Purification and kinetic characterization of glucose-6-phosphate dehydrogenase from cellular slime mold Dictyostelium discoideum

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PURIFICATION AND KINETIC CHARACTERIZATION
OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM CELLULAR SLIME MOLD,
Dictyostelium discoideum

By
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Glucose-6-phosphate dehydrogenase from Dictyostelium discoideum was purified and kinetically characterized. The enzyme exhibited stoichiometry in catalyzing the reaction, which is irreversible in vitro. The purified enzyme was shown to be specific for NADP⁺. Michaelis constants for glucose-6-phosphate and NADP⁺ were 0.024 mM and 0.043 mM, respectively. Initial velocity and product inhibition studies suggested that this enzyme follows an ordered Bi:Bi sequential mechanism with glucose-6-phosphate binding first and NADPH released last. The Ki values for 6-phosphogluconate and NADPH were 61 mM and 0.115 mM, respectively, suggesting that 6-phosphogluconate exerts a minor effect on enzyme activity under physiological conditions. The optimal pH was around 8. Different temperature profiles for the enzymatic activity were seen when incubation occurred in the presence and absence of glucose-6-phosphate. Protection of enzyme activity against heat inactivation by the substrates was concentration dependent, but could be lost completely at high levels of substrates. The enzyme showed no absolute requirements for divalent cations.

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A special thanks is extended to my wife, Hu Xiaofei whose constant support and encouragement is one of the most valuable assets I have. I would like to dedicate this manuscript to her—my wife and best friend.
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Chapter I

INTRODUCTION

*Dictyostelium discoideum* is a soil amoeba in forest detritus that feeds on the bacteria of decaying fallen leaves and other matter in nature. It divides by binary fission much like any other soil amoebae. This organism was first discovered by Raper (1) in the woods of North Carolina and found in similar ecological environments throughout the world afterwards (2, 3, 4, 5). It is a unique organism due to its simple and typical life cycle. Most importantly, it differentiates without concomitant growth in the absence of bacteria and exogenous substrates, therefore, it can be used as a model to study the mechanisms of differentiation.

A. Life Cycle of *Dictyostelium discoideum*:

In laboratories, *Escherichia coli* B/r or *Klebsiella aerogenes* are most frequently used as food source for *D. discoideum*. The bacteria grow rapidly throughout the medium until nutrients are exhausted. The amoebae of the slime mold continue to grow with a doubling time of about 4 hours until they have ingested most of the bacteria and have cleared the plate. It has been shown that folic acid released by the bacteria is responsible for attracting the amoebae (6, 7). The bacteria are ingested by their predators via
phagocytosis. Once the environment is depleted of the food source, cells begin to collect in large streaming patterns to form groups consisting of up to $10^5$ cells. Cyclic AMP has been identified as the signalling substance excreted by cells (8) under the starving conditions. Cells respond to cAMP and migrate toward the region of high concentration of cAMP (9). Increase in excretion of cAMP is due to inhibition or inactivation of activity of cAMP phosphodiesterase (10) rather than increase in adenyl cyclase activity (11, 12). In addition, Ca$^{2+}$ may also be involved in aggregation process (13, 14). Aggregating cells synthesize a specific protein which is inserted in the plasma membrane and facilitates cellular cohesion. Meanwhile, a surface sheath is formed shortly after initiation of differentiation to cover the aggregates, which soon transform into fingerlike pseudoplasmodia. Throughout this stage Dictyostelium continuously produces sheath components during migration. The sheath components secreted by cells are mainly composed of polysaccharides containing glucose, mannose, N-acetylglucosamine, and galactose and a few other sugars. Cellulose may account for about 85% of the polysaccharide. Some proteins with small molecular weights and rich in asparagine are also found in the sheath. D. discoideum in pseudomodial stage could remain migratory for several days under favorable conditions. After migration stops, the anterior end of the pseudoplasmodia moves up vertically and
culmination starts. During this stage, the peripheral cells at the neck of the anterior tip synthesize and deposit a ring of cellulose at local areas forming a tubular sheath. Such tubular structure is called stalk. Cells within the sheath vacuolize and increase in volume 3-4 fold. When the elongating stalk comes in contact with the solid support, further expansion is restricted to the apical direction. As the stalk continues to extend, cells at the anterior end move into the funnel of the stalk sheath where they undergo vacuolization and secrete cellulose to form angular walls. While some of the cells at the base of the stalk vacuolize, and are self-degraded, and differentiate into hard walled support cells, others in the same region travel vertically up to the newly formed stalk. The peripheral spore cells begin to encapsulate. As culmination proceeds further, more and more cells initially at the posterior end undergo encapsulation forming heavy walled spores with noticeable changes in cytological structures. The culmination stage ends with the formation of a fruiting body, when all cells have either entered the stalk or become encapsulated. Stalk cells are no longer viable after vacuolization. The spores, however, are capable of tolerating extreme environmental conditions, such as dehydration and elevated temperature, for a relatively long period of time. Germination of the spore is prevented under such conditions by the inhibitor called N,N-dimethylguanosine (15). Shortly after being
placed under favorable conditions, spores undergo germination and are split longitudinally and release amoeba, but they smaller than normal. The differentiation process may take 20-24 hours to complete.

B. RNA Metabolism in Dictyostelium discoideum:

When the slime mold depletes the nutrients in the medium, it starts to utilize endogenous macromolecules for energy and for precursors of accumulating end products. It has been shown that both soluble glycogen (16) and RNA (17, 18) are degraded through all the developmental stages. Net RNA degradation does not result in an increase in the size of ribose-5-phosphate pool. This implies that R5P must be metabolized at a high rate, comparable to that of RNA degradation. The pentose phosphate pathway in D. discoideum is mainly involved in conversion of R5P to the hexose phosphates which serve as precursors for end product polysaccharide synthesis.

C. The Pentose Phosphate Pathway in Dictyostelium discoideum:

In the 1930's, a direct oxidative pathway of G6P catabolism, distinct from the glycolytic route, was found. Since then, the complete pentose phosphate pathway has been identified in various organisms (19, 20, 21, 22, 23). The pentose phosphate pathway in the slime mold began to be
noticed when the ratio of "CO₂ from 6-¹⁴C glucose to 1-¹⁴C glucose was determined (24). This ratio was found to vary during cell development, and the value is above unity only at precultivation stage (amoeba, 0.74; pseudoplasmodium and precultivation, 1.3; young sorocarp, 0.53; old sorocarp, 0.22). "CO₂ from 1-¹⁴C glucose with which cells are incubated is considered to be mainly produced via the pentose phosphate pathway, since negative results were consistently obtained in assaying for the presence of the splitting enzyme for 2-keto-3-deoxy-6-phosphogluconate, an unique member in the Entner-Doudoroff pathway. The ratio, therefore, indicates a relative activity of the pentose phosphate pathway when compared with the glycolytic pathway during differentiation.

D. Computer Modelling of Dicytostelium discoideum metabolism:

Computer modelling has been used to simulate in vivo metabolism. Such computer models basically consist of information determined experimentally, for instance, change of concentrations of metabolites, enzyme mechanisms, etc. The advantage of using a mathematical model is to organize the data, clarify compatible factors, find inconsistent data and provide conceptual insights. For example, enzyme activities determined in vitro are rarely found to reflect activities in the intact cell or organism (16). The enzymes
that are masked, partially inhibited, or bound to their substrates in vivo may show higher activities when measured in vitro. On the other hand, enzymes which are depleted of their activators in experimental preparations may have lower activities in vitro than in vivo. A computer model, however, is able to predict a relatively valid activity of an enzyme in vivo, based upon the concentrations of substrates and products, flux measured by means of tracer experiments, kinetic constants and enzyme mechanism determined in vitro. The model may also aid in the analysis of phenomena that are not easily explained through experiments (16). Additionally, some useful, even critical predictions with regard to the mechanism underlying differentiation may be made with these models. Such predictions include, 1.) changes in an enzyme activation function \([Vv(t)]\) 2.) enzyme and metabolite compartmentalizations (25), 3.) the effects of substrates, and/or products, and 4.) the effect of other unknown factors on enzyme activation. These predictions may be of help in designing future experiments to substantiate them.

Two kinds of mathematical models are currently being used in our laboratory. The first, TFLUX, is specifically used to simulate steady state metabolism at various differentiation stages in *D. discoideum*. The organism, at each stage over brief periods of time, presumably maintains unchanged levels of all metabolites, i.e., the pools remain constant in size over the course of an experiment, or change
insignificantly as a result of the addition of tracer levels of exogenous substrates. The purpose of this type of model is to determine all fluxes between the metabolite pools at each differentiation stage in vivo. This is done by empirically setting metabolite concentrations, unknown reaction rates, and predicting metabolite compartmentation, so that the computer output matches the specific radioactivities of metabolites that have been assessed experimentally as a function of time. The second type of model, METASIM, is a transition model that was developed to simulate in vivo multi-enzyme systems undergoing long-term changes in metabolite concentrations and enzyme activities, i.e., an organism that is under nonsteady state conditions. Therefore, METASIM can be used not only in differentiation studies, but also in the study of other long-term metabolic transformation such as those that occur in aging and cancer, or in studying perturbations of metabolic systems, eg. by drugs (26). This model consists of a metabolic pathway map, initial metabolite concentrations, enzyme kinetic expressions and constants, enzyme activation functions, and time functions giving independent metabolite concentrations. Output of this model must also be consistent with metabolite accumulation patterns observed experimentally during differentiation and with flux values determined in vivo, or through analysis of the TFLUX model for the same pathway.
E. Glucose-6-Phosphate Dehydrogenase in Dictyostelium discoideum:

G6P dehydrogenase (E.C. 1.1.1.49) catalyzes the oxidation of G6P with NADP⁺ as the electron acceptor in the oxidative portion of the pentose phosphate pathway. This

\[
\text{G6P + NADP}^+ \rightarrow \text{6PG + NADPH}
\]

enzyme has attracted a great deal of attention and has been purified and studied in many organisms (27, 28, 29, 30). Thomas (31) has shown that G6P dehydrogenase activity remains relatively constant over the course of differentiation in crude extracts of this cellular slime mold. The enzyme was shown to possess a mass-action ratio far displaced from its equilibrium, suggesting that it may be acting at a rate-limiting step in the overall pathway.

The present work was designed to further purify this enzyme from D. discoideum, to characterize and elucidate its kinetic mechanism, and to provide input for a computer model of pentose metabolism.
Chapter II

Materials and Methods

A. Materials and standardizations:

All chemicals and chromatographic materials were obtained from Sigma Chemical Co. (St. Louis, MO). The concentrations of the substrates and products were determined by means of enzymatic reactions. G6P and NADP were assayed with commercially available G6P dehydrogenase as described by Lowry and Passonneau (32). NADPH was assayed according to Bergmeyer (33) with 50 mM Tris-HCl buffer, pH 8.1 (adjusted at 25°C), replacing the NaOH solution. The NADPH was prepared in 5 mM Tris-HCl, pH 8.1, just prior to use. Alpha ketoglutarate and NH\textsubscript{4}Cl were increased to 2.5 mM and 5 mM, respectively. NADPH was added last to start the reaction. 6PG was assayed as previously described (32). The reaction mixture contained 50 mM imidazole acetate buffer, pH 7.0, 5 mM MgCl\textsubscript{2}, 0.5 mM dithiothreitol, 30 mM ammonium acetate, 1 mM EDTA and 1 mM NADP\textsuperscript{+}. Addition of appropriate amount of 6PG was followed by 3 \textmu l of commercially obtained 6PG dehydrogenase to start the reaction.

B. Cell growth:

D. discoideum strain NC-4 (ATCC 24697) was grown as previously described (34) in co-culture with Escherichia
**coli** on nutrient agar sheets in an humidified incubator at 23°C with a relative humidity of 75%. Cells were incubated for about 48 hours, and were harvested in a solution containing 10 mM potassium phosphate, pH 7.6, 10% glycerol (v/v), 1 mM EDTA, and 1 mM β-mercaptoethanol. Bacteria were removed by centrifugation at 1,000g. Cell pellets were stored frozen at -50°C; freezing did not result in significant loss of enzymatic activity.

**C. Determination of protein:**

Protein was measured by the bicinchoninic acid (BCA) protein assay method using bovine serum albumin as the standard (35). Protein samples from each preparation step were diluted to proper concentrations in 100 µl giving O.D. readings at 562 nm within the standard range. BCA solution was mixed with 4% CuSO₄ at ratio of 50 to 1 volume. Two ml of the mixture solution was added to each sample, and incubated in a water bath at 37°C for 30 minutes.

**D. Purification of the enzyme:**

All purification steps were conducted either on ice or at 4°C. Cells were lysed using a nitrogen pressure cell (Yeda Instruments, Rehovot, Israel) pressurized to 160 psi. The cell lysate was centrifuged at 27,000g for 20 min, and the pellet was discarded. Solid ammonium sulfate was slowly added to 45% saturation, and the solution was stirred in the
cold room for 20 min. The precipitated protein was pelleted by centrifugation at 19,000g for 20 min and discarded. Solid ammonium sulfate was slowly added to 65% saturation as previously described. The resulting pellet was resuspended in the smallest possible volume of 50 mM Tris-HCl buffer, pH 7.5 at 25°C, containing 1 mM DTT and 0.4 mM EDTA (buffer A). The suspended sample was dialyzed against 1 liter of buffer A for 2 hours with three changes of buffer. The dialyzed sample was applied to a reactive red 120-agarose column previously equilibrated with buffer A. The column was washed with buffer A until absorbance at 280 nm was zero. G6P dehydrogenase was eluted from the column using a linear gradient of 0-2 M NaCl in buffer A at a flow rate of 20 ml/hr. Fractions of 2 ml were collected. The total gradient size was 200 ml. The fractions with G6P dehydrogenase activity giving changes of O.D. greater than 0.04 per minute were pooled and stored at -20°C until used. G6P dehydrogenase was fairly stable frozen; however, repeated freezing and thawing caused significant loss of activity.

E. Enzyme assay:

The standard assay mixture contained 50 mM Tris-HCl buffer, pH 8.0, and 1 mM NADP⁺ (32). The enzyme fraction was added and the reaction was started by the addition of 10 μl of 100 mM G6P. The final assay volume was 0.6 ml. The increase in absorbance was followed at 340 nm using a
continuous recording spectrophotometer. One unit was defined as the amount of enzyme catalyzing the reduction of 1 μmol NADP⁺ per minute at room temperature.

F. Kinetic Analysis:

Kinetic studies were carried out by varying substrate and product concentrations, and measuring initial velocity as described in the previous part of this section. Michaelis constants (Km) for substrates and inhibition constants (Ki) for products were determined by the equation as:

\[
v = \frac{V'_{ab} - V'_{pq}}{\frac{K_mb}{K_i'K_m^a} + \frac{K_mp}{K_m^bK_i^q} + \frac{q}{K_i^q}}
\]

\[
\text{DENOMINATOR} = 1 + \frac{a}{K_i^a} + \frac{K_m^b}{K_i'K_m^a} + \frac{K_m^q}{K_iK_m^b} + \frac{q}{K_i^q}
\]

\[
+ \frac{ab}{K_i'K_m^a} + \frac{K_m^ap}{K_i'K_m^aK_i^q} + \frac{K_m^bpq}{K_i'K_m^aK_i^q}
\]

\[
+ \frac{pq}{K_m^bK_i^q} + \frac{abp}{K_i'K_m^aK_i^q} + \frac{bpq}{K_i^qK_m^bK_i^q}
\]

where \( v \) = net initial forward velocity of the reaction

\( V' \) = forward Vmax

\( V' \) = backward Vmax

\( a, b \) = concentrations of substrates

\( p, q \) = concentrations of products

\( K_m \) = Michaelis constant for substrate and product

\( K_i \) = inhibition constant for substrate and product
The values of $K_m$ and $K_i$ are calculated by the Lineweaver-Burk double reciprocal plot and replot of slopes and intercepts versus concentration. The method of calculation of kinetic constants can be elucidated clearly by simplifying and rearranging equation 1. For example, when the initial velocity is measured in the absence of products, equation 1 can be simplified by eliminating those components that contain $p$ and $q$. When $[A]$ and $[B]$ are varied, the equation becomes:

$$\frac{1}{V} = \frac{1}{V'} \left( \frac{K_i'K_m^*}{b} + K_m^* \right) \frac{1}{a} + \frac{1}{V'} \left( \frac{K_m^*}{b} + 1 \right)$$

(2)

Then the slopes and intercepts can be expressed as:

$$\text{Slope} = \frac{K_i'K_m^*}{V'} \frac{1}{b} + \frac{K_m^*}{V'}$$

(3)

$$\text{Intercept} = \frac{K_m^*}{V'} \frac{1}{b} + \frac{1}{V'}$$

(4)

Replots of slopes or intercepts versus $[B]$ give the values of the $K_m$s for $A$ and $B$.

The enzyme mechanism is deduced based on product inhibition patterns. When no product is present, a family of intersecting lines of Lineweaver-Burk plots indicates a sequential mechanism. The inhibition patterns of products with respect to the substrates give the order of the binding of substrates and the release of products.
Chapter III

RESULTS

A. Purification of Glucose-6-phosphate dehydrogenase:

G6P dehydrogenase has been purified from other sources using different immobilized triazine dyes (36). Reactive red 120-agarose affinity chromatography has proven useful in the purification of NADP⁺-dependent dehydrogenases (37, 38). The triazine dye is coupled to the agarose, and form stable conjugates. The high capacity of this dye column makes it very useful in large-scale purification of enzymes. G6P dehydrogenase from D. discoideum was successfully purified using this dye-affinity chromatography and eluting activity with a NaCl gradient (Fig. 1). The purification steps and enzymatic activity assays are summarized in Table 1. The enzyme collected from the affinity chromatograph column exhibited stoichiometry in catalyzing the conversion of G6P to 6PG with NADP⁺ as the coenzyme (Table 2). Purified G6P dehydrogenase did not exhibit any activity with NAD⁺ as the coenzyme, in contrast to other studies (30, 39, 40, 41). Neither 6PG dehydrogenase nor phosphoglucose isomerase activity was found. The reaction was apparently irreversible in vitro with either low or high concentrations of 6PG and NADPH as substrates.
Figure 1. Purification of Glucose-6-Phosphate Dehydrogenase.

Glucose-6-phosphate dehydrogenase was eluted with a 200 ml linear gradient of 0 - 2M NaCl in 20mM Tris-HCl buffer pH 7.6 from a reactive red 120-agarose column (1.5 cm x 9.5 cm) that was previously equilibrated in the same buffer. ●-●, Glucose-6-phosphate dehydrogenase activity; o-o, Absorbance at 280nm.
# Table 1.

## Purification of Glucose-6-Phosphate Dehydrogenase

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (units/mg of protein)</th>
<th>Yield (%)</th>
<th>Purification factor (n)</th>
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<tr>
<td>Crude Extract</td>
<td>19</td>
<td>1552</td>
<td>90.0</td>
<td>0.058</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>((NH_4)_2SO_4) 0-45% Saturation Supernatant</td>
<td>10.5</td>
<td>606</td>
<td>34.0</td>
<td>0.056</td>
<td>38</td>
<td>0.97</td>
</tr>
<tr>
<td>((NH_4)_2SO_4) 45-65% Saturation Pellet</td>
<td>3</td>
<td>257</td>
<td>22.8</td>
<td>0.089</td>
<td>25</td>
<td>1.5</td>
</tr>
<tr>
<td>Reactive Red 120-Agarose</td>
<td>45</td>
<td>2.16</td>
<td>2.43</td>
<td>1.125</td>
<td>2.7</td>
<td>19.4</td>
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Table 2.
Stoichiometry

<table>
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<th>nmol/μl</th>
<th>Calculated*</th>
<th>Enzymatically assayed* (%)</th>
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<tr>
<td>G6P left</td>
<td>3.09</td>
<td>3.00</td>
</tr>
<tr>
<td>NADPH produced</td>
<td>0.076</td>
<td></td>
</tr>
<tr>
<td>6PG produced</td>
<td></td>
<td>0.072</td>
</tr>
</tbody>
</table>

* Known amount of G6P was added to an assay mixture containing sufficient amount of NADP\(^+\) and purified enzyme to start the reaction. The reaction was monitored by reading O.D. at 340 nm, and stopped by heating at boiling temperature for 2 min.

* The heated mixture was allowed to cool on ice and an appropriate amount was transferred for measuring concentrations of G6P and 6PG with commercially obtained G6P dehydrogenase and 6PG dehydrogenase.
B. Effects of pH and temperature on Glucose-6-phosphate dehydrogenase activity: Enzymatic activity was assayed from pH 5.7 to 9.9 using different kinds of buffers (Fig. 2). Maximal activity was observed around pH 8. The optimal temperature was found to be 37°C under the assay conditions in the presence of G6P. When the enzyme was incubated alone, however, the activity remained constant at a level slightly lower than the maximal activities measured under the above condition (Fig. 3), and no activity was seen at 45°C. In contrast, the enzyme was still partially active at this temperature in the presence of G6P. Subsequently, maximal protection of the enzyme from heat inactivation at 45°C (Fig. 4A and B) was found either at 0.1 mM NADP' or 0.5 mM G6P while the other substrate was fixed at 0.2 mM. However, at 45°C the enzyme lost activity completely in the presence of higher concentrations of the substrates (G6P at 10 mM and NADP' at 0.35 mM). The assay for enzymatic activity was carried out at room temperature, because D. discoideum dies at 30°C and only grows well at 23-25°C.

C. Effects of calcium, magnesium, and EDTA:

Ca" and Mg" had little effect on the activity of G6P dehydrogenase, but both caused slight activation at concentrations of up to 10 mM CaCl₂ and 5 mM MgCl₂. Inhibition of the enzymatic activity was seen when Ca" and Mg" were present at 50 mM and 10 mM, respectively. The
Figure 2. Effect of pH on Purified Glucose-6-phosphate Dehydrogenase.

The assay mixture contained 0.1M of each buffer as follows: (●), 2[N-Morpholino]ethane Sulfonic acid (MES); (♦), PIPES; (♦), Tris-HCl; (♦), Bis Tris Propane; (○), Carbonate-Bicarbonate buffer. Measurement of enzymatic activity was in duplicate. The pH of assay mixture was checked before and after each measurement with a pH meter.
Figure 3. Effect of Temperature on Glucose-6-Phosphate Dehydrogenase.

The enzyme was assayed in a mixture containing 0.21 mM of NADP' and 0.22 mM of G6P. (●), Enzyme preparation was incubated at various temperatures for 15 minutes in the presence of 0.22 mM G6P, and was assayed for activity by adding NADP'. (○), Incubation of the enzyme was carried out in the absence of G6P, and the activity was assayed by adding both substrates.
Figure 4. Protection of Enzymatic Activity Against Heat Inactivation by Substrates.

(A) Protection by NADP⁺; (B) Protection by Glucose-6-Phosphate. G6P dehydrogenase was incubated at 45°C for 15 min in the presence (Ap) and absence (Aa) of each substrate at various concentrations, and was assayed for activity immediately by adding the other substrate. As control (Ac), the activity of G6P dehydrogenase was also assayed with corresponding concentrations of substrates without incubation. The percentage of protection was then calculated as in the equation of (Ap - Aa) x 100/Ac. One of the substrates was present at a fixed level of 0.2mM while the other which was studied for protection ability was varied at different levels.
presence of EDTA at concentrations up to 10 mM had no effect on the enzyme, and the concentration of 20 mM or higher was inhibitory (Table 3). These results suggest that the enzyme has no absolute requirement for these divalent ions, similar to the enzyme isolated from yeast (42).

D. Effects of NAD\(^+\) and NADH:

Approximately double activity was seen when the assay was carried out in the presence of both NAD\(^+\) and NADH up to 0.2 mM and 5 mM (Table 4).

E. Fructose-6-Phosphate inhibition:

G6P dehydrogenase from D. discoideum is neither activated nor inhibited by F6P, in contrast to the observation of Benziman and Mazover (43).

F. Kinetic analysis:

Enzyme was added to a final concentration of approximately 0.008 U/ml for kinetic characterization experiments. The activity was measured with each substrate concentration varied as shown (Fig. 5). The double reciprocal plot of initial velocities versus concentrations yielded a family of intersecting lines, indicating that this enzyme follows a sequential mechanism. Appropriate replots of slopes and intercepts were used to calculate the Kms. Product inhibition studies were done to further
### Table 3.

Effects of Ca\(^{2+}\), Mg\(^{2+}\), and EDTA

<table>
<thead>
<tr>
<th>Ca(^{2+}) (mM)</th>
<th>Activity(^{a})</th>
<th>Mg(^{2+}) (mM)</th>
<th>Activity</th>
<th>EDTA (mM)</th>
<th>Activity</th>
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<tbody>
<tr>
<td>0.001</td>
<td>0.060</td>
<td>0.001</td>
<td>0.066</td>
<td>0.0001</td>
<td>0.077</td>
</tr>
<tr>
<td>0.01</td>
<td>0.072</td>
<td>0.005</td>
<td>0.071</td>
<td>0.001</td>
<td>0.075</td>
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<tr>
<td>0.05</td>
<td>0.070</td>
<td>0.01</td>
<td>0.068</td>
<td>0.01</td>
<td>0.070</td>
</tr>
<tr>
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<td>0.05</td>
<td>0.069</td>
<td>0.05</td>
<td>0.085</td>
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<tr>
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<td>0.069</td>
<td>0.1</td>
<td>0.077</td>
</tr>
<tr>
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<tr>
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<td>0.023</td>
<td>50</td>
<td>0.021</td>
<td>20</td>
<td>0.073</td>
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<tr>
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<td>75</td>
<td>0.008</td>
<td>40</td>
<td>0.045</td>
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</tbody>
</table>

\(^{a}\) Enzymatic activity is represented by initial velocity expressed as \(\mu\)mol/ml/min. Assays were conducted in the presence of 0.2 mM G6P and 0.3 mM NADP\(^{+}\).
Table 4.

Effects of NAD' and NADH

<table>
<thead>
<tr>
<th>NAD' (mM)</th>
<th>Activity'</th>
<th>NADH (mM)</th>
<th>Activity</th>
</tr>
</thead>
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<td>0</td>
<td>0.058</td>
</tr>
<tr>
<td>0.01</td>
<td>0.119</td>
<td>0.01</td>
<td>0.122</td>
</tr>
<tr>
<td>0.05</td>
<td>0.117</td>
<td>0.05</td>
<td>0.124</td>
</tr>
<tr>
<td>0.1</td>
<td>0.119</td>
<td>0.1</td>
<td>0.128</td>
</tr>
<tr>
<td>0.5</td>
<td>0.119</td>
<td>0.2</td>
<td>0.120</td>
</tr>
<tr>
<td>1</td>
<td>0.115</td>
<td>0.4</td>
<td>0.097</td>
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<tr>
<td>5</td>
<td>0.110</td>
<td>0.5</td>
<td>0.066</td>
</tr>
<tr>
<td>10</td>
<td>0.090</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

* The activity is represented by initial velocity expressed as μmol/ml/min. The assay mixture contained 0.2 mM G6P and 0.31 mM NADP'.

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Figure 5. Double Reciprocal Plot of Initial Velocity of Glucose-6-Phosphate Dehydrogenase vs Substrate Concentrations.

Double reciprocal plots and secondary replots for G6P dehydrogenase were constructed. The G6P concentrations used were 0.016 mM, 0.032 mM, 0.064 mM, 0.129 mM, and 0.193 mM, while NADP' was at 0.012 mM (•), 0.024 mM (○), 0.048 mM (▲), 0.096 mM (+), and 0.145 mM (□). Inset figure: Replot of slope and intercepts versus NADP' concentrations.
characterize the kinetic mechanism. 6PG was shown to be a mixed-type inhibitor against both substrates when either of the substrates were present at unsaturated concentrations (Fig. 6A and B). To obtain the Ki for 6PG, 6PG concentrations were 0-80 mM and NADP' concentrations were varied as shown. Three separate experiments were conducted with G6P held constant at 0.1 mM, 0.15 mM or 0.244 mM. Each set of conditions generated a series of curves as shown in figure 6A for 0.15 mM G6P. The first replot plotted the y-axis intercepts versus the concentrations of 6PG. Each set of curves generates a single line on this replot. Thus three parallel lines result (Fig. 7). A secondary replot is made by plotting each of the x-intercepts versus the reciprocal of G6P concentrations (inset Fig. 7). The resulting y-intercept is the Ki for 6PG (44). NADPH was also shown to be a mixed-type inhibitor with respect to NADP' when G6P was unsaturated (Fig. 8A). G6P is competitively inhibited by NADPH at all NADP' concentrations (only 1 mM NADP' is shown in Fig. 8B). The inhibition constant of NADPH was calculated by replotting slopes versus concentrations of NADPH. The product inhibition patterns, Kms and Kis are summarized in tables 5 and 6, respectively.

When NADP' was present at concentrations higher than 0.5 mM, the activity of G6P dehydrogenase was inhibited by its substrate.
Figure 6. Product Inhibition by 6-Phosphogluconate.

(A) NADP' concentrations were varied and unsaturated G6P was constant (0.15 mM). 6PG was present at 0 mM (●), 30 mM (●), 50 mM (▲), and 60 mM (▲); (B) G6P was varied and unsaturated NADP' was constant (0.3 mM). 6PG was present at 0 mM (●), 35 mM (●), and 65 mM (▲).
Figure 7. Determination of the Ki for 6-Phosphogluconate.

A replot of intercepts versus the concentrations of 6PG from Figure 6A was constructed. A secondary replot (inset) of X-intercepts versus the reciprocal of the concentration of G6P, 0.244 mM (•), 0.150 mM (○), and 0.100 mM (▲), was constructed to determine the Ki.
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Figure 8. Product Inhibition by NADPH

(A) NADP$^+$ was varied and G6P was constant at 1 mM. NADPH was present at 0.2 mM (●), 0.3 mM (○), 0.4 mM (△), and 0.5 mM (□); (B) G6P was varied and NADP$^+$ was constant at 1 mM. NADPH was 0.05 mM (●), 0.1 mM (○), 0.2 mM (△) and 0.3 mM (□). The Ki for NADPH was determined from a replot of slope versus NADPH concentration (inset).
Table 5.

Product Inhibition Pattern

<table>
<thead>
<tr>
<th>Varied</th>
<th>G6P</th>
<th>NADP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Const.</td>
<td>unsat.</td>
<td>'NADP*</td>
</tr>
<tr>
<td>6PG</td>
<td>MT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>--&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>NADPH</td>
<td>C&lt;sup&gt;f&lt;/sup&gt;</td>
<td>C</td>
</tr>
</tbody>
</table>

<sup>a</sup> The substrate was present at unsaturated concentration.

<sup>b</sup> The substrate was present at saturated concentration.

<sup>c</sup> MT = mixed-type inhibition.

<sup>d</sup> The product inhibition experiment could not be conducted due to substrate inhibition.

<sup>e</sup> C = competitive inhibition.
<table>
<thead>
<tr>
<th></th>
<th>Km (mM)</th>
<th>Ki (mM)</th>
</tr>
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<tbody>
<tr>
<td>G6P</td>
<td>0.024</td>
<td>--</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>0.043</td>
<td>--</td>
</tr>
<tr>
<td>6PG</td>
<td>--</td>
<td>61</td>
</tr>
<tr>
<td>NADPH</td>
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<td>0.115</td>
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</tbody>
</table>
Chapter IV

DISCUSSION

The simple differentiation process renders *D. discoideum* an excellent material for study of metabolic models. During differentiation, metabolite precursors (soluble glycogen and RNA) are converted to the end products by a series of changes in enzyme activities and metabolite fluxes. Differentiation of *D. discoideum* is characterized by accumulation of some macromolecules such as trehalose, cell wall glycogen, and mucopolysaccharides starting around the culmination stage of development (45). Soluble glycogen, which accumulates during the growth phase of the life cycle, is used in the differentiation phase as a carbon source for the synthesis of the end products (16). Total carbohydrate level per cell aliquot, however, remains constant for lack of gluconeogenesis (46). Further examination of the quantitative changes in these macromolecules (47, 48, 49) revealed that the increase in the amount of end products can roughly be accounted for by the loss in the contents of soluble glycogen (50) and RNA. As soon as differentiation is initiated, *D. discoideum* begins to degrade RNA at a rate higher than RNA synthesis (17, 18). RNA degradation is carried out at a fairly constant rate following first-order kinetics (51) for some reason(s) other than for supplying
oxidizable substrate (52). Thus, it was shown that when a large amount of cellular soluble glycogen is initially present due to external glucose provided in the medium, the RNA content doesn't change during development (51).

Quantitative studies of RNA degradation revealed that cells lose 40% of their RNA at aggregation by the time they become sorocarps (51). Net RNA degradation doesn't result in increases in the sizes of nucleoside pools, nor effect the total carbohydrate level during differentiation, because the R5P moiety serves as one of the sources for end products synthesis via the pentose phosphate pathway.

The existence of the pentose phosphate pathway in D. discoideum was postulated based on the observed ratio of $^{13}CO_2$ from 6-$^{13}$C glucose to 1-$^{13}$C glucose (24). A ratio of 1-$^{13}$CO$_2$ to 6-$^{13}$CO$_2$ equal to one can be expected as a result of glycolysis alone. However, a ratio greater than one was observed and was considered to be due to activity of the pentose phosphate pathway, since the attempt to detect the presence of the splitting enzyme for 2-keto-3-deoxy-6-phosphogluconate was not successful. Therefore it was assumed that the relative activities of the pentose phosphate pathway and the glycolytic pathway during cell differentiation was indicated by the ratio of radioactive 1-$^{13}$CO$_2$ to 6-$^{13}$CO$_2$. As differentiation proceeds to the end, the change of the ratio reveals elevation in the activity of the pentose phosphate pathway (24). Like most other organisms,
this pathway in *D. discoideum* participates in anabolism to generate metabolites and reducing equivalents (NADPH) for biosynthesis. This is supported by a 140% increase in NADPH concentration between early and late stages of differentiation (53). In *D. discoideum*, however, this cycle also appears to play an important role in processing the products of RNA degradation. The R5P from RNA must be efficiently metabolized by the pentose phosphate pathway, because there is no significant increase in its cellular pool, even though there is a net degradation of about 40% of the RNA. Thus, the study of this pathway becomes extremely important in understanding biochemical differentiation of the cellular slime mold.

G6P dehydrogenase has been studied in crude extracts of *D. discoideum* (31). It is one of the key enzymes in differentiation, for it catalyzes the production of 6PG from G6P, and G6P is a branch point metabolite which also serves as a precursor for glucose-1-phosphate. The calculated mass action ratio of the G6P dehydrogenase-catalyzed reaction was found to be far displaced from its equilibrium constant \((10^4)\) towards 6PG synthesis (31). This is interesting in view of the necessity of sparing G6P for G1P and end product synthesis during differentiation. The extremely high value of the Ki, 61 mM, for 6PG implies that this metabolite has little effect on the activity of the enzyme under physiological conditions, as 6PG is present at levels
between 6 μM at aggregation and 18 μM at culmination (31). This suggests, if inhibition of this enzyme occurs, it would be due to the presence of metabolites other than 6PG. It has been shown that G1P is a competitive inhibitor of the enzyme with respect to G6P in *Leuconostoc mesenteroides* (54).

The optimal pH for the activity of G6P dehydrogenase from *D. discoideum* was determined to be around 8. Most bacterial enzymes show optimal activity around pH 9 (27, 28, 30,). G6P dehydrogenase from bass liver has maximal activity at pH 7.2 with form B, and pH 6.5 to pH 8.5 with form A (55). The enzyme showed different temperature profiles when it was incubated at various temperatures under different conditions. In the absence of G6P, the temperature curve was relative flat over a temperature range of 8°C through 40°C; however, the enzyme was denatured at 45°C. When the incubations occurred in the presence of G6P, the enzyme exhibited a peak activity at 37°C. Moreover, relatively high activity was still found at 45°C. The divalent cations, Ca²⁺ and Mg²⁺, did not have much effect on the enzymatic activity, nor did EDTA. This implies that the enzyme has no requirement for cations.

Inhibition and loss of protection of the enzymatic activity against heat inactivation by higher levels of substrates revealed by the experimental data is in agreement with the observations of Kurlandsky *et al.* (41). The secondary binding site theory proposed by Markus *et al.* (56)
could account for the experimental results. Activation of the activity by NAD’ and NADH is not clearly understood at present. NAD’ might be serving as an indirect electron acceptor, or possibly replace the position of NADP’ on the protein molecule as a reaction proceeds. Such binding is impossible, however, when no NADP’ is present.

The enzyme shows kinetics consistent with an ordered Bi:Bi mechanism as revealed by the product inhibition patterns. Unlike the enzymes from Leuconostoc mesenteroides (57) and Acetobacter xylinum (43), our data show that NADPH exhibits competitive inhibition with respect to G6P and mixed-type with respect to NADP’, suggesting that G6P is the first substrate that binds to the enzyme and NADPH is the last product released. Thus, these enzymes carry out the catalysis of the same reaction in distinctive orders of substrate binding and product release, although all of the enzymes follow the same sequential mechanism.
Chapter V

SUMMARY

A. Conclusions:

1. The purified G6P dehydrogenase from reactive red 120-agarose chromatography exhibited stoichiometry in catalyzing conversion of G6P to 6PG with NADP⁺ as a coenzyme.

2. The enzyme of the slime mold had an optimal pH around 8, different from many other bacterial sources, which have optimal pH around 9.

3. The results of heat inactivation experiments indicated that protection by the substrates are concentration-dependent. Higher levels of substrates does not necessarily provide better protection against heat inactivation. In fact, the data showed that the substrates lost such abilities when present in higher concentrations.

4. The effects of Ca²⁺, Mg²⁺, and EDTA on the activity of the enzyme were not significant under physiological conditions, indicating no requirement of this dehydrogenase for divalent cations.

5. The values of Km of the enzyme for G6P and NADP⁺ were 0.024 mM and 0.043 mM, respectively. The initial velocity experiment showed evidence that the enzyme follows a sequential ordered Bi:Bi mechanism.

6. The Ki value for 6PG, one of the products in the
reaction, is extremely high indicating that this metabolite has little effect on regulation of G6P dehydrogenase activity. The Ki value for NADPH is 0.115 mM.

7. Product inhibition studies further revealed the order of binding of the substrates and release of the products. Since competitive inhibition was only seen between NADPH and G6P with NADP* at all levels, it could be concluded without a doubt that G6P is the first substrate binding to the active site of the enzyme and NADPH is the last product liberated from the complex.

8. Although no NAD*-linked activity was found for G6P dehydrogenase from Dictyostelium discoideum, activation effects on the enzyme activity were seen when both NAD* and NADH were present at levels up to 5 mM and 0.2 mM, respectively, in the assay mixture containing 0.2 mM G6P and 0.31 mM NADP*.

B. Suggestions:

The regulation of the activity of G6P dehydrogenase could be very important in carbohydrate metabolism in Dictyostelium discoideum during differentiation, since the large amount of R5P produced from RNA degradation must be efficiently metabolized. The pentose phosphate pathway may play equally important roles in generating six carbon units for end products synthesis as well as reducing power, NADPH, throughout development. Flux from the G6P pool to ribulose-
5-phosphate pool may be restricted by G6P dehydrogenase to the degree that only ensures maintenance of the pathway.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>NADP'</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>6PG</td>
<td>6-Phosphogluconic acid</td>
</tr>
<tr>
<td>R5P</td>
<td>Ribose-5-phosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
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<td>Ethylenediamine tetraacetic acid</td>
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<td>Maximal velocity</td>
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<tr>
<td>NAD'</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
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<td>F6P</td>
<td>Fructose-6-phosphate</td>
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<td>G1P</td>
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REFERENCE


