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REDUCED NICOTINAMIDE-ADENINE-DINUCLEOTIDE-PYROPHOSPHORYLASE: A NOVEL ENZYME IN SEEDS

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Key Word Index—*Pisum sativum*; Leguminosae; pea; seeds; ATP; $NADH_2$; germination; AMP phosphorylation.

Abstract—An enzyme which splits reduced NAD has been partially purified from pea (*Pisum sativum*, Kelvedon Wonder) seeds. The activity requires orthophosphate and the products are ADP and probably NMN (dihydro NMN?). The enzyme splits the $NADH_2$ at the pyrophosphate bond and incorporates the phosphate into the AMP residue. NAD, NADP or $NADPH_2$ could not replace $NADH_2$. The enzyme is unstable during storage, is activated by Mg^{2+} and by Mn^{2+}, and inhibited by Ca^{2+}. K^+, Li^+ and NH_4^+ have no effect. The possible role of this enzyme in the synthesis of ATP in seeds at the early stage of germination is discussed.

INTRODUCTION

The phosphorylation of AMP to ATP in seeds in the early stages of germination is a result of two enzymes: pyruvate kinase (EC 2.7.1.40), which uses ADP and phosphoenolpyruvate (PEP) as substrates; and adenylate kinase (EC, 2.7.4.3), which uses ATP and AMP resulting in ADP [1–5]. AMP is known to be present in seeds [6–10]. Phosphoenolpyruvate seems to be a product of two very active enzymes found in all seeds so far examined, namely, malate dehydrogenase (EC 1.1.1.37) [11–13] and PEP-carboxytransphosphorylase (EC 4.1.1.38) [14, 15].

In order to start the phosphorylation cycle from AMP to ATP, one molecule of ADP or ATP is needed as a 'spark'. In preliminary work [16] it was shown that an enzyme which splits reduced NAD (a product of the malate dehydrogenase) to ADP and to a nicotinamide compound is present. The present report describes some characteristics of this enzyme.

RESULTS AND DISCUSSION

The dependence of the ATP synthesis on reduced NAD and orthophosphate is demonstrated in Fig. 1. The presence of PEP seems to be necessary to activate the trapping system of pyruvate kinase which is present in the enzyme source, thus resulting in higher amounts of ATP from the ADP obtained from $NADH_2$. In the presence of PEP and the absence of orthophosphate, significant activity was noted, probably because of the presence of free orthophosphate in the PEP solution. If the incubation of the mixture was conducted in the absence of PEP and if at various time intervals, PEP was added for a short time, increasing amounts of ATP appeared, up to *ca* 20 min of incubation, after which the amount of ATP decreased. This indicates a lack of activity (which is seen in the whole reaction mixture) as well as instability of ADP in the reaction mixture. NAD, NADP or $NADPH_2$ could not replace $NADH_2$ for ATP synthesis. The enzyme activity is inhibited by Ca^{2+}, unaffected by NH_4^+, Li^+, K^+, and Na^+, and stimulated by Mg^{2+} and Mn^{2+}.

The enzyme appears to be very unstable even at low temperatures and its loss of activity at two temperatures, as compared with pyruvate kinase activity found in the same protein mixture, is depicted in Fig. 2. This may explain in part the decrease in activity during incubation. Fig. 3 demonstrates reciprocal plots of the rate of enzyme activity vs the concentrations of $NADH_2$, ortho-

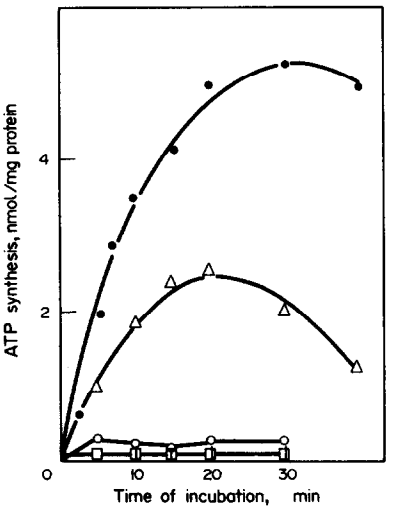


Fig. 1. Some characteristics of the $NADH_2$-pyrophosphorylase enzyme. The enzyme activity was examined in a full system (●), in the absence of $NADH_2$ (□) and in the absence of PEP (○) throughout the incubation, or by the addition of PEP at the indicated times followed by an additional incubation for 5 min (△). For details see Experimental.

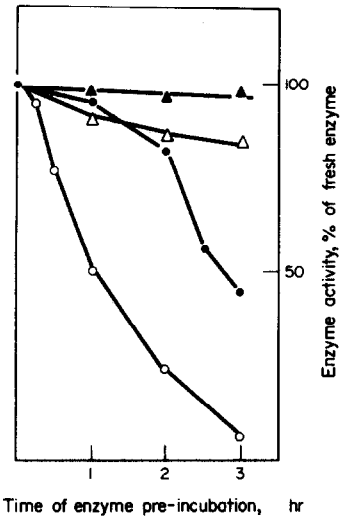


Fig. 2. The effect of storage on the $NADH_2$-pyrophosphorylase activity. The loss of $NADH_2$-pyrophosphorylase activity after enzyme storage at 4° (●) or 25° (○) compared with the loss of pyruvate kinase activity stored at 4° (▲) or at 25° (△). For details see Experimental.

phosphate and Mg^{2+}, the three substrates required for this enzyme activity.

Using chromatography and electrophoresis to separate the products of the enzyme activity, we found that in the presence of radioactive orthophosphate in the routine reaction mixture, the labelled phosphate appeared in the ATP; however, if PEP was omitted, radioactivity appeared in the ATP and a small amount in the ADP. This indicates that cleavage occurs at the pyrophosphate bond and that the phosphate is incorporated in the AMP, resulting in ADP. The residue is probably NMN (dihydro?) which could not be detected because of the minute amount liberated. The appearance of radioactive ADP and ATP indicates that the primary product was ADP, which was partially converted to ATP by adenyl kinase activity present, or completely converted to ATP in the presence of PEP by the present pyruvate kinase activity (Fig. 2).

In an attempt to follow the degradation of $NADH_2$ by the decrease of absorbance at 345 nm, we noted that the partially purified protein oxidizes $NADH_2$ very rapidly (data not shown). Using an oxygen monitor we found that $NADH_2$ is indeed oxidized by an oxygenase using oxygen as substrate. The activity was *ca* 0.8 μmol/min/mg of protein. This may explain the high concentration of $NADH_2$ necessary for the phosphorylase activity, as well as the relatively short period of activity during incubation (in addition to the enzyme instability), and is expressed in the unusual curve obtained at low concentration of $NADH_2$ (Fig. 3).

Although the presence of various pathways for the synthesis of minute amounts of ATP in seeds is not excluded, the enzyme described here fits in well into the whole system for ATP synthesis at the early stage of germination, as follows. Malate is pooled into the seeds during ripening [17] and is well known as a major component in fruits ('apple acid', the original name for malate). Malate dehydrogenase and PEP-carboxytransphosphorylase have been shown to be relatively very active in seeds of various cultivars [15, 16, 18]. These enzymes produce PEP, $NADH_2$ and CO_2. $NADH_2$ is reoxidized to NAD by oxygen, enabling the dehydrogenase activity to continue with small amounts of NAD present in seeds. This part of the system may explain the so-called 'alternate respiration' (see [10]) (uptake of oxygen and release of CO_2). The pyruvate kinase and the adenylate kinase were present in seeds of all cultivars so far examined in our laboratory (i.e. [5]). The appearance of $NADH_2$ during seed wettening results in small amounts of ADP (one molecule is enough!) by the enzyme described in this report. This ADP serves as a 'spark' to activate the combined pyruvate kinase–adenylate kinase to produce ATP from AMP. The whole system is summarized in Scheme 1.

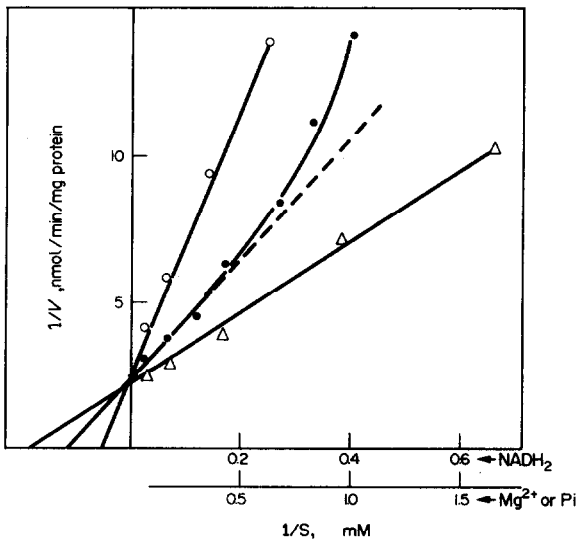
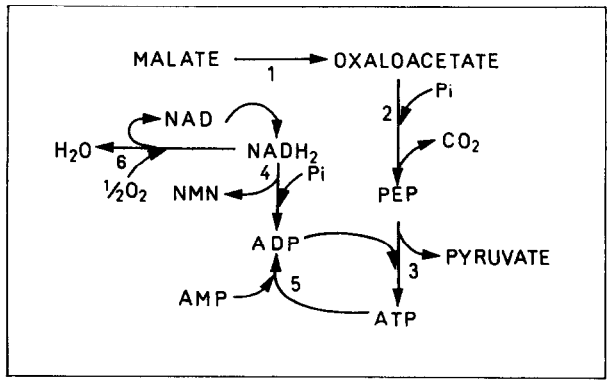


Fig. 3. The effect of substrate concentrations on the $NADH_2$-pyrophosphorylase activity. Reciprocal plots of the rate of enzyme activity vs concentrations of $NADH_2$ (●), orthophosphate (△) and Mg^{2+} (△). For further details see Experimental.



Scheme 1. 1, Malate dehydrogenase; 2, PEP-carboxylase; 3, pyruvate kinase (PK); 4, $NADH_2$, pyrophosphorylase; 5, adenylate kinase (AK); 6, $NADH_2$ oxygenase.

EXPERIMENTAL

All expts described were conducted with pea (*Pisum sativum*, cv Kelvedon Wonder) seeds. For enzyme prepn, seeds were powdered and ground as described previously [5]. The extract was centrifuged at 18000 g for 10 min. The supernatant was treated with protamine sulphate (2 mg/mg protein), centrifuged as before and from the supernatant the protein fraction was resuspended and used as the enzyme source within 1 hr after its prepn.

The ATP-synthesizing system included in a final vol. of 0.25 ml, unless otherwise stated, the following compounds: KPi buffer, pH 6.6, 25 mM; $NADH_2$, 20 mM; PEP, 10 mM; $MgCl_2$, 5 mM; and enzyme source, 250–500 μg protein. The amount of ATP was examined 5, 10, and 15 min after incubation in 50 μl of the reaction mixture. For ATP examination, a modified method of the luciferin–luciferase system described in refs. [19, 20] was used.

Identification of the reaction products. In a final vol. of 0.2 ml, the above-mentioned concns of $NADH_2$, PEP, $MgCl_2$ and enzyme were included but 0.1 mM buffer and *ca* 2×10^5 cpm of ^{32}P-orthophosphate were added. After 30 min incubation at 25°, 50 μl of the reaction mixture was separated by TLC on cellulose using 95% EtOH–NH_4OAc, pH 5, 1 M (7:3) or by electrophoresis (pyridine–acetate, pH 5.4, 12 V/cm). After the plates were dried, the cellulose from 1 cm segments of the plate was scraped off and counted for radioactivity in a liquid scintillation counter.

For the localization of the products, internal (non-radioactive) or separate (radioactive) ATP, ADP and NMN, as well as orthophosphate, were run in the TLC and electrophoresis systems. Localization was done under short wave UV or by radioactivity as described above. For controls, the products of reaction mixtures in the absence of $NADH_2$ or PEP were also examined.

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