REGULATION OF PENTOSE PHOSPHATE PATHWAY DEHYDROGENASES BY $NADP^+$/NADPH RATIOS

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SUMMARY: Equilibrium dialysis indicates that rat liver glucose-6-P dehydrogenase binds two molecules of $NADP^+$ per subunit with a dissociation constant of 0.6×10^{-6} M. The $NADP^+$ free enzyme will not bind glucose-6-P indicating a compulsory order of substrate binding. Development of an isotopic assay allowed a direct measurement of the effect of physiological alterations in the $NADP^+$/NADPH ratio on the activity of glucose-6-P and 6-phosphogluconate dehydrogenases. A combination of enzyme induction and altered $NADP^+$/NADPH ratios could produce 30-50 fold changes in the capacity of these enzymes to produce NADPH during alterations in the nutritional state of the animal.

A comparison of equilibrium constants and mass action ratios suggests that glucose-6-P dehydrogenase (E C 1.1.1.49) may be a control point in the oxidative portion of the pentose phosphate pathway (1). There are at least two distinct types of regulation of the pentose phosphate pathway dehydrogenases. In liver, the concentrations of both glucose-6-P dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD, E C 1.1.1.44) change drastically with the nutritional state of the animal (2-6). The $NADP^+$/NADPH ratio also changes under these conditions (1,7) and this ratio has been shown to regulate the activity of G6PD (8,9) and 6PGD (10). Thus, both induction of enzymes and modulation of enzyme activity at the substrate level may be important in regulating the capacity of the pentose phosphate pathway dehydrogenases to produce NADPH for biosynthetic reactions. We report here the use of an isotopic assay which allowed a direct measurement of the regulation of pentose phosphate pathway dehydrogenase activity at $NADP^+$ and NADPH levels observed *in vivo* at different nutritional states.

METHODS: Assays for G6PD, 6PGD, and protein were as previously described (5, 10). Here we report some properties of these enzymes under more physiological conditions of pH 7.4, 37° and a constant ionic strength of 0.25. One unit of enzyme assayed at pH 8 and 30° equals 1.47 and 1.51 units of 6PGD and G6PD respectively when assayed at pH 7.4 and 37°.

Preparation of G6PD free of $NADP^+$. Purified G6PD (11) was incubated with 3×10^{-3} M glucose-6-P and 10 mg of bovine serum albumin for 2 hr at 0° and passed through a Sephadex G-25 column equilibrated with 0.05 M sodium acetate buffer pH 7.4. Under these conditions bound $NADP^+$ was converted to NADPH and separates from the enzyme.

Equilibrium dialysis. A multichamber apparatus as described by Furlong *et al.*, (12) was used and chambers were separated by dialysis tubing. One side of the chamber held 0.1 ml of [^{14}C]$NADP^+$ (specific activity 22 μ Ci/μmole) in 0.05 M sodium acetate-bovine serum albumin (5 mg/ml) pH 7.4. The opposite side held

0.1 ml of $NADP^+$ free G6PD in bovine serum albumin (total protein equals 5 mg/ml). Equilibration was for 16 hr at 4°.

Assay of G6PD and 6PGD at physiological ratios of $NADP^+$/NADPH. Each assay mixture contained 300 µmoles of Tris-acetate buffer pH 7.4, 1.7 µmoles of magnesium acetate and sodium acetate pH 7.4 to a constant ionic strength of 0.25. The $NADP^+$ + NADPH was kept constant at 0.5×10^{-4} M and the ratio $NADP^+$/NADPH was varied between 0.0025 and 0.02. Each reaction mixture contained 3.8×10^{-9} moles of [^{14}C]$NADP^+$ (specific activity 25 µ Ci/µmole) as a portion of the total $NADP^+$ present. Sugar phosphates were present at 3×10^{-4} M. Reaction mixtures were incubated at 37° for 10 min prior to starting the reaction by adding enzyme. One-half ml aliquots were withdrawn at 30 sec, 3, 6, 9 and 12 min, added immediately to a test tube containing 1.5 µmoles of non-radioactive $NADP^+$, and boiled for 3 min. Each sample was then diluted to 5 ml with water and put onto a DEAE-cellulose column (0.9 x 2 cm) for separation of $NADP^+$ from NADPH by the method of Silverstein (13). The $NADP^+$, NADPH and [^{14}C]$NADP^+$ were all purified as described by Silverstein (13) prior to use in these experiments. In all cases the reactions were linear for 12 min and converted less than 10% of the $NADP^+$ present.

Materials. The [^{14}C]$NADP^+$ and [^{14}C]glucose-6-P (specific activity 107 µ Ci/µmole) were purchased from Amersham/Searle. Dialysis tubing was purchased from Arthur H. Thomas Co. All other materials were described previously.

Results and Discussion

The regulation of pentose phosphate pathway dehydrogenase activity at the substrate level appears to be exerted primarily by the $NADP^+$/NADPH ratio (8-10). We have used kinetic data and the appropriate rate equation to calculate the activity of 6PGD at several $NADP^+$/NADPH ratios observed *in vivo* (10). While attempting to obtain similar data for G6PD it became obvious that extrapolating kinetic data to *in vivo* conditions could lead to serious errors. For example, although the kinetic data suggested that G6PD had a random order of substrate binding (unpublished observations), equilibrium dialysis experiments indicated an ordered mechanism. Figure 1 shows a Scatchard plot (14) for the binding of $NADP^+$ to G6PD. Approximately 2.4 $NADP^+$ bind to each subunit with a dissociation constant of 0.6×10^{-6} M. There may have been up to 20% inactive enzyme in this preparation and if it contributed to the binding of $NADP^+$ this would overestimate the number of binding sites per subunit. Therefore, we believe that 2 moles of $NADP^+$ bound to each mole of G6PD subunit is a reasonable estimate. In a separate experiment 1 mg of $NADP^+$ free G6PD was equilibrated with 5×10^5 cpm of [^{14}C]glucose-6-P at a concentration of 2.5×10^{-5} M. With a Km for glucose-6-P equal to 8×10^{-5} M under these conditions (unpublished observation), at pH 7.4, one would have expected about 11,000 cpm of glucose-6-P to bind to G6PD if there was a random order of substrate binding. In this experiment there was no significant amount of glucose-6-P bound to the enzyme and this strongly suggests that there is an ordered mechanism of substrate binding to G6PD. These uncertainties about the correct kinetic mechanism for G6PD coupled with the recent demonstration by Cavalieri and Sable (15) that high

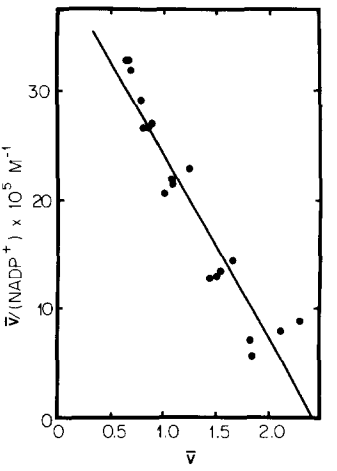

Figure 1. Scatchard plot for the binding of $[^{14}C]NADP^+$ to G6PD. The concentration of $[^{14}C]NADP^+$ was varied between 0.78 and 6.25 x 10^{-6} M. G6PD free of $NADP^+$ (specific activity 256) was present at a concentration of 0.51 x 10^{-10} moles of subunit of molecular weight 64,000 (12). This represents 0.92 units of G6PD activity. The line was calculated by the method of least squares. All other details are given in the methods section.

concentrations of NADPH in spectrophotometric assays can lead to large errors in calculating inhibition constants for NADPH, led us to develop a more direct and accurate method to assay G6PD and 6PGD at physiological ratios of $NADP^+$/ NADPH.

A discontinuous assay followed the rate of conversion of $[^{14}C]NADP^+$ to $[^{14}C]$NADPH under conditions which approach physiological. Since even small amounts of $NADP^+$ contaminating NADPH solutions would lead to large errors in $NADP^+$/NADPH ratios of 0.02-0.0025, the $NADP^+$ and NADPH were repurified for these experiments. Figures 2 and 3 show the effect of increasing $NADP^+$/NADPH ratios on the activity of G6PD and 6PGD, respectively. In both cases the enzymes are extremely inhibited at low $NADP^+$/NADPH ratios and the activity of both enzymes is very responsive to changes in this ratio.

In Table I we calculate the capacity of the pentose phosphate pathway dehydrogenases to produce NADPH at three different nutritional states. The $NADP^+$/ NADPH ratios in rat liver cytoplasm as measured by Greenbaum et al. (1) were approximately 0.006, 0.01 and 0.02 for fasted animals or rats fed control or lipogenic diets respectively. The % Vm observed at these three $NADP^+$/NADPH ratios was calculated for both dehydrogenases from the data in Figures 2 and 3. Thus, 0.00485 units of G6PD will produce 0.00485 μmoles of NADPH/min/ml of assay mixture with saturating concentrations of both substrates and this approximates

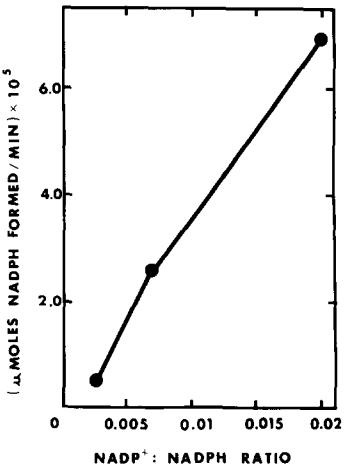

Figure 2. The effect of alterations in the $NADP^+$/NADPH ratio on the activity of G6PD. The conditions for the assay were kept as close to physiological as possible: A constant ionic strength of 0.25, pH 7.4, 37° C, $NADP^+$ plus NADPH equals 0.5 x 10^{-4} M, and the $NADP^+$/NADPH ratios were 0.0025, 0.007 and 0.02. The reaction was initiated with 0.00485 units of $NADP^+$ free G6PD (specific activity 256) per ml of assay mixture. The enzyme activity is based upon assay at 37° and pH 7.4. Further details are provided in the methods section.

Vm. At a $NADP^+$/NADPH ratio of 0.02, 0.00485 units of enzyme produced 6.9 x 10^{-5} μmoles of NADPH/min/ml of assay mixture and this represents 1.4% of Vm. This demonstrates that at physiological concentrations of glucose-6-P, $NADP^+$, and NADPH, G6PD is over 98% inhibited. Figure 3 illustrates that the same degree of inhibition holds for 6PGD.

The approximate capacity of each enzyme to produce NADPH in liver was calculated from the amount of each enzyme found in liver and the % Vm observed at each nutritional state. These calculations illustrate that the combination of alterations in the $NADP^+$/NADPH ratio and in the amount of enzyme present with the nutritional state of the animals can produce a 30-50 fold variation in the capacity for each enzyme to produce NADPH.

Both factors would appear to be important but would function on different time scales. Presumably the $NADP^+$/NADPH ratio could change rapidly with alterations in the demand for reducing equivalents by the cell. A rapid rate of fatty acid synthesis, for example, could utilize NADPH rapidly and increase the $NADP^+$/NADPH ratio. This would rapidly increase the activity of both dehydrogenases and tend to restore the $NADP^+$/NADPH ratio back to its normal value. Alterations in the amount of enzyme present occur in 2-4 days and are due to alterations in the rates of synthesis for both enzymes (5,6). Thus, a long

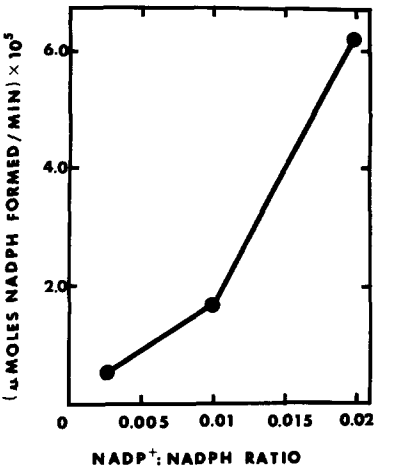

Figure 3. The effect of alterations in the $NADP^+$/NADPH ratio on the activity of 6PGD. All details are identical to those described in the legend to Figure 2 except that 0.00485 units of 6PGD (specific activity 40) and $NADP^+$/NADPH ratios of 0.0025, 0.01 and 0.02 were used.

term demand for high rates of fat synthesis, such as in rats fed a lipogenic diet, increases the concentration of both dehydrogenases.

Since both dehydrogenases are extensively inhibited under in vivo conditions one must ask if the capacity to produce NADPH calculated in Table I appears sufficient to meet the needs of the cell. Windmueller and Spaeth (16) have estimated the maximum rate of fatty acid synthesis in liver to be 0.12 μmoles/g liver/min. If one assumes that 50% of the 14 NADPH molecules required for the synthesis of one molecule of palmitic acid comes from the pentose phosphate pathway dehydrogenases, then a rate of fatty acid synthesis of 0.12 μ moles/min/g liver would represent a requirement for 0.84 μmoles of NADPH/min/g liver from the pentose phosphate pathway dehydrogenases. This is reasonably close to our estimated capacity of these enzymes to produce NADPH in rats fed a lipogenic diet. In starved rats the rate of fatty acid synthesis decreases about 50 fold so there also seems to be sufficient capacity to produce NADPH in these animals. However, the estimates calculated in Table I must be regarded as maximum estimates since they are based upon initial reaction velocity measurements. If the presence of products or some other cellular constituent inhibits either enzyme then some mechanism for the activation of these enzymes may be required. The recent discovery by Eggleston and Krebs (9) that oxidized glutathione could reverse the inhibition of G6PD by NADPH might represent a means to increase the activity of these enzymes in vivo.

Sapag-Hagar et al. (8) have reported that at $NADP^+$/NADPH ratios encountered

in vivo there is an apparent physiological unbalance in liver because the ratio 6PGD/G6PD activity is as low as 0.01. These results do not agree with those reported here. In fact, there appears to be a remarkable balance in the capacity of the two enzymes to produce NADPH under all of the conditions listed in Table I. The combination of alterations in enzyme synthesis and $NADP^+$/NADPH ratios with changes in the nutritional state of the animal appear to produce effects on both dehydrogenases which insure a balance in the capacity of each to provide reducing equivalents to meet the changing demands of the cell.

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