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Glucose-6-Phosphate Dehydrogenase from Rat Liver (II)

The Evidence for a Specific Function of Lysine Residue. Substrate Specificity and Isoelectric Point of the Enzyme.

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Introduction

Glucose-6-phosphate dehydrogenase is widely distributed in nature throughout both the animal and plant kingdoms. The enzyme first discovered in 1931 by Warburg and Chrisitian, and named as the "Zwischenferment". The discovery of this enzyme caused the coenzyme, nicotinamide adenin dinucleotide phosphate to be known.[10]

This enzyme is the first regulatory enzyme in pentose phosphate pathway, which is known as a second way in glucose utilization. The fatty acids, cholesterol, reduced glutation and steroid hormones are required $NADPH+H^+$, for thier synthesis, which is generated by the activity of this enzyme.[10, 13] If the enzyme deficient, these four biosynthesis can not take place in the cells. In this case erythrocytes are not able to survive and may be hemolysed.

It is rather important to know the mechanism of the catalytic activity of this enzyme, and which amino acids are involved in the active center of the enzyme. Probable amino acids which link with coenzyme $NADP^+$ were discussed in the previous paper.[6]

The aim of the presenet paper, is to discuss the amino acid to which substrate glucose-6-phosphate binds in the active center and also to discuss some physical features of the enzyme.

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Pyridoxal 5'-phosphate has been utilized to detecet the ε-amino group of lysine at the active site of various enzymes, including glucose-6-P dehydrogenase from *Leuconostoc mesenteroides*,[12] and *Candida utilis*.[5] Glutamate dehydrogenase,[17] and tryptophanase[9] from *Escherichia coli* and ribonuclease from bovine panceras[11] have been used for further studies of pyridoxal 5'-phosphate (PL 5'-P) binding to the ε-amino group of the lysine residue. These enzymes were first treated with PL 5'-P, then reduced with sodium borohydrid ($NaBH_4$) and these derivatives were digested with trypsin and chymotrypsin. From these mixtures small peptides, containing 5'-phosphopyridoxyl residue were isolated and the amino acid sequences were determined. PL 5'-P were found to be bounded to the ε-amino group of the lysine residue in all theree experiments.[9, 11, 17]

The isoelectric point and substrate specificity of glucose-6-P dehydrogenase are also discussed in this paper.

Materials and Methods

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC. 1.1.1.49) from rat liver was used in all experiments in purified form. Fairly pure enzyme perparation was a gift by Dr Holten.

One unit of glucose-6-phosphate dehydrogenase activity is the amount required to form 1 micromole of $NADPH + H^+$ in one minute in the assay employed.[2] The initial reaction velocity was expresssed as micromoles of $NADPH + H^+$ formed per minute. Glucose-6-phosphate dehydrogenase activity was measured at 340 mμ wavelength by the initial rate of reduction of $NADP^+$ at 30° C.

All solutions were prepared and used frehly and all assays were done in duplictate. In kinetic studies a Gilford model 240 spectrophometer was used and for routine assays, a Beckman model DB-G. The other details and experimental conditions were described in the previous paper.[6]

Electrofocussing Experiment

The experiment was designed according to the manual book of LKB-Laboratories. A 110 ml capacity electrofocussing column was used and glycerol was applied as a fillling material instead of sucrose. Glycerol was prepared in twenty four different concentrations, between 2.5 to 60 % and the amount was 4.6 ml of each. Ampholyne was mixed with glycerol as a carrier substance (Ampholyne, LKB-Producter AB, Stockholm) The best working pH range, was 4-6. Sulfuric acid was first added as a

anodal buffer and then glycerol-ampholyne mixture was applied to the column very carefully in decreasing amounts starting by 60 %. 2.5 % mixture was added finally and the triethanolamine was applied as a cathodal buffer top of the column. 74 units of dialysed pur enzyme were applied to the middle of the column. Two electrodes were connected with a power supply and the experiment was continued for fifty hours with a 900 volts electric current. The column content was removed in 1 ml quantities from the column by a fraction collector. Each sample was tested for pH and enzyme activity. The column tube was enclosed in a glass water jacket through which water at 4° C. was circulated. This was necessary for preventing overheating by high voltage.

Chemicals

Glucose-6-phosphate, $NADP^+$, and 2-deoxy-D-glucose-6-phospate were obtained from Boehringer Mannheim Corporation New York. Galactose-6-phosphate and pyridoxal 5'-phosphate from Sigma Chemical Company St. Louis, Missoury, ampholyne LKB-Laboratory Stockholm and the other chemicals used were analytical reagent grade from Mallincrodt Chemical Company St. Louis Missouri, U.S.A.

Results

Inhibition of Glucose-6-Phosphate Dehydrogenase by Pyridoxal 5'-Phosphate

Pyridoxal 5'-phosphate was used by several investigators to determine the ε -amino froup of the lysine residue in the active center of different enzymes. Pyridoxal 5'-phosphate (PL 5'-P) was found to inhibit glucose-6-phosphate dehydrogenase (G-6-PDH) from rat liver. 2.7×10^{-2} units/ml of enzyme were incubated for 15 minutes at 0° C in the dark with different concentrations of PL 5'-P, and the enzyme activity was measure at 30° C by the addition of glucose-6-phosphate and $NADP^+$ at the same time. The enzyme was incubated with PL 5'-P in dark, in order to prevent the decomposition of PL 5'-P by light. The concentration dependence of pyridoxal 5'-phosphate inhibition in bicine buffer at pH 7.9 was illustrated in Figure 1. 3.5×10^{-4} M. PL 5'-P produces 50 % inhibition under the experimental conditions.

Six different concentrations of glucose-6-P, and two different concentrations of PL 5'-P were incubated for 15 minutes at 0° C in the dark and 1×10^{-3} M. $NADP^+$ was added last and enzyme activity was measured. The inhibition of PL 5'-P was found to be competitive with glucose-6-P. The result of this experiment was applied to Line-Weaver-

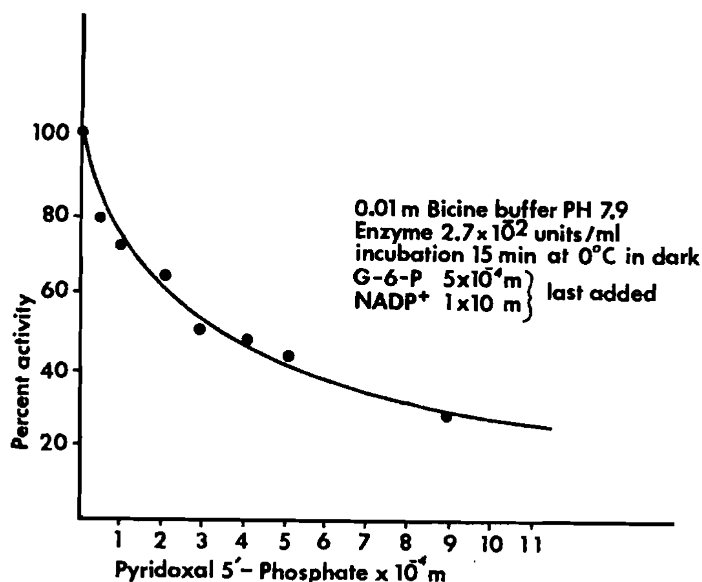

Figure 1

The concentration dependent inhibition effect of pyridoxal 5'-phosphate on glucose-6 -phosphate dehydrogenase. The enzyme was incubated for 15 min. at 0° C in the dark in 0.01 M. bicine buffer at pH 7.9 and at the indicated concentration of PL 5'-P 0.01 ml quantities were removed and the enzyme activity was measured in the routine assay conditions.

Burk plot as shown in Figure 2 The results suggest that PL 5'-P binds to the glucose-6-P binding site on the Enzyme surface. In this experiment the Km value of glucose-6-P was found as Km= 5.3 x 10^{-5} M. and the inhibiton constant for PL 5'-P was found as Ki= 5.6 x 10^{-5} M.

In a second experiment 2.7 x 10^{-2} units/ml of enzyme were incubated in five different concentrations of $NADP^+$ and two different concentrations of PL 5'-P for 15 minutes in the dark at 0° C in bicine buffer at pH 7.9. 2 x 10^{-3} M. glucose-6-P was added finally and enzyme activity was measured at 30° C. PL 5'-P showed non-competitive inhibition with respect to $NADP^+$. This result indicates that the PL 5'-P binding site is different from the $NADP^+$ binding site on enzyme surface. The Km value of $NADP^+$ was found as Km= 7.27 x 10^{-6} M. and the inhibition constant of PL 5'-P was found as Ki... 5.6 x 10^{-5} M. The result is summarized as shown in Figure 3.

The studies with PL 5'-P, strongly suggested that the ε -amino group of the lysine residue was one of the binding sites for glucose-6-P.

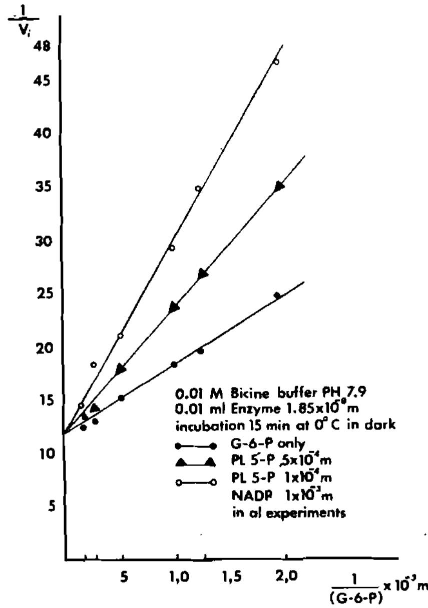

Figure 2
A double riciprocal plot showing competitive inhibition by pyridoxal 5'-phosphate with glucose-6-P in glucose-6-P dehydrogenase. The enzyme was incubated for 15 min. at 0° C. in the dark, in 0.01 M. bicine buffer at pH 7.9 Glucose-6-P and pyridoxal 5'-P concentrations were as indicated. 1 x 10^{-3} M. $NADP^+$ was added last and enzyme activity was measured.

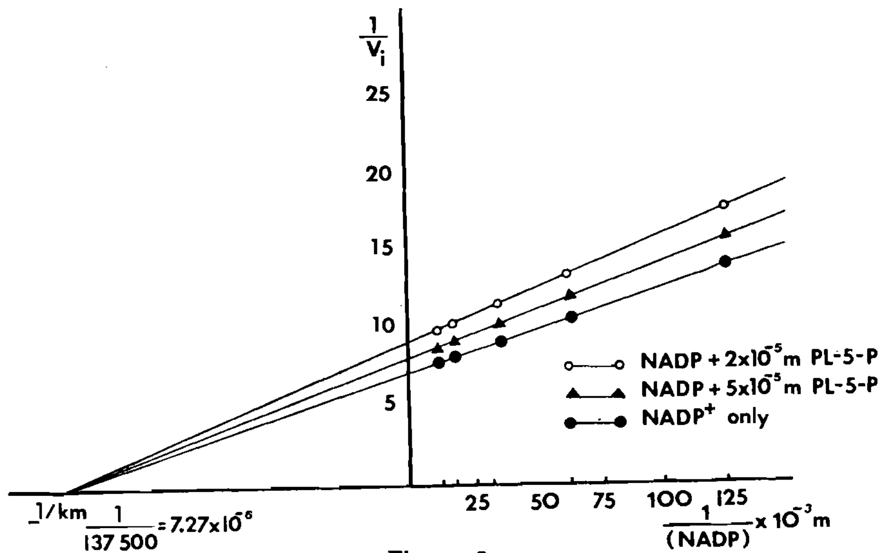

Figure 3
A double reciprocal plot showing non-compatitive inhibition by pyridoxal 5'-phosphate with to $NADP^+$. The enzyme was incubated for 15 min. at 0° C. in the dark, in 0.01 M. bicine buffer at pH 7.9. Pyridoxal 5'-P and $NADP^+$ concentrations were as indicated. 1 x 10^{-3} M. glucose-6-P was added last and enzyme activity was mesured.

Substrate Specificity of Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase from rat liver uses glucose-6-P as a natural subtrate. The enzyme was found to utilize 2-deoxy-D-glucose-6-P and galactose-6-P in addition to glucose-6-P. The relative rates of oxidation of these two compounds were rather low as compaired with that of glucose-6-P. The result is summarized in Table I.

TABLE I

The Relative Oxidation Rates by Glucose-6-Phosphate Dehydrogenase for Glucose-6-Phosphate, 2-Deoxy-D-Glucose-6-Phosphate and Galactose-6- Phosphate.

Substrate	Concentration	Per cent Activity
Glucose-6-P	5×10^{-3} M.	100
2-deoxy-D-glucose-6-P	5×10^{-3} M.	7.5
Galactose-6-P	5×10^{-3} M.	8

Seven different concentrations of glucose-6-P, two different concentrations of 2-deoxy-D-glucose-6-phosphate and 3.6×10^{-2} units/ml of enzyme were mixed and 1×10^{-3} M. $NADP^{+}$ was added immediately and the enzyme activity was measured. The experiment result is presented in a Line-Weaver-Burk plot in fig. 4. The kinetic behaviour of 2-deoxy-D-glucose-6-P was competitive against to glucose-6-P. The Km value was found as $Km = 6.7 \times 10^{-5}$ M. and the inhibition constant Ki value was found as $Ki = 5 \times 10^{-4}$ M.

Isoelectric Focussing Experiment

The isoelectric focussing experiment was carried out as described in the material and methods section. Ampholyne is a substance which acts as an acid in a strongly acidic medium and as an alkali in a strongly basic medium. During a period of 50 hours under 900 volts a pH gradient is established in the column. The pH is 1 at the bottom of the column and 10 at the top. The enzyme in the column will move up to a definite zone where pH value is equal to its electric charge. In the zone where the pH is equal to the isoelectric point of the enzyme no further migration occurs. At the end of experimental period 1 ml mixture was removed from the column by a fraction collector. pH value and enzyme activity was determined for each tube and result is represented in fig. 5. The maximal activity was found as 2.8 units/ml of enzyme in the tube number 44.

The pH curve intersects the maximal activity peak point at a pH value of 5.3 which is called the isoelectric point (pI) of the enzyme. Two small peaks appeared on both sides of the optimal peak the pH value of which could not be determined because they were too close to the optimal point.

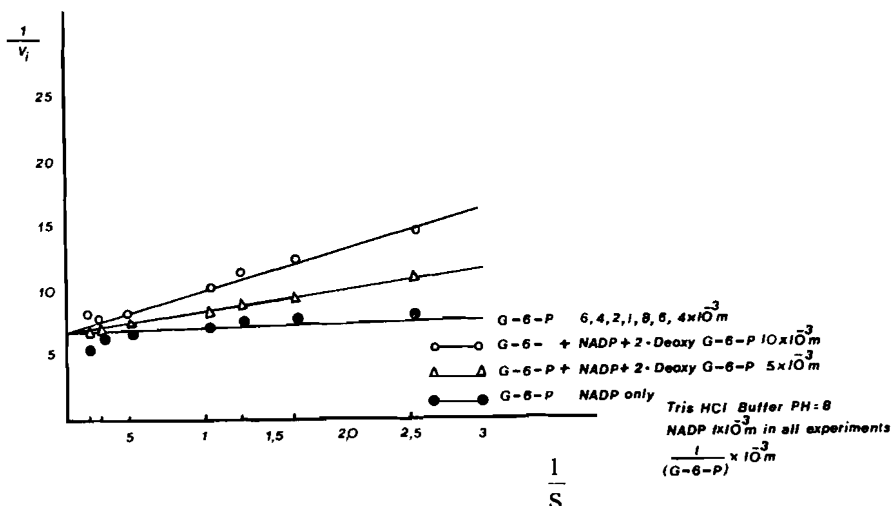

Figure 4

A double reciprocal plot showing competitive inhibition of glucose-6-P dehydrogenase by 2-deoxy-D-glucose-6-P with glucose-6-P. 3.6 units/ml of enzyme were mixed with two different concentrations of 2-deoxy-D-glucos-6-P and six different concentrations of glucose-6-P. Finally 1 x 10^{-3} M. $NADP^{+}$ was added immediately and the enzyme activity was measured. $\frac{1}{v}$ is plotted against to $\frac{1.}{S}$

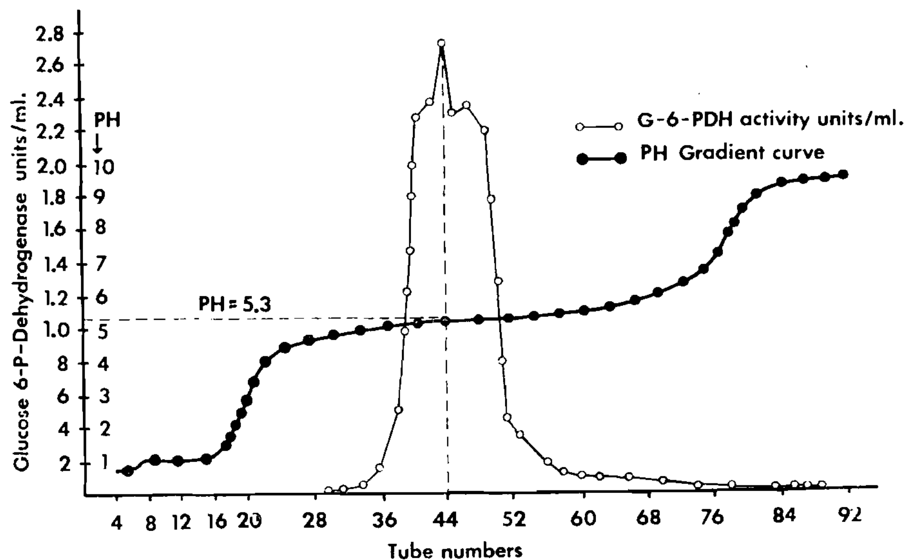

Figure 5

pH curve and the enzyme activity curve was drown after the isoelectric focusing experiment as explained in the text. The isoelectric point of glucose-6-phosphate dehydrogenase from rat liver was found 5.3 as shown in the graph.

Discussion

Pyridoxal 5'-phosphate was used to label the active lysine residues in several enzymes.[5, 9, 11, 12, 17] Pyridoxal 5'-P showed competitive inhibition with glucose-6-P and non-competitive inhibition with $NADP^+$ as shown in fig. 3 and 4. Glucose-6-phosphate dehydrogenase isolated from *Candida utilis* by Domischke and Domagk was traeted with pyridoxal 5'-phosphate (PL 5'-P) and also showed competitive inhibition with glucose-6-P[5]. The same result was also obtained by Olive, Geroch and Levy with glucose-6-P dehydrogenase isolated from *Leuconostoc mesenteroides* by using PL 5'-P in a kinetic method.[12] Olive, Geroch and Levy suggested that the ionization of the phosphate group of glucose-6-P which has a pK' of 6.1 and should exist in the anionic form in order to bind to the protonated ε -amino group of the lysine residue. The second probablitiy is that the aldehyde group of the PL 5'-P forms a Schiff's base with the ε -amino group of a specific lysine residue of the enzyme protein.

To determine the pyridoxyllysine complex in different enzymes some detailed studies were done by several authors. Glutamate decarboxylase isolated from *Escherichia coli* by Strausbach and Fischer,[17] tryptophanase, by Kagamiyama, Morino and Snell[9] and ribonuclease from bovine pancreas by Means and Fenney[11] were incubated with PL 5'-P and then reduced with sodium borohydride ($NaBH_4$). These complexes were digested with trypsin and chymotrypsin and small peptides containing N^{ε}-pyridoxyllysine residue were isolated. The amino acid sequence of the small peptides were determined by these authors. PL 5'-P was found to be covalently bound to an ε-amino group of the lysyl residue. The amino acid sequences are indicated as follows.

Glutamate decarboxylase Ser-Ile-Ser-Ala-Gly-His-Lys-Phe
/
PL 5'-P

Tryptophanase .... Ser-Ala-Lys-Lys-Asp-Ala
/
PL 5'-P

In conclusion pyridoxal 5'-P and glucose-6-P were bound to the same site on glucose-6-P dehydrogenase and showed competitive inhibition. The evidence strongly suggests that the ε -amino group of the lysine residue is one of the probable binding sites for glucose-6-P in the structure of glucose-6-P dehydrogenase form rat liver.

Glucose-6-P dehydrogenase was found to utilize 2-deoxy-D-glucose-6-P and galactose-6-P as a substrate. The oxidation rates were found as low as 7.5 and 8 % as compared with glucose-6-P.

The enzyme isolated from human erythrocyte by Yoshida was found to utilize these two compounds as a substrate.[18] Scott and Tatum observed-that the enzyme isolated from *Neurospora crassa* also utilized these two substances. Therefore, in this respect there are similarities between the human erythrocyte, rat liver and *Neurospora carssa* Enzymes. From these experiment it is concluded that both substrates can not play an important role in physiological conditions.

Recently isoelectric focussing techniques have been developed for separation of isoenzymes and proteins. These techniques are also avaliable for determination of the isoelectric point of proteins. In our experiment the isoelectric point (pI) of glucose-6-P dehydrogenase was found to be 5.3 as showen in Figure 5, but the two points at both sides of this value remained undetermined. For the enzyme isolated from *Neurospora crassa*, three bands of protein in the pH range of 4.8 to 5.2 were consistently observed by Scott.[15] He estimated a difference in isoelectric point of less than 0.2 pH unit. The resolving power of isoelectric focussing is demonstrated by an experiment of Albers and Scanul. The authors used a narrow pH gradient for human serum lipoproteins and they separated fraction B from C with a pI difference of only 0.09 pH units. Probably using a narrow pH gradient in our experiment it is possible to separate the close points from the main peak.

Acknowledgements

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Summary

Glucose-6-phosphate dehydrogenase from rat liver was used in purified form in all experiments.

The binding site of glucose-6-P to the active center of the enzyme was tested with pyridoxal 5'-phosphate by using a kinetic method Pyridoxal 5'-phosphate showed a competitive inhibition with glucose-6-P in the experimental conditions. This result indicates that glucose-6-P and Phyridoxal 5'-phosphate (PL 5'-P) binds to the same site on the enzyme surface. The ε -amino group of the lysine residue was found to be one of the probable binding sites of the substrate glucose-6-P.

Glucose-6-P dehydrogenase from rat liver was found to utilize 2-deoxy-D-glucose-6-P and galactose-6-P in addition to glucose-6-P. The relative oxidation rates of two compounds were very low (7.5 and 8 %) as compared with its natural substrate and ware thus thought not to be important in physiological conditions. 2-deoxy-D-glucose-6-P was run as a second substrate and showed a competitive inhibition with glucose-6-P.

In order to determine the isoelectric point of glucose-6-P dehydrogenase from rat liver an isoelectric focussing experiment was done in a glycerol-ampholyne mixture at a high voltage for 50 hours. The isoelectric point of the enzyme was found 5.3 in our experimental conditions.

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