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Biotinyl-Glucose-6-Phosphate Dehydrogenase

Preparation, Kinetics, and Modulation by Avidin

THAT T. NGO,* HOWARD M. LENHOFF, AND JEANNE IVY

Department of Developmental and Cell Biology, University of California, Irvine, California 92717

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Abstract

The kinetics of free glucose-6-phosphate dehydrogenase (G-6-PDH), biotinylated G-6-PDH, and biotinylated G-6-PDH complexed with avidin were investigated. The kinetics of the free enzyme were consistent with a sequential rather than a ping-pong mechanism. The kinetics of the biotinylated enzyme were similar to that of the free enzyme, but the kinetic constants were different; the K_m value for NADP was halved, whereas the K_m for G-6-P decreased only slightly. In the presence of avidin, the K_m of biotinylated G-6-PDH for G-6-P nearly doubled whereas the K_m for NADP did not change significantly. Avidin complexed with biotinylated G-6-PDH inhibited the enzyme from acting. Based upon these reactions, it was possible to devise assays for either free biotin or free avidin using biotinylated G-6-PDH as the indicator enzyme. Concentrations of biotin between 40 and 60 mg/mL, or of 25–95 μg/mL of avidin could be measured within 2 min through the use of biotinylated G-6-PDH.

Index Entries: Enzyme modulation; modulation, enzyme; avidin; biotin; glucose-6-phosphate dehydrogenase; biotinylated enzyme; enzyme, biotinylated.

Introduction

The attachment of low molecular weight ligands to enzymes by covalent bonds yields stable ligand–enzyme conjugates (*1*, *2*). It would appear that the enzyme activity of such conjugates could be affected by substances that combine with the bound ligand. In some instances, for example, the activity can be modulated by

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antibodies to the bound ligand (*3–5*). Whether the modulation is stimulatory or inhibitory depends on the nature of the enzyme and of the attached ligand. For example, antibodies to morphine inhibit the activity of the morphine–malate dehydrogenase conjugate (*4*), whereas antithyroxine antibodies activate thyroxine–malate dehydrogenase (*5*).

The purpose of the present study was to determine whether the activity of ligand–enzyme conjugates can be modulated by receptor molecules for the bound ligand other than antibodies. The ligand–receptor pair we chose was the biotin–avidin system, with biotin serving as the bound ligand. The kinetics of biotinylated G-6-PDH and its reactions with avidin were studied in detail. From these investigations, we also demonstrated that the biotinylated enzyme can be used rapidly to determine either biotin or avidin.

Methods and Materials

Reagents used were: glucose-6-phosphate, NADP, D-biotin, avidin, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, *N*-hydroxysuccinamide and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (Sigma Co.); *N*-hydroxysuccinimidobiotin (Pierce Co.); D-[carbonyl-^{14}C]-biotin (Amersham, Co.); and Aquasol scintillation fluid (New England Nuclear). Other reagents used were of analytical grade.

Preparation of ^{14}C-Labeled N-Hydroxysuccinimidobiotin

To a solution consisting of 1.5 mL dry *N,N*-dimethylformamide and 1 mL dry tetrahydrofuran, we added 0.2 mmol D-biotin, 1 μmol D-[carbonyl-^{14}C]-biotin, 0.2 mmol 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide, 0.2 mmol *N*-hydroxy-succinamide, and 0.2 mmol triethylamine, and stirred the solution at room temperature for 24 h. The specific activity of the resultant ^{14}C-labeled *N*-hydroxysuccinimide-biotin was 0.25 μCi/μmol.

Covalent Labeling of Glucose-6-Phosphate Dehydrogenase with Activated Biotin

We dialyzed glucose-6-phosphate dehydrogenase (5.3 mg) against two changes of 2 L of 0.1*M* sodium phosphate, pH 7.6 at 4°C for a total of 24 h, and incubated the dialyzed enzyme (3.2 mg/mL) with *N*-hydroxysuccinimidobiotin dissolved in dimethylsulfoxide. The concentration of biotin used was in excess. The molar concentration of biotin to enzyme was 100 : 1. We allowed the reaction of *N*-hydroxysuccinimidobiotin with the enzyme to proceed at room temperature for 30 min to 2 h. We separated the unreacted *N*-hydroxysuccinimidobiotin from the enzyme by means of a gel filtration chromatography in a Sephadex G-50 column (1.5 × 45 cm) previously equilibrated with 0.1*M* sodium phosphate, pH 6.7. We used the same buffer as the eluant, the volume in each fraction being 5 mL. We pooled fractions containing the enzyme and dialyzed them against 4 L of 0.1*M* sodium phosphate, pH 7.6 for 12 h at 4°C.

We determined the number of biotin molecules covalently bound to one molecule of glucose-6-phosphate dehydrogenase by reacting the enzyme with ^{14}C-labeled *N*-hydroxysuccinimidobiotin in a manner similar to the one using unlabeled *N*-hydroxysuccinimidobiotin. After removing the unreacted ^{14}C-labeled *N*-hydroxysuccinimidobiotin from the labeled enzyme by means of gel filtration chromatography and dialysis, we determined the number of biotins labeled to one molecule of the enzyme by measuring the amount of radioactivity of the ^{14}C-biotin labeled enzyme. The number of biotins per molecule of enzyme ranged from 16 to 24.

Enzyme Assay

We measured the initial rate of glucose-6-phosphate dehydrogenase at 25°C in 1 mL 0.1*M* sodium phosphate, pH 7.6, containing glucose-6-phosphate and NADP. We started the reaction by adding 20 μL of enzyme solution, and recorded the increases in absorbance at 340 nm continuously. For the rate calculations, we used a molar extinction coefficient of 6270 M^{-1} cm^{-1} for NADPH (*6*). The exact concentrations of enzyme, glucose-6-phosphate, and NADP are specified in legends to the figures. We expressed the initial rates (v) in the double reciprocal plots as change in absorbance at 340 nm per min.

We determined protein concentration according to Lowry et al. (*7*).

Results

When NADP was the variable substrate and G-6-P was the changing fixed substrate, the double reciprocal plots of G-6-PDH catalyzed reactions showed a family of straight lines intersecting to the left of $1/v$ axis (Fig. 1a). We observed a similar pattern when G-6-P was the variable substrate and NADP was the changing fixed substrate (Fig. 1b). From Figs. 1a and 1b, and from replots of the intercepts against the concentrations of the changing fixed substrate (inserts, Fig. 1a and 1b), we calculated the maximal rate of G-6-PDH to be 0.79 μmol/min/μg enzyme and the Michaelis constants of the enzyme for G-6-P and NADP to be 0.77 m*M* and 33 μ*M*, respectively.

With either NADP (Fig. 2a) or G-6-P (Fig. 2b) as the variable substrate and either G-6-P or NADP, respectively, as the changing fixed substrate, the double reciprocal plots for biotinylated G-6-PDH also showed families of straight lines intersecting to the left of $1/v$ axis. From the double reciprocal plots and the secondary plots (Fig. 2a, 2b, and inserts), the maximal rate of biotinylated G-6-PDH was calculated to be 0.03 μmol/min/μg enzyme and the Michaelis constants of the biotinylated enzyme for G-6-P and NADP were 0.63 m*M* and 14.5 μ*M*, respectively.

By adding increasing concentrations of avidin to biotinylated G-6-PDH, the enzyme activity was inhibited in a dosage-dependent manner (Fig. 3). At avidin concentrations greater than 5 μg/mL, the enzyme exhibited less than 10% of its original activity. Avidin, however, did not inhibit the native, i.e., non-biotinylated, G-6-PDH.

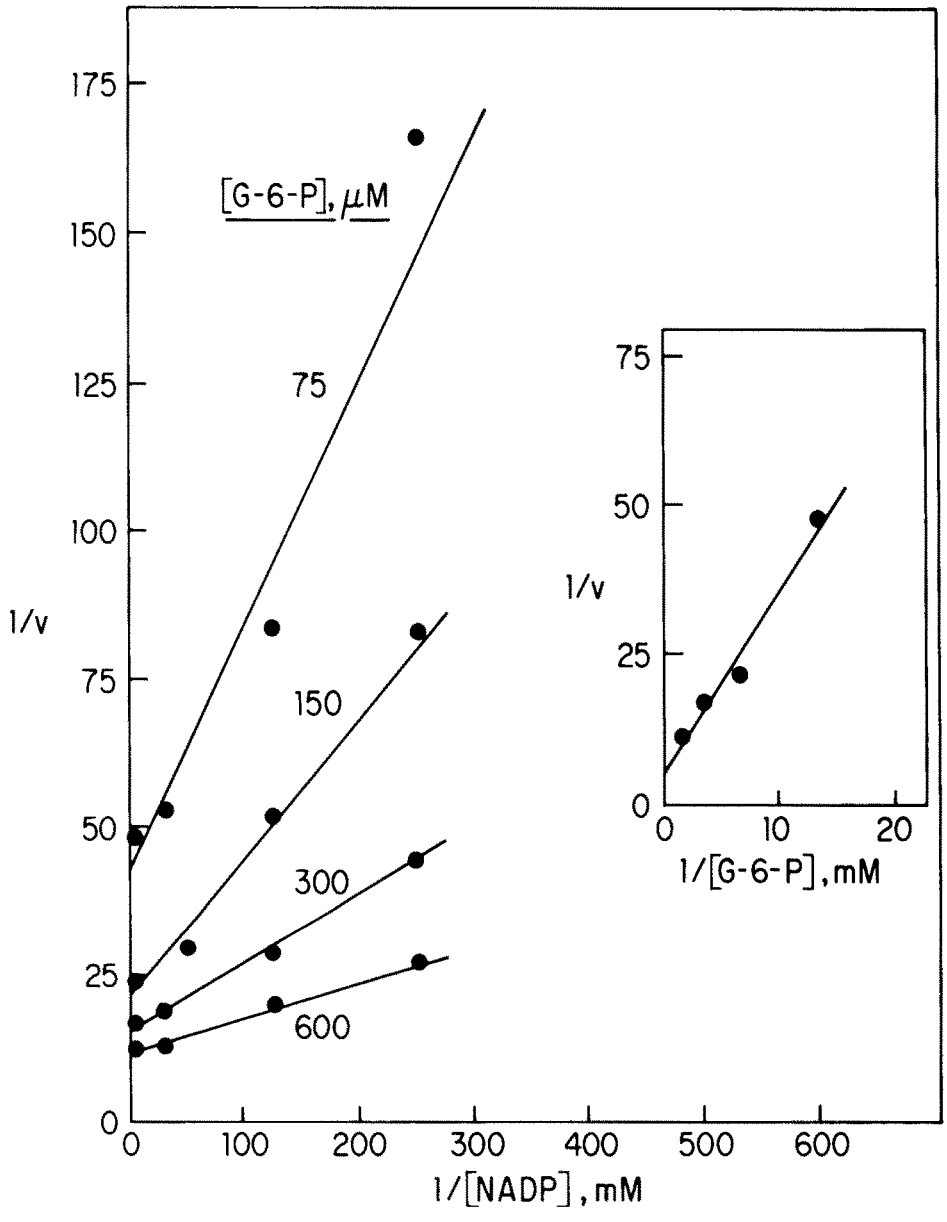


Fig. 1a. Kinetics of free glucose-6-phosphate dehydrogenase. The reaction was carried out in 1 mL 0.1*M* sodium phosphate pH 7.5, at 25°C, and was initiated by adding 20 μL enzyme (0.1 μg). (a) Double reciprocal plots with NADP as the variable substrate and G-6-P as the changing fixed substrate. The insert is a plot of $1/v$ intercepts versus concentration of G-6-P.

D-Biotin, when added to a solution of biotinylated G-6-PDH, was able to neutralize the inhibitory effect of avidin that was added subsequently. The inhibitory effect of avidin added under these conditions decreased when increasing amount of D-biotin was first added to the biotinylated G-6-PDH solution (Fig. 4).

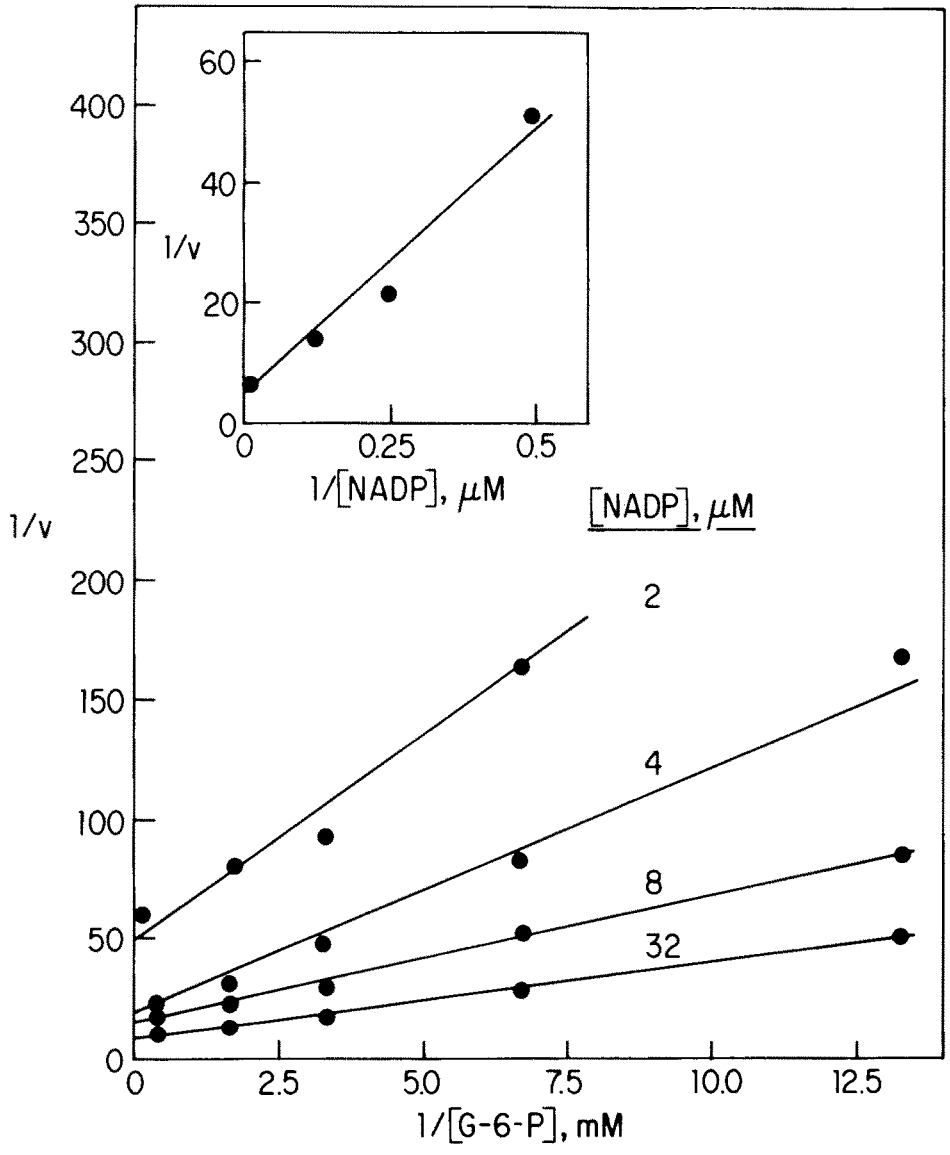


Fig. 1b. Double reciprocal plots with G-6-P as the variable substrate and NADP as the changing fixed substrate. The insert is a plot of $1/v$ intercepts versus concentration of NADP.

Avidin inhibited biotinnylated G-6-PDH by means of a mixed-type competitive and noncompetitive mechanism (Fig. 5). In addition, Dixon plots of the inhibition of biotinylated G-6-PDH by avidin were nonlinear.

The pH–rate profiles for G-6-PDH, biotinylated G-6-PDH, and biotinylated G-6-PDH in the presence of avidin, showed pH optima of 9.5, 10, and 9, respectively (Fig. 6).

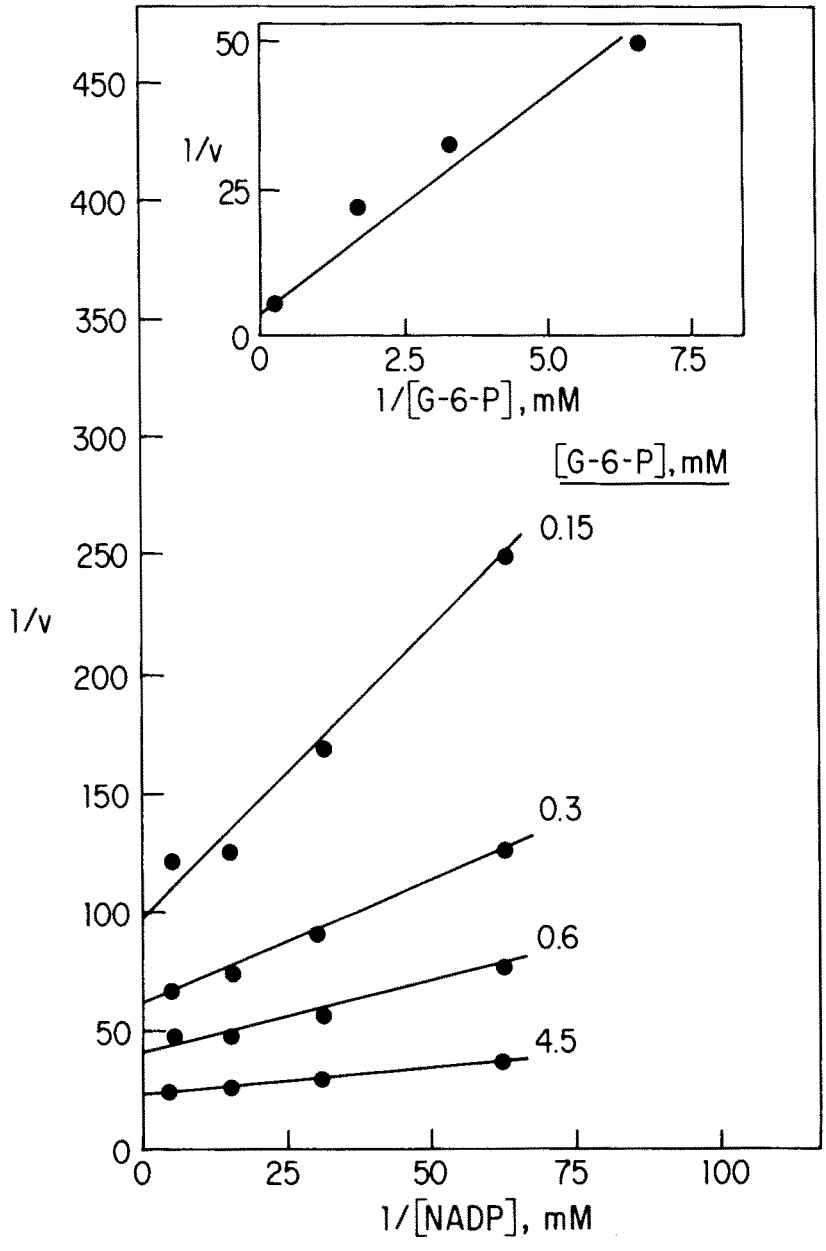


Fig. 2a. Kinetics of biotinylated glucose-6-phosphate dehydrogenase. The reaction was carried out in 1 mL 0.1*M* sodium phosphate, pH 7.5, at 25°C, and was initiated by adding 20 μL enzyme (0.5 μg). (a) Double reciprocal plots with NADP as the variable substrate and G-6-P as the changing fixed substrate. The insert is a plot of $1/v$ intercepts versus concentration of G-6-P.

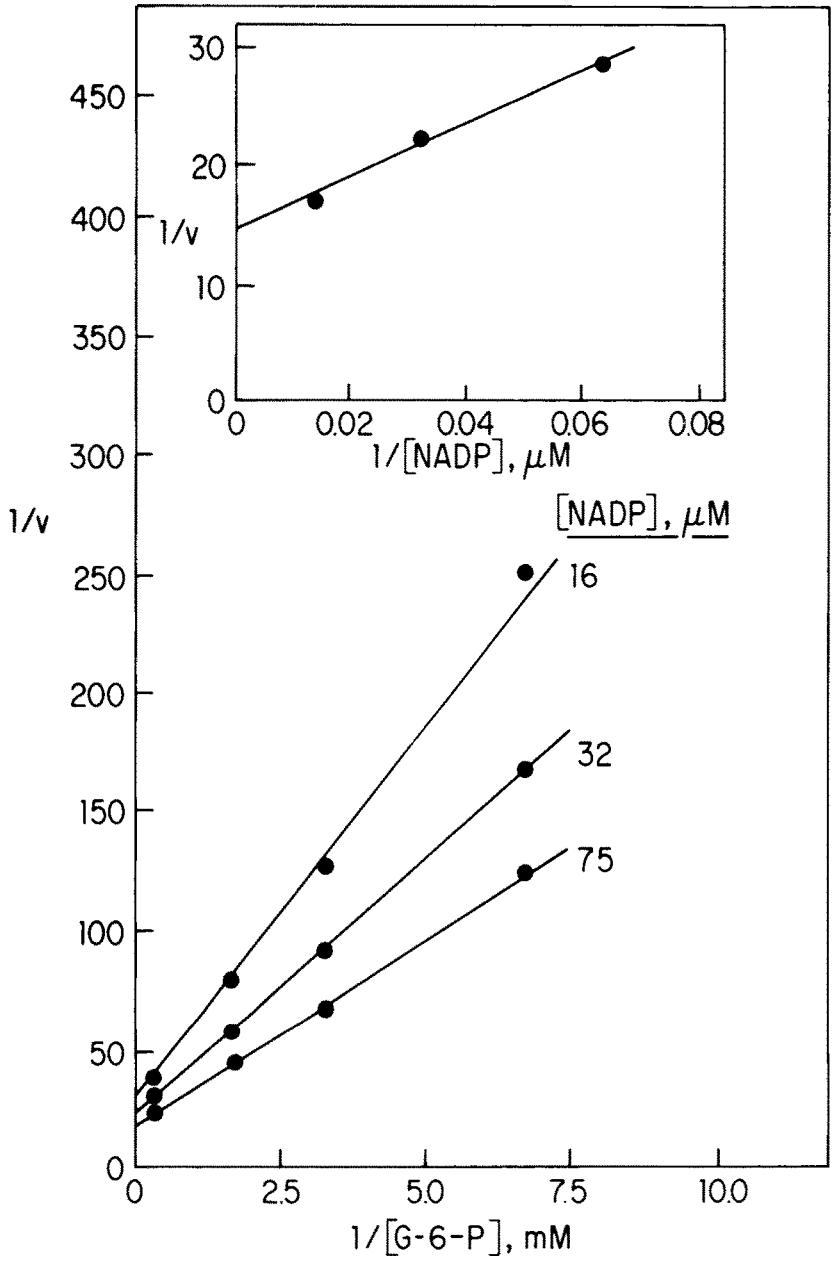


Fig. 2b. Double reciprocal plots with G-6-P as the variable substrate and NADP as the changing fixed substrate. The insert is a plot of $1/v$ intercepts versus concentration of NADP.

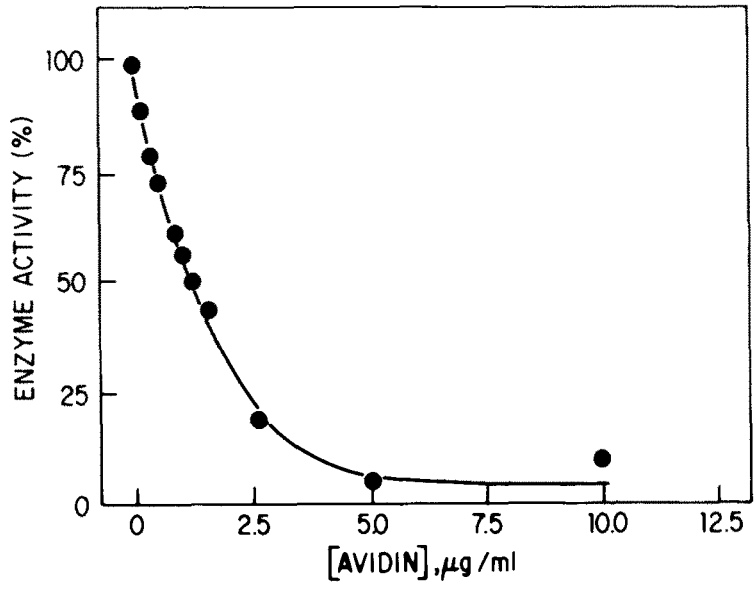


Fig. 3. Standard curve for determining avidin. Avidin solution (100 μL) was added to 0.9 mL substrate solution containing 5 μmol G-6-P and 1 μmol NADP. The reaction was initiated by adding 20 μL (0.5 μg) biotinylated G-6-PDH. The absorbance change per min at 340 nm was 0.18.

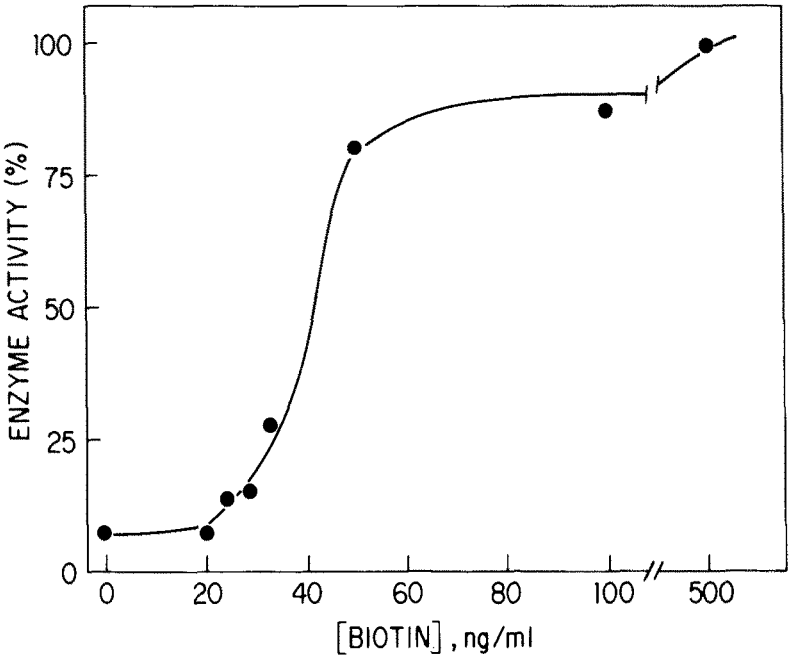


Fig. 4. Standard curve for determining biotin. To 100 μL solution of biotin, 20 μL (0.5 μg) of biotinylated G-6-PDH and 100 μL avidin (10 μg) were added sequentially. The solution was briefly mixed. Substrate solution (0.9 mL) was then added, the solution was mixed, and the absorbance at 340 nm was immediately monitored. The composition of substrate solution was as described in Fig. 3. The absorbance change per min was 0.15.

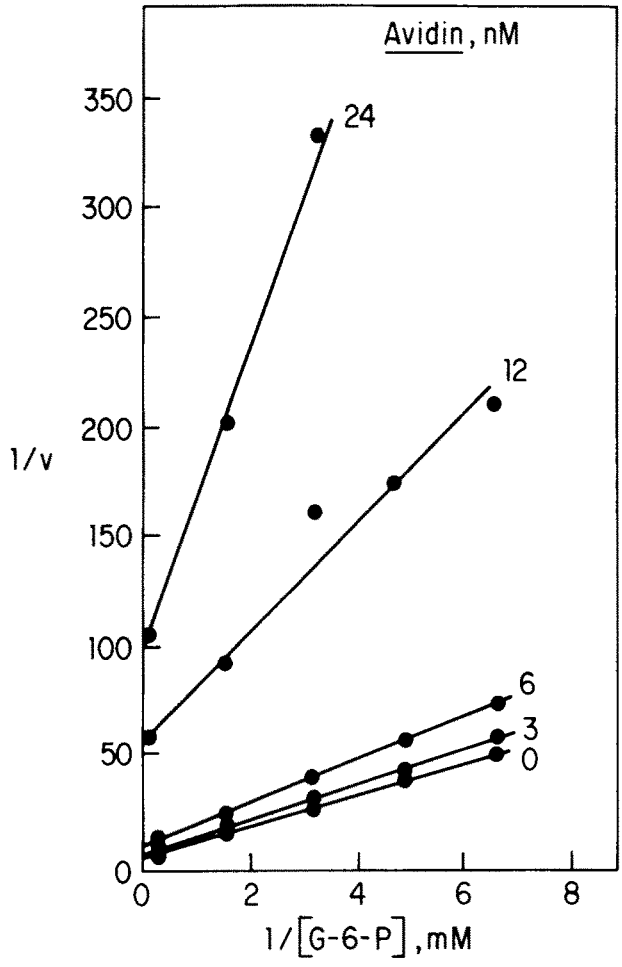


Fig. 5. Kinetics of biotinylated glucose-6-phosphate dehydrogenase in the presence of avidin. The concentration of NADP was 35 μM. The concentrations of glucose-6-phosphate and avidin are indicated in the figure. The concentration of biotinylated G-6-PDH was 0.545 μg/mL.

Discussion

Double reciprocal plots of the initial rates of G-6-PDH gave straight lines intersecting to the left of $1/v$ axis when either G-6-P or NADP was the variable substrate and when either NADP or G-6-P was the changing fixed substrate (Fig. 1a and 1b). Such double reciprocal plots indicate that the initial rate data are consistent with a sequential rather than a ping-pong kinetic mechanism. (*8*). A sequential mechanism for G-6-PDH is also consistent with the kinetics of other dehydrogenases (*9*). Data on initial rates alone, however, do not permit further differentiation among the various classes of sequential mechanisms (*8*).

The kinetics of biotinylated G-6-PDH showed similar patterns (Fig. 2a and 2b) except that the kinetic constants of the biotinylated G-6-PDH differed from those of the nonbiotinylated enzyme. As shown in Table 1, K_m values for G-6-P were 0.77

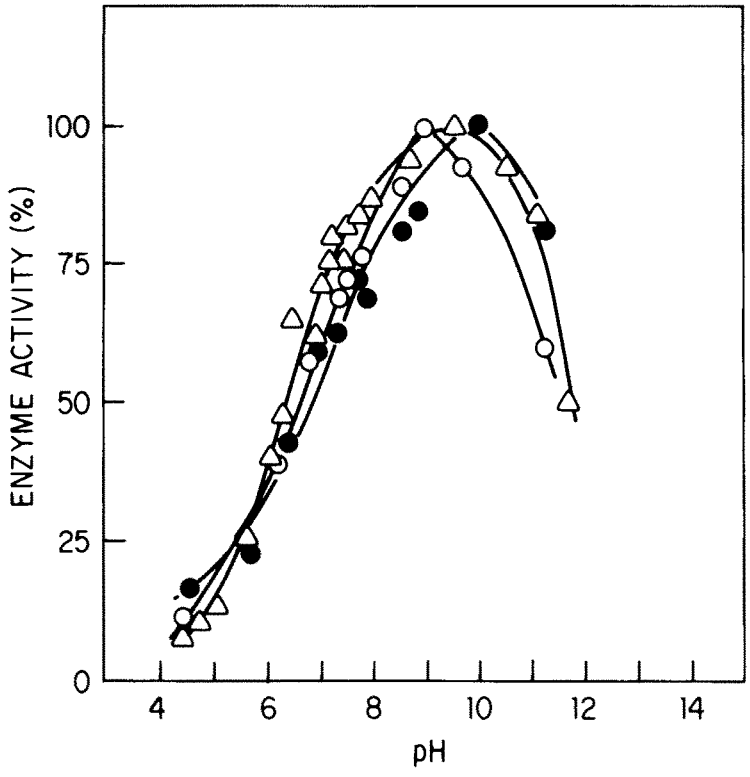


Fig. 6. Effect of pH on the activity of glucose-6-phosphate dehydrogenase (△), biotinylated glucose-6-phosphate (●), and biotinylated glucose-6-phosphate in the presence of avidin (○).

and 0.63 mM, respectively, for the nonbiotinylated and biotinylated enzyme, and K_m values for NADP were 33 and 14.5 μM, respectively, for the non-biotinylated and biotinylated enzyme. The covalent attachment of biotin G-6-PDH, therefore, halved the K_m for NADP, but did not significantly change the K_m for G-6-P. Furthermore, the specific activity of G-6-PDH decreased 26-fold after it was labeled with biotin.

TABLE 1
Michaelis Constants (K_m) for Glucose-6-phosphate Dehydrogenase and Its Biotinylated Derivative[a]

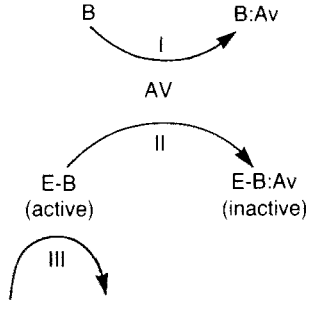
		K_m, mM	
Enzyme	Avidin	Glucose-6-phosphate	NADP
Glucose-6-phosphate dehydrogenase	0	0.77	0.033
Biotinylated glucose-6-phosphate dehydrogenase	0	0.63	0.014
Biotinylated glucose-6-phosphate dehydrogenase	0.3 μg/mL	1.11	0.018

[a]All kinetic parameters were obtained from linear regression analyses of experimental results with correlation coefficients better than 0.93.

In the presence of avidin, the K_m of biotinylated G-6-PDH for G-6-P nearly doubled, whereas the K_m for NADP did not change significantly (Table 1). Our analysis of the kinetics of the avidin-induced inhibition of biotinylated G-6-PDH revealed that it does not follow any simple inhibition mechanism. The double reciprocal plots of initial rate against the concentration of G-6-P at several levels of avidin concentrations showed a mixed type inhibition (Fig. 5). Dixon plots of the same data, however, show a couple nonlinear relationship (figure not shown). It thus appears that the mechanism of that inhibition could involve a multiplicity of factors such as conformational transitions, steric effects, charge effects, and so on. Rowley et al. (*4*) interpret the inhibition of malate dehydrogenase covalently linked with morphine by antimorphine antibodies as resulting from a conformational "freezing" of the enzyme that occurs when the antibody combines with the morphine residues.

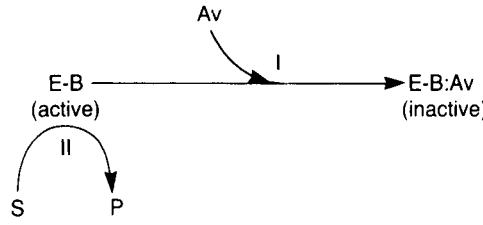
It is possible, through two different kinds of competition experiments, to use the biotinylated G-6-PDH that we describe for determining small amounts of either biotin or avidin.

The competitive binding reactions for quantifying biotin (B) are shown in Scheme I. This method uses biotin covalently linked to enzyme (G-6-PDH). The enzyme–biotin conjugates (E–B) are enzymatically active and compete effectively (reaction II) with the analyte biotin (B) from the sample (reaction I) for a limited concentration of avidin (Av). When avidin binds the enzyme–biotin conjugate (reaction II), an enzymatically inactive complex, E–B : Av is formed. Free biotin (B), however, would compete for binding sites on the avidin, leaving more E–B uncomplexed and free to catalyze the conversion of substrates to products (reaction III). Thus, the net result of an increase in the concentration of biotin is an increase in enzyme activity. Figure 4 shows the standard curve for determining biotin by this method. The assay is especially sensitive at biotin concentrations between 40 and 60 mg/mL. It is rapid, with each determination achieved in less than 2 min.



Scheme I

Scheme 2 shows that avidin can also be quantified by using biotinylated G-6-PDH for the indicator reaction. As in Scheme 1, the enzymatically active enzyme-biotin conjugates (E–B) in the presence of avidin (Av) form enzymatically inactive complexes of E–B : Av (reaction I).



Scheme 2

Thus, the addition of increasing amounts of avidin to a fixed concentration of biotinylated G-6-PDH (E–B) would result in decreasing enzyme activity (Fig.3). A single measurement of avidin in a solution by such a system can be completed within 2 min.

There is at present no simple, straightforward way to explain the observed effects of pH on the activity of the various forms of G-6-PDH (Fig. 6). Further experiments using spectroscopic methods may be required.

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