Kinetic Mechanism of Glucose-6-phosphate Dehydrogenase from the Lactating Rat Mammary Gland

IMPLICATIONS FOR REGULATION

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The steady state kinetic mechanisms for glucose-6-P dehydrogenase from lactating rat mammary glands were derived for the NADP-linked and NAD-linked reactions. Initial velocity studies and inhibition patterns using NADPH and glucosamine-6-P are consistent with sequential mechanisms with random order addition of glucose-6-P and coenzyme. No evidence was found for enzyme isomerization or dead-end complexes. Both NADP* and glucose-6-P were found to quench the fluorescence of the enzyme. The dissociation constant for NADP* derived from fluorescence quenching titrations was similar to the kinetically derived binding constant, consistent with random order binding. Analysis of fluorescence quenching by glucose-6-P revealed the presence of two classes of binding sites on the enzyme with different affinities for the substrate. Isotope effects were determined using both deuterium and tritium. From these it was concluded that the transfer of hydrogen from glucose-6-P to NADP* limits the overall reaction rate by only 15 to 19%. We conclude that the mechanism for the reaction, with either NADP* or NAD* as coenzyme, is probably partial rapid equilibrium random. Such a mechanism would be advantageous for mammary glucose-6-P dehydrogenase because it would permit glucose-6-P to counteract NADPH inhibition. Support for this idea was provided by experiments in which increased concentrations of glucose-6-P partially reversed the inhibition of purified mammary glucose-6-P dehydrogenase at high NADPH/NADP* ratios.

In mammalian tissues glucose-6-P dehydrogenase catalyzes the first step in the hexosemonophosphate shunt. The regulation of this pathway is not well understood, although it is generally agreed that glucose-6-P dehydrogenase catalyzes the rate-limiting step and that modulation of this enzyme's activity is critical to the control of the shunt pathway. The high concentration ratio, NADPH/NADP*, in many mammalian tissues and the potent NADP* -competitive inhibition by NADPH demonstrated for many mammalian glucose-6-P dehydrogenases has posed problems in explaining not only how these enzymes are regulated, but how they can even function under physiological conditions. Eggleston and Krebs described a physiological mechanism whereby rat liver glucose-6-P dehydrogenase may be deinhibited by oxidized glutathione and a labile cofactor (1). Their results suggested that this mechanism could operate in other rat tissues, but not in the lactating mammary gland (1).

The purpose of this study was to gain some insight into the regulation of lactating rat mammary glucose-6-P dehydrogenase by examining the kinetic mechanism of the enzyme. There were two reasons why we believed that such an investigation might prove to be useful. First, it appeared likely, in view of previous kinetic studies with other mammalian glucose-6-P dehydrogenases (2), that the mammary enzyme would catalyze some form of a sequential mechanism. Whether, in such a mechanism, the addition of substrates is ordered or random affects critically the mechanism whereby NADPH inhibition can be counteracted. Because of the high, intracellular NADPH/NADP* ratio, it was important to determine the binding order. Second, like all mammalian glucose-6-P dehydrogenases tested, the rat mammary enzyme is NADP-prefering (2), although it can utilize NAD* (3). Glucose-6-P dehydrogenase from Leuconostoc mesenteroides displays dual nucleotide specificity (4). The kinetic mechanisms of the NAD- and NADP-linked reactions in the latter enzyme differ (5), a fact which appears to play an important role in its regulation (6).

It was of interest, therefore, to examine the mechanisms of both the NAD-linked and NADP-linked reactions catalyzed by mammary glucose-6-P dehydrogenase to ascertain whether these differed.

EXPERIMENTAL PROCEDURES

Preparation and Assay of the Enzyme—Standard assays (except for kinetic studies, see below) were performed as previously described (7). Protein concentration was determined from absorbancies at 260 and 280 nm (8). The enzyme was purified as previously described (9). During this purification it is eluted from an affinity matrix by NADP* (10). Most of the coenzyme was removed from the pure enzyme by precipitating with ammonium sulfate, dialyzing, or gel-filtering the dissolved precipitate using Buffer A (35 mM potassium phosphate, 7 mM 2-mercaptoethanol, 1 mM EDTA, 20% (v/v) glycerol, pH 7.5). The concentration of NADP* in the enzyme solution was determined by adding excess glucose-6-P to a measured quantity of enzyme, monitoring the increase in fluorescence at 460 nm (excitation wavelength, 340 nm), and comparing this with a standard curve of fluorescence versus NADPH concentration. The final ratio of the concentration of NADP*/enzyme dimer ranged from 0.34 to 3.0 in various preparations. Due to the low concentrations of enzyme used in the assays during the kinetic and isotope studies, the final concentration of NADP* in the assay due to the NADP* in solution with the enzyme.

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1 The nomenclature employed to describe quaternary structure is that used in Ref. 2. Thus, a dimer is that form of the enzyme containing two subunits. This nomenclature differs from that used previously (e.g. in Ref. 7).
enzyme was less than 0.1 nM. The lowest coenzyme concentration used in an assay during a kinetic or isotope study was 0.21 pM; therefore, the NADP+ added to the assay mixtures with the enzyme did not affect the rate of the reaction.

The buffer used for all standard and kinetic assays was 67 mM Tris/acetate, pH 8.6. Enzyme assays were performed routinely at 25°C by monitoring the absorbance of the reduced coenzyme at 340 nm using a spectrophotometer equipped with either 3-cm light paths or 15-mL cuvettes with 5-cm light paths. Some assays were performed by monitoring the fluorescence of NADPH at 460 nm, while exciting at 340 nm, using a Perkin Elmer MFP-3L fluorescence spectrophotometer. For all assays of the NAD-linked reaction, NAD, glucose-6-P and glucosamine-6-P were first neutralized with sodium bicarbonate. The concentrations of all solutions of glucose-6-P, NAD+, and NADP+ were determined enzymatically. The concentrations of all solutions of NADPH were determined from absorbance readings at 339 and 295 nm, using the appropriate extinction coefficients (11). The assays performed at the lowest concentrations of the varied and the changing fixed substrate were done in quadruplicate; all other assays were done in duplicate.

Quenching of Protein Fluorescence of the Enzyme by NADP+ and Glucose-6-P—These experiments were done following the procedure of Grove et al. (12) with the following modifications. Titrations of the enzyme were performed in 67 mM Tris/acetate, pH 8.6. Control titrations were performed on glycy1-4-tropanyl in the same buffer. Ligands were added in increments of 1 to 10 μL. The concentration of enzyme dimer was 0.786 μM. The excitation band width was 1.5 nm. Deuterium and Tritium Isotope Effects—[1-3H]Glucose-6-P and [1-2H]glucose were prepared from [1-3H]glucose (98% 3H) and [1-2H]glucose, respectively, using the procedure of Lowenstein (13). The purified [1-3H]glucose-6-P was titrated to pH 7.5 with NaOH and concentrated by lyophilization before use.

The deuterium isotope effect was determined in both 67 mM Tris/acetate pH 8.6, and 3.3 mM glycy1glycine, pH 7.5, by varying the concentrations of glucose-6-P and [1-3H]glucose-6-P at a fixed NADP+ concentration. The isotope effects on V and V/K (where K = Kcat for glucose-6-P) then were determined from reciprocal plots of the initial velocity data. In the determination of the tritium isotope effect on V/K, a 5-mL reaction mixture containing 3.3 mM glycy1glycine (pH 7.5), 73 μM NADP+, and 184 μM [1-3H]glucose-6-P. The reactions were monitored using a Zeiss PMQ II spectrophotometer and stopped, after 35% of the NADP+ had been reduced, by the rapid addition of 0.5 ml of carbon tetrachloride followed by vigorous mixing (14). After removal of the carbon tetrachloride the reaction mixture was again monitored spectrophotometrically to ensure that the reaction had stopped. The radioactive NADP+ then was isolated using DEAE-cellulose (Whatman DE52) by the procedure of Pastore and Friedkin (15). The concentration of [1-3H]glucose-6-P was determined enzymatically using mammalian glucose-6-P dehydrogenase. The concentration of [1-2H]glucose-6-P was determined enzymatically using glycy1glycine (pH 7.4), 5 mM α-ketoglutarate, and 0.025 M ammonium acetate (16). The radioactivities of the labeled glucose-6-P and NADPH were determined using liquid scintillation counting. Each sample was diluted to 1 ml with 10 mM glycy1glycine, pH 7.5, before counting.

Treatment of Data—Kinetic data were calculated using a computer program based on the Fortran program of Cleland (17). This program could not be used when NAD+ was the varied substrate because the Kcat for NAD+ is so high (see below) that all the NAD+ concentrations used were below 0.4 Kcat, concentrations which were too low to adequately define a rectangular hyperbola. Whenever NAD+ was the varied substrate the experimental data were fitted to lines using linear regression. All inhibitor replots were also drawn using linear regression.

The method of Klotz et al. (18) was used to plot the ligand binding data calculated from the quenching of enzyme fluorescence by NADP+. The line was fitted to the data using the computer program described above. For each titration with NADP+ or glucose-6-P the maximum fluorescence quenching (ΔFmax) was determined from a complete reciprocal of the change in fluorescence corrected for dilution, versus the reciprocal of the total ligand concentration (19). The concentrations of bound and free ligand were calculated (19) using the enzyme active site concentration of 1.57 μM, assuming that the fluorescence quenching proportional to the concentration of bound ligand. Because the dissociation constant for NADP+ is low, in the same range as the concentration of enzyme active sites, ΔFmax in the titrations with NADP+ was calculated using only that portion of the double reciprocal plot representing quenching data when the enzyme was more than 75% saturated, as specified by Luisi et al. (19). The validity of this procedure was indicated (19) by the fact that the number of NADP+ molecules binding/active site, calculated from the Klotz plots, was 1.00 ± 0.05 (mean ± 2 standard deviation from six experiments).

Materials—Fisher rats (CDF strain, pregnant or lactating with pups), were obtained from Charles River Breeding Laboratories; rat mammary glands were removed from the rats and dissected free of connective tissue. Glucose-6-P dehydrogenase, at 259 and 398 nm (21). The isolated material was 98% pure, based on the absorbance at 259 and 296 nm and, after complete reduction by glucose-6-P and glucose-6-P dehydrogenase, at 259 and 398 nm (21).

RESULTS

Kinetic Studies on the NADP-Linked Reaction—Initial velocity studies on the NADP-linked reaction indicate that the enzyme catalyzes a sequential mechanism (22). Lineeweaver-Burk plots with either glucose-6-P (Fig. 1) or NADP+ (not shown) as varied substrate give intersecting patterns. Product inhibition studies show that NADPH is a competitive inhibitor with respect to both NADP+ (Fig. 2) and glucose-6-P (Fig. 3).

These product inhibition patterns are consistent with a random mechanism (22), as are the patterns of inhibition with glucosamine-6-P, a dead-end inhibitor competitive with respect to glucose-6-P (Fig. 4). The apparent K, for glucosamine-6-P when glucose-6-P is the varied substrate is 0.82 mM. Fig. 5 shows that glucosamine-6-P is a noncompetitive inhibitor with respect to NADP+. The intercept and slope replots reveal apparent K, values for glucose-6-P of 4.6 and 1.4 mM, respectively. Glucosamine-6-P was not a substrate for the NAD-linked reaction under the conditions used here.

Ringler and Hilf (23) reported kinetic data for the NADP-linked reaction of the lactating mammary gland enzyme, indicating two catalytic binding sites for glucose-6-P, which differed 7-fold in apparent K, values. We confirmed this finding in an experiment in which the concentration of glucose-6-P was varied from 10.9 μM to 3.26 mM (0.4 K, to 123 K,). At 20.8 μM NADP+ (22 K,) the double reciprocal plot from this experiment was biphasic, yielding apparent K, values of 32.8 and 88.2 μM with concentrations of glucose-6-P below 0.12 and above 0.45 mM, respectively. The molar activity for the NADP-linked reaction (Table I) was calculated from the apparent Vmax obtained at low glucose-6-P concentration.

Kinetic Studies on the NADP-Linked Reaction—Experiments performed to determine the kinetic mechanism of the NADP-linked reaction were complicated by a hysteretic activation of the enzyme during the assay. Assays of the NADP-linked reaction showed an increase in reaction rate with time for the first 1 to 3 min after initiation; thereafter, the rate remained constant until one of the substrates approached depletion. The constant rate was approximately 25% faster than the initial rate. Enzyme that had undergone activation in the NADP-linked reaction had NADP-linked activity approximately 25% greater than enzyme that had not been activated. The specific activity (before hysteretic activation) of the enzyme used in these experiments was approximately 25% lower than that of fully active enzyme. No hysteresis was observed with the NAD-linked reaction (22).

The abbreviation used is: S-NADP+, thionicotinamide adenine dinucleotide phosphate.
Kinetic Mechanism of Mammary Glucose 6-P Dehydrogenase

seen with the NADP-linked reaction under any circumstances. The hysteresis was seen with both pure enzyme and partially purified enzyme that had not been subjected to affinity chromatography; it was, therefore, not a result of the affinity chromatography, the presence of NADP⁺ in the purified enzyme, or its subsequent removal from the enzyme. The activation was seen at different concentrations of glucose-6-P and NAD⁺ and was not due to a change in the pH or ionic strength during the assay. Preincubation of the enzyme with glucose-6-P, NAD⁺, NADP⁺, NADH, or NADPH, or performing the assay in the presence of 2-mercaptoethanol, did not alleviate the hysteresis. All kinetic data reported for the NAD-linked reaction represent rates taken after activation was complete.

Initial velocity studies were performed for the NAD-linked reaction. As with the NADP-linked reaction intersecting patterns resulted, indicating a sequential mechanism. Alternate product inhibition studies were performed with NADPH. Low concentrations of NADPH had to be used to avoid sigmoid behavior in the NAD⁺ kinetics caused by NADPH shifting the equilibrium between two dimeric enzyme forms that have different ratios of NADP⁻ to NAD-linked activity (7). NADPH is a competitive inhibitor with respect to NAD⁺ (Fig. 6) and also a competitive inhibitor with respect to glucose-6-P (Fig. 7). Fig. 7 demonstrates the sigmoid behavior induced by NADPH in the kinetics of the NAD-linked reaction (7). The competitive inhibition patterns in both Figs. 6 and 7 indicate that the NAD-linked, like the NADP-linked reaction, operates via a random mechanism.

Also consistent with a random mechanism for the NAD-linked reaction are the patterns of inhibition with glucosamine-6-P. As with the NADP-linked reaction, glucosamine-6-P is a dead-end inhibitor competitive with respect to glucose-6-P. The apparent Kᵢ for glucosamine-6-P when glucose-6-P is the varied substrate is 3.3 mM. Glucosamine-6-P is a non-competitive inhibitor with respect to NAD⁺. The apparent Kᵢ values from the intercept and slope replots are 2.3 and 13 mM, respectively. Glucosamine-6-P was not a substrate for the NAD-linked reaction under the conditions used here.

The kinetic constants derived from these studies are listed in Table I. Table II summarizes the inhibition patterns for the NADP- and NAD-linked reactions.

Quenching of Protein Fluorescence by Glucose-6-P and NADP⁺—Both glucose-6-P and NADP⁺ quench the protein fluorescence of the enzyme. Fig. 8A shows a representative plot of the reciprocal of the total NADP⁺ concentration. From plots of this kind the maximum fluorescence quenching for NADP⁺ was found to average 39.5% ± 1.3% from five titrations. Fig. 8B shows the Klotz plot (19) derived from the data in Fig. 8A. Klotz plots were used to obtain values for the dissociation constant (see Table I).

When the data from enzyme fluorescence quenching by glucose-6-P were plotted in double reciprocal form, distinctly biphasic plots were seen, as illustrated in Fig. 9. This suggests that the enzyme contains two classes of glucose-6-P binding sites. Rough estimates of the binding parameters were made from these plots. The dissociation constants for the tight and weak binding sites were 88 ± 33 μM and 1.66 ± 0.62 mM, respectively, and the maximum fluorescence quenching were 16.9 ± 1.7% and 69 ± 22%, respectively (data from three experiments).

Deuterium and Tritium Isotope Effects—The V and V/K deuterium isotope effects were 1.79 and 1.90, respectively (Fig. 10). These values were determined using 3.3 mM glycyglycine buffer, pH 7.5, and 21 Kᵢ NADP⁺. The deuterium isotope effects were also determined using 67 mM Tris/acetate buffer, pH 8.6, and three different NADP⁺ concentrations: 1 Kᵢ, 5 Kᵢ, and 20 Kᵢ. The values determined for both the V and the V/K deuterium isotope effects, using Tris/acetate, varied randomly with NADP⁺ concentration from 1.28 to 1.77 and from 1.17 to 1.43, respectively, showing that the magnitude of the deuterium isotope effect on V or V/K does not significantly depend on NADP⁺ concentration.

The V/K tritium isotope effect was determined by incubating the enzyme with [1⁻²H]glucose-6-P at 79 Kᵢ NADP⁺ in 10 mM glycyglycine, pH 7.5. The reaction was stopped after 35% of the limiting substrate, NADP⁺, was reduced. The NADP⁺ concentration at the time the reaction was terminated corresponded to 51 Kᵢ, thereby ensuring that NADP⁺ remained saturating during the time course of the reaction, a necessity if a true measure of the V/K effect was to be made (24). The specific activity of the [1⁻²H]glucose-6-P used in the experiment was 2.09 × 10⁶ cpm/μmol and the specific activity of the [⁶²H]NADP⁺ produced, separated from the [1⁻²H]glucose-6-P remaining in the reaction mixture, was 7.43 ± 10⁶ cpm/μmol. Performing the calculations as described by Cleland (24), we determined the tritium isotope effect on V/K to be 3.25.

It was calculated that the intrinsic deuterium isotope effect on V is between 5.20 and 6.35, using the tables and equations published by Northrop (25), the correction factor of 1.27 reported for the glucose-6-P dehydrogenase reaction by Schimerlik et al. (26), and the deuterium and tritium isotope effects determined using glycyglycine buffer. Using these values, calculations (27) were performed that indicate that the transfer of hydrogen from glucose-6-P to NAD⁺ limits the

**Table I**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Constant</th>
<th>NADP reaction</th>
<th>NAD reaction</th>
</tr>
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<tr>
<td>Coenzyme</td>
<td>Kᵢ</td>
<td>0.92 μM</td>
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<td></td>
<td>Kᵢ</td>
<td>5.0 μM</td>
<td>15 mM</td>
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<tr>
<td></td>
<td>Kᵢ</td>
<td>2.86 ± 1.5 μM</td>
<td>(5)²</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>Kᵢ</td>
<td>26 μM</td>
<td>0.31 mM</td>
</tr>
<tr>
<td></td>
<td>Kᵢ</td>
<td>49 μM</td>
<td>0.59 mM</td>
</tr>
<tr>
<td></td>
<td>Kᵢ</td>
<td>(see text)</td>
<td>(see text)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Kᵢ, varying coenzyme</td>
<td>1.4 μM</td>
<td>0.64 μM</td>
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<tr>
<td></td>
<td>Kᵢ, varying glucose-6-P</td>
<td>5.7 μM</td>
<td>1.2 μM</td>
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<tr>
<td></td>
<td>Molar activity, kat./ mol of subunit</td>
<td>113</td>
<td>90</td>
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</table>

* Mean ± standard deviation (number of determinations); see text.

*² Not determined.

**Table II**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Inhibitor</th>
<th>Varied substrate</th>
<th>Type of inhibition</th>
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</thead>
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<td>NADP-linked</td>
<td>NADPH</td>
<td>NADP⁺</td>
<td>Competitive</td>
</tr>
<tr>
<td>NADPH</td>
<td>Glucose-6-P</td>
<td>NADP⁺</td>
<td>Competitive</td>
</tr>
<tr>
<td>Glucosamine-6-P</td>
<td>Glucose-6-P</td>
<td>NADP⁺</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>NAD-linked</td>
<td>NADPH</td>
<td>NAD⁺</td>
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<td>NAD⁺</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>Glucosamine-6-P</td>
<td>Glucose-6-P</td>
<td>NAD⁺</td>
<td>Competitive</td>
</tr>
</tbody>
</table>
by the fact that for NADP⁺ the kinetic constants and the $K_r$ value obtained from fluorescence quenching measurements are similar to each other. The simplest mechanism consistent with the experimental results with both the NADP-linked and the NAD-linked reaction is a random one with no dead-end complexes. It is often difficult to distinguish unequivocally among steady state, rapid equilibrium, and partial rapid equilibrium random mechanisms (22, 30). Deuterium and tritium isotope experiments with mammary glucose-6-P dehydrogenase indicate that the transfer of hydrogen from glucose-6-P to NADP⁺ limits the overall reaction rate only 15 to 19%. This fact, our failure to detect any curvature near the ordinate in the initial velocity plots, and the fact that NADPH is a competitive inhibitor with respect to both NADP⁺ and glucose-6-P all suggest that the mechanism is most likely partial rapid equilibrium random, although a steady state random mechanism cannot be excluded. A Theorell-Chance type of mechanism can be ruled out for the NADP-linked reaction by the fact that various steroids are uncompetitive dead-end inhibitors with respect to both NADP⁺ and glucose-6-P (31). The most likely explanation of these inhibition patterns is that the steroids bind to the ternary complex and thus demonstrate the presence of a kinetically significant concentration.

The fact that NADPH inhibits competitively with respect to both NADP⁺ and NAD⁺ indicates that both coenzymes bind to the same enzyme form and that enzyme isomerization is not a kinetically significant step in either mechanism.

The kinetic experiments performed on the NAD-linked reaction of mammalian glucose-6-P dehydrogenase were complicated by a hysteretic activation of the enzyme during the initial part of the assay. The facts that this activation resulted in approximately a 25% increase in both the NAD- and NADP-linked activities and that the enzyme used had a specific activity approximately 25% lower than fully active enzyme suggest that some form of inactive enzyme, perhaps inactive subunits (32), was being reactivated by binding NAD⁺ and glucose-6-P.

Table I contains a summary of the kinetic and binding constants for mammalian glucose-6-P dehydrogenase. It can be seen that NADP⁺ binding is significantly enhanced by glucose-6-P. Tightening of NADP⁺ binding by glucose-6-P is observed in a number of glucose-6-P dehydrogenases which catalyze ordered mechanisms, such as that from rat liver (33) and, to a small extent, that from pig liver (34) and human erythrocytes (35), but not in the glucose-6-P dehydrogenases from human platelets (36), bovine adrenals (37), or L. mesenteroides (12) (glucose-6-P tightens NAD⁺ binding in L. mesenteroides glucose-6-P dehydrogenase). In the case of glucose-6-P dehydrogenase from Saccharomyces carlsbergensis, which operates via a random mechanism, there is no enhancement of NADP⁺ binding by glucose-6-P (38).

In confirmation of the report by Ringler and Hilf (23) we find that there are two classes of glucose-6-P binding sites as revealed by two $K_r$ values. Protein fluorescence quenching studies also show two distinct classes of glucose-6-P binding sites (Fig. 9). The binding constants derived from these studies, although only approximate, suggest that the tight sites are responsible for glucose-6-P binding during the NADP-linked reaction. The two classes of glucose-6-P sites may relate to the two forms of the enzyme, which react preferentially with either NADP⁺ or NAD⁺ and which exist in equilibrium with each other (7), but further studies are needed to examine this question. Kuby et al. (38) observed two classes of glucose-6-P binding sites on glucose-6-P dehydrogenase from S. carlsbergensis using equilibrium dialysis; only the tighter binding sites were revealed by kinetic studies.

The results of fluorescence quenching experiments are con-

<table>
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<tr>
<th>Glucose-6-P</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td></td>
<td>No NADPH</td>
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</tr>
<tr>
<td></td>
<td>NADPH</td>
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</tr>
<tr>
<td>mM</td>
<td>nmol/min</td>
<td>nmol/min</td>
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<tr>
<td>0.030</td>
<td>3.42</td>
<td>1.03</td>
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<td>0.075</td>
<td>1.54</td>
<td>61</td>
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<tr>
<td>0.152</td>
<td>1.88</td>
<td>60</td>
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<td>0.304</td>
<td>4.62</td>
<td>2.31</td>
</tr>
<tr>
<td>0.456</td>
<td>1.03</td>
<td>2.31</td>
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</table>

Table III

Effect of glucose-6-P concentration on NADPH inhibition of mammary glucose-6-P dehydrogenase

Reaction mixtures contained glucose-6-P dehydrogenase (157 ng), 20 μM S-NADP⁺, the indicated concentrations of glucose-6-P, with and without NADPH as indicated, and either 67 mM Tris/acetate, pH 8.6 (Experiment 1), or Krebs-Ringer bicarbonate (Experiment 2). The reduction of S-NADP⁺ was monitored at 400 nm.

**DISCUSSION**

Initial velocity studies with lactating rat mammary glucose-6-P dehydrogenase demonstrate that this enzyme catalyzes a sequential mechanism with either NADP⁺ or NAD⁺ as the coenzyme. Inhibition studies with both NADPH and glucose-samine-6-P indicate that the coenzyme (NADP⁺ or NAD⁺) and glucose-6-P add to the enzyme in random order. For the NADP-linked reaction, random-order addition is supported overall rate of the reaction only 15% to 19%.

Reversal of NADPH Inhibition by Glucose-6-P—A random mechanism predicts that either NADP⁺ or glucose-6-P should reverse NADPH inhibition of mammary glucose-6-P dehydrogenase. A critical test of this prediction, reversal of NADPH inhibition by glucose-6-P, was performed as follows. In order to facilitate the measurement of coenzyme reduction in the presence of a large excess of NADPH, such as that normally found in mammary gland cells (28), S-NADP⁺ was used instead of NADP⁺ and the reaction was monitored at 400 nm. S-NADP⁺ was found to have an apparent $K_r$ of 2.2 μM, measured under standard assay conditions in the presence of 0.272 mM glucose-6-P, and a molar activity of 47.4 kat/mol. Thus, S-NADP⁺ has a somewhat lower affinity for the enzyme than NADP⁺ and is a less effective oxidant for glucose-6-P (see Table I). In the first experiment illustrated in Table III, the rate of S-NADP⁺ reduction was measured at various concentrations of glucose-6-P in the absence and presence of 130 μM NADPH in 67 mM Tris/acetate, pH 8.6. The concentration of S-NADP⁺ (20 μM) was considered to be equivalent (based on relative $K_r$ values) to 5.5 μM NADP⁺ and, therefore, to provide an effective ratio of reduced/oxidized coenzyme of 15.3. These conditions approximate those found by McLean in the mammary gland of a rat on the 18th day of lactation (29). The glucose-6-P concentration ranged from 1.17 to 17.5 times its $K_r$ concentration for the NADP-linked reaction or from 0.2 to 3.0 times the concentration found by Greenbaum et al. in isolated rat mammary gland cells incubated for 60 min under specified conditions (28). Increasing glucose-6-P concentrations reduce the inhibition by NADPH from 70% at 30.4 μM glucose-6-P to 38% at 456 μM glucose-6-P (Table III). Inclusion of 7.7 mM NAD⁺ and 2.8 mM NADH in the reaction mixtures containing S-NADP⁺ and NADPH had no effect on rates of reaction. In the second experiment, performed in Krebs-Ringer bicarbonate buffer (pH 7.6), the effect of 0.6 mM NADPH was tested; this provided an effective ratio of reduced/oxidized coenzyme of 70.6, approximately that found by Greenbaum et al. in isolated rat mammary cells (28). Partial reversal of NADPH inhibition was again observed as the glucose-6-P concentration was raised (Table III).
sistent with the kinetic data, indicating that the enzyme binds its substrates in a random fashion. NADP⁺ and NADPH have been shown to quench the protein fluorescence of other glucose-6-P dehydrogenases (12, 28, 38), but the quenching of enzyme fluorescence by glucose-6-P has not been previously reported for any glucose-6-P dehydrogenase. The fluorescence quenching by glucose-6-P could not have been caused by the NADP⁺ contained in the enzyme because, after the initial addition of glucose-6-P, which reduces the NADP⁺ to NADPH, the fluorescence of the enzyme is progressively quenched by further additions of glucose-6-P. Also the kinetic data give no evidence that an enzyme-glucose-6-P-NADPH dead-end ternary complex can be formed since NADPH is a competitive inhibitor with respect to glucose-6-P (Fig. 3). The quenching of protein fluorescence by NADP⁺ and glucose-6-P most likely is due to a conformational change altering the environment of tryptophan residues or a change in the fluorescence lifetime of the tryptophan residues.

The fluorescence quenching data are consistent with the binding of 1.0 mole of NADP⁺/glucose-6-P dehydrogenase active site (see "Experimental Procedures"). Holten et al. (40) using equilibrium dialysis to show that 2.4 molecules of NADP⁺/enzyme subunit bound to rat liver glucose-6-P dehydrogenase with a Kd of 0.6 µM. Glucose-6-P did not bind to the enzyme, confirming the ordered addition of substrates proposed by Thompson et al. (33). Kuby et al. (38) showed that both glucose-6-P and NADP⁺ bound to S. carlsbergensis glucose-6-P dehydrogenases, confirming the proposed random mechanism.

Determination of deuterium and tritium isotope effects must be made under similar conditions because these effects may be sensitive to changes in pH, temperature, and other factors. Also, with enzymes such as glucose-6-P dehydrogenases where there is an isotope effect on the equilibrium constant of the enzymatic reaction, a single value for the intrinsic isotope effect cannot be calculated (27). This is due to differences in the stiffness of the C–H bond for the transferable hydrogen in glucose-6-P compared to NADPH (26). Instead, a range of values is calculated using a correction factor that takes into account the isotope effect on the equilibrium constant of the enzymatic reaction (25). Albory and Knowles (41) have pointed out that the usefulness of Northrop’s method is greatest when the observed deuterium isotope effect is approximately 2. For the NADP-linked reaction of mammary glucose-6-P dehydrogenase, a deuterium isotope effect of 1.79 was found for V (Fig. 10). Calculations performed using the deuterium and tritium isotope effects determined for the lactating rat mammary gland enzyme indicate that the transfer of hydrogen from glucose-6-P to NADP⁺ limits the overall rate of the reaction only 15 to 19%. Surprisingly, in view of similar studies with other dehydrogenases (27), the magnitude of the deuterium isotope effects on V and V/K showed no dependence on NADP⁺ concentration.

The results of our kinetic studies on mammary glucose-6-P dehydrogenase may have important implications for the regulation of this enzyme. First, the enhancement of NADP⁺ binding by glucose-6-P helps in catalyzing the reaction in vivo, where the NADP⁺ concentration is relatively low (28). Second, the fact that binding of glucose-6-P and NADP⁺ can occur in random order should enable glucose-6-P to compete with NADPH and, therefore, to counteract inhibition by NADPH, which is present in much higher concentration than NADP⁺ (28). Support for this prediction is provided by experiments in which increasing glucose-6-P concentrations were effective in partially reversing the inhibition by NADPH at high ratios of NADPH to S-NADP⁺ (Table III). Although it is apparent that glucose-6-P reverses NADPH inhibition under our assay conditions (Fig. 3), we wished to establish this under more physiological conditions. It should be noted, also, that, even at low glucose-6-P concentration, inhibition by an effective NADPH to NADP⁺ ratio of over 70 was not complete. This is in striking contrast to the finding by Eggleston and Krebs (1) that rat liver glucose-6-P dehydrogenase is almost totally inhibited at NADPH to NADP⁺ ratios above 6. In those other mammalian glucose-6-P dehydrogenases which have been examined kinetically, all of which bind NADP⁺ first, glucose-6-P cannot overcome NADPH inhibition. Schachet and Squire (37) proposed a random mechanism for beef adrenal glucose-6-P dehydrogenase, but, as pointed out elsewhere (2), this conclusion was not supported by their data, which suggest, instead, that the mechanism is probably ordered. Our results are the first, unequivocal demonstration of a random mechanism for any mammalian glucose-6-P dehydrogenase. It may be relevant, in this connection, that the mammary enzyme was the only glucose-6-P dehydrogenase among eight tested in rat tissues which failed to respond to dehydrogenation by glutathione, suggesting to Eggleston and Krebs that regulation of this enzyme may proceed via a different mechanism (1).

It is interesting that all mammalian glucose-6-P dehydrogenases examined display some activity with NAD⁺ and that a number of microbial glucose-6-P dehydrogenases react with both coenzymes under physiological conditions (2). Glucose-6-P dehydrogenase from L. mesenteroides, for example, reacts with both NAD⁺ and NADP⁺ in vitro (4) as well as in vivo (42). Like the rat mammary enzyme, L. mesenteroides glucose-6-P dehydrogenase exists as two conformational forms which react with NAD⁺ and NADP⁺, respectively; isomerization between these forms is an obligatory step in its kinetic mechanism (5), whereas no such isomerization can be detected kinetically for the mammary enzyme. Glucose-6-P promotes the binding of NAD⁺, but not of NADP⁺, by L. mesenteroides glucose-6-P dehydrogenase (12) and appears to play a role in regulating the relative reactivities of the NAD- and NADP⁺-linked reactions of this enzyme (6, 43). Thus, glucose-6-P may play a regulatory role in different glucose-6-P dehydrogenases via different mechanisms.

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