Communication

Substrate-Induced Stability of Glyceraldehyde 3-Phosphate Dehydrogenase from Mung Beans (Vigna radiata L.)

Srinivasan*, Arvind M. Kayastha, and O. P. Malhotra

Biotechnology Centre, Indian Agricultural Research Institute, New Delhi-110 012, India (S.); and School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi-221 005, India (A.M.K., O.P.M.)

ABSTRACT

Time-dependent thermal inactivation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) present in the extract of mung beans at different periods of germination showed biphasic kinetics in the 12-h germinated seeds but single exponential decay at 24 h of germination. The glyceraldehyde 3-phosphate (G-3-P) concentration in the deproteinated extracts was found to increase with period of germination up to 36 h, parallel to that of GAPDH activity. G-3-P was found to offer protection of the enzyme against thermal inactivation and trypsin digestion. It is suggested that accumulation of G-3-P in germinating mung beans may be of physiological significance and it might offer protection to the enzyme in vivo against thermal inactivation and proteolysis.

NAD-GAPDH\(^1\) (o-glyceraldehyde 3-phosphate: NAD\(^+\) oxidoreductase [phosphorylating]; EC 1.2.12), an enzyme of the glycolytic pathway, has been extensively studied (5). GAPDH isolated from a variety of sources are all tetrameric proteins, each made of chemically identical monomers (5). Thermal inactivation kinetics have been successfully employed for studying pea (Pisum sativum), spinach (Spinacia oleracea), and mung bean (Vigna radiata L.) GAPDHs (8–11).

The phenomenon of stabilization of enzyme proteins by the respective substrates has been recognized for a long time. Examples of the enhanced stability of enzyme-substrate complexes over the free enzyme toward denaturants are well documented (2, 6, 13). In the present communication, we report some experiments that suggest that in germinating mung beans, the substrate G-3-P offers protection against thermal inactivation and proteolysis.

MATERIALS AND METHODS

GAPDH of mung beans (Vigna radiata L.) was purified as described earlier (12). This preparation is homogeneous on PAGE and SDS-PAGE and is free from any bound NAD\(^+\), NADH, \(\Delta\)-glyceraldehyde 3-phosphor acid diethylal

\(^1\) Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G-3-P, \(\Delta\)-glyceraldehyde 3-phosphate; TEA, triethanolamine hydrochloride; % \(A_{340}\), percentage amplitude of slow phase; % \(A_{240}\), percentage amplitude of fast phase.

barium salt, and TEA were purchased from Sigma Chemical Co. All the other chemicals used were of analytical reagent grade. Only double-distilled water from an all-quartz assembly was used in these studies.

Mung beans were procured from the regular food markets. For germination, seeds were washed thoroughly, soaked in water at 30°C for 2 to 3 h, and were later spread over moist filter paper on a sand bed in a seed germinator at 30°C.

Enzyme Assay

Aqueous solutions of the NAD\(^+\) and the substrate G-3-P were prepared and standardized as described by Ferdinand (4). The enzyme activity was measured as described earlier (12). Protein was estimated by the method of Lowry et al. (7).

Thermal Inactivation

The enzyme solution in desired buffer was maintained at the specified temperatures (±0.1°C) in a LKB water bath. Small aliquots (50 \(\mu\)L) were withdrawn at different time intervals, chilled immediately, and tested for enzyme activity at 30°C (10–20 \(\mu\)L) using routine assay.

Assay of G-3-P

Increase in absorbance at 340 nm was noted on additions of 0.01 mL of 20-fold diluted G-3-P solution to a solution containing 0.69 mL of assay buffer (40 mm TEA, pH 8.6, 25 \(\mu\)M EDTA free acid, and 50 mm orthophosphoric acid), 0.05 mL of NAD\(^+\), and 0.05 mL purified enzyme (50–100 \(\mu\)g). In preliminary experiments, it was found that the reaction was completed in less than 3 min. Therefore, in the routine assay, 3 min was allowed for completion of the reaction before recording the final absorbance value. The average of three such observations was used to determine the amount of G-3-P present. \(\Delta\)NADH at 340 nm is 6.2 \(\times\) 10\(^5\) M\(^{-1}\) cm\(^{-1}\). For assay of G-3-P in crude extract, the pH of the extract was brought to 2.5 with slow addition of 2 m HCl with constant stirring. The resulting suspension was then kept in a boiling water bath for 3 min and then chilled. The suspension was centrifuged to get a clear solution. Aliquots from this solution were then utilized for G-3-P estimation enzymically. A so-
RESULTS AND DISCUSSION

Thermal Inactivation of GAPDH in the Crude Extracts of Mung Beans

The preliminary reports from this laboratory show that the kinetic pattern of thermal inactivation of GAPDH in the extracts of mung beans is different from that of the purified enzyme and that the pattern varied with the period of germination (15). For example, if the extract was prepared from ungerminated seeds, the inactivation followed biphasic kinetics. However, the enzyme activity in the extract prepared from germinated seeds (36 h) was destroyed in a single exponential decay on heating. This has now been studied in greater detail.

Time-dependent thermal inactivation of GAPDH present in the extract prepared from seeds after different periods of germination are shown in Figure 1. The enzyme activity in the extract of ungerminated seeds is destroyed in a biphasic manner with each phase accounting for approximately half of the initial activity, i.e. \( \% A_{\text{slow}} = \% A_{\text{fast}} \approx 50\% \) (curve 1). After 12 h of germination, the kinetics of inactivation are biphasic but percentage amplitude of slow phase (\( \% A_{\text{slow}} = 74\% \)) is larger than \( \% A_{\text{fast}} \) (curve 2). However, at 24 h of seed germination, the kinetics show a single exponential decay of activity (curve 3). In the extracts obtained from seeds...
that were allowed to germinate for 36 h, a lag is observed before the inactivation of GAPDH commences. The changes in kinetic pattern in curves 1, 2, and 3 are similar to those observed when GAPDH is heated in the presence of increasing G-3-P concentrations (11). Therefore, the crude extracts obtained after different periods of germination were assayed for G-3-P for a quantitative comparison. Results are shown in Figure 2, in which the levels of GAPDH activity in the extracts are also shown. It is interesting that the levels of enzyme and its substrate change parallel to each other. Further, it was found that changes in the kinetic pattern were quantitatively the same as those expected on the basis of the earlier data (11).

Any lag period similar to that observed in curve 4 of Figure 1 has not been reported previously. In this experiment, G-3-P concentration in the solution being heated was 102 μM, which is higher than the substrate concentrations employed in earlier studies. Therefore, the kinetics of purified mung bean GAPDH were investigated at higher G-3-P concentrations. Qualitatively similar results were obtained as in curve 4 of Figure 1, namely a lag period followed by a single exponential decay and the same rate constant. The lag period varied with the G-3-P concentration as shown in Table I.

G-3-P is not very stable at near neutral pH. We have now studied its decomposition in 20 mM TEA buffer, pH 7.3, at 52°C. G-3-P disappears in an exponential decay with a t1/2 equal to 12 min. It is now possible to calculate the loss of G-3-P during the lag period. The G-3-P concentration at the end of the lag period is always in the range of 95 to 100 μM. Thus, the enzyme is fully protected against thermal denaturation even at 52°C when G-3-P concentration is around 100 μM or more. At the ambient temperature, the inactivation will be much slower than at 52°C.

Effect of G-3-P on the Inactivation of GAPDH with Trypsin

Purified GAPDH was incubated with trypsin at pH 7.5 at 30°C in the absence and presence of different concentrations of G-3-P. Aliquots withdrawn at different time intervals were assayed for enzyme activity. The results are shown in Figure 3. Loss of GAPDH activity in these experiments is almost entirely due to proteolytic attack, because no inactivation is observed under these conditions in the absence of trypsin. The protective effect of substrate against proteolysis is evident. Furthermore, a lag period is observed when G-3-P concentration is equal to or higher than 110 μM. Thus, GAPDH is almost fully protected against thermal inactivation and proteolysis at these concentrations.

Protection against proteolysis is of particular physiological significance because proteases are ubiquitous in germinating leguminous seeds. These are induced on germination and are required for mobilization of the storage proteins (1, 3, 14).

---

Table I. Relationship between G-3-P Concentration and Lag Period Observed in the Thermal Inactivation of Mung Bean GAPDH at pH 7.3 and 52°C

<table>
<thead>
<tr>
<th>Initial G-3-P (mM)</th>
<th>Lag Period (min)</th>
<th>Residual G-3-P after Lag Period (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>0.08</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>0.11</td>
<td>2</td>
<td>0.095</td>
</tr>
<tr>
<td>0.12</td>
<td>5</td>
<td>0.090</td>
</tr>
<tr>
<td>0.18</td>
<td>10</td>
<td>0.100</td>
</tr>
<tr>
<td>0.36</td>
<td>23</td>
<td>0.098</td>
</tr>
<tr>
<td>1.20</td>
<td>45</td>
<td>0.090</td>
</tr>
</tbody>
</table>

* Mean residual G-3-P at the end of lag period = 0.095 ± 0.005 mM.

---

LITERATURE CITED

9. Malhotra OP, Srinivasan (1985) pH-dependent conformational...
transformation in mung bean glyceraldehyde 3-phosphate dehydrogenase. Arch Biochem Biophys 236: 775–781