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Active Site Phosphopeptides from Pea Seed Nucleoside Diphosphate Kinase

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ABSTRACT

Nucleoside diphosphate kinase from pea seed was incubated with (^{32}P)ATP, inactivated with alkali and digested with trypsin. From the digest the main part of the bound phosphorus was isolated as two phosphopeptides, both containing 14 amino acid residues. These phosphopeptides had an identical amino acid sequence Asx-Val-Ile-His-Gly-Ser-Asx-Ala-Val-Glx-Ser-Ala-Asx-Lys, as determined by dansyl-Edman technique. The only difference found between the two phosphopeptides was a different lability to acid of the phosphoryl bond. The possibility that the appearance of two phosphopeptides was due to a specific migration of the phosphoryl bond within the peptide chain from 1-phosphohistidine to 3-phosphohistidine is discussed.

INTRODUCTION

Pea seed nucleoside diphosphate kinase (ATP: nucleoside diphosphate phosphotransferase, EC 2.7.4.6) has been shown to consist of four subunits which probably have an identical primary structure and are phosphorylated by ATP (1, 2). Data from rapidmixing experiments and initial velocity analysis indicate that the phosphoryl enzyme is an intermediate of the enzyme reaction (3).

The phosphoryl binding site of nucleoside diphosphate kinases has hitherto been studied by alkaline hydrolysis of the phosphorylated enzyme. In an alkaline hydrolysate of phosphorylated nucleoside diphosphate kinase from baker's yeast the main part of the bound phosphate is isolated as 1-phosphohistidine, while in alkaline hydrolysates of other nucleoside diphosphate kinases, a few phosphopeptides accounted for most of the bound phosphate (4, 5, 12).

The phosphopeptides from bovine liver nucleoside diphosphate kinase are probably 1-phosphohistidine peptides (13). Similar phosphopeptides are obtained from pea seed nucleoside diphosphate kinase (5), suggesting similarities in the amino acid sequence around the phosphoryl binding site of the two enzymes.

Since a main part of the bound phosphate of these two enzymes cannot be isolated as a single phosphoamino acid after alkaline hydrolysis alternative degradation methods are of importance in order to establish the nature of the phosphoryl binding. One approach seemed to be the proteolytic degradation of the phosphorylated enzyme. By analysing the amino acid sequence of these fragments a rational basis may be obtained for the choice of methods for further degradation of the phosphopeptides to a free phosphoamino acid, accounting for most of the bound phosphate. Sequence data would also give further support to previous results on the identity of the subunits of the nucleoside diphosphate kinase.

In the present study alkali-inactivated, ^{32}P-labelled pea seed nucleoside diphosphate kinase was digested with trypsin and the main part of the bound radioactivity was isolated as two phosphopeptides. The amino acid sequence was the same for both phosphopeptides as determined by dansyl-Edman technique. A transformation of one of the phosphopeptides into the other is discussed on the basis of phosphoryl group migration.

MATERIALS

The enzyme from pea seed was purified as described earlier (5). (^{32}P)ATP labelled at the γ-P, was prepared according to Engström (6). The specific radioactivity of the (^{32}P)ATP ranged from 0.2 to 1.0×10^{6} counts·min^{-1}·nmole^{-1}. Sephadex (G-50 and G-25) and DEAE-Sephadex (A-50 and A-25) were purchased from Pharmacia Fine Chemicals. Trypsin (EC 3.4.21.4) code TRTPCK, was obtained from Worthington. Precoated polyamide

thin layer sheets for identification of dansyl amino acids were obtained from Cheng Chin Trading Company, Taiwan, Taipei. They were washed with formic acid before use (11). Dansyl chloride, phenyl isothiocyanate and trifluoracetic acid were obtained from Pierce Chemical Company. The phenyl isothiocyanate was stored under nitrogen at −20°.

Pyridine was purchased from Mallinckrodt. It was refluxed for 3 hours over phtalic anhydride and distilled off at 114° to 117° and then stored under nitrogen at −20°. Butylacetate from Merck was distilled from K_2CO_3 at 127°. Dansylamino acids were obtained from BDH Chemicals Ltd., Poole, England, with exception for ε-dansyllysine and α-dansylhistidine, which were obtained from Pierce Chemical Company. Histidyllysine was from Sigma. All chemicals were of highest grade available.

METHODS

All preparations were carried out at 5° unless otherwise stated. Ninhydrin analysis was performed with alkaline hydrolysis essentially according to Hirs (8).

400 μl of sample corresponding to 20 to 40 nmoles of amino acid residues were diluted with 200 μl of 12 M sodium hydroxide and heated on a boiling water bath for 2.5 hours. After chilling 200 μl of concentrated acetic acid were added, followed by 400 μl of a ninhydrin reagent (10). The solution was then heated in a boiling water-bath for 15 min, chilled and diluted with 500 μl of 50% (v/v) ethanol and the absorbance at 570 nm was measured. Glycylglycin was used as a standard. Radioactivity and stability to acid of the phosphoryl bond were assayed as described previously (14). Amino acid analysis was carried out on a BioCal 2 000 amino acid analyzer using the two column system. About 50 nmoles of peptide were hydrolyzed for 24 and 72 hours in 6 M HCl at 110° under reduced pressure in sealed ampouls.

The tryptophan content of the peptides was assayed by measuring the fluorescence intensity at 350 nm after exitation at 280 nm using an Aminco Bowman fluorescence spectrophotometer. Quantitation was achieved by using a standard solution of tryptophan equimolar to the peptide solution. Amino acid sequence determinations were made essentially according to Hartley (17). At the identification of dansylhistidine, a hydrolysate of dansylated histidyllysine was used as a reference. All spectrophotometric measurements of absorbance were made using a Zeiss PMQ II spectrophotometer.

RESULTS

Phosphorylation of the enzyme with (^{32}P)ATP and digestion of the alkali inactivated ^{32}P-labelled enzyme with trypsin

About one μmole, i.e. 70 mg, of pea seed nucleoside diphosphate kinase in 0.01 M triethanolamine-acetic acid buffer (pH 7.4) was diluted in the same icecold buffer (pH 7.4) to a final concentration of 0.2 mg per ml. The solution was kept in an ice-water bath. To one tenth of this solution were added 25 μl to 50 μl of 1 mM solution of (^{32}P)ATP in the same buffer (pH 7.4). These conditions were chosen to optimize ^{32}P-phosphate incorporation from (^{32}P)ATP into the enzyme. To the rest of the nucleoside diphosphate kinase solution was added a solution of ATP in the same buffer (pH 7.4) giving a molar excess of ATP to nucleoside diphosphate kinase of 50 to 100 in different preparations and a final concentration of about 0.2 mM ATP. This was done to obtain optimal conditions for phosphate incorporation. The incubations were stopped after 40 seconds by the addition of 2.5 mM sodium hydroxide to a final concentration of 0.1 M in each incubation mixture. The two incubation mixtures were pooled and kept in an ice-water bath for 2.5 hours to obtain complete denaturation.

A 2 M potassium hydrogen carbonate solution was then added to a four-fold molar excess over the sodium hydroxide added giving a pH 9.3 to 9.4 at 25°. The solution containing 0.3 mg enzyme per ml was then kept at 25°. A fresh solution of trypsin, 1 mg per ml 1 mM hydrochloric acid, was added giving a trypsin to nucleoside diphosphate kinase ratio of 1 : 10 (w/w). The mixture was gently stirred for 3 hours.

Purification of phosphopeptides

First chromatography on Sephadex G-50. The digestion was interrupted by chromatography of this mixture on a (6.5×48 cm) Sephadex G-50 column, equilibrated and eluted with a 5 mM potassium hydrogen carbonate buffer (pH 9.4) collected in 30 ml fractions. After elution of about 0.6 column volume, the main part of radioactivity appeared as one broad peak corresponding to about 60% of the total radioactivity of the (^{32}P)ATP used. The rest of the radioactivity was eluted after about one column volume and only background activity could be found with the void volume. The peak fractions from the peak appearing after 0.6 column volume were pooled. Two to three moles of phosphate were incorporated into the pooled material per mole of enzyme incubated with (^{32}P)ATP of known specific activity.

Chromatography on DEAE-Sephadex A-50. The pooled material was applied to a (2.0×15 cm) DEAE-Sephadex A-50 column, equilibrated with 5 mM potassium hydrogen carbonate buffer (pH

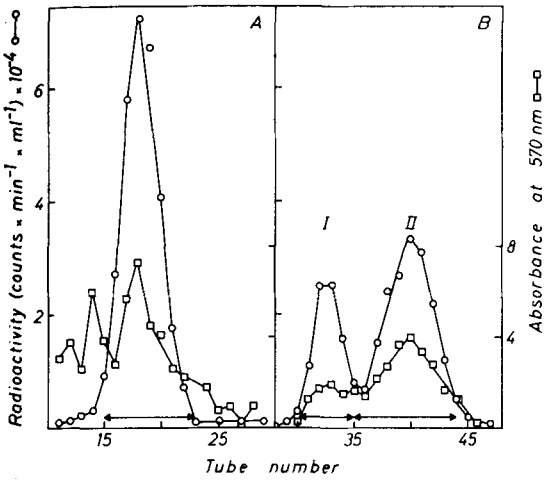

Fig. 1. Two successive chromatographies of tryptic phosphopeptides from pea seed nucleoside diphosphate kinase on DEAE-Sephadex columns. The columns were in A a (2.5×17.5 cm) DEAE-Sephadex A-50 column and in B a (1.4×17.5 cm) DEAE-Sephadex A-25 column, eluted first with 100 ml and 50 ml, respectively, of 0.01 M potassium hydrogen carbonate buffer (pH 9.4) and then with a linear gradient (total volume 1 l) formed from 0.01 M and 0.2 M potassium hydrogen carbonate buffer (pH 9.4). 15 ml fractions were collected after starting the gradient. ○—○, ^{32}P radioactivity. □—□, Ninhydrin positive material assayed as described under Methods. The figures correspond to the amount of material present in 1 ml. Fractions were pooled as indicated in the figure.

9.4). The radioactive material was eluted as described in the legend of Fig. 1 A, which shows a typical chromatogram. Almost all of the radioactive material appeared as one peak and was pooled.

Chromatography on DEAE-Sephadex A-25. The pooled material was rechromatographed on a (6.5× 48 cm) Sephadex G-50 column in 5 mM potassium hydrogen carbonate buffer (pH 9.4) in order to decrease the ionic strength of the sample before its application to a (1.4×17.5 cm) DEAE-Sephadex A-25 column, equilibrated with the above-mentioned buffer (pH 9.4). The main part of the radioactivity appeared in two peaks, called fractions I and II in order they were eluted from the column (Fig. 1 B). The fractions were pooled as indicated in the figure. 30% of the radioactivity applied to the column appeared in fraction I, and 66% in fraction II. The duration of the preparation was eight days from the first chromatography on Sephadex G-50 to the chromatography on DEAE-Sephadex A-25. At two other preparations taking 7 and 22 days, the ratio of the radioactivity of fraction I to fraction II was 0.1 and 1.5 respectively. The overall yield of radioactivity in fractions I and II together in relation to the pooled material from the first chromatography on Sephadex G-50 varied between 60% and 80%, mean value 70%, in four different preparations.

Chromatography on Sephadex G-25. The pooled fractions were freeze-dried and then dissolved in water to a volume of 5 to 10 ml. The solutions were then desalted by chromatography on a (2.1× 180 cm) Sephadex G-25 column equilibrated and eluted with 0.01 M ammonium hydrogen carbonate. Almost all of the radioactivity pooled from the DEAE-Sephadex A-25 chromatography appeared after about 0.4 column volume and was pooled and concentrated. The pooled material was freeze-dried and dissolved in water. From this solution samples were taken for amino acid and amino acid sequence analysis. Calculated from data obtained with amino acid analysis (Table I) on aliquots from these pools about 2.5 moles of peptide were obtained per mole of enzyme incubated with ATP.

Properties of the phosphopeptides

The stability to acid of the phosphoryl bond. In samples from the peak fractions of the DEAE-

Table I. *Amino acid composition of the phosphopeptide fractions I and II from the second chromatography on DEAE-sephadex*

The values given are mean values from two different preparations hydrolyzed for 24 and 72 hours. Each value is calculated as moles of amino acid per mole of peptide (tryptophan could neither be detected in fraction I nor in fraction II)

	Fraction I	Fraction II	Residues per lysine residue
Lysine	1.0	1.0	1
Histidine	0.8	0.7	1
Aspartic acid	2.8	2.9	3
Serine[a]	1.7	1.9	2
Glutamic acid	1.1	1.2	1
Glycine	1.3	1.3	1
Alanine	1.9	2.0	2
Valine[b]	1.7	1.8	2
Isoleucine[b]	1.2	1.0	1
Total			14

[a] The values were obtained by extrapolation to zero time of hydrolysis.
[b] 72-hours hydrolysis value.

Table II. *Amino acid analysis data for samples from fractions I and II after the third and fourth Edman degradation step*

The peptides were hydrolyzed for 24 hours. Fraction number is indicated by roman and degradation step by arabic numerals. Numbers given are mole of amino acid per mole of peptide where the nearest integer is given within brackets

Amino acid	I 3rd	I 4th	II 3rd	II 4th
Lysine	0.6 (1)	0.6 (1)	0.7 (1)	0.6 (1)
Histidine	1.0 (1)	0.3 –	0.7 (1)	0.2 –
Aspartic acid	2.2 (2)	2.3 (2)	1.9 (2)	1.8 (2)
Serine	2.3 (2)	2.3 (2)	1.9 (2)	1.8 (2)
Glutamic acid	1.2 (1)	1.2 (1)	1.0 (1)	1.0 (1)
Glycine	1.1 (1)	1.0 (1)	1.1 (1)	1.0 (1)
Alanine	2.1 (2)	2.2 (2)	1.7 (2)	1.6 (2)
Valine	1.1 (1)	1.2 (1)	1.0 (1)	1.0 (1)
Isoleucine	0.2 –	0.2 –	0.3 –	0.3 –

Sephadex A-25 chromatography, 4% of the radioactivity in fraction I and 40% in fraction II was split off as $^{32}P_i$ during treatment for 20 seconds with a molybdate solution containing 0.5 M sulphuric acid (14). The values given are the mean values of four different preparations.

Amino acid sequence analysis. An identical amino acid sequence, Asx-Val-Ile-His-Gly-Ser-Asx-Ala-Val-Glx-Ser-Ala-Asx-Lys, was found for both fractions I and II. In the identification system used, some problems were encountered in demonstrating α-dansylhistidine in the presence of ε-dansyllysine. Therefore, amino acid analysis was also performed on aliquots after the third and fourth Edman degradation steps. The results are given in Table II, and were in accordance with the findings obtained with the chromatographic method, i.e. histidine was demonstrated as the fourth amino acid residue. From amino acid analysis data and the known sequence of the thirteen additional amino acid residues lysine was assumed to be C-terminal. This would also be expected from the known specificity of trypsin.

DISCUSSION

One phosphoryl group is bound to each subunit of pea seed nucleoside diphosphate kinase, presumably as 1-phosphohistidine, during its action (1, 2, 3, 5). In the present investigation the aim was to isolate and determine the structure of a part of the active site of the enzyme containing this histidine residue. When ^{32}P-labelled pea seed nucleoside diphosphate kinase was digested with trypsin after inactivation with alkali, the main part of the bound radioactivity was recovered as two phosphopeptides both having the same sequence.

As far as the amino acid sequence is concerned the present work supports the previous suggestion of the existence of identical subunits in the peak seed nucleoside diphosphate kinase.

The appearance of two phosphopeptides with phosphoryl linkage with different stability to acid at first seemed to contradict this conclusion. However, during the isolation procedure, the most labile phosphopeptide, with a lability similar to 1-phosphohistidine gave rise to a more stable form. It was also found that the more rapidly the isolation was carried out, the more the most labile form dominated. It is therefore suggested that the most labile phosphopeptide represents the phosphoryl binding site of the phosphorylenzyme and that the phosphoryl group is bound as 1-phosphohistidine.

The most plausible explanation for the appearance of the more stable phosphopeptide is a phosphoryl group migration from 1-phosphohistidine. Hultquist found that 3-phosphohistidine is formed at alkaline pH in a solution containing 1-phosphohistidine and therefore the more stable phosphopeptide may be a 3-phosphohistidine peptide (12). However, since N-ε-phospholysine has also been a product of alkaline hydrolysis of phosphorylated nucleoside diphosphate kinases (4, 5, 12) the presence of this phosphoamino acid in the more stable phosphopeptide cannot be excluded. In the present work the nature of the phosphoryl linkage has not been further investigated. This problem is preferably approached by further proteolytic degradation, as discussed below.

The dansyl-Edman technique does not discriminate between an acidic amino acid residue and its amide. The transition between the two phosphopeptides may therefore be explained by deamidation, making the more stable phosphopeptide the most acid. This explanation is, however, not valid since the stable phosphopeptide was found to be the least acid one, as judged by its migration on DEAE-Sephadex chromatography.

Mainly ^{32}P-orthophosphate and only small amounts of 1-(^{32}P) phosphohistidine were obtained when the ^{32}P-phosphopeptides obtained from an alkaline hydrolysate of the ^{32}P-labelled bovine liver

nucleoside diphosphate kinase were further hydrolyzed in alkali (13).

Since the tryptic phosphopeptides correspond to the main part of the radioactivity bound to the enzyme it is evident that proteolytic degradation does not labilize the phosphoryl bond to the same extent as alkaline hydrolysis does. It therefore seems reasonable to believe that further proteolytic degradation is the more suitable way to definitely establish the nature of the phosphoryl bond of the phosphorylated nucleoside diphosphate kinase of pea seed at present.

Earlier studies of the phosphoryl bond of phosphorylated pea seed and bovine liver nucleoside diphosphate kinase by alkaline hydrolysis pointed to similarities in the amino acid sequence of the phosphorylated binding site (5, 12). The approach used in the present work would then be useful in the search for ways of studying other nucleoside diphosphate kinases, as well as other phosphorylated enzymes containing the same type of phosphoryl bond.

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