The Regulatory Properties of Purified *Phaseolus aureus* Sucrose Synthetase

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ABSTRACT

*Phaseolus aureus* sucrose synthetase, purified to homogeneity, was assayed in the presence of a variety of biological compounds to test for possible regulatory effectors. The oxidized form of nicotinamide adenine dinucleotide phosphate, as well as indoleacetic acid, gibberellic acid, and pyrophosphate were found to activate the forward reaction (sucrose degradation) and inhibit the reverse reaction (sucrose synthesis). The reduced form of nicotinamide adenine dinucleotide phosphate antagonizes the effect of the oxidized form. Fructose 1-phosphate and divalent cations inhibit the forward and activate the reverse reaction. Pyrophosphate and fructose 1-phosphate are effective only in the presence of magnesium chloride. Uridine triphosphate inhibits both the forward and reverse reactions. All effectors except gibberellic acid are active only in the millimolar range of concentrations; maximal stimulation for any effector is approximately 2-fold. The effects of combinations of effectors are roughly additive. Using pyrophosphate in the presence of magnesium chloride as an effector, results of kinetic studies offer a model by which an effector can activate an enzymatic reaction in one direction and inhibit in the reverse direction.

Delmer and Albersheim (2) studied the distribution of sucrose synthetase in seedlings of the mung bean, *Phaseolus aureus*, and found that levels of this enzyme were high only in nonphotosynthetic tissues of the plant. Such a result suggests that sucrose synthetase, at least in *P. aureus*, functions predominantly in the degradation of sucrose translocated from leaves to the nonphotosynthetic tissues. A similar suggestion has been made by Gibbs (5) and by Pavlinova (11). The enzyme has been reported to exist in a wide variety of plant tissues, most of which were nonphotosynthetic (3, 7). Furthermore, the results of many workers (1, 8–10, 13, 14) have suggested that sucrose synthetase may play a major role in the conversion of sucrose to starch in ripening seeds.

Recently this author (3) has succeeded in purifying to homogeneity a sucrose synthetase from etiolated *R. aureus* seedlings and has characterized the physical as well as some basic kinetic properties of the enzyme. The purified enzyme can catalyze the following reversible reactions.

\[
\text{Sucrose } + \text{nucleoside-DP } \rightleftharpoons \text{nucleoside-DP-glucose } + \text{fructose}
\]

where the nucleoside may be uridine, adenosine, thymidine, cytosine, or guanidine. Kinetic data (3) indicate a random mechanism for the enzyme. The \(K_m\) values for all five possible nucleoside diphosphates are similar, but the \(V_{\text{max}}\) of the forward reaction is markedly higher when UDP is the substrate. For the reverse reaction, the \(V_{\text{max}}\) is similar regardless of substrate, but the \(K_m\) for UDP-glucose is 10-fold lower than for the other nucleoside diphosphate glucoses (6).

Since the enzyme is potentially capable of catalyzing the synthesis of many of the major precursors for plant polysaccharides (or, alternately, of sucrose synthesis), it seemed important to search for possible modes of regulation of this enzyme's activity. It was of particular interest to see if one could find effectors which might differentially affect the catalytic efficiency for the various nucleoside diphosphate substrates or which might differentially alter the reaction rates of the forward and reverse reactions. A number of effectors of the enzyme's activity have been found, and this paper presents the results of these studies.

MATERIALS AND METHODS

GENERAL

U-\(^{14}\)C-Sucrose was purchased from Schwartz BioResearch Co. \(^{14}\)C-UDP-Glucose (label in glucose only) was purchased from International Chemical and Nuclear. All unlabeled nucleoside diphosphate glucoses, nucleoside diphosphates, and pyridine nucleotides were purchased from Sigma Chemical Company. Solutions of the following chemicals were made fresh each day used: IAA, NADH, and NADPH. All solutions were adjusted to pH 7.5 prior to use.

PREPARATION OF ENZYME

Purified enzyme was prepared as described previously (3). All preparations were checked for purity before use by acrylamide gel electrophoresis as described previously (3). The final enzyme preparation was in 30 mM tris, pH 7.5, containing 0.1 mM EDTA and dithiothreitol. All substrates and effectors (with the exception of sodium pyrophosphate and MgCl\(_2\)) were made up in this same buffer.

ASSAYS

Forward Reaction. U-\(^{14}\)C-Sucrose, 0.1 \(\mu\)c (100 \(\mu\)c/\(\mu\)mole), 0.001 \(\mu\)mole of UDP, effector (if present) at concentration indicated, and enzyme were incubated at 25 °C in drawntip Pasteur
Table I. Effects of Various Compounds on Activity of Purified Sucrose Synthetase

<table>
<thead>
<tr>
<th>Compound Tested</th>
<th>Forward Reaction Suc + UDP → UDP-Glu + Fructose</th>
<th>Reverse Reaction UDP-Glu + Fructose → Suc + UDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MgCl₂</td>
<td>2 mM MgCl₂</td>
</tr>
<tr>
<td>PP</td>
<td>99</td>
<td>227 (122)²</td>
</tr>
<tr>
<td>Fructose-1-P</td>
<td>85</td>
<td>61</td>
</tr>
<tr>
<td>NADPH</td>
<td>70</td>
<td>98</td>
</tr>
<tr>
<td>NADH</td>
<td>60</td>
<td>57</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>100</td>
<td>113</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>155</td>
<td>142</td>
</tr>
<tr>
<td>IAA</td>
<td>141</td>
<td>134</td>
</tr>
<tr>
<td>GA</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>UTP</td>
<td>61</td>
<td>148 (83)²</td>
</tr>
</tbody>
</table>

₁ Present at 60 μM.
² Number in parentheses is relative to minus MgCl₂ control.

pipettes in a final volume of 25 μl. The reaction was terminated by spotting onto discs of Whatman DE-81 filter paper. Studies show the reaction terminates immediately upon spotting. The discs were then placed on filters and the unreacted ³C-sucrose washed off with three successive 10-ml washes of distilled H₂O. The filters were dried and bound ³C-UDP-glucose was counted in standard toluene liquid scintillation fluid. Counting efficiency was approximately 50%. All effectors used were tested for possible interference with UDP-glucose binding. At the concentrations utilized, none interfered. All reactions were run in duplicate for each time point; only data from linear portions of the reaction are presented. Maximum deviations for the assay were of the order of 10% of stated values.

Reverse Reaction. ³C-UDP-Glucose, 0.05 μc, (95 μc/μmole), 0.005 μmole of fructose, effector (if present), and enzyme were incubated in a final volume of 25 μl. Otherwise the reactions were run by a procedure identical to that of the forward reaction, except that rates were determined by disappearance of ³C-UDP-glucose from the discs rather than its appearance.

Km Determinations. For these experiments only, the forward reaction was assayed by a previously described Assay C (3). The reverse reaction was assayed by the method of Grimes et al. (6).

RESULTS AND DISCUSSION

In screening for possible compounds which might affect the enzyme's activity, all reactions were run at limiting substrate concentrations to avoid missing the effects of any compounds which might act competitively with the substrates. A final concentration of 2 mM was arbitrarily chosen for testing compounds. The following compounds were tested and found to have no effect upon the rate of either the forward or the reverse reaction: glucose-1-P, glucose-6-P, fructose-6-P, fructose-1,6-diP, ribose-5-P, ribulose-1,5-diP, P-enolpyruvate, 3-P-glycerate, P₃ citrate, AMP, ATP, KCl, NaCl, chondroitin sulfate, and 6-furfurylamino purine.

The Effect of Divalent Cations. When tested at 2 mM, MgCl₂, CaCl₂, and MnCl₂ were found to inhibit the forward reaction (sucrose + UDP → UDP-glucose + fructose) and activate the reverse reaction. To cite representative duplicate reactions, examples of these results for the forward reaction, containing no divalent cations catalyzed the conversion of 5290 and 5230 cpm of ³C-sucrose into ³C-UDP-glucose under the standard reaction conditions described under "Methods." In the presence of 2 mM MgCl₂, the incorporation was reduced to 2400 and 2460 cpm in duplicate samples, representing an average inhibition of 46%. For the reverse reaction, control reactions in the absence of MgCl₂ converted 10,515 cpm and 10,780 cpm from ³C-UDP-glucose to ³C-sucrose. In the presence of 2 mM MgCl₂, 16,955 and 16,555 cpm, respectively, were converted, representing an activation of 157% over control values. A similar effect of MnCl₂ has been reported for potato sucrose synthetase by Pressey (12). For simplicity, in the subsequent experiments reported, data on effectors will be shown as a percentage of control activity. Control rates were generally similar to the data presented in this section.

The Interaction of MgCl₂ and Other Effectors. Certain compounds were found to have no effect on either the forward or reverse reaction when tested alone, but had significant effects when tested in combination with MgCl₂. Compounds which exhibited this property were fructose-1-P and sodium pyrophosphate (PP₃). Table I shows these results. In this case, fructose-1-P is an inhibitor of the forward reaction and an activator of the reverse reaction only in the presence of equimolar MgCl₂. Similarly, PP₃ is an effective activator of the forward and a slight inhibitor of the reverse reaction only when MgCl₂ is present. The optimum interaction has been found to occur when the molar ratio of Mg²⁺ to PP₃, or fructose-1-P is between 1 and 2. In the case of PP₃, much of the activation relative to a plus MgCl₂ control is almost certainly due to chelation of the inhibitory MgCl₂. However, PP₃ may be regarded as a true activator of the forward reaction in the presence of MgCl₂ since it raises this activity above minus MgCl₂ control levels. To avoid misinterpretation, the remaining data on PP₃/Mg are always calculated relative to a control minus MgCl₂. On the other hand, data for effects of the fructose-1-P/Mg combination are always compared to the plus MgCl₂ control.

Effectors not Dependent upon MgCl₂. Table I also shows the effects of other compounds which are relatively unaffected by the presence of MgCl₂. Many of these compounds (the most

Fig. 1. Effect of varying effector concentrations on the rate of the forward reaction of purified sucrose synthetase. Control activity represents that reaction rate obtained in the absence of any effector. One exception to this is for the fructose-1-P/Mg combination where per cent control activity represents the rate at any particular concentration of fructose-1-P/Mg relative to the activity obtained with that same concentration of MgCl₂ alone. Where PP₃/Mg and fructose-1-P/Mg are varied, the concentration indicated represents the molarity of each species.
noticeable exception being UTP) have differential effects on the rates of the forward and reverse reactions. The effect of IAA seems relatively specific since L-tryptophan has been shown to be completely ineffective and indole less effective than IAA (115% of control activity for the forward reaction at 2 mM).

**Varying the Effector Concentration.** Two millimolar is probably a relatively high level of most of these compounds physiologically. Therefore, experiments were run to test the effect of these compounds on the rate of the forward reaction at varying concentrations. Figure 1 shows these results for fructose-1-P/Mg, PP/PP, MgCl₂, PP₁, NADP⁺, NADPH, IAA, and GA. With the exception of GA, which is effective at relatively low levels, the other compounds show virtually no activation or inhibition below 0.1 mM.

**Combinations of Effectors.** Although certain of these effectors alone may be weakly effective at physiological concentrations, the possibility exists that several compounds in combination could produce significant effects. The results of experiments where different combinations of two positive effectors were tested at various concentrations are shown in Figure 2. The open circles are the experimental values obtained; the dotted line shows what the expected results would be if the effects of the two compounds in question were additive. Below about 3 mM, the effectors are at least additive in their effects. The inhibition by the NADP⁺/IAA combination at 4 mM is quite reproducible, and may indicate some interaction between the binding sites for these two effectors. The combined effect of IAA, NADP⁺, and PP₁/Mg was tested at only one concent-

![Fig. 2. Activity of the forward reaction in the presence of combinations of effectors. For A, B, and C, the ratio of any two effectors remains constant at 1:1, and the absolute concentration indicated by different points represents the concentration of each effector. In D, GA was held constant at 40 mM and only IAA varied as indicated. The closed circles represent experimental values obtained. The dotted line represents the expected results (calculated from simultaneous experiments performed with individual effectors) if the effects of the two compounds were additive.](image)

![Fig. 3. Varying the ratio of NADP⁺ to NADPH. The forward reaction was measured. NADP⁺ was held constant at 1.67 mM, and NADPH varied to give the molar ratios indicated.](image)

![Table II. Effect of Activators in Presence of Different Nucleoside Diphosphate Substrates](table)

The forward reaction was measured as described under "Methods," except that the nucleoside diphosphate substrate was varied as indicated.

<table>
<thead>
<tr>
<th>Second Substrate</th>
<th>PP₁/Mg (40 mM)</th>
<th>IAA (4.0 mM)</th>
<th>NADP⁺ (1.46 mM)</th>
<th>GA (33 μM)</th>
<th>% Control Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP</td>
<td>175</td>
<td>200</td>
<td>140</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>UTP</td>
<td>171</td>
<td>182</td>
<td>173</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>TDP</td>
<td>190</td>
<td>167</td>
<td>202</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>CDP</td>
<td>120</td>
<td>95</td>
<td>204</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>GDP</td>
<td>131</td>
<td>60</td>
<td>99</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

![Table III. Effect of PP₁/Mg on Kinetic Parameters of P. aureus Sucrose Synthetase](table)

$K_m$ values and $V_{max}$ were obtained from Lineweaver-Burk plots. PP₁ and MgCl₂ were both present at 4.0 mM. $K_{eq}$ was determined at pH 7.5 and 25°C.

<table>
<thead>
<tr>
<th>$V_f/V_b$</th>
<th>$K_m/UDP$</th>
<th>$K_m/Glucose$</th>
<th>$K_m/Ethane$</th>
<th>$K_{eq}$</th>
<th>$K_{eq}$ Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>-PP₁/Mg</td>
<td>1.23</td>
<td>0.19</td>
<td>17.0</td>
<td>0.21</td>
<td>2.0</td>
</tr>
<tr>
<td>+PP₁/Mg</td>
<td>5.1</td>
<td>0.50</td>
<td>17.0</td>
<td>0.26</td>
<td>0.75</td>
</tr>
</tbody>
</table>

 superscript 1 Mean with standard deviation of five separate determinations.

 superscript 2 One determination only.
Varying the Ratio of NADP+/NADPH. The fact that opposite effects are obtained with the oxidized and reduced forms of NADP* suggests that the reducing state of the cell may play some role in controlling the relative rates of the forward and reverse reactions. Figure 3 shows that the oxidized and reduced forms of NADP when present in combination do compete with one another for effector activity. When an equimolar ratio is present, these compounds have no effect whatsoever on sucrose synthetase activity.

The Effect of Activators in the Presence of Different Nucleoside Diphosphate Substrates. Table II shows how four different activators affect the rate of the forward reaction when different nucleoside diphosphates serve as the second substrate. With one exception, there are no striking differences in effect. The exception is the case of IAA and GA when GDP is the substrate. In this case, these compounds inhibit rather than activate the forward reaction.

The Mechanism of PPi/Mg Effector Activity. One might ask just how a compound can activate a reversible reaction in one direction and inhibit in the reverse direction. A number of years ago, in a theoretical paper, Frieden (4) proposed that such an effect was mechanistically possible for an enzyme which catalyzes a reversible reaction by a random mechanism. The explanation invokes the use of the Haldane relationship which states that, for an enzyme with a random mechanism, the following formulation holds.

\[ K_{eq} = \frac{V_i K_m K_m'}{V_i K_m K_m'_{eq}} \]

where, for a bimolecular reaction, \( K_m \) and \( K_m' \) are taken to be the \( K_m \) values for the substrates in the back reaction, \( K_m' \) and \( K_m'' \), those of the substrate of the forward reaction, and \( V_i \) and \( V_i' \), the respective \( V_{max} \). An examination of this equation shows that the equilibrium relationship can be maintained when \( V_i/V_i' \) varies, providing that the \( K_m \) values are changed by the effector in such a way as to maintain \( K_{eq} \) constant. Since \( P. aureus \) sucrose synthetase has been shown to utilize a random mechanism where such a Haldane relationship applies (3), this seemed the most logical explanation for the mechanism by which these effectors act. Experiments using PPi/Mg as effector support this conclusion. Table III summarizes these results. \( K_m \) values and \( V_{max} \) were measured in the presence of 4 mM PPi/Mg. Similar numbers for the nonactivated state as well as \( K_m' \) have been reported in the author’s previous publication (3). In the presence of effector (as is true in its absence) the enzyme exhibits typical Michaelis-Menten kinetics, and activation (or inhibition) is observed at every concentration of substrate. Hill plots for all substrates yield an \( n \) value of approximately 1. When the data of Table III are fitted to the Haldane relationship, a very close approximation to the experimentally determined \( K_{eq} \) is obtained. In the case of PPi/Mg, a rise in \( V_i/V_i' \) is compensated for by concomitant changes in the \( K_m \) values for UDP, fructose, and, to a lesser extent, UDP-glucose. In this regard, therefore, PPi/Mg acts as an uncompetitive effector for these substrates, altering both the \( K_m \) and \( V_{max} \), and a noncompetitive inhibitor with respect to sucrose, where only the \( V_{max} \) is altered. The fact that, within experimental error, the \( K_m' \) is the same in the presence of PPi/Mg as in its absence indicates that these compounds do not participate as substrates. Further support for this comes from the results of an experiment which showed that PPi/Mg was not hydrolyzed or altered in concentration during the course of the reaction. (Similarly, it has been shown that NADP* or NADPH do not change oxidation states when they are present as effectors.)

In conclusion, what, if any, is the physiological significance of any or all of these effectors is a difficult question to resolve. For some of the compounds, most notably IAA, the levels required for significant activation or inhibition are almost certainly higher than those encountered by the enzyme in vivo. However, the fact that, at lower concentrations, the effects of combinations of effectors are at least additive may mean that, although the variation in levels of any single effector may elicit only a small response, a simultaneous variation in a number of effector concentrations inside the cell may have rather significant effects. Unfortunately, such a situation is difficult to test experimentally, and, for the present, the data probably warrant no major physiological interpretation.

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LITERATURE CITED