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KINETIN EFFECT ON ATP SYNTHESIS AND ON ADENYLATE KINASE ACTIVITY IN PEA SEEDS

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Key Word Index—*Pisum sativum*; Leguminosae; pea seed; kinetin; ATP; adenylate kinase; pyruvate kinase.

Abstract—Pea seed powder incubated in the presence of AMP and phosphoenolpyruvate (PEP) accumulated relatively large amounts of ATP. The rate of accumulation increased in the presence of kinetin. The amount of ATP accumulated at relatively low temperatures after 1 hr incubation was about double in the presence of kinetin as compared with the control mixture. Two fractions of adenylate kinase (AK) were separated from pea seed extracts. One fraction was stimulated by kinetin (10^{-5}–10^{-3} M) in the direction of ATP synthesis from ADP and was inhibited in its reverse reaction (ATP + AMP → 2ADP). This protein fraction bound kinetin (33 μmol/mg protein). The amount of bound hormone was slightly diminished by 5′-AMP, unaffected by ATP but decreased to 40% in the presence of 2′-AMP, a potent modifier of the AK activity.

INTRODUCTION

One of the most intriguing problems in seedling development is the origin of ATP at the early stage of germination. Many scientists have demonstrated the lack of oxidative phosphorylation, which is the main system for ATP production by aerobic respiration, as well as the inadequate ability of ATP synthesis by presently known anaerobic pathways, which might explain the relatively high amounts of ATP in seeds at their early stage of germination (reviewed in [1] and [2]).

Seed powder of various species incubated in the presence of phosphoenolpyruvate (PEP) and AMP results in a linear accumulation of relatively large amounts of ATP up to 30 min [3, 4]. In cell-free extracts the enzymic activities responsible for ATP synthesis have been established. The system consists of pyruvate kinase (PK) (EC 2.7.1.40), adenylate kinase (AK) (EC 2.7.4.3) [5], and a novel enzyme which splits reduced NAD into ADP and an unidentified nicotinamide component (a dihydronicotinamide mononucleotide is suggested [6, 7]). This system needs oxygen to reoxidize $NADH_2$ to NAD. Therefore it may explain the response of adenine nucleotide changes to anaerobiosis as described by Pradet and coworkers [8]. Nevertheless, the participation of an oxidative-phosphorylation-like system is not excluded.

This paper describes an ATP-synthesizing system in pea seed powder and the effect of kinetin on the whole system, as well as the effect of cytokinin on partially purified AK, an enzyme involved in the ATP synthesis.

RESULTS AND DISCUSSION

The ATP-synthesizing system in pea seed powder is demonstrated in Fig. 1. ATP accumulation is dependent on the presence of PEP and AMP. In the absence of AMP small amounts of ATP are accumulated reflecting the internal AMP concentration. The activities of PK and AK in partial purified enzyme fractions are depicted in Fig. 2A and the activity of the NADH-splitting enzyme in crude extract in a partial purified protein fraction is shown in Fig. 2B.

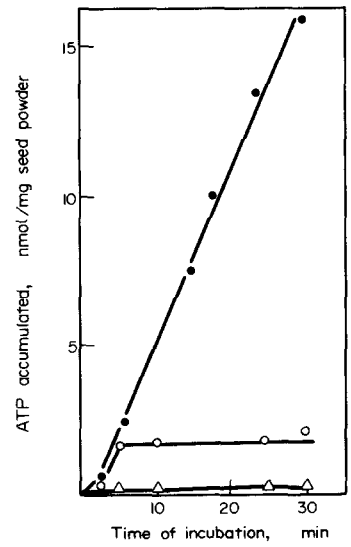

Fig. 1. ATP accumulation in pea seed powder in the presence of AMP (Δ), PEP(○) and AMP + PEP(●).

The separation of various proteins on a DEAE-Sepharose column revealed two fractions with AK activities (Fig. 3). The low ionic strength extractable fraction (fraction A) has also PK activity, while the second fraction (fraction B) does not. In fraction A the AK activity is inhibited (*ca* 20%) in the presence of PEP. The NADH-splitting activity was completely lost through the column purification. The two AK fractions differ slightly in their optimal pH (fraction A: 6.2; fraction B: 7.0) and their affinity to ADP (K_m values, 0.25 mM for A and 0.15 mM for B). In an attempt to recover higher PK activities, we found, surprisingly, that 0.1 mM fructose-

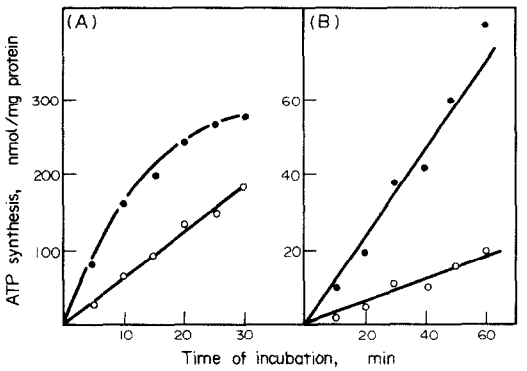

Fig. 2. (A) Pyruvate kinase (PK) (●) and adenylate kinase (AK) (○) activities in ammonium sulfate precipitates of pea seed extracts. (B) The ATP synthesis from NADH by crude pea extracts (●) and by $(NH_4)_2SO_4$ precipitates (○). For details see Experimental.

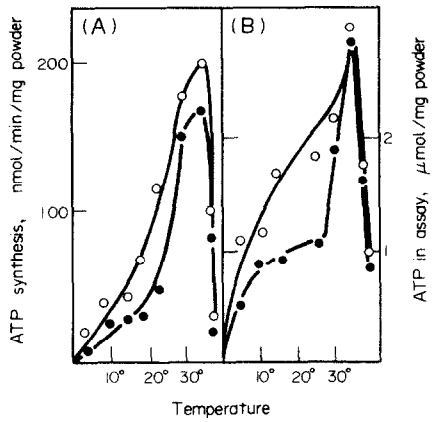

Fig. 4. (A) The effect of kinetin on the rate of ATP accumulation in pea seed powder in the presence of PEP and AMP at various temperatures: (●) no kinetin; (○) + kinetin (2.5×10^{-4}). (B) As for (A), after 1 hr incubation. For details see Experimental.

1,6-diphosphate increases the AK activity about three-fold but has no significant effect on the PK activity (not shown). Both fractions require either Mg^{2+} or Mn^{2+} and are stimulated by Co^{+}. Li^{+}, Na^{+}, K^{+} and NH_3^{+} have no effect up to 5 mM concentrations. Fraction A is inhibited by NADP and 5'-AMP, while 2'-AMP, 3'-AMP, kinetin and benzyladenine have stimulatory effects if measured in the direction of ATP synthesis from ADP (2ADP → ATP + AMP).

The entire system for ATP synthesis is found in dry seeds. Thus, it may play a role in the early stage of seed germination, and therefore may be controlled by plant hormones. The accumulation of ATP in seed powder is indeed enhanced by kinetin and benzyladenine, slightly enhanced by gibberellin, but is not affected by abscisic acid (not shown). The rate of ATP accumulation at various temperatures is always higher in the presence of kinetin (Fig. 4A); if the accumulation is examined after a relatively long period of incubation (1 hr), large differences are found at relatively low temperatures (< 30°) (Fig. 4B). The hormone is highly effective between 15° and 25°. At the optimal temperature for ATP accumulation (30°), the visible hormone effect is minimal. This may account either for a hormone effect on the enzyme structure which does not occur at higher temperatures, or for a hormone stimulation in ATP synthesis which is uncovered only if utilization of ATP is moderated by lower temperatures.

Using the column purified enzyme fraction, it was shown that one fraction (fraction A) is stimulated in its adenylate activity by kinetin while the second fraction (fraction B) is not. There is a sharp peak of ATP accumulated in the reaction mixture at the equilibrium point in the presence of apparent 'saturated' concentrations of kinetin (Fig. 5). This indicates an increase in the enzyme activity toward ATP synthesis (from ADP) at a relatively high ratio of [ATP] [AMP]/[ADP] in the reaction mixture. Moreover, by examining the AK reaction in the presence of ATP, measuring the AMP-dependent decrease of ATP (ATP + AMP → 2ADP), it is shown that kinetin not only inhibits the rate of ATP decrease, but also increases the amount of

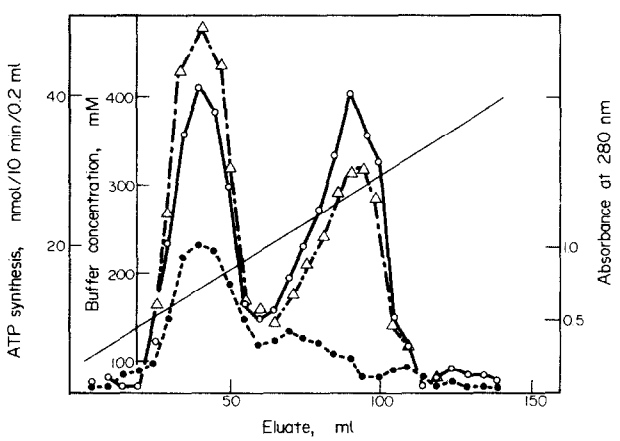

Fig. 3. Separation of protein fractions by ion exchange. (●) A at 280 nm; (○) AK; (Δ) PK; (–) buffer concentration. For details see Experimental.

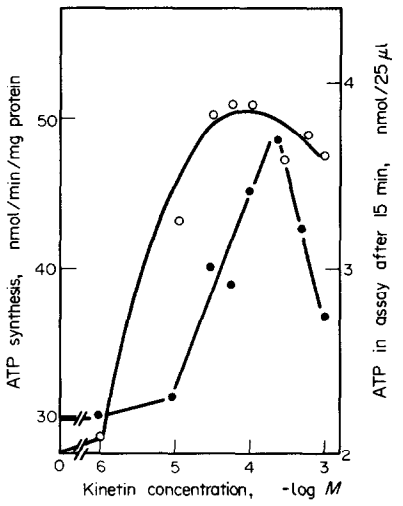

Fig. 5. The effect of various kinetin concentrations on the rate of ATP synthesis by fraction A. In the presence of ADP (●) (AK); ATP concentration in the AK reaction mixture 15 min after incubation (○).

ATP in the reaction mixture at the steady-state equilibrium point of the reaction (Fig. 6). The AMP-dependence of ATP degradation is clearly demonstrated; thus a kinetin effect on an ATPase-like activity is excluded. By reciprocal plots of ADP concentration vs rate of ATP synthesis, it was found that kinetin increases the affinity toward ADP from a K_m value of 220 µM to 40 µM (not shown). This may explain the mode of the hormone action.

An attempt was made to correlate the kinetin effect on AK activity to the hormone's ability to bind to the protein. The binding of [^{14}C]-8-kinetin (Radiochemical Centre, Amersham) was estimated by equilibrium dialysis. The binding was examined with the kinetin-affected fraction (A) in the presence of 5'-AMP, ATP and 2'-AMP (2 µM each). As shown in Fig. 7, 5'-AMP has some inhibitory effect, and 2'-AMP lowers the amount of bound kinetin at infinite hormone concentration from 33 to 5 nmol/mg protein. ATP has no significant effect. Fraction B binds only about 23 % of the amount of kinetin that bound to fraction A (per mg protein).

The data presented indicate that the ATP synthesis system as described schematically for seeds [5, 7] at their early stage of germination, may be controlled by kinetin. The experiments lead to the conclusion that the hormone binds to the protein fraction that includes one AK enzyme inhibiting the reaction toward ATP degradation, or rather increasing the affinity to ADP, thus, stimulating ATP synthesis at relatively low ADP concentrations. Such a control mechanism will cause ATP accumulation in spite of the relatively high AMP and low ADP concentrations in seeds at the start of germination.

EXPERIMENTAL

Seeds of pea (*Pisum sativum* L. cv Kelvedon wonder) were used.

The ATP-synthesizing system in seed powder was examined as described in ref. [3]. 20 mg of the powdered seed was incubated with 0.2 ml substrate mixture which included KPi buffer, pH 6.6, 10 mM; phosphoenolpyruvate (PEP) 5 mM; AMP 1 mM. After various time intervals of incubation at 24–26°, 10 µl of concentrated/$HClO_4$ was added. The tube was kept on ice for at least 30 min then neutralized with 1 M KOH. After precipitation of the perchlorate, 50 µl from the supernatant were examined for ATP with a modified luciferin–luciferase method [3] described in ref. [9]. All materials included in the assay mixtures were examined for their effect on this system.

Adenylate kinase (*AK*) was examined in 0.25 ml of an assay mixture including: KPi, pH 6.6, 10 mM; ADP 0.5 mM; MgCl and $MnSO_4$ 1 mM each; and $CoSO_4$ 0.5 mM; enzyme source 0.1–0.2 mg protein of crude enzyme or 0.05–0.1 protein of partial purified enzyme. At various time intervals between 0 and 10 min, 20 µl of the reaction mixture was examined for ATP as described above.

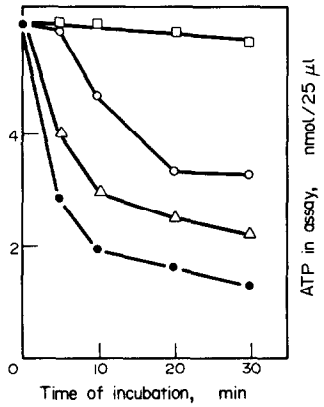

Fig. 6. The effect of kinetin on AK in fraction A. (ATP + AMP → ADP). The reaction mixture contains (in 0.25 ml): AMP, 2 mM; ATP, 1 mM; $MgSO_4$ and $MnSO_4$ 1 mM each; KPi buffer, pH 6.6, 50 mM; and 40 µg protein of fraction A. At the indicated time intervals ATP was quantified in 25 µl from the reaction mixture as described in Experimental. (□) No AMP; (●) full system; (Δ) full system + 10^{-5} M kinetin; (○) full system + 2.5 × 10^{-4} M kinetin.

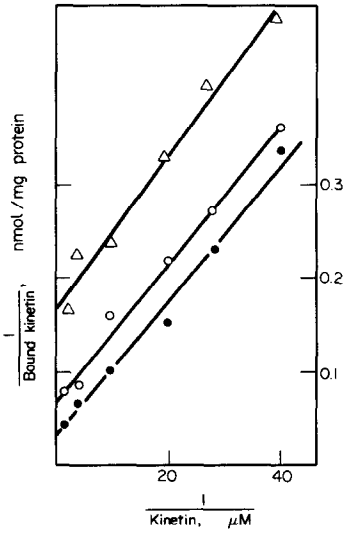

Fig. 7. Reciprocal plots of kinetin concentrations vs the amount of kinetin bound to fraction A. (●) No additions; (○) + 2 µM 5'-AMP; (Δ) + 2 µM 2'-AMP. For details see Experimental.

Pyruvate kinase (PK) was examined as AK but 5 mM PEP were included in the reaction mixture.

Purification and separation of proteins with $(NH_4)_2SO_4$ *and by ion exchange.* To crude extract protamine sulfate was added (2 mg/mg protein). After 20 min on ice, the mixture was centrifuged and from the supernatant the protein precipitated between 20 and 80% $(NH_4)_2SO_4$ and dialysed against 3 l. KPi pH 6.6, 25 mM at 4°. *Ca* 1 g of $(NH_4)_2SO_4$ precipitated and dialysed protein was layered on a DEAE-Sepharose column (0.6 × 35 cm, pre-equilibrated with 50 mM KPi buffer pH 6.6) and eluted with a continuous KPi buffer gradient (0.1–0.4 M). Five-ml fractions were collected and each fraction was examined for *A* at 280 nm, AK activity, PK activity and for NADH-splitting enzyme.

The binding of kinetin to protein. One ml (~2 mg protein) of column purified enzyme was dialysed at 15° against a soln of 50 mM KPi, pH 6.6, containing 0.01% NaN_3 and various concns of radioactive kinetin (10^3 cpm/nmol) (10–500 μM). Equilibrium was established within 18 hr. The dialysis was stopped after 30 hr and radioactivity was determined inside and outside the dialysis bag. The excess of radioactive kinetin inside the bag was used to determine the amount of bound kinetin.

Protein was determined in crude extracts in the TCA-insoluble fraction by the method of ref. [10].

The ATP synthesis from NADH was examined as PK but instead of AMP, NADH was included (10 mM).

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