A PEPTIC PHOSPHOPEPTIDE FROM THE ACTIVE SITE OF PEA SEED NUCLEOSIDE DIPHOSPHATE KINASE

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1. Introduction

ATP: nucleoside diphosphate phosphotransferases (EC 2.7.4.6) (NDP kinase) from different sources are phosphorylated by their substrate ATP [1], and kinetic evidence, including rapid mixing experiments [2–4], has shown that the enzyme is intermediately phosphorylated. The phosphoryl bond of the phosphorylated enzyme has been studied by alkaline hydrolysis and tryptic digestion of the phosphoryl-enzyme [1,5]. From alkaline hydrolysates of phosphorylated mammalian enzymes and the pea seed enzyme small amounts of 1-phosphohistidine, 3-phosphohistidine and *N*-ε-phospholysine have been obtained [5,6]. Phosphopeptides, presumably 1-phosphohistidine peptides, are the main phosphorylated products from the hydrolysates [7]. In the alkaline hydrolysate of phosphorylated NDP kinase from baker's yeast 1-phosphohistidine was the main phosphorylated product, while no phosphopeptides were detected, but small amounts of 3-phosphohistidine and *N*-ε-phospholysine were also found [8]. By tryptic digestion of the alkali inactivated, phosphorylated pea seed enzyme two phosphopeptides have been obtained, both containing the same 14 amino acids with the same amino acid sequence [5]. The two phosphopeptides differed with regard to the acid lability of the phosphoryl linkages [5]. The labile phosphopeptide has also been shown to give rise to the stable one [5]. The labilities to acid of the two phosphopeptides were about the same as for 1-phosphohistidine and 3-phosphohistidine, respectively. Since 1-phosphohistidine has been shown to give rise to 3-phosphohistidine at an alkaline pH, this could explain the appearance of the phosphopeptide stable to acid [9]. It could not be ruled out, however, that the latter phosphopeptide was formed by transfer of the phosphoryl group from histidine to the lysine residue of the peptide [5]. With regard to the possible migration of phosphoryl groups at an alkaline pH, it seemed of interest to study the phosphoryl binding site of pea seed NDP kinase after inactivation with acid and degradation at a low pH, instead of alkaline inactivation and tryptic digestion. By using peptic degradation it should also be possible to further explore the amino acid sequence at the active site. In the present investigation, a peptic phosphopeptide has been isolated from phosphorylated pea seed NDP kinase and its amino acid sequence determined. This phosphopeptide has been further digested by trypsin and subtilopeptidase A, yielding a phosphopeptide containing a histidine residue but no other basic amino acid residue. The results provide evidence that the phosphoryl group is bound to the same histidine residue as that of the dominating tryptic phosphopeptide.

2. Materials

NDP kinase from pea seed was purified as described earlier [6]. [^{32}P]ATP with a specific activity of 15 000 cpm · nmole^{-1} · min^{-1} was prepared according to Engström [10]. Radioactivity was measured from the Čerenkov radiation emitted from ^{32}P using an Intertechnique LS 30 liquid scintillation counter. Trypsin was obtained from Worthington (code TRTPCK) pepsin (code 15445 EPBK) from Boehringer–Mannheim GmbH and Subtilopeptidase A from Sigma. Sephadex and Sephadex ion exchangers were obtained from

Pharmacia Fine Chemicals, Uppsala, Sweden. The materials used for the sequence analysis were the same as previously reported [5].

3. Methods and results

All chromatographic steps were performed at +5°C.

3.1. *Phosphorylation of the enzyme with [^{32}P]ATP. Inactivation with acid and digestion of the ^{32}P-labelled enzyme with pepsin*

All incubations were performed in an ice-water bath. To 0.5 μmoles (35 mg) of pea seed NDP kinase, dissolved in 33 ml of 0.01 M triethanolamine–acetic acid buffer, pH 7.4, 15 μmoles of [^{32}P]ATP were added in 3 ml of the same buffer, pH 7.4. One min later 3.5 ml of 1 M HCl were added, followed after another 10 sec by 7 ml of 0.5 M HCOOH and 3.5 ml of 1.25 M sodium acetate buffer, pH 5.5. 20 mg of pepsin dissolved in 1 ml of 1 mM HCl were added immediately to the mixture. After 30 min the digestion mixture, about 60 ml, was chromatographed at 5°C on a Sephadex G-50 column (7.1 × 73 cm) equilibrated and eluted in 20 ml fractions with 5 mM $KHCO_3$–K_2CO_3 buffer, pH 9.4. A radioactive peak appeared after 0.6 column volume. This material was collected.

No radioactivity was eluted with the void volume. The amount of radioactivity collected corresponded to 1.2 moles of phosphate per mole of enzyme originally incubated with [^{32}P]ATP.

3.2. *First chromatography on DEAE-Sephadex*

The pooled material, about 600 ml, was applied to a (2.0 × 15 cm) DEAE-Sephadex A-50 column equilibrated with the same buffer as above, pH 9.4. The column was eluted with 100 ml of 0.01 M $KHCO_3$–K_2CO_3 buffer, pH 9.4, and a linear gradient of 1 litre formed from 0.01 M and 0.3 M $KHCO_3$–K_2CO_3 buffer, pH 9.4, collected in 20 ml fractions. Almost all of the radioactivity appeared as one peak.

3.3. *Second chromatography on DEAE-Sephadex*

The peak fractions (140 ml) were collected and diluted with 20 vol of water. This material was applied to a (1.4 × 16 cm) DEAE-Sephadex A-25 column, equilibrated with 5 mM $KHCO_3$–K_2CO_3 buffer, pH 9.4, and eluted with 50 ml of 0.01 M $KHCO_3$–K_2CO_3 buffer, pH 9.4, and a linear gradient, total vol 1 litre, formed from 0.01 M and 0.2 M $KHCO_3$–K_2CO_3 buffer, pH 9.4. 10 ml fractions were collected. The radioactivity appeared as one main peak, preceded by a smaller one (fig. 1A). The fractions of the main peak, corresponding to 65% of the radioactive material applied to the column, were pooled.

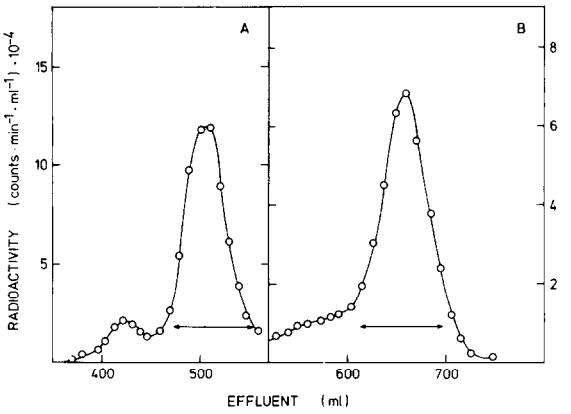

Fig. 1. Anion exchange chromatographies of a peptic phosphopeptide from pea seed NDP kinase. In A) a (1.4 × 16 cm) DEAE-Sephadex A-25 column was used and in B) a (2.1 × 16 cm) QAE-Sephadex A-25 column. For details see text. (o–o–o) Radioactivity eluted.

3.4. *Chromatography on QAE-Sephadex*

The pooled fractions (about 90 ml) were diluted with 360 ml of water and the material was applied to a (1.4 × 16 cm) QAE-Sephadex A-25 column. The column was eluted with a linear gradient, total volume 1 litre, formed from 0.01 M and 0.3 M $KHCO_3$–K_2CO_3 buffer, pH 9.4. 18 ml fractions were collected. The fractions of the main radioactivity peak, corresponding to 70% of the radioactive material applied to the column were pooled (fig. 1B).

3.5. *Desalting of the phosphopeptides*

The pooled fractions, about 90 ml, were lyophilized and then dissolved in 10 ml of water and chromatographed on a (2.1 × 110 cm) Sephadex G-25 column, equilibrated and eluted with 0.01 M NH_4HCO_3. The fraction volume was 5 ml. Almost all of the radioactivity applied to the column appeared in one peak.

The peak fractions were pooled and lyophilized. The material collected represented a total yield of 64% of the material pooled from the first Sephadex G-50 chromatography, or 0.8 moles of phosphate per mole of enzyme originally incubated with [^{32}P]ATP.

3.6. *Further digestion of the peptic phosphopeptide with trypsin and subtilopeptidase A*

Pea seed NDP kinase was incubated with [^{32}P]ATP and digested with pepsin as described above. The incubation mixture was adjusted to pH 9.5 with 1.3 M NaOH and 10 mg trypsin in 1 ml of 1 mM HCl were added. After digestion for 30 min at 22°C, 10 mg of subtilopeptidase A in 1 ml of 0.01 triethanolamine–acetic acid buffer, pH 7.4, were added. The digestion with subtilopeptidase A was performed in order to get smaller contaminant peptides. One hour later the digestion mixture was chromatographed on a (6.6 × 39 cm) Sephadex G-25 column, equilibrated and eluted with 5 mM $KHCO_3$–K_2CO_3 buffer, pH 9.4, eluted in 30 ml fractions. The radioactive material appearing as one peak after 0.5 column volume was pooled. No radioactivity was eluted with the void volume and the pooled material was well separated from [^{32}P]ATP and $^{32}P_i$. The pooled material corresponded to about 1.8 moles of phosphate per mole of enzyme originally incubated with [^{32}P]ATP, as calculated from the specific radioactivity of the [^{32}P]ATP used.

3.7. *Chromatography on DEAE-Sephadex*

This material (65 ml) was applied to a (1.4 × 16 cm) DEAE-Sephadex A-25 column, equilibrated with 5 mM $KHCO_3$–K_2CO_3 buffer, pH 9.4, and eluted (9 ml fractions) with 50 ml of 0.01 M $KHCO_3$–K_2CO_3 buffer, pH 9.4, and a 1 litre linear gradient formed from 0.01 M and 0.4 M $KHCO_3$–K_2CO_3 buffer, pH 9.4. The peak radioactive fractions were pooled as shown (fig. 2A). This material corresponded to 70% of the material applied to the column.

3.8. *Chromatography on QAE-Sephadex*

The pooled material (35 ml) was diluted with 70 ml of water and applied to a (2.1 × 16 cm) QAE–Sephadex A-25 column equilibrated with 5 mM $KHCO_3$–K_2CO_3 buffer, pH 9.4. The column was eluted in 10 ml fractions with 50 ml of 0.01 M $KHCO_3$–K_2CO_3 buffer, pH 9.4, and a 1 litre linear gradient formed from 0.01 M and 0.4 M $KHCO_3$–K_2CO_3 buffer, pH 9.4. The peak fractions were pooled as indicated in fig. 2B. The pooled material corresponded to 82% of the material pooled from the previous step. The overall yield from the first chromatography on Sephadex G-25 was 60%. The pooled material was desalted as reported above for the peptic phosphopeptide and the desalted material was used for amino acid sequence analysis.

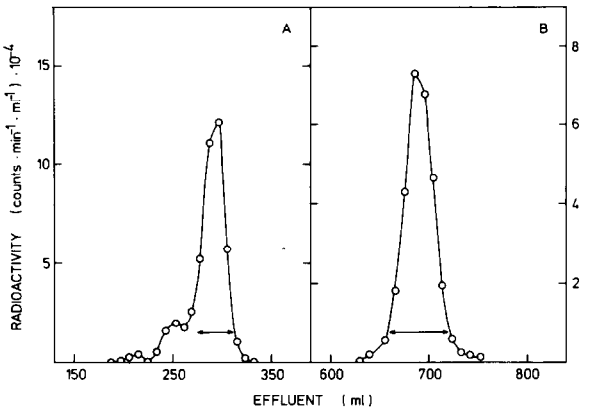

Fig. 2. Anion exchange chromatographies of a phosphopeptide obtained by digestion of phosphorylated pea seed NDP kinase by pepsin, trypsin and subtilopeptidase A. In A) a (1.4 × 16 cm) DEAE-Sephadex A-25 column was used and in B) a (2.1 × 16 cm) QAE-Sephadex A-25 column. For details see text. (o–o–o), Radioactivity eluted.

3.9. *Stability to acid of the phosphoryl bond*

The ^{32}P-labelled enzyme was inactivated with alkali or acid followed by digestion with trypsin or with pepsin, trypsin and subtilopeptidase A respectively, on a scale one tenth of that described for the tryptic phosphopeptides [5] and in the present paper. The digestion mixtures were chromatographed on (1.2 × 49 cm) Sephadex G-50 and G-25 columns, respectively, equilibrated and eluted with 5 mM $KHCO_3$–K_2CO_3 buffer, pH 9.4. The labilities to acid of the peak phosphopeptide fractions eluted were tested. 53% of the radioactivity of the tryptic phosphopeptide material and 55% of the radioactivity of the phosphopeptide material obtained after digestion with pepsin, trypsin and subtilopeptidase A was split off as $^{32}P_i$ during treatment for 20 sec with a molybdate solution containing 0.5 M sulphuric acid [11].

Table 1
Amino acid composition of phosphopeptides from the active site of pea seed NDP kinase

Amino acid	Peptide P	P 5th	P 6th	Peptide PTS	PTS 3rd	PTS 4th
Aspartic acid	3.0(3)	1.9(2)	1.7(2)	2.0(2)	1.0(1)	1.1(1)
Serine*	1.1(1)	1.0(1)	0.9(1)	0.9(1)	1.0(1)	1.1(1)
Glycine	2.0(2)	1.2(1)	1.0(1)	1.0(1)	1.0(1)	0.8(1)
Alanine	1.0(1)	0.2 –	0.1 –	–	–	–
Valine	1.0(1)	0.6(1)	0.6(1)	1.0(1)	0.1 –	0.1 –
Isoleucine	3.0(3)	0.7(1)	0.6(1)	0.9(1)	0.1 –	0.1 –
Histidine	1.0(1)	0.8(1)	0.7(1)	1.0(1)	0.9(1)	0.1 –
Arginine	1.1(1)	0.7(1)	0.2 –	–	–	–
Total	13	8	7	7	4	3

The peptic phosphopeptide P was further digested by trypsin and subtilopeptidase A to give the phosphopeptide PTS. The substractive Edman degradation steps are indicated in the table. Amino acid analysis was performed on 50 nmoles of peptide P hydrolysed for 24 and 72 hr, and on 5–10 nmoles of peptide PTS or Edman degraded peptides hydrolysed for 24 hr in 6 M HCl at 110°C in a sealed evacuated ampoul. A single column Durrum amino acid analyser was used. The numbers given are mole of amino acid per mole of peptide where the nearest integer is given within brackets.

* The values were obtained by extrapolation to zero time of hydrolysis.

3.10. *Amino acid sequence analysis*

The amino acid sequences of the phosphopeptides were investigated with the dansyl–Edman technique according to Hartley [12]. Amino acid analysis was also performed on the 5th and 6th degradation steps of the peptic phosphopeptide and the 3rd and 4th degradation steps of the peptic phosphopeptide digested with trypsin and subtilopeptidase A. The results from amino acid analysis are given in table 1. Since the peptide Asx–Val–Ile–His–Gly–Ser–Asx obtained after digestion of the peptic phosphopeptide by trypsin and subtilopeptidase A is part of the peptic phosphopeptide Ala–Ile–Asx–Ile–Gly–Arg–Asx–Val–Ile–His (Gly, Ser, Asx) it is concluded that the full sequence of the peptide is Ala–Ile–Asx–Ile–Gly–Arg–Asx–Val–Ile–His–Gly–Ser–Asx.

4. Discussion

The sequence of the peptic phosphopeptide Ala–Ile–Asx–Ile–Gly–Arg–Asx–Val–Ile–His–Gly–Ser–Asx isolated from the acid inactivated phosphoenzyme overlaps by seven amino acid residues that of the tryptic phosphopeptides Asx–Val–Ile–His–Gly–Ser–Asx–Ala–Val–Glx–Ser–Ala–Asx–Lys purified from the alkali inactivated phosphoenzyme. Digestion of the peptic phosphopeptide with trypsin and subtilopeptidase A leaves a smaller phosphopeptide containing the overlapping sequence Asx–Val–Ile–His–Gly–Ser–Asx mentioned. This phosphopeptide does not contain a lysine residue, or other basic amino acid residue, to which the phosphoryl group could migrate, as discussed with the tryptic phosphopeptides [5]. It also has the same lability to acid of the phosphoryl bond as the tryptic phosphopeptides from the alkali inactivated phosphorylenzyme at an early stage of preparation, i.e. that of phosphohistidine.

Since the same amino acid sequence has been obtained around the phosphohistidine residue when the phosphoenzyme has been inactivated by two opposite methods of inactivation, acid and alkali, it would seem clear that this histidine residue is phosphorylated under normal catalysis. The amino acid sequence around this histidine residue should then be Ala–Ile–Asx–Ile–Gly–Arg–Asx–Val–Ile–His–Gly–Ser–Asx–Ala–Val–Glx–Ser–Ala–Asx–Lys.

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