EVIDENCE FOR IDENTICAL SUBUNITS IN PEA SEED NUCLEOSIDE DIPHOSPHATE KINASE

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1. Introduction

All nucleoside diphosphate kinases (NDP kinase) (EC 2.7.4.6) studied have been shown to be phosphorylated by their substrate ATP, incorporating more than 3 moles of phosphate per mole of enzyme [1–3]. The phosphorylated pea seed enzyme has been shown to dissociate into four subunits of equal size when treated with detergent. This suggests that each subunit of this enzyme is phosphorylated [4]. Evidence for this should be at hand if subunits could be shown to be identical.

From amino acid analysis of the enzyme and molecular weight of its subunits the average number of lysine and arginine residues in each subunit can be calculated. Provided the subunits are identical, the maximal number of tryptic peptides would be one more than the sum of arginine and lysine residues of each subunit. This possibility has been investigated in the present work.

2. Experimental procedure

Pea seed NDP kinase was purified as described [3]. Amino acid analysis was performed on 0.1–0.3 μmoles of pea seed NDP kinase. The enzyme was hydrolyzed for 24 and 72 hr at 110°C in 6 M hydrochloric acid in a sealed ampoule. The amino acid analyzer used was a BioCal 2000, equipped with a two column system. The N-terminal amino acid was assayed for by the methods of Gray [5, 6] and Stark [7].

The enzyme was prepared for tryptic digestion by denaturation in the following way. 1 mg of enzyme was dissolved in 0.5 ml of ice-cold 0.01 M triethanolamine acetic acid buffer, pH 7.4, mixed with 50 μl of 1 M sodium hydroxide and incubated for 2.5 hr in an ice-water bath. After this period, 100 μl of 0.5 M hydrochloric acid was added, the enzyme precipitated, and the enzyme suspension was carefully dialyzed against 0.1 M ammonium hydrogen carbonate. Ten μl of a solution containing 1 mg trypsin (Worthington, code TR TP CK) in 1 ml 1 mM hydrochloric acid was added to the suspension which became a clear solution after 3 hr at 37°C.

After lyophilization, the residue was suspended in 50 μl of a pyridine acetic acid buffer [8], pH 5.5. 10 μl of the suspension was spotted on a 18 × 18 cm Whatman cellulose CC41 thin layer plate (500 μm thick) and electrophoresis was conducted in the pyridine–acetate buffer at 350 V for 1.5 hr at 10°C using a Desaga 12 12 00 equipment. The plate was dried in an air stream, and a second dimension ascending chromatography was performed using the system of Waley and Watson [9]. For detection of peptide spots the plate was sprayed with a collidine–ninhydrin spray [10]. Arginine-containing peptides were detected under ultraviolet light (354 nm) after using a phenantrene quinone reagent [11].

Isoelectric focusing of pea seed NDP kinase in polyacrylamide gel was performed according to Conway-Jacobs and Lewin [12]. Pea seed NDP kinase, equine heart myoglobin (two times crystallized from Calbiochem), human serum albumin (Kabi) and ovalbumin, grade V, (Sigma) were added, separately or in mixture, to the gel solution. The final concentration was 0.1 mg per ml of each protein. A few microliters of a Patent blue V solution (2 mg/ml) were also added [12]. The gels were allowed to polymerize in bright light, and subjected to electrophoresis at 110 V for

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16 hr in the cold room (+ 5°C). Clearly visible precipitation discs were formed by all proteins tested, and the isoelectric point of NDP kinase was calculated as described [12].

3. Results and discussion

The result of the amino acid analysis on NDP kinase from pea seed is given in table 1. The amino acid analysis data further supports the view that the linkages between the subunits are noncovalent [4], since the amount of cysteine found was to small to equal one half-cystine residue per subunit. This was verified by performic acid oxidation of the enzyme [3], and amino acid analysis which displayed less than 0.3 residues of cysteic acid per subunit.

Table 1
Amino acid composition of pea seed nucleoside diphosphate kinase.

Amino acid	Number of residues found
Lysine	9.9 (10)
Histidine	3.1 (3)
Arginine	5.9 (6)
Aspartic acid	14.0 (14)
Threonine*	7.0 (7)
Serine*	12.8 (13)
Glutamic acid	15.2 (15)
Proline	7.3 (7)
Glycine	15.7 (16)
Alanine	14.5 (15)
Half-cystine**	–
Valine**	12.0 (12)
Methionine**	1.9 (2)
Isoleucine***	14.0 (14)
Leucine***	7.6 (8)
Tyrosine	3.8 (4)
Phenylalanine	8.1 (8)
Tryptophan†	2.7 (3)

The values given are the mean values from 5 different analyses, and calculated as number of residues per subunit (17000) of the enzyme. Nearest integer is given within parentheses.

* Values were obtained by extrapolation to zero time hydrolysis.

** Only trace amounts of half-cystine were found even after performic acid oxidation of one sample before amino acid analysis. The anount of methionine sulphone was found correspondent to the value given above for methionine.

*** 72-hr hydrolysis value.

† Determined in duplicate by a spectrophotometric method [16] on two different NDP kinase preparations.

The results represent independent evidence for close identity of the subunits. Further support for the existence of such an identity would have been the finding of four identical N-terminal amino acids per enzyme molecule [5–7]. However, no N-terminal amino acid could be detected. The reason for this is not clear.

The number of ninhydrin positive peptide spots (20, 16 of them salient) found by the fingerprint technique, are in close agreement with the expected number (maximally 17) from amino acid analysis data, since on the average 10 lysine and 6 arginine residues are found in each subunit. In line with this finding 6 peptides containing arginine were detected (figs. 1 and 2).

Previous studies on the pea seed NDP kinase revealed that the enzyme had high purity. The present data give independent support for this result. Recent-

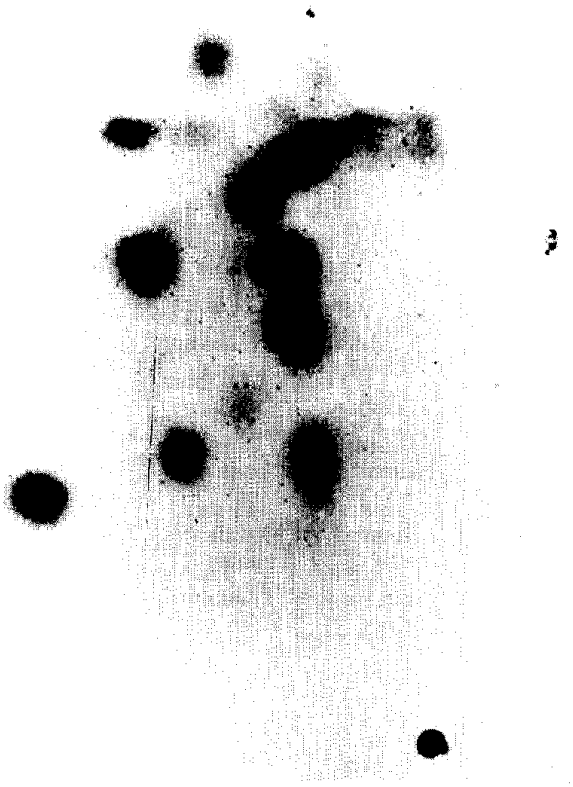

Fig. 1. Map of tryptic peptides from pea seed NDP kinase. For details, see Experimental procedure.

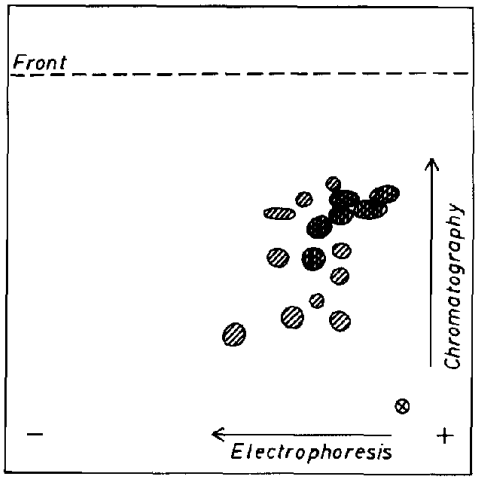

Fig. 2. Composite map of tryptic peptides from pea seed NDP kinase. Oblique lines: Ninhydrin positive peptides. Vertical lines: Arginine containing peptides. For details see Experimental procedure.

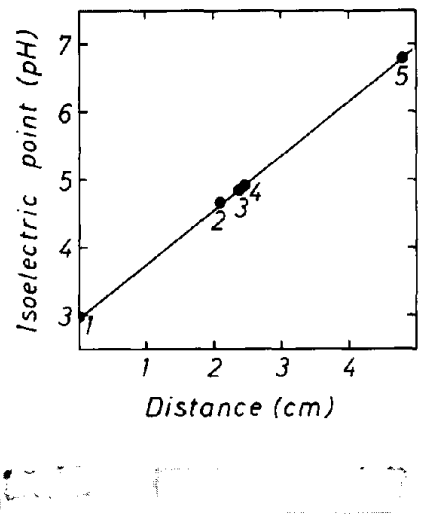

Fig. 3. Isoelectric focusing of pea seed NDP kinase in a polyacrylamide gel. The enzyme formed a single precipitation disc as shown by the photograph. In the figure is shown the distance from Patent blue V (pI 3.0) [1] at which the following proteins form precipitation discs. 2: Ovalbumin (pI 4.7), 3: pea seed NDP kinase, 4: Human serum albumin (pI 4.9) and 5: Myoglobin (pI 6.8). For details see Experimental procedure.

ly, however, several NDP kinases have been investigated with respect to their isoelectric points, and the results have diverged greatly even when the enzymes were isolated from the same source [14, 15]. It was therefore of interest to test the purity of the enzyme also with respect to the isoelectric point. The enzyme was found to form a single band at pH 4.9 (fig. 3).

In conclusion, the present work presents strong evidence that the four subunits of pea seed NDP kinase are identical. Together with previous findings [4], this indicates that each subunit can bind one phosphoryl group.

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