Unusual Kinetic Transition in Honeybee Glyceraldehyde Phosphate Dehydrogenase*

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ABSTRACT: D-Glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) (GPDH) has been isolated from honeybees (*Apis mellifera*) and its kinetic properties have been examined. Titration of the enzyme with either NAD^+ or glyceraldehyde 3-phosphate (G 3-P) yields kinetic curves which appear normal over a NAD^+ concentration range of 0 to 16×10^{-5} M; however between 16×10^{-5} and 18×10^{-5} M NAD^+ a kinetic transition is observed. This transition may be abolished by titrating the crystalline honeybee enzyme with NAD^+ in the presence of 4×10^{-4} M G 3-P instead of 43×10^{-4} M G 3-P, by performing the experiment with enzyme that has been partially inhibited with *N*-ethylmaleimide, or by preincubation of the enzyme with 43×10^{-4} M G 3-P plus 1×10^{-3} M NAD^+. Preincubation with either substrate alone does not abolish the transition. Equilibrium dialysis experiments have been performed with honeybee GPDH and the dissociation constants for the four NAD^+ binding sites, in the absence of G 3-P, have been determined. On the basis of experimental results it is concluded that the sigmoid curve observed in the region of 16×10^{-5} to 18×10^{-5} M NAD^+ in the saturation kinetics is not due to a cooperative interaction of the GPDH subunits. It is postulated that a kinetic activation of a metastable state of the enzyme may be responsible for the observed transition.

Recent reports in the literature have described several enzymes which display saturation curves characterized by an intermediary plateau region in the "zero time" velocities (Corwin and Fanning, 1968; Levitski and Koshland, 1969; and Somero, 1969).

Teipel and Koshland (1969) have attempted to analyze curves of this type using a general model based on cooperative interactions, which also assumes that the rate of equilibration between ligand and enzyme is more rapid than the catalytic rate. Although they were able to generate mathematically the intermediary plateau by appropriate choices of parameters, certain of the experimental curves, the most notable case being CTP synthetase, cannot be reproduced quantitatively if the number of ligand binding sites chosen for purposes of calculation is restricted to a reasonable value. Thus, the apparent "cooperativity" exhibited in the saturation curve is clearly greater than the model permits when one employs a reasonable number of binding sites. They concluded that in these cases "either the rate of ligand binding or the rate of conformational change within the enzyme are comparable with or slower than the catalytic process." They also pointed out that it was not possible to generate curves with an intermediate plateau region from the types of models proposed by Sweeney and Fisher (1968) based on nonallosteric effects. It would appear therefore that certain of the anomolous kinetics associated with what have been regarded as allosteric proteins cannot be rationalized by popular models.

Studies of the kinetic properties of glyceraldehyde phosphate dehydrogenase have suggested that this enzyme possesses certain cooperative characteristics. Kirschner *et al.* (1966) have shown that yeast GPDH exhibits hyperbolic kinetics at 20° but weakly sigmoid kinetics at 50°. More recently Conway and Koshland (1968) have proposed a mechanism of negative cooperativity with respect to NAD^+ binding to explain the kinetics observed for rabbit muscle GPDH. However neither the yeast enzyme nor the rabbit muscle enzyme was observed to exhibit an intermediate plateau region.

It was reported earlier (Gelb and Nordin, 1969a) that the GPDH from both *Camponotus pennsylvanicus* (carpenter ant) and *Apis mellifera* (honeybee) do exhibit intermediary plateau regions in their saturation curves. This report details kinetic observations on GPDH from the honeybee. The intermediary plateau region is also clearly beyond interpretation in terms of the usual models, but experiments have been performed which allow a perturbation of the plateau region.

Materials and Methods

DL-Glyceraldehyde 3-phosphate (G 3-P)[1] diethyl acetal, monobarium salt, β-NAD^+, β-NADH, ATP, fructose 1,6-diphosphate, *N*-ethylmaleimide, idooacetic acid, rabbit muscle GPDH, and aldolase were purchased from Sigma Chemical Company. The DL-G 3-P diethyl acetal was converted into the free acid before use (Sigma Chemical Company, 1961). D-G 3-P was prepared using aldolase, by the method

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[1] Abbreviations used are: G 3-P glyceraldehyde 3-phosphate; GPDH, glyceraldehyde 3-phosphate dehydrogenase; NEM, *N*-ethylmaleimide.

of Hall (1960). [^{14}C]β-NAD$^+$ (carbonyl labeled) was purchased from Amersham–Searle Company and had a specific radioactivity of 20.6 mCi/mmole. Crystalline 3-phosphoglyceric acid kinase and 3-phosphoglyceric acid were obtained from Calbiochem. Sephadex gels were purchased from Pharmacia Fine Chemicals Inc. Honeybees were a gift from the Entomology Department, University of Massachusetts. The molecular weight of the honeybee enzyme was determined by the method of Whitaker (1963) using Sephadex G-200. Polyacrylamide gel electrophoresis was performed using a modified procedure of Hedrick and Smith (1968). Protein concentrations were determined spectrophotometrically at 280 mμ. Sedimentation values were obtained using a Spinco Model E ultracentrifuge at 16°.

Glyceraldehyde Phosphate Dehydrogenase Assay. Activity measurements in the direction of G 3-P to 1,3-diphosphoglyceric acid were performed by the method of Velick (1955). The reaction mixture contained 50 mM sodium pyrophosphate, 5 mM sodium arsenate, 1 mM EDTA, pH 8.5, 1 mM NAD$^+$, and 4.3 mM G 3-P[2] in a final volume of 1 ml. Assays were performed using a Gilford Model 240 recording spectrophotometer. The absorbance change at 340 mμ between 10 and 30 sec was extrapolated to zero time. Under these conditions one unit of activity is defined as a change of 0.01 optical density unit per minute at 25° and specific activity as units per optical density unit at 280 mμ (A_{280}). The reverse reaction starting with 1,3-diphosphoglyceric acid was studied by the method of Wu and Racker (1959). The assay contained 50 mM Tris, 9 mM $MgCl_2$, pH 7.4, 15 mM ATP, 25 mM 3-phosphoglyceric acid, and 2.5 μg/ml of 3-phosphoglyceric acid kinase. Increments (5 μl) of 50 mM NADH were added to the 1-ml reaction mixture and the rate of decrease in absorbance at 340 mμ was observed.

Stopped-flow experiments were performed using a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corp., Palo Alto, Calif.) equipped with a Tektronix storage oscilloscope (Tektronix Inc., Portland, Ore.). The assay system contained standard assay buffer pH 9.0, 1.76 mM G 3-P, 2.5 μg/ml of honeybee GPDH, and varying amounts of NAD$^+$ in a final reaction volume of 0.2 ml. Sensitivity was varied from 40 to 160 mV full scale and time of recording from 0.2 to 0.5 sec per cm. Time constants were always kept smaller than 0.1 of the scanning time. The offset adjustment was used to give an initial per cent transmittance near 100% at the different millivolt settings employed. Transmittance data were obtained directly from photographs of the oscilloscope pattern and converted into optical density with the aid of a computer programmed for this purpose.

Preparation of Honeybee GPDH. GPDH was obtained from honeybees, *Apis mellifera*, by a slight modification of the procedure of Marquardt *et al.* (1968). In the present studies whole honeybees were killed by freezing. They were then ground in an Omni-Mixer and column chromatography was performed with DEAE-Sephadex A-25 in place of DEAE-cellulose.

Equilibrium Dialysis. Equilibrium dialysis experiments were performed in a 0.3-ml dialysis cell at 4° for 20 hr. [^{14}C]NAD$^+$ was diluted with appropriate amounts of unlabeled NAD$^+$ to obtain the desired chemical concentration and specific radioactivity. Specific radioactivities were then determined by enzymatically reducing the diluted [^{14}C]NAD$^+$ with rabbit muscle GPDH and assaying for NADH using an extinction coefficient of 6.22×10^3 l. mole^{-1} at 340 mμ (Murdock and Koeppe, 1964). Radioactivity measurements were made in a Tri-Carb liquid scintillation spectrometer. For the separation of NAD$^+$ from the enzyme, acid-washed Norit A was equilibrated with 0.5 mM EDTA, pH 6.5, and poured into a column, 0.7 × 2.0 cm. The column was washed with several volumes of 0.5 mM EDTA and the enzyme solution passed through the column (Murdock and Koeppe, 1964). The enzyme was then desalted on a Sephadex G-25 column (1.3 × 15 cm) which was previously equilibrated with 0.05 M sodium pyrophosphate and 1 mM EDTA, pH 8.5, at 22°. This also served to equilibrate the enzyme in the equilibrium dialysis buffer. Protein concentrations were determined at 280 mμ using extinction coefficients of 1.06 and 0.815 cm^2 mg^{-1} for holo- and apoenzyme, respectively (Murdock and Koeppe, 1964; Marquardt *et al.*, 1968). Each dialysis was performed in quadruplicate and the average values were used in calculating dissociation constants.

[2] All G 3-P concentrations given are for the DL racemic mixture unless stated otherwise.

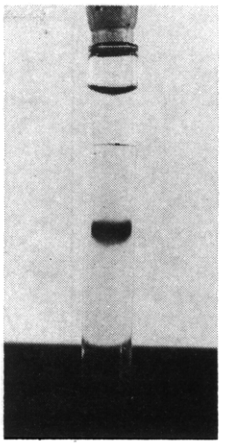

FIGURE 1: Disc gel electrophoresis of crystalline honeybee glyceraldehyde phosphate dehydrogenase. The gel contained 75 μg of protein and was run at pH 8.3 for 1 hr with a constant current of 4 mA.

Results

The purification procedure for honeybee GPDH is summarized in Table I. The final crystals were white with no trace of the yellow color observed by Marquardt *et al.* (1968). Specific activities for the purified enzyme ranged between 38,000 and 80,000 units per A_{280}; these values correspond to 61 and 125 μmoles/min per mg and are within the range of specific activities for GPDH isolated from other sources (Allison and Kaplan, 1964; Trentham, 1968). The highest specific activities were obtained with freshly recrystallized enzyme. All preparations of the purified enzyme were examined by disc gel electrophoresis at pH 8.3 and in all cases were found to migrate as a single band. Figure 1 shows a typical result obtained with crystalline honeybee GPDH of specific activity 40,000 units/A_{280}. Attempts to perform disc gel electrophoresis at pH 4.3 were unsuccessful because of precipitation of the enzyme at this pH.

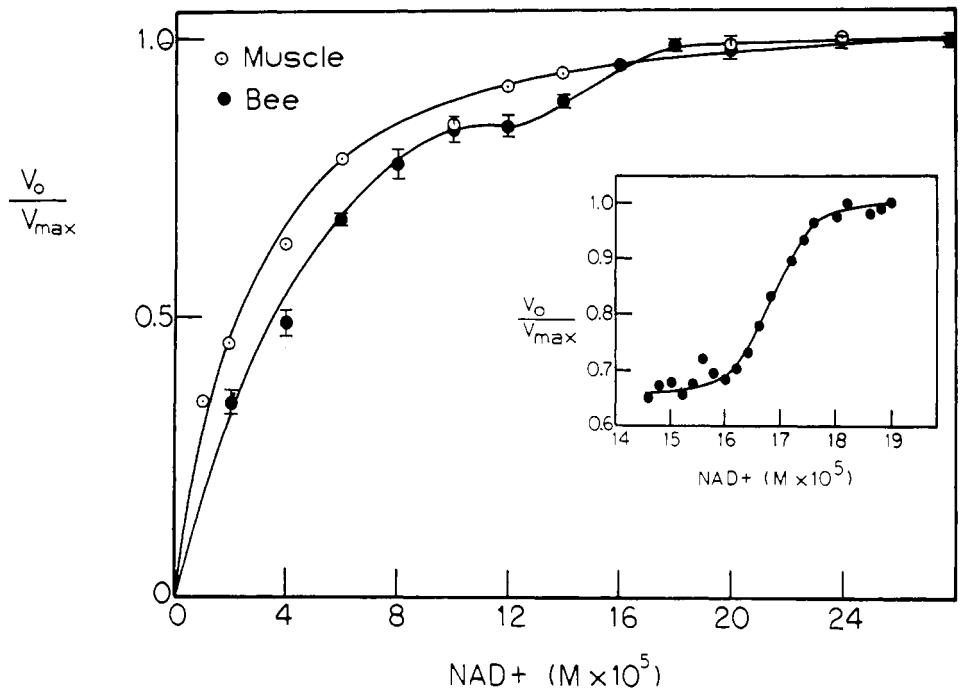

FIGURE 2: Comparison of the saturation kinetics of rabbit muscle and honeybee glyceraldehyde phosphate dehydrogenases. Conditions were: 50 mM pyrophosphate, 5 mM arsenate, 1 mM EDTA, pH 9.0, 4.3 mM DL-G 3-P, in a final volume of 1 ml. All reactions were initiated by the addition of NAD^+. All assays were performed at 30°; (O——O) rabbit muscle enzyme, (●——●) honeybee GPDH; the error bars represent the maximum deviation for four individual assays. The inset shows a more detailed representation of the transition area for honeybee enzyme obtained in a separate experiment. The absolute maximum velocities for both enzymes were identical.

The enzyme's molecular weight was found to be 140,000 by Sephadex gel chromatography (Whitaker, 1963) which is the value reported for the enzyme from other sources (Allison and Kaplan, 1964) and from honeybees (Marquardt *et al.*, 1968). The enzyme, which was homogeneous on Sephadex G-200 chromatography and in the ultracentrifuge, had a sedimentation constant of 7.5 S. Marquardt *et al.* (1968) have previously reported a value of 7.6 S for the honeybee enzyme.

TABLE I: Purification of D-Glyceraldehyde Phosphate Dehydrogenase from Honeybees; A_{280} = optical density at 280 mμ.[a]

Procedures	Protein (A_{280})	Total Activity (Units × 10^6)	Specific Activity (Units/A_{280})	Per Cent Recovery
Crude extract	34,000	11.3	300	100
60–100% $(NH_4)_2SO_4$	3,100	9.9	3,200	88
DEAE-Sephadex A-25	1,840	9.0	4,900	80
First crystals	425	5.6	13,000	50
Second crystals	132	4.3	31,000	40
Sephadex G-100	132	4.6	35,000	42
Third crystals	112	4.2	38,000	39

[a] Units of activity and specific activity are defined in Methods and Materials section.

Kinetic Studies. All kinetic experiments were performed with honeybee and rabbit muscle GPDH which had specific activities of approximately 40,000 units/A_{280}. Each assay contained between 1 and 3 μg of enzyme per ml unless otherwise indicated.

Figure 2 shows a comparison between the kinetic curves for rabbit muscle and honeybee GPDH. As can be seen the honeybee enzyme exhibits gross hyperbolic shape below 16 × 10^{-5} M NAD^+, and a seemingly cooperative transition between 16 × 10^{-5} and 18 × 10^{-5} M NAD^+. The rabbit muscle enzyme does not show this latter phenomenon. Repeated recrystallization of the honeybee enzyme did not eliminate the intermediary plateau region. When the reaction was studied in the reverse direction (1,3-diphosphoglyceric acid + NADH → G 3-P + NAD^+) the transition occurred over the NADH concentration range of 1.7 × 10^{-3} to 2.2 × 10^{-3} M. The relative increase in initial velocity from the first apparent V_{max} to the second V_{max} over this NADH concentration range was approximately 25%. In order to make a more precise evaluation of the transition region a separate experiment was run in the range 14.5 × 10^{-5} to 19.0 × 10^{-5} M NAD^+ and more experimental points were obtained. These data are shown in the inset portion of Figure 2. The initial velocities show an increase of 30 to 35% from the first apparent V_{max} to the second V_{max}. The transition from the first to the second V_{max} (ΔV_{max}) is greater as shown in the inset when compared to the saturation over a wide range of NAD^+ concentration. A range of ΔV_{max} values of from 15 to 35% have been observed with variation occur-

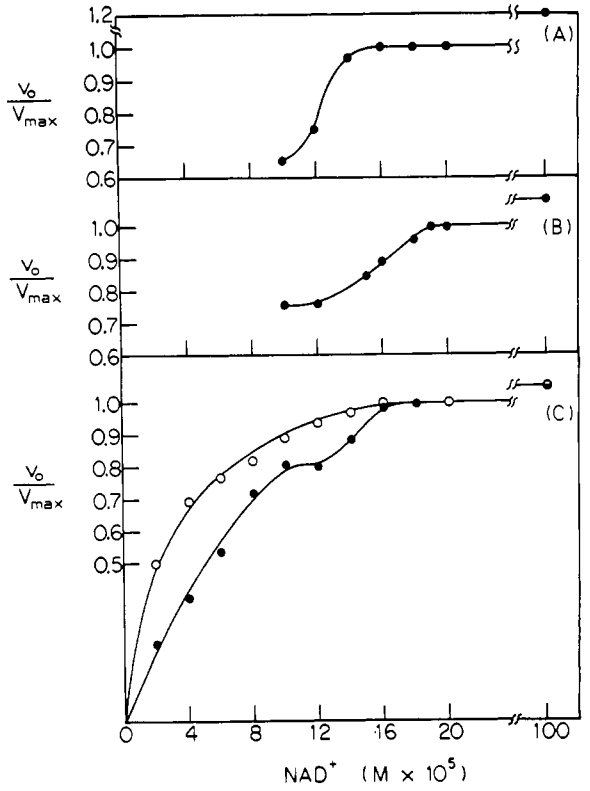

FIGURE 3: The titration of rabbit muscle GPDH and honeybee GPDH with NAD^+ in the Durrum–Gibson stopped-flow spectrophotometer: rabbit muscle GPDH (O--O), honeybee GPDH (●--●). Each experiment was conducted by stopped-flow mixing of an enzyme, NAD^+ solution with a G 3-P solution. Final concentrations were: enzyme 2.5 μg/ml, and DL-G 3-P 1.76 mM. Increasing quantities of NAD^+ were employed to obtain its desired final concentration. Each point was obtained from four superimposable oscilloscope traces.

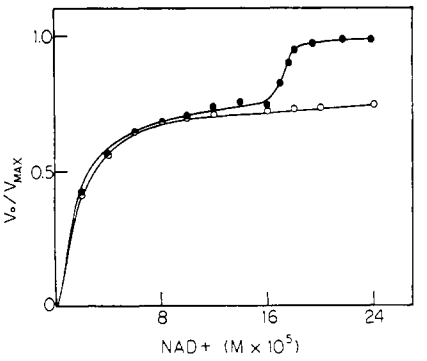

FIGURE 4: Comparison of the saturation kinetics of honeybee GPDH at two substrate concentrations. Conditions were the same as described in Figure 2. (O—O) 0.4×10^{-4} M G 3-P, (●—●) 4.3×10^{-4} M G 3-P. All reactions were initiated with NAD^+.

ring from one experiment to another. It should be noted that from the data presented in the inset portion of Figure 2, the V_{max} occurs over a range of 2×10^{-5} M NAD^+ which would imply an extremely cooperative transition. When a Hill plot is made using the data from the inset in Figure 2 a coefficient (n) of nearly 40 is obtained. However, Atkinson (1966) has pointed out that the Hill equation is valid only when the velocity of an enzymic reaction is proportional to the fractional saturation of the enzyme. Evidence from binding studies presented below indicates that this is not the case with honeybee GPDH.

Stopped-flow experiments were performed in order to study the early time course of the reaction. In the time interval of approximately 0.05 to 2.00 sec the optical density change was found to be linear, allowing the direct calculation of initial velocities without detectable product or arsenate (Teipel and Koshland, 1970) inhibition. These studies confirmed the results obtained with the Gilford spectrophotometer. The results of three typical stopped-flow experiments are shown in Figure 3. In comparing Figures 2 and 3 it can be seen that the transition occurs at a slightly lower NAD^+ concentration when the experiment is performed using stopped-flow techniques. The reason for this is unknown; however it is clear that generation of the velocity transition is reproducible and that the rabbit muscle enzyme does not behave in the same manner.

Over a time course of 1 sec after initiation of the reaction it was determined that at NAD^+ concentrations of 2.0×10^{-5}, 10.0×10^{-5}, and 18.0×10^{-5} M, 7, 4, and 3% of the total reactions were completed, respectively. Therefore within the transition region, initial velocities were obtained when less than 4% of the reaction had been completed. Thus, the possibility that the transition arises from an artifact caused by differing extents of reaction at the various NAD^+ concentrations used in this study, or by product inhibition, is eliminated. This argument is further supported by the fact that no transition is observed with the rabbit muscle GPDH under identical experimental conditions.

Experiments were undertaken to attempt to alter the plateau region in the kinetic curves. Figure 4 shows the results of a kinetic experiment in which initial velocities were determined as a function of NAD^+ concentration at two different concentrations of G 3-P. The curve with open circles represents a saturation curve at a low concentration of G 3-P (4×10^{-4} M) and the curve with closed circles represents the saturation kinetics at a high G 3-P concentration (43×10^{-4} M). Two things should be noted: the transition occurs only in the presence of high G 3-P and between NAD^+ concentrations of 16×10^{-5} and 18×10^{-5} M. Secondly the first apparent V_{max} has already been reached at a NAD^+ concentration of 12×10^{-5} M (top curve, Figure 4).

When a G 3-P saturation experiment was performed in the presence of high (100×10^{-5} M) and low (10×10^{-5} M) NAD^+ (Figure 5), similar results were obtained. In the presence of high NAD^+ a sharp increase in the initial velocity was observed between 6.2×10^{-4} and 7.2×10^{-4} M D-G 3-P. However when the titration was performed in the presence of a NAD^+ concentration (10×10^{-5} M), lower than that required for the transition in the NAD^+ saturation curve, no transition was observed in the G 3-P saturation curve. These data implicate both NAD^+ and G 3-P in the transition phenomenon.

Equilibrium Dialysis. In order to determine whether these unusual kinetics are due to a binding phenomenon, equilibrium dialysis experiments were undertaken. The techniques employed are essentially those described by Conway and Koshland (1968).

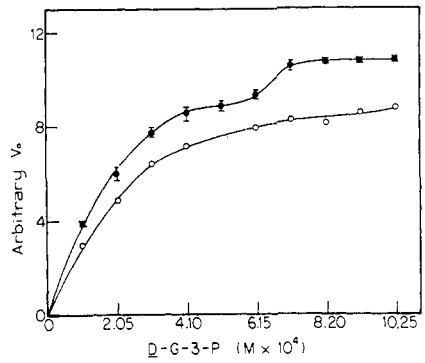

FIGURE 5: Comparison of the saturation kinetics of honeybee GPDH at two NAD^+ concentrations. Conditions were the same as in Figure 2. All assays contained the same quantity of enzyme (2 μg): (○—○) 10 × 10^{-5} M NAD^+, (●—●) 100 × 10^{-5} M NAD^+. Error bars indicate the maximum deviation of four separate determinations for each point. All reactions were initiated with G 3-P. The choice of arbitrary units was made to demonstrate the kinetics of the enzyme under conditions of low NAD^+ showed both a loss of the transition and a lower maximum velocity as compared to the enzyme assayed in the presence of high NAD^+.

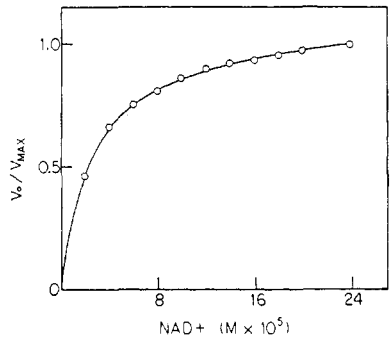

FIGURE 7: Effects of *N*-ethylmaleimide on the saturation kinetics of honeybee GPDH. The enzyme (10 μM) was incubated at 25° in 10 mM sodium pyrophosphate, 100 μM *N*-ethylmaleimide at pH 6.5. After 12 min the excess inhibitor was removed by chromatographing the mixture on Sephadex G-25. Assays were performed at 30°, pH 9.0, in the presence of 4.3 mM DL-G 3-P. All reactions were initiated by the addition of NAD^+.

Since the molecular weight of honeybee GPDH is known to be 140,000, molar concentrations of enzyme could be determined by obtaining absorbance measurements at 280 mμ for enzyme solutions. Radioactivity measurements of both the protein and nonprotein compartments in the dialysis cell allowed determination of the molar concentration of NAD^+ in the respective compartments. Thus the parameters D_b (NAD^+ bound to enzyme), D_f (free NAD^-), E_t (total enzyme concentration), and D_b/E_t (the molar ratio of bound NAD^+ to total enzyme) could be determined. The set of dissociation constants of the four NAD^+ sites which were obtained from the binding data is the following: 1 × 10^{-6}, 3 × 10^{-5}, 3 × 10^{-5}, and 6 × 10^{-4} mole $l.^{-1}$ for the first, second, third, and fourth dissociation constants, respectively. The value for the weakest site is very approximate since accurate binding data at the high concentrations necessary to saturate this site could not be obtained.

These dissociation constants were then used in the Adair equation (Adair, 1925), and a computer generated curve of degree of saturation (D_b/E_t) *vs.* free NAD^+ concentration was obtained (Figure 6). Experimental points (open circles) taken directly from equilibrium dialysis data are also shown. The Adair equation describes the binding of four ligands to a protein while making no assumption about conformational changes. The sharp transition as seen in the titration of the bee enzyme in Figure 2 (inset) cannot be explained on the basis of NAD^+ binding to honeybee GPDH. A comparison of Figures 2 and 6 shows that in the region of 16 × 10^{-5} to 18 × 10^{-5} M NAD^+ the initial velocity of the enzyme catalyzed reaction is not proportional to the fractional saturation of the enzyme with NAD^+. Whereas there is a 15–35% rise in the initial velocity in this NAD^+ range there is only a 2–3% increase in the amount of NAD^+ bound.

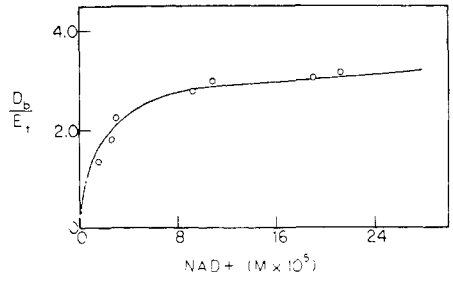

FIGURE 6: Theoretical curve and experimentally determined points for NAD^+ binding to honeybee GPDH. The degree of saturation (D_b/E_t) was calculated at specific NAD^+ concentrations by using the dissociation constants obtained from equilibrium dialysis, in the Adair equation (Adair, 1925). The open circles represent data taken directly from equilibrium dialysis.

Inhibitor Studies. It has been demonstrated that various sulfhydryl inhibitors affect GPDH differently. Iodoacetate preferentially inactivates GPDH when NAD^+ is bound (Segal and Boyer, 1953; Racker, 1965), while *N*-ethylmaleimide (NEM) tends to inactivate GPDH to a greater extent with NAD^+ removed from the enzyme (Racker, 1965). Racker has shown that there is 100% interaction of NEM with glutathione and 97% interaction between apo-GPDH and NEM, while only 26% interaction between NEM and GPDH with NAD^+ bound. Other experiments (Racker, 1965) indicate that NEM is reacting with sulfhydryl groups which are part of or closely associated with the catalytic site of the enzyme. Honeybee GPDH (10 μM) which has 3 moles of NAD^+ bound per mole of enzyme was treated with 0.1 mM NEM in 10 mM pyrophosphate buffer, pH 6.5, at 20°. Under these conditions the bee enzyme was 40% inactivated. Following treatment with NEM, excess inhibitor was removed by passing the solution over a Sephadex G-25 column. Figure 7 shows the NAD^+ saturation kinetics of the NEM treated enzyme under conditions where a transition is normally observed. It can be seen that the transition is abolished by this treatment. Similar experiments were attempted using iodoacetate as the sulfhydryl inhibitor, but kinetic experiments could not be performed because, with iodoacetate, inactivation was almost complete and accompanied by precipitation of the enzyme. Marquardt *et al.* (1968) have made a similar observation.

It was considered that the enzyme might exist in two states, a metastable state and a thermodynamically stable

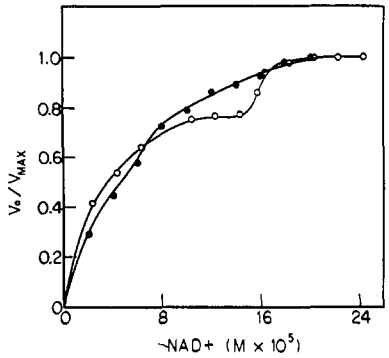

FIGURE 8: Effects of preincubation of honeybee GPDH with NAD^+ or NAD^+ plus G 3-P. Conditions were: (O——O) enzyme (1 μM) was preincubated in assay buffer containing 50 mM pyrophosphate, 5 mM arsenate, 1 mM EDTA, pH 9.0, 3.0 mM NAD^+ for 1 min at 30°. The mixture was diluted 1:100 in assay buffer at 30° and initial velocities were measured in the presence of 4.3 mM G 3-P. Reactions were initiated by the addition of NAD^+. (●——●) Enzyme (5 μM) was preincubated as described above in assay buffer at 30°, pH 9.0, containing 4.3 mM G 3-P and 1.0 mM NAD^+. The mixture was diluted 1:500 in assay buffer at 30° and pH 9.0 and initial velocities were measured in the presence of 4.3 mM G 3-P. These reactions were also initiated with NAD^+. The absolute maximum velocities for both reactions were identical.

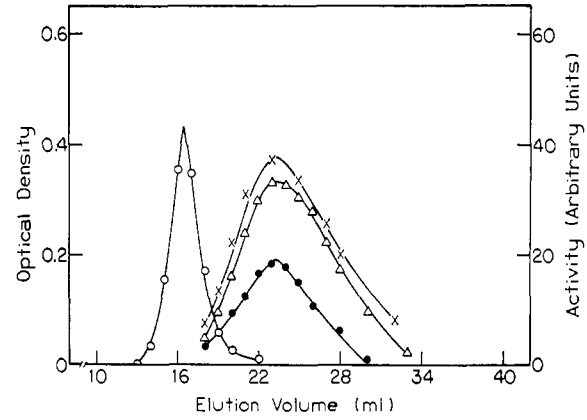

FIGURE 9: Sephadex G-200 chromatography of honeybee enzyme. Conditions were: all experiments were performed at 22° in a column (1.4 × 25 cm) preequilibrated with 5 mM pyrophosphate, 5 mM arsenate, 1 mM EDTA, plus any substrate which the enzyme was preincubated with at pH 9.0. Fractions were collected every minute with a flow rate of 45 ml/hr. (O——O) Blue dextran was measured at 650 mμ and emerged at the void volume (V_0). Enzyme alone (●——●) and enzyme in the presence of 1 mM NAD^+ (Δ—Δ) were determined at 280 mμ. Both emerged at an elution volume of 23.5 ml. Enzyme chromatographed in the presence of 1 mM NAD^+ and 4.3 mM G 3-P (×——×) was located by the standard assay procedure, and was eluted at a volume of 24.0 ml.

state, and the existence of these two states might be involved in the transition. If this is true, enzyme which was incubated in a high concentration of NAD^+, diluted, and reassayed would not be expected to exhibit the transition in the saturation curve if the conversion from the metastable into the thermodynamically stable state were dependent upon NAD^+ concentration alone. Figure 8 shows that the transition remains after preincubation of enzyme in high NAD^+. Preincubation with NADH under similar conditions followed by dilution before assay to lower the NADH concentration also failed to abolish the transition. However, preincubation in the presence of high concentrations of both NAD^+ and G 3-P abolished the transition when the diluted incubation mixture was then assayed under normal conditions (Figure 8). The dilution procedure employed here decreased the concentration of oxidized and reduced substrate and cofactor so that they may be considered negligible during the subsequent assays.

In order to rule out the possibility that the transition phenomenon is due to enzyme aggregation or dissociation, it was chromatographed on Sephadex G-200 under conditions employed in the perturbation of the transition. Figure 9 yields good evidence that no association or dissociation reactions are occurring with added NAD^+, or with NAD^+ plus G 3-P, since the enzyme is eluted at the same volume with respect to blue dextran under all conditions.

Discussion

Comparative studies of GPDH suggest that the enzyme has undergone little change during the course of evolution (Marquardt *et al.*, 1968; Allison and Kaplan, 1964). Other studies indicate that GPDH from both mammals and yeast probably consists of four identical subunits (Harrington and Karr, 1965; Perham and Harris, 1963; Harris and Perham, 1965). Although the amino acid sequence of the honeybee enzyme has not been determined, its evolutionary position would allow one to assume that the honeybee enzyme is also composed of four identical or nearly identical subunits. If similarity of structure is assumed it might be expected that the honeybee enzyme would also display negative homotropic interactions with NAD^+. The NAD^+ dissociation constants obtained in the present studies are consistent with this view since the binding of the first NAD^+ to the protein makes it more difficult for the subsequent NAD^+ molecules to bind. However the second and third NAD^+ dissociation constants for the honeybee enzyme are about the same while in the case of the rabbit muscle enzyme the binding of the third mole of NAD^+ is more difficult than the second (Conway and Koshland, 1968). Thus with the bee enzyme the binding of the first NAD^+ is affecting ligand binding to the second and third NAD^+ sites to approximately the same degree.

The negative cooperativity theory (Conway and Koshland, 1968) can explain the sequential order of NAD^+ binding to honeybee GPDH, but a binding phenomenon cannot explain the unusual transition found in the kinetic curve (Figure 2). As shown in Figure 6, NAD^+ binding to the protein as revealed by equilibrium dialysis appears normal even in the range of NAD^+ where the kinetic transition occurs.

In the present study, honeybee GPDH has been purified to homogeniety as evidenced by three criteria: disc gel electrophoresis at pH 8.3 (the enzyme precipitates at pH 4.5 and thus precludes analysis at that pH), sedimentation velocity in the ultracentrifuge, and chromatography on Sephadex G-200. Lebherz and Rutter (1967) have examined GPDH from the three body segments of the honeybee by electrophoresis and found the same isozyme in all three body segments. While it is still conceivable that in the present study two isozymes could copurify and cocrystalize and the preparation still appear homogeneous by the methods

employed, it is impossible for the transition in the kinetic saturation curve to be due to the presence of two isozymes. The possibility of one isozyme having "normal" kinetics and a second with a larger K_m and greater sensitivity to NEM but which displays a cooperative type of saturation with G 3-P or NAD^+ has been ruled out by direct experiment. One isozyme (that which would bind both G 3-P and NAD^+ in a cooperative manner) would have to catalytically bind between 20 and 40 moles of NAD^+ per mole of enzyme to give the observed "cooperative" transition. This has been found not to be the case (Figure 6) by equilibrium dialysis experiments. Secondly, it has been shown that preincubation of the honeybee GPDH with NAD^+ (1 mM) and G 3-P (4.3 mM) followed by dilution and reassay abolishes the transition (Figure 8). Due to the "irreversibility" of this process the "metastable" form (present before incubation with high G 3-P and high NAD^+) would have to catalytically bind between 20 and 40 moles of NAD^+ per mole of enzyme and after incubation revert to binding three to four moles of NAD^+. Equilibrium dialysis experiments (Figure 6) clearly show the enzyme in the metastable state (displaying the transition in its saturation kinetics) does not bind more than four moles of NAD^+ per mole of enzyme. Therefore an isozyme is not responsible for the observed phenomenon.

It does not seem likely that the observed effects are due to artifacts arising from changes in pH or ionic strength. Under the conditions of assay there was no observable change in the pH of the reaction mixture. Changes in ionic strength were negligible due to addition of substrates since the concentration of the pyrophosphate buffer (50 mM) was 200-fold greater than that of any substrate added.

The fact that the same results were obtained employing two different instruments rules out the possibility that the transition is due to an artifact arising from a particular spectrophotometer.

The kinetic data imply that certain concentrations of G 3-P and NAD^+ are required for the transition. For example, when kinetic saturation experiments were performed at high G 3-P (4.3 mM) the transition is observed during the titration with NAD^+. When the identical experiment is performed using a final G 3-P concentration of 0.4 mM no transition is obtained in the NAD^+ titration (Figure 4). When the converse experiment is performed at high (1.0 mM) and low (0.1 mM) NAD^+ a transition is observed upon titration of the enzyme with G 3-P at high [NAD^+] but not at low [NAD^+] (Figure 5). These experiments were performed with both DL-G 3-P and D-G 3-P to exclude the possibility of an artifact arising from the use of the L isomer of G 3-P in the assay system. It was found that two different treatments of the enzyme abolish the transition. The first of these, preincubation of the enzyme with high (above transition) levels of G 3-P and NAD^+ together, are consistent with the hypothesis that a metastable state of the enzyme is being isolated and that it is converted into a more active form by exposure to both substrate and cofactor. However preincubation of the enzyme with either ligand alone failed to abolish the transition so one ligand is not sufficient to cause the interconversion.

The abolition of the plateau region by preincubation of the enzyme in NEM suggests that sulfhydryl groups in the enzyme are somehow involved in the transition between forms. Anomalous zero time velocities resulting in intermediary plateau regions have also been reported to occur with cytidine triphosphate (CTP) synthetase (Levitski and Koshland, 1969), pyruvate kinase (Somero, 1969), phosphoenol pyruvate carboxylase (Corwin and Fanning 1968), and glutamate dehydrogenase (LeJohn and Jackson, 1968). In the latter case, a model for sequential binding of ligands proposed by Teipel and Koshland (1969) will explain the observed plateau. However the anomalies displayed by CTP synthetase and PEP carboxylase and the observations detailed in the present study cannot be explained by their model since the sharpness of the transition is too great to be consistent with the number of binding sites, and since the saturation curve (of honeybee GPDH) is not reversible once the enzyme passes through the intermediary plateau region.

It would seem that this type of kinetic behavior of enzymes may be of some general occurrence and conceivably could be of physiological significance. It is interesting to note that the anomalous kinetics of pyruvate kinase observed by Somero (1969) has been related to a temperature dependent interconversion of two forms of one isozyme with two different Michaelis constants for substrate. The effect of this is to provide the poikilothermic organism (Alaskan king crab) with one enzyme molecule that has a low K_m for substrate over a range of temperatures in which it normally exists.

While the significance of these observations is unknown, perhaps some of the insect GPDH exists *in vivo* in a cellular location loosely bound to a membrane and this binding dictates a certain conformation of the protein. Isolation of the enzyme may result in the presence in the pure preparation of a portion of the molecules in a conformation that is less active than others. This latent activity is only expressed after the protein undergoes a structural change that results in the restoration of full activity. The conformational change would only take place after exposure to certain levels of both G 3-P and NAD^+.

With regard to the above possible explanation, latent hexokinase is present in brain mitochondria (Wilson, 1968) and activities are demonstrable in various pools defined by their availability to assay. These latent forms can be made assayable with or without solubilization by exposure to detergents and substrate. Latent cytochrome oxidase of brain mitochondria has also been reported (Koch and Lindall, 1966).

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Kinetic Studies on the Reaction Mechanism of *p*-Hydroxybenzoate Hydroxylase*

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ABSTRACT: The reaction mechanism of *p*-hydroxybenzoate hydroxylase has been investigated by the "flow methods" as well as by the overall reaction measurements. No intermediate enzyme species, such as a semiquinoid flavin, is detected at the steady state of the reaction. The reaction scheme of this enzyme is: (1) the holoenzyme is activated by binding with the substrate (*p*-hydroxybenzoate), (2) the enzyme–substrate complex is then reduced by NADPH to yield the reduced enzyme–substrate complex and NADP through transitory formation of a ternary complex such as an oxidized enzyme–substrate–NADPH complex, and (3) the reduced enzyme–substrate complex forms another ternary complex with molecular oxygen and it breaks down to the oxidized enzyme, product (protocatechuate), and water. The binding of the substrate to the enzyme is assumed to occur by a two-step process, which facilitates an interpretation of the inhibition mechanism of the reaction under excess substrate conditions.

Many investigators reported on the purification and properties of oxygenases, including both dioxygenases and monooxygenases (Nozaki *et al.*, 1963; Kita *et al.*, 1965; Fujisawa and Hayaishi, 1968; Yamamoto *et al.*, 1965; Hosokawa and Stanier, 1966), but only a few reports which contain kinetic studies have been published (Goldstein *et al.*, 1968; Sparrow *et al.*, 1969).

In previous papers (Yano *et al.*, 1966, 1969a,b,c), it was reported that *p*-hydroxybenzoate hydroxylase, a monooxygenase, can be induced by adding *p*-hydroxybenzoate as a sole carbon source in the culture medium of *Pseudomonas desmolytica* IAM 1123, and that the enzyme can be crystallized in two forms, *i.e.*, the holoenzyme–*p*-hydroxybenzoate complex and the free holoenzyme. This enzyme contains 1 mole of FAD per mole of enzyme, whose molecular weight was estimated to be 68,000, and it catalyzes the hydroxylation of *p*-hydroxybenzoate (usually referred to as the substrate) to yield protocatechuate (the product) in the presence of NADPH and molecular oxygen. It was also reported that one molecule of *p*-hydroxybenzoate could activate one enzyme molecule, so that the anaerobic reduction rate, v_{red}, of the bound FAD of the activated enzyme was increased about 10^4 times over that of the holoenzyme. The activation could be attributed to the conformational changes in the active site, which was caused by binding with the substrate.

By stoichiometric analyses and spectrophotometric investigations, the following reaction sequence was postulated for the overall reaction of the enzyme, where E_{ox}, E_{ox}–S, E_{red}, and E_{red}–S stand for oxidized enzyme, oxidized enzyme–*p*-hydroxybenzoate complex, re-

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