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Effects of Chemical Modification of Lysine, Tyrosine and Tryptophan Residues in Pea Seed Nucleoside Diphosphate Kinase and Inhibition of the Enzyme with Antibodies

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ABSTRACT

Pea seed nucleoside diphosphate kinase (NDP kinase) is an oligomeric, tetrameric enzyme which has been shown to be phosphorylated by its substrate ATP, presumably forming an 1-phosphohistidine at its active site. Recently has also a reactive lysine residue been demonstrated at the active site. In the present investigation nitration of a tyrosine residue is shown to inactivate the enzyme. There seems only to be a slight structural change in the enzyme on modification of these essential lysine and tyrosine residues, since double diffusion experiments with an inhibitory antiserum shows no difference in reactivity between the native enzyme and the modified enzyme. It is also found that modification of all tryptophan residues in the enzyme reduces the enzyme activity only to a small degree, indication that these hydrophobic amino acid residues are not directly involved in the catalytic process.

INTRODUCTION

ATP:nucleoside diphosphate kinase transphosphorylase (EC 2.7.4.6) (NDP kinase) from pea seed is an oligomeric, tetrameric enzyme with a molecular weight of 70 000 daltons (1). It is intermediately phosphorylated by its substrate ATP, presumably forming an 1-phosphohistidine residue at its active site (2,3). The amino acid sequence around this phosphohistidine residue has been investigated and found to contain a lysine residue (4,5).

In a recent report the native enzyme has been treated with reagents specific for certain amino acid residues, and in addition to a reactive histidine residue, has a reactive lysine residue of importance for enzyme activity been found (6).

No aromatic amino acid residue apart from histidine has been found in the sequence of twenty amino acid residues from the active site of pea seed NDP kinase (5), and there is no evidence for more than one polypeptide chain in the enzyme (1). However, the present investigation was made since there might still be a tyrosine or tryptophan residue in the active site of the enzyme

at some distance in the primary structure from the histidine residue that is phosphorylated. Thus the native enzyme has been treated with 2-OH-5-NO_2-benzylbromide (HNB-bromide) and tetranitromethane (TNM) to react with tryptophan and tyrosine residues respectively (7,8).

It also seemed to be of interest to study if antibodies against the enzyme were inhibitory to enzyme action and if chemical modification altered its antigenicity, as has been found with other enzymes, for a review see (9).

In the present investigation the effect of antibodies on enzyme activity has been studied as well as the reactivity in an immunodiffusion test of native NDP kinase and enzyme modified by fluoro-2,4-dinitrobenzene (FDNB) and TNM.

The effect of chemical modification and antibodies on enzyme activity are discussed.

MATERIALS AND METHODS

Pea seed NDP kinase was prepared as previously described (3). Enzyme activity was measured according to Monrad and Parks, using dGDP for nucleoside diphosphate (10). Protein was measured by a Folin method, using human serum albumin as standard (11). Photometric measurements were made, using a Zeiss PMQII spectrophotometer, equipped with an automatic cell unit recorder. 2-OH-5-NO_2-benzylbromide (HNB-bromide) and tetranitromethane (TNM) were from Sigma. Immunodiffusion experiments were performed according to Ouchterlony (12). Agarose l'Industrie Biologique Francaise (0,8% w/v) in a sodium diemal buffer (75 mmoles/l, pH 8.6) containing calcium lactate (2 mmoles/l) was used. After visible precipitation lines had formed, usually within 24 hours at room temperature, the agarose layers were extracted with NaCl (0,9% w/v) dried and stained with Coomassie Brilliant Blue R-250 essentially according to Laurell (13).

Reaction of tryptophan residues with HNB-bromide. 1 ml aliquots of a NDP kinase solution, 1 mg per ml of a phosphate buffer (0.1 moles/l, pH 7,4) were incubated with 25-50 μl of HNB-bromide (0,2-0,4 moles/l) dissolved in acetone. The HNB-bromide solutions were made ex tempore using redistilled acetone. The incubation mixtures were then cooled in an ice-water bath and the HNB-hydroxide formed was separated by centrifugation at 4000 rpm for 5 min. The supernatant solutions were chromatographed at room temperature on 0,9x60 cm Sephadex G-50 columns, equilibrated and eluted with sodium borate buffer (0,01 moles/l, pH 8,5). The enzyme activity, protein concentration and absorbancy at 410 nm of the void volume fractions were determined. A control experiment was run in parallel with no HNB-bromide added. It was found that the enzyme activity was unaffected by the acetone concentrations used (up to 5% v/v). The specific activity of each test sample was then compared to that

of the control experiment and the number of tryptophan groups modified per subunit was calculated using a molar absorbance value of 18 000 cm^{-1} for the HNB-groups in the protein (7).

Nitration of tyrosine residues with TNM. To 3,5 ml of NDP kinase (31 µmoles/l) in sodium borate buffer (0,1 moles/l, pH 8.5) were added 50 µl of a solution of TNM in ethanol (1 mole/l). After mixing was the incubation mixture kept at 30°C. 0,5 ml samples were taken at time invervals, as indicated in Fig. 2, and chromatographed on 0,9x60 cm Sephadex G-50 columns, equilibrated and eluted at room temperature with sodium borate buffer (10 mmoles/l, pH 8.5). The absorbancy at 428 nm of the void volumes was determined, as well as protein concentration. The number of nitrotyrosine residues formed per subunit was calculated from the molar absorbance of 4 300 cm^{-1} for a nitrotyrosine group in a protein (14) and the protein content. The specific enzyme activity was determined for each sample and compared to that of a control sample treated in the same way but without TNM added.

Production of antiserum. Antibodies against NDP kinase were rised in rabbits by injecting subcutaneously 1 ml of a 1:1 suspension of Freund's complete adjuvant (Difco, USA) and pea seed NDP kinase (2 mg/l) in a sodium phosphate buffer (0,1 moles/l, pH 7.4) containing NaCl (0,15 moles/l). One week later an intramuscular injection of the same amount of enzyme was made, but now using Freund's incomplete adjuvant (Difco, USA), and a booster dose was given in the same way after another week. The rabbits were starved overnight and bled through the ear vein to harvest serum. Protein concentration of the antiserum was 85 g/l.

Inactivation of pea seed NDP kinase by antibodies. 25-200 µl of rabbit antiserum were added to 20 µg of pea seed NDP kinase in 100 µl of a triethanolamine-acetic acid buffer (10 mmoles/l, pH 7,4). The incubation mixtures were kept at 37° for 30 min, followed by dilution in an ice-cold tris-acetic acid buffer (0,15 moles/l, pH 7,4). The specific activities were compared to that of a sample incubated at 37°C for 30 min without antiserum added. The specific activity of the enzyme (1500 units/mg) was unaffected by this treatment. All enzyme tests were performed in duplicate.

Reactivity of inhibitory antibodies against the native enzyme and enzyme treated with ^{14}C-FDNB and TNM. The enzyme was treated with ^{14}C-FDNB essentially as described before (5), except that only a twofold molar excess of ^{14}C-FDNB (17 mCi/mmole) over NDP kinase was used. Samples were taken after a reaction time of 12 hours, when 0,8 moles of ^{14}C-DNP-lysine residues had been formed per mole of subunit, and when 25% of the enzyme activity remained. A sample was also taken before the addition of FDNB (control sample). The immunological reactivity of the enzyme was tested as described in Fig. 4 A.

NDP kinase was also inactivated by nitration with TNM as described above.

A control sample was taken before addition of TNM and treated in the same way. Samples were taken 95 min after the addition of TNM, when 1,0 mole of tyrosine residues had reacted per mole of subunit of the enzyme (2), 5% of the enzyme activity remaining. The samples were tested for immunological reactivity as described in Fig. 4 B.

RESULTS AND DISCUSSION

There seems to be a tyrosine residue more reactive to tetranitromethane than the other tyrosine residues in each subunit of the enzyme, and nitration of this tyrosine residue leads to inactivation of the enzyme, indicating that this tyrosine residue is of importance for enzyme activity as seen in Fig. 1.

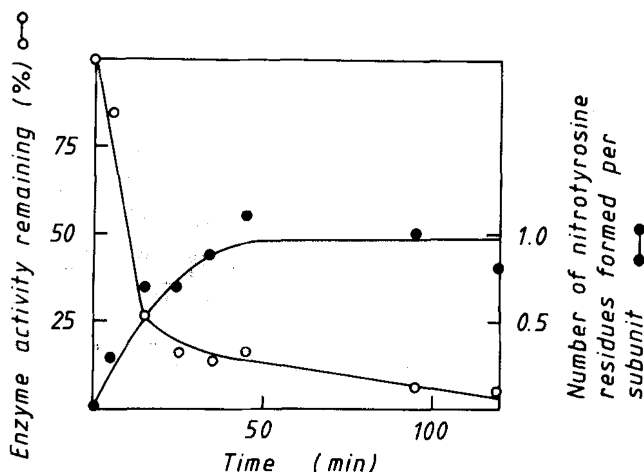

Fig. 1. Inactivation of pea seed NDP kinase by nitration of tyrosine. Open circles indicate enzyme activity remaining, filled circles indicate number of nitrotyrosine residues formed per subunit. For details see text.

The product ADP (4 mmoles/l) did not protect the enzyme from inactivation. This is in contrast to its protection of the enzyme from inactivation by 2,4-dinitrofluorobenzene (6), 2,4,6-trinitrobenzene sulfonic acid (6) and diethylpyrocarbonate (6). Neither did the nitroenzyme separate from the native enzyme on chromatography on Sephadex G-200, indicating that the enzyme was not dissociated into its subunits on nitration.

Fifty percent of the enzyme activity remained after reaction of all three tryptophan residues in each subunit by HNB-bromide (Fig. 2).

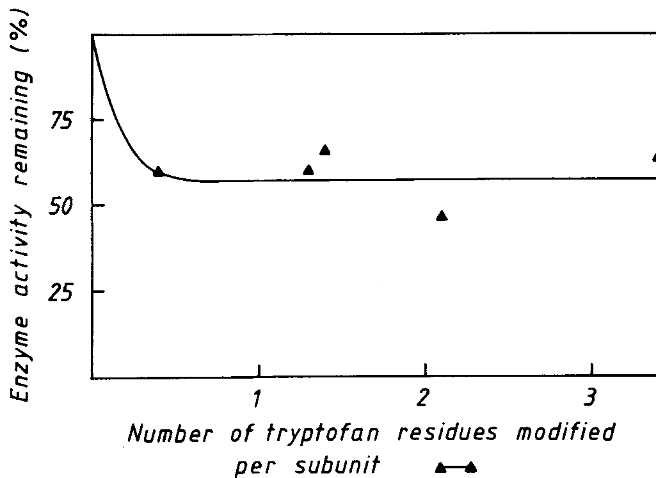

Fig. 2. Modification of pea seed NDP-kinase with 2-OH-5-NO_2-benzyl bromide. Filled triangels indicate enzyme activity remaining after modification of the number of tryptophan residues per subunit given on the abscissa. For details see text.

Therefore, modification of the tryptophan residues does not seem to directly involve the active site.

As seen in Fig. 3, the native enzyme was inactivated in direct proportion to the relative amount of antiserum added, strongly indicating antigenic sites involving the active site of the enzyme. A certain restriction of substrate diffusion or trapping of active enzyme in antigen-antibody complexes seem to have occurred since some enzyme activity was regained by using a large excess of antiserum. Thus, 30% of enzyme activity remained using 850 μg of antiserum per μg of antiserum per μg of NDP kinase.

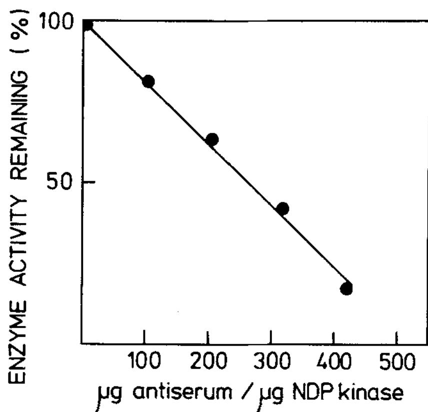

Fig. 3. Inactivation of pea seed NDP kinase by specific rabbit antiserum. Filled circles indicate enzyme activity after incubation of native enzyme with antiserum. For details see text.

In Ouchterlony double diffusion tests the enzyme inactivated by modification of a lysine residue (1) (Fig. 4 A) or a tyrosine residue (as shown above) (Fig. 4 B), in each subunit, showed complete fusion bands with the native enzyme, using antiserum against the native enzyme (Fig. 4).

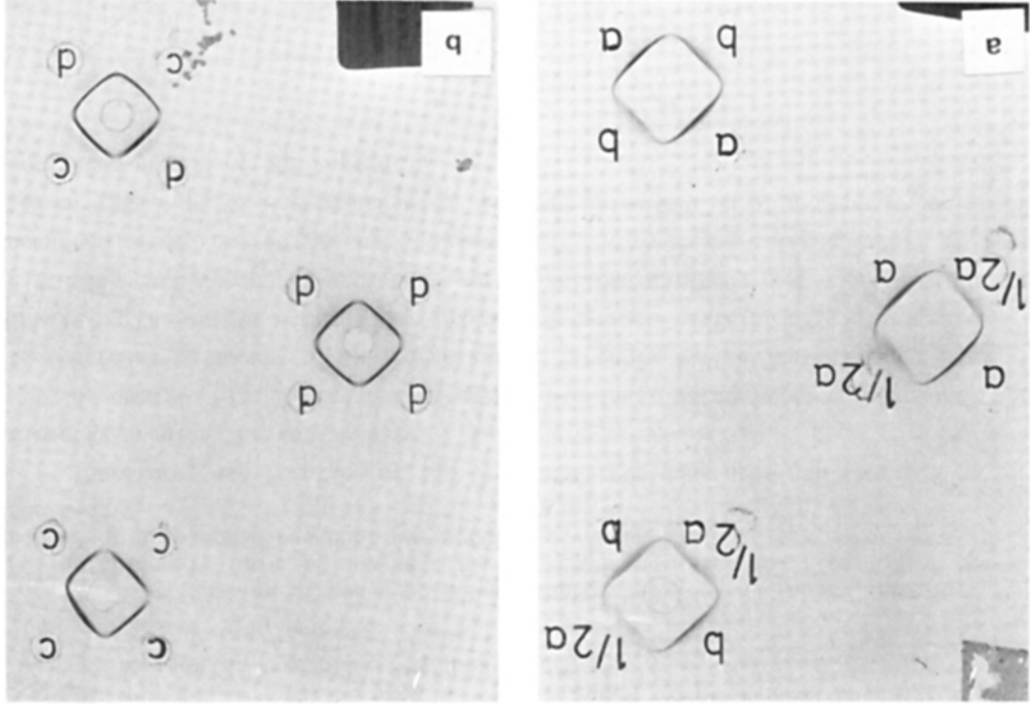

Fig. 4 A. Double immunodiffusion test of native pea seed NDP kinase and the enzyme inactivated with ^{14}C-FDNB. 5 µl samples were added to each well. Centre wells: Rabbit antiserum, 85 g/l. Periferal wells: a = 0,2 g/l and 1/2 a = 0,1 g/l control sample, b = 0,2 g/l ^{14}C-FDNB-treated enzyme. Photographs of stained precipitation lines, for details see text.

Fig. 4 B. Double immunodiffusion test of native pea seed. NDP kinase and the enzyme inactivated by TNM. 10 µl samples were added to each well. Centre wells: Rabbit antiserum, 85 g/l. Periferal wells: c = 0,4 g/l control sample and d = 0,5 g/l of enzyme treated for 95 min with TNM. Photographs of stained precipitation lines, for details see text.

Thus apart from the histidine residue phosphorylated during enzyme action (2,3) there seem to be a lysine (5) and a tyrosine residue essential for enzyme activity in pea seed NDP kinase.

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