 6.5 two histidine residues reacted before the enzyme was completely inactivated. The enzyme from beef heart cytosol has also been inactivated by carbetoxylation (17).

The trinitrophenylation of pea seed NDP kinase leads to rapid inactivation of the enzyme, supporting the view that the enzyme contains a lysine residue which is essential for its activity (Fig. 3). However, on prolonged incubation, all ten lysine residues in each subunit were modified, showing that all of them became accessible to TNBS.

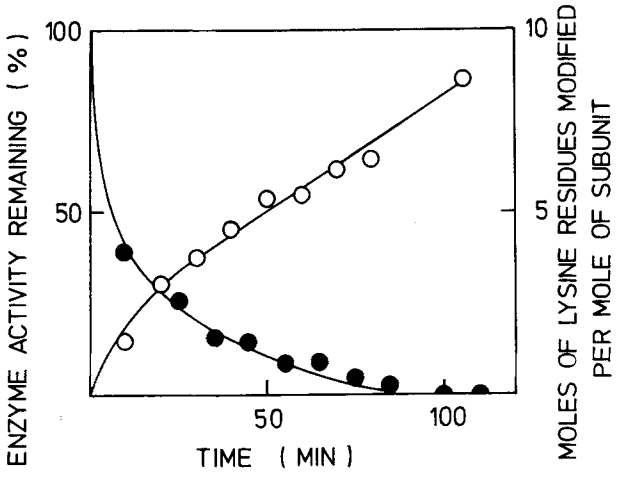

Fig. 3. Inhibition and modification of pea seed NDP kinase by TNBS. Filled circles represent enzyme activity remaining and open circles trinitrophenyl groups incorporated per subunit after the indicated incubation period.

The fact that the substrate ATP and the product ADP protect, at least partially, the enzyme from inactivation by the reagents used here, indicates that the reactions involve the active site of the enzyme (Table 1). Nucleotides have also been shown to protect some NDP kinases from inactivation by p-chloromercuribenzoat (pCMB) (18,19,20,21). It has been discussed that such a sulfhydryl group may be essential for the qurtenary structure of the enzyme and not a part of the active site of the enzyme (18,21). Pea seed NDP kinase does not contain any SH-group at all (2). Thus there seems to be a reactive lysine residue in addition to a reactive histidine residue in the active site of pea seed nucleoside diphosphate kinase.

Table 1. Effect of ATP and ADP on the inactivation of pea seed NDP kinase by FDNB, DPC and TNBS. In each series a control experiment was made with no addition of nucleotides. In the control experiments the enzyme showed the same specific activity (1 200 units/mg) as when the enzyme was kept in triethanolamine-acetic acid buffer (0.01 moles/l, pH 7.4). The mean values obtained in duplicate experiments are given below. For details see text.

Additions	Residual enzyme activity (per cent)
ATP (control)	100
FDNB	47
FDNB + ATP	80
ADP (control)	100
FDNB + ADP	84
ATP (control)	100
DPC	4
DPC + ATP	39
ADP (control)	100
DPC + ADP	75
ATP (control)	100
TNBS	58
TNBS + ATP	81
ADP (control)	100
TNBS + ADP	75

ACKNOWLEDGEMENTS

This work was supported by the Swedish Medical Research Council (Project No. 13X-50). The skilful technical assistance by Mrs. Jill Ekström is gratefully acknowledged.

REFERENCES

1. Edlund, B.: Tetrameric structure of nucleoside diphosphate kinase from pea seed. FEBS Letters 13:56-58, 1971.
2. Edlund, B.: Evidence for identical subunits in pea seed nucleoside diphosphate kinase. FEBS Letters 38:222-224, 1974.
3. Edlund, B. & Wålinder, O.: Evidence for an intermediary phosphorylation of nucleoside diphosphate kinase from pea seed. FEBS Letters 38:225-228, 1974.

4. Edlund, B., Rask, L., Olsson, P., Wålinder, O., Zetterqvist, Ö. & Engström, L.: Preparation of crystalline nucleoside diphosphate kinase from baker´s yeast and purification of 1-phosphohistidine as the main phosphorylated product of an alkaline hydrolysate of enzyme incubated with adenosine (^{32}P)-triphosphate. Eur J Biochem 9:451-455, 1969.
5. Edlund, B.: Active site phosphopeptides from pea seed nucleoside diphosphate kinase. Uppsala J Med Sci 79:143-147, 1974.
6. Edlund, B. & Engström, L.: A peptic phosphopeptide from the active site of pea seed nucleoside diphosphate kinase. FEBS Lett 47:279-283, 1974.
7. Hultquist, D.E.: The preparation and characterization of phosphorylated derivatives of histidine. Biochim Biophys Acta 153:329-340, 1968.
8. Edlund, B.: Purification of nucleoside diphosphate kinase from pea seed and phosphorylation of the enzyme with adenosine (^{32}P)triphosphate. Acta Chem Scand 25:1370-1376, 1971.
9. Wålinder, O., Zetterqvist, Ö. & Engström, L.: Purification of a bovine liver protein rapidly phosphorylated by adenosine triphosphate. Isolation of 1-^{32}P-phosphohistidine, 3-^{32}P-phosphohistidine and N-ε-^{32}P-phosphohistidine from ^{32}P-labelled protein. J Biol Chem 243:2793-2798, 1968.
10. Hirs, C.H.W.: Reaction with reactive aryl halides. In: Methods in Enzymology (ed. C.H.W. Hirs), 11: p. 551. New York, Academic Press, Inc., 1967.
11. Habeeb, A.F.S.A.: Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal Biochem 14:328-336, 1966.
12. Haynes, R., Osuga, D.T. & Feeney, R.E.: Modification of amino groups in inhibitors of proteolytic enzymes. Biochemistry 6:541-547, 1967.
13. Pradel, L.-A. & Kassab, R.: Site actif des ATP:guanidine phosphotransferases. II. Mise an évidence de rêsidue histidine essentiels au moyen du pyrocarbonate d´éthyle. Biochim Biophys Acta 167:317-325, 1968.
14. Thomé-Beau, F., Lè-Thi-Lan, Olomucki, A. & van Thoai, N.: Essential histidyl residues in arginine oxygenase (decarboxylating). Comparison with amino acid oxidases. Eur J Biochem 19:270-275, 1971.
15. Mourad, N. & Parks J:r, R.E.: Erythrocytic Nucleoside Diphosphokinase. II. Isolation and kinetics. J Biol Chem 241:271-278, 1966.
16. Henkart, P.: The chemistry and identification of im-dinitrophenyl histidine. J Biol Chem 246:2711-2713, 1971.
17. Colomb, M.G., Chéruy, A. & Vignais, P.V.: Nucleoside diphosphokinase from beef heart cytosol. II. Characterization of the phosphorylated intermediate. Biochemistry 11:3378-3389, 1972.
18. Agarwal, R.P. & Parks, J:r, R.E.: Erythrocytic nucleoside diphosphokinase. V. Some properties and behavior of the pI 7,3 isoenzyme. J Biol Chem 246:2258-2264, 1971.

19. Palmieri, R., Yue, R.H., Jacobs, H.K., Maland, L., Wu, L. & Kuby, S.A.: Nucleoside triphosphate-nucleoside diphosphate transphosphorylase (Nucleoside diphosphokinase). III. Subunit structure of the crystalline enzyme from brewer's yeast. J Biol Chem 248:4486-4499, 1973.
20. Colomb, M.G., Chĕrnuy, A. & Vignais, P.V.: Adenosine diphosphate as a regulatory ligand in beef heart cytosol nucleoside. Biochemistry 13:2269-2277, 1974.
21. Minh Duc, D., Lascu, I., Porumb, H., Gozia, O., Schell, H.D. & Barzu, O.: Differential sensitivity to p-chloromercuribenzoate and urea of soluble and Sepharose-bound pig heart nucleoside diphosphate kinase. FEBS Lett 127:281-284, 1981.

Address for reprints:

Docent Bror Edlund
Department of Clinical Chemistry
University Hospital
S-750 14 Uppsala 14
Sweden