Properties of Uridine and Thymidine Phosphorylating Enzymes of Zea Mays

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The studies support the previous conclusion that two uridine phosphorylating enzymes exist during germination of Zea mays, of which only one is active in vitro during the initial 48 hours of germination. The early appearing enzyme is less heat stable and of higher molecular weight than the second one, which becomes active after this time.

The evidence presented indicates that the second enzyme is identical with thymidine kinase. It consists of two components of equal molecular size, each of which is slightly smaller than bovine serum albumin. Both are inactivated at temperatures above 55 °C, but component T is somewhat more stable than component P. The latter is already present in small amounts during early germination stages. Competitive inhibition of thymidine phosphorylation by uridine and uridine phosphorylation by thymidine occurred, the $K_M$s for thymidine and uridine being equal to the respective $K_M$s with similar turn-over numbers for both reactions.

Thymidine kinase of Zea mays is dissociable into two parts, component P and component T, which could be separated by precipitation with ammonium sulphate or electrophoresis. A partial dissociation of the enzyme was also observed under assay conditions. Biosyntheses of the two components evidently occur independently of each other. Component P is present from the beginning of germination, whereas component T begins to appear 48 hours later. The enzyme activity is mainly controlled by the component which is present in limiting amounts. Uridine kinase was also resolvable into two components by the same procedures employed for thymidine kinase, an observation which, together with the finding that component P of both enzymes exhibited the same electrophoretic mobility, suggested that these two kinases are structurally related. However, the presence of a very high uridine kinase activity during the initial 36 hours of germination, which seemed to be caused by another phosphorylating enzyme, made it impossible to get a clear picture of this relationship by germination experiments. It was the aim of the investigations presented here to obtain a better insight in this problem.

Methods

The procedures which have been followed, were already described in detail in previous work. Standard incubations were performed at 30 °C for 30 minutes. 0.1 ml of the assay mixture contained 1.6 x 10⁻⁴ moles [¹⁴C]-nucleotide (uridine or thymidine), 2 x 10⁻⁴ moles ATP* (d-sodiumsalt), 2 x 10⁻⁴ moles MgCl₂, 20 x 10⁻⁴ moles phosphate buffer (K, Na) pH 7 and enzyme preparation at a convenient dilution.

Results

1. Heat treatment

Heat stabilities of the enzymes extracted from corn seedlings germinated for 18 or 96 hours at 25 °C were compared (fig. 1). The uridine kinase of seedlings germinated for 18 hours was strongly inactivated at temperatures of 40 °C and higher. The uridine kinase activity of the extract of 96 hours old seedlings was also depressed by pretreatment at this temperature, but only by about 40%; further elevation of the temperature to 55 °C exerted only a slight additional effect, whereas above this temperature, inactivation soon became complete. Thymidine kinase, which was rather stable until about 50 °C was also inactivated completely at 60 °C.

In a second experiment, an extract from 96 hours germinated seedlings was heated at 59 °C for different times. Thymidine and uridine kinase were inactivated almost completely within 5 min (fig. 2) with no significant recovery being produced by addition of component T to the enzyme mixture; the

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Footnotes:

* The following abbreviations were used: ATP = adenosinetriphosphate, TdR = deoxythymidine, UR = uridine, dTMP = deoxythymidylic acid, UMP = uridylic acid.
addition of component P, however, resulted in a very strong reactivation of the heat treated extracts, thus indicating that the heat inactivation was primarily caused by the selective degradation of this component in the extract. Component T's activity was only depressed by about 40% after 30 min. pretreatment at 59 °C but it was also inactivated completely if a temperature of 61 °C was applied for 30 minutes. The increase of the enzyme activities in the untreated extracts on addition of component P and sometimes also on addition of component T can be ascribed to the presence of an excess amount of the complementary component and a partial dissociation under assay conditions as has already been stated previously 2.

A similar experiment was carried out with an extract from 25 hours old seedlings. A temperature of 47 °C was chosen in this case because of the lower stability of this enzyme. It was completely inactivated during the course of a 30 min. lasting treatment (fig. 2). No reactivation was observed on addition of component T or P respectively. The lowered activity observed after addition of the component T might be attributed to unknown factors in the preparations and to the fact that the enzyme activity can be reduced very easily, for example by freezing and thawing. Since a specific interaction of one of the two components with the heat labile uridine kinase seems improbable, the enzyme here must therefore be quite different from the one in the paragraph above.

2. Fractionation by Sephadex chromatography

Another difference between the two uridine phosphorylating enzymes was found by gel-filtration on Sephadex G 200. Two enzymes were discovered in the eluate. One apparently had a molecular weight of 600,000 or higher (fig. 3), as it was eluted immediately after the void volume 4. The second

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Fig. 3. Gel filtration on Sephadex G 200 of an extract from seedlings terminated for 96 hours. A Sephadex column with a bed volume of 260 ml was equilibrated with 0.2 M phosphate buffer pH 7. 10 ml buffer extract (3 seedlings/ml) were applied and 10 ml samples were collected. — — — Extinction at 280 nm. Assay mixtures contained per 100 µl: ● — ● and △ — △ 25 µl eluate + 25 µl buffer; ○ — ○ and △ — △ 25 µl eluate + 25 µl component P. Circles represent values for uridine kinase and triangles for thymidine kinase. The arrow indicates the elution peak of a bovine serum albumin marker. The enzyme peak appeared slightly after the position of a bovine serum albumin reference. From a rough evaluation according to the method of Andrews\(^5\) one obtains a molecular weight between 5 and \(6 \times 10^4\). Only the kinase with the high molecular weight was present in the extracts obtained from young seedlings (fig. 4). Once more the similarity between thymidine kinase and the low molecular weight uridine kinase is indicated by the observation that the elution patterns are practically identical for both enzyme activities, except for the fact that the uridine kinase activity of the fractions is appreciably lower than the thymidine kinase activity. Besides the active enzymes, there is a remarkable excess of component T present in this fractions, which was recovered by adding component P to the assay mixture. On the other hand, the presence of component P in fractions of the extracts prepared from young seedlings was demonstrable for both enzymes by the addition of component T. This could not be proved for uridine kinase previously because of the very high activity of the labile high molecular weight enzyme in crude extracts. The somewhat contradictory observation that no significant difference was found between the elution pattern of the whole enzyme and its components would appear to be another indication for a partial dissociation of the enzyme in solution (see above). It is known, however, that the partition of molecules by Sephadex chromatography is also influenced by factors other than the molecular size. Chromatography of the isolated components on Sephadex G 100 again showed the same distribution patterns for both enzyme activities (figs. 5 and 6). They appeared again slightly behind the serum albumin marker. There is also practically no difference between the elution patterns of component P and component T, so that the conclusion seems justified that both components are of similar size. The uridine kinase peak of the T-fraction eluted together with the high molecular weight proteins is due to contamination by the high molecular weight uridine kinase, which was unusually high in this particular experiment.

3. Enzyme kinetics

To study kinetics a mixture of equivalent amounts of the two components was prepared. The Michaelis constants (\(K_M\)) for the nucleosides as derived from Lineweaver-Burk plots were \(1.25 \times 10^{-3}\) moles\(\cdot l^{-1}\) for thymidine and \(5.5 \times 10^{-3}\) moles\(\cdot l^{-1}\) for uridine (fig. 7). Similar values for thymidine kinase were obtained with crude enzyme extracts. The corresponding maximum velocities were \(1.46 \times 10^{-5}\) and \(1.84 \times 10^{-5}\) moles\(\cdot l^{-1}\) min\(^{-1}\), giving a ratio of 0.79. In other experiments ratios of 0.6 and 1.1 have been found.

Fig. 5. Gel filtration of component T on Sephadex G 100. Component T was prepared as described previously and 2 ml were applied to a column with a bed volume of 200 ml. Samples of 7.5 ml were collected. Further details as in fig. 3.

Fig. 6. Gel filtration of component P on Sephadex G 100. Component P was prepared as described previously and 8.5 ml were applied to a column with a bed volume of 200 ml. Samples of 7.5 ml were collected. Further details as in fig. 3 except, that component P was replaced by component T.

The remarkable similarities between the two dissociable phosphorylating enzymes justify the question of whether two different enzymes actually do exist or whether both reactions are catalysed by one aspecific enzyme. In the latter case a mutual competitive inhibition of thymidine and uridine phosphorylation by uridine and thymidine respectively would be expected. Thus thymidine should inhibit uridine phosphorylation competitively and visa versa. In order to test this possibility, it is helpful to regard the "inhibition" of the phosphorylation of labelled thymidine by unlabelled thymidine itself as prototype of a competitive inhibition, the inhibitor constant $K_i$ being represented by the $K_M$. Then thymidine should inhibit the phosphorylation of labelled uridine and thymidine present at given low concentration in the same way if a mutual competition does exist. A semilogarithmic plot of the reaction velocity $v$ against the "inhibitor" concentration $i$ allows a comparison of both reactions. The dependence of $v$ on $i$ is given by the equation:

$$v = \frac{V_{max} \cdot s \cdot K_i}{K_M \cdot K_i + K_M \cdot i + K_i \cdot s}.$$  

(1)

The term $K_i \cdot s$ can be neglected at substrate concentrations $s \ll K_M$ and (1) becomes then:

$$v = \frac{V_{max} \cdot s \cdot K_i}{K_M \cdot K_i + i}.$$  

(2)

The shape of the curve of the semilogarithmic plot is determined by the second term of the right side. $v$ becomes 1/2 at "inhibitor" concentration $i = K_i$.

Theoretical curves are presented in fig. 8 by making use of the $K_M$s as $K_i$. The assumption can be made that the reaction is "uninhibited" ($= 100\%$) at very low nucleoside concentrations (this "100\%" is actually about $99\%$ at concentrations which are a factor $10^{-2}$ below the $K_i$ — this or lower concentrations are requisite for a successful application of the plot). The figure shows a good agreement between the theoretical curves and the experimental data.

The correspondence of the $K_M$s with the mutual $K_i$s makes a competitive inhibition extremely likely, but it does not prove it definitely. The inhibition of thymidine phosphorylation by uridine was therefore investigated at two different thymidine concentrations. Table 1 shows, that the inhibition is more efficient at low substrate concentrations. The same is the case in the inhibition of the uridine phosphorylation by thymidine. It can be concluded from these experiments that a mutual competitive inhibition by the two substrates does exist.

6 H. Lineweaver and D. Burk, J. Amer. chem. Soc. 56, 658 [1934].


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Fig. 7. Inverse plot of the dependence of the phosphorylation velocity on the nucleoside concentration. Uridine kinase: + + and lower scale on the abscissa; thymidine kinase: × × × and upper scale on the abscissa.

![Graph]

Fig. 8. Semilogarithmic plots of the phosphorylation velocities of the labelled nucleosides against the concentration of the unlabelled nucleosides. K_Ms were used as K_M for calculation of the theoretical curves. —— concentrations of thymidine; K_I = 1.25 x 10^{-2} moles·l^{-1}; —— concentration of uridine; K_I = 5.5 x 10^{-3} moles·l^{-1}.

x = ^{14}C-thymidine phosphorylation; + = ^{14}C-uridine phosphorylation. 28.3% of the added thymidine (1.6 x 10^{-5} moles·l^{-1}) and 9.9% of the added uridine were phosphorylated in the "uninhibited" reactions. These values were set equal to 100%, no corrections for v_o were made.

Table I. Inhibition of thymidine and uridine phosphorylation by uridine and thymidine respectively at different substrate concentrations.

<table>
<thead>
<tr>
<th>[M]</th>
<th>moles dTMP formed/min·l</th>
<th>% inhibition</th>
</tr>
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<tr>
<td>16 x 10^{-6} TdR</td>
<td>0.166 x 10^{-6}</td>
<td>32.5</td>
</tr>
<tr>
<td>10 x 10^{-6} TdR</td>
<td>0.112</td>
<td></td>
</tr>
<tr>
<td>+5 x 10^{-3} UR</td>
<td>7.65</td>
<td>24.5</td>
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<tr>
<td>1 x 10^{-3} TdR</td>
<td>5.79</td>
<td></td>
</tr>
<tr>
<td>+5 x 10^{-3} UR</td>
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<td></td>
</tr>
<tr>
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<td>7.65</td>
<td>24.5</td>
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<tr>
<td>+5 x 10^{-3} UR</td>
<td>5.79</td>
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<tr>
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Discussion

The previous conclusion that two uridine phosphorylating enzymes might exist in germinating corn seedlings is proved by the results reported above. The high uridine kinase activity during the initial germination time is attributable to only one enzyme with a molecular weight of at least 600,000, which is inactivated at temperatures of 40 °C and by freezing and thawing in 0.2 molar phosphate buffer pH 7. The enzyme activity decreases strongly during the first 48 hours of germination, but small amounts of the high molecular weight enzyme are still present after 96 hours.

At 96 hours a second uridine phosphorylating enzyme is detectable. It is this enzyme, which, like thymidine kinase, can be split into components P and T. Gel filtration studies indicated that a small amount of component P is present during early germination time before the appearance of component T. On the other hand there is an excess of the T component at later germination stages. This is in complete agreement with what has been observed for thymidine kinase. Further relationship between the two enzymes emerges from the fact, that they become labile at approximately 55 °C and that in both cases component P is slightly more labile than component T. The heat inactivation profiles indicate that both constituents consist of proteins as essential
chemical compounds, a finding which has not been established previously.

The partition by gel filtration on Sephadex further revealed that the molecular sizes of the components are the same for thymidine kinase and for uridine kinase. Moreover, no significant difference in size exists between component P and component T, both being slightly smaller than bovine serum albumin. From previous experiments it was concluded that the enzyme might be partly dissociated in solution. This interpretation is strengthened by the present observation that no significant difference exists between the distribution pattern of the whole enzyme and the two components. Furthermore the degree of dissociation can be expected to be exaggerated under the conditions of gel filtration; and therefore the true molecular size of the active enzyme might be a multiple of that found by this method. The conclusion seems justified that the differences in electrical charge and the ionic strength play an important role in the binding of the two components. Bresnick and coworkers have observed a complete disaggregation of thymidine kinase from Walker carcinoma into subunits at about the same ionic concentration, namely 0.2 M KCl. However, there were no evident differences between the subunits which would permit separation, such as is possible in the kinase of plants. In this respect the studies of Gerhart and Pardee on aspartate transcarbamylase, which also consists of subunits are of special interest. Physical properties and activity of this enzyme were strongly affected by low molecular weight compounds, especially those which were somehow related to the normal substrates. Similar experiments on thymidine kinase of corn have not been reported so far.

One of the questions of the present investigation was whether the P- or T-component might be common to both enzymes. Indeed the many similarities strongly suggest that they both are constituents of one and the same enzyme, carrying out the two different reactions by virtue of an incomplete specificity of the substrate binding site. In this case the thymidine kinase reaction would have to be inhibited competitively by uridine, with a $K_i$ equal to the $K_M$ for uridine in the uridine kinase reaction. This case is clearly demonstrated by the experiments and the same is true for the opposite case, namely the competitive inhibition of the uridine phosphorylation by thymidine. It seems unlikely that the presence of the $-\text{OH}$ or $-\text{H}$ group in the 2'-position has a significant influence on the energy requirement of the reaction and therefore the turnover numbers in both reactions should be similar. The $V_{\text{max}}$ of a given enzyme preparation was indeed found to be very similar for both enzyme reactions. Thymidine, however, is preferentially phosphorylated because of its lower $K_M$. An enzyme which can phosphorylate uridine and cytidine was found in ascites tumor cells. But this kinase was not able to catalyse the phosphorylation of deoxyribonucleosides. A similar enzyme seems to exist in sea urchin eggs. It seems less likely that one enzyme should phosphorylate nucleosides and deoxyribonucleosides because of the different functions of the products. Also an experimental argument exists against the assumption, that we deal with one aspecific enzyme: The ratio of thymidine phosphorylation to uridine phosphorylation can vary within a range of 3 to 5 in preparations which are free of the high molecular weight uridine kinase. Other factors, unknown till now, may be responsible for this discrepancy, but this has still to be demonstrated. Okazaki and Kornberg have observed pronounced effects of phosphorylated nucleoside derivatives on the $K_M$ of the thymidine kinase of E. coli. The state of purification of the components must be improved, however, before similar experiments can successfully be performed with enzyme from corn. Current investigations are designed for elucidating whether the aspecificity is limited to thymidine kinase and uridine kinase or whether other nucleosides and deoxyribonucleosides are included.

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