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Catalytic Sites in Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase. Their Number and Their Kinetic and Spectral Properties†

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ABSTRACT: Fast kinetic measurements of D-glyceraldehyde 3-phosphate oxidation, catalyzed by rabbit muscle glyceraldehyde-3-phosphate dehydrogenase in 0.05 M imidazole buffer at pH 7.0 and 25°, indicate that all four NAD binding sites are catalytically active within a single turnover. The subsequent dissociation of NADH from all sites on the acylated enzyme may be described by a single rate constant (about 170 sec^{-1}), as is NADH dissociation from nonacylated enzyme (k = 98 ± 9 sec^{-1} at 25° and 13.5 ± 0.6 sec^{-1} at 4°). The value of the rate constant with acylated enzyme, however, is less certain until the effect of enzyme acylation on the absorptivities of enzyme–coenzyme complexes are measured. Spectrophotometric titrations of apoenzyme with coenzymes give absorptivities of 1.22 and 5.12 (cm mM)$^{-1}$ for NAD–enzyme and NADH–enzyme complexes, respectively, at 340 nm and 3°. The equilibrium constant for hydration of glyceraldehyde 3-phosphate decreases from 26 at pH 8.6 in 0.2 M triethanolamine to 2 at pH 7 in 0.05 M imidazole–0.1 M KCl buffer. It is suggested that previous indications of only two catalytic sites for the rabbit muscle enzyme are the consequence of particular experimental conditions.

Glyceraldehydephosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle is a tetramer composed of apparently identical polypeptide chains (Harris and Perham, 1965; Harrington and Karr, 1965). Successive NAD equivalents, however, appear to bind less tightly to the enzyme (Conway and Koshland, 1968; De Vijlder and Slater, 1968), an effect termed "negative cooperativity."

This apparent nonequivalence of NAD binding sites raises questions regarding the existence and nature of asymmetry in the catalytic rates. Published kinetic studies of this question have been mainly limited to use of pseudosubstrates, or the reactions of NAD alone with enzyme. Teipel and Koshland (1970) found only small changes in intrinsic rate constants in going from enzyme$(NAD)_1$ to enzyme$(NAD)_4$ with glyceraldehyde, acetaldehyde, and propionaldehyde. Malhotra and Bernhard (1968), however, found that 2 equiv of the chromophoric pseudosubstrate, β-(2-furyl)acryloyl phosphate, acylate dehydrogenase 20 times faster than the next 1.5 equiv and conclude from this and other data (MacQuarrie and Bernhard, 1971) that only two of the four sites are catalytic in nature. On the basis of the different spectral properties found for the fourth NAD bound to dehydrogenase and the magnitudes of the inhibition constants, K_i, for NAD and NADH, Boers *et al.* (1971) conclude that only the fourth coenzyme molecule bound is catalytically active in dehydrogenase.

Except for the measurements from Bernhard's laboratory, the above studies were also deficient in that they were made with enzyme of low specific activity, typically less than 65% of the highest values obtainable (195 IU/mg). Using enzyme with high specific activity, Bloch (1970) finds that the molar absorptivity of the Racker band for the fourth NAD molecule bound to dehydrogenase is the same as for the first three, in contrast to earlier measurements indicating zero absorptivity (DeVijlder and Slater, 1968) or reduced absorptivity (Boers *et al.*, 1971) for the fourth NAD bound. Bloch (1970) also describes slow first-order reactions (relaxation times ≃20 msec), which follow the stopped-flow mixing of dehydrogenase with NAD, but these transitions are abolished when 2 moles of NAD per mole of enzyme are incubated with dehydrogenase. Hammes *et al.* (1971) report temperature-jump relaxation measurements on equilibrium (incubated) mixtures of NAD and dehydrogenase. Only processes with relaxation times less than 5 msec were found, but these indicated sequential conformational changes of the enzyme accompanying the binding of NAD. Bloch (1970) also presents a few

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measurements of D-G3P[1] oxidation by NAD and enzyme, with and without incubation of 2 NAD moles per mole of dehydrogenase. He finds that only 2.2 moles of NADH are produced per mole of dehydrogenase, but more importantly, that the enzyme-catalyzed oxidation of D-G3P can occur more rapidly than any of the inferred isomerization steps and is unaffected by incubation of NAD with dehydrogenase. In contrast to his extensive and most elegant study of the reactions of NAD alone with enzyme, Bloch's measurements of D-G3P oxidation, however, are too limited in substrate concentration range to reveal the maximum number of D-G3P oxidizing sites and to permit chemical identification of each reaction phase.

Trentham (1971a,b) has completed fast kinetic measurements on lobster and sturgeon dehydrogenase. Only a single rate constant was required to describe the oxidation of D-G3P at 3.1 sites per enzyme molecule, and similarly only one rate constant was required to characterize the reductive dephosphorylation of 1,3-diphosphoglycerate at 3.7 sites per molecule of tetrameric enzyme, although asymmetry in equilibrium dissociation constants of NAD from lobster enzyme have been reported (DeVijlder *et al.*, 1969). Substantial molecular differences do exist among the D-glyceraldehyde-3-phosphate dehydrogenases from yeast, lobster, sturgeon, and mammalian sources (Trentham, 1971a), in spite of common homology around the active site. The primary purpose of this paper is to report on the direct evaluation of the number of active sites in rabbit muscle enzyme, using an extensive concentration range of the natural substrates NAD and D-G3P and enzyme preparations with high specific activities. In addition we present evaluations of rate and equilibrium constants for dehydration of D-glyceraldehyde 3-phosphate, rate constants for enzymatic reduction of NAD and release of enzyme-bound NADH, and absorptivities of NAD and NADH complexes with glyceraldehyde-3-phosphate dehydrogenase enzyme.

Experimental Section

Materials. DL-Glyceraldehyde 3-phosphoric acid solution (50 mg/ml), dithiothreitol, and fluorescent grade imidazole were from Sigma Chemical Co. The concentration of the D isomer of G3P, determined with dehydrogenase at pH 8.9 in an excess of NAD and arsenate, was 47 ± 1% of the specified DL-G3P concentration. No loss of D-G3P was detected in stock solutions, adjusted to pH 7.0 in the 0.05 M imidazole buffer used for kinetic studies, during a day at 4° or for a week at −18°. ChromatoPure β-NAD and β-NADH from P-L Biochemicals were used for coenzyme solutions which were prepared and the pH was adjusted just prior to use. Their concentrations were determined enzymatically (Klingenberg, 1963). Other chemicals were of the highest grades commercially available. Solutions were prepared in glass-distilled water.

Holo- and Apoenzyme Preparations, Properties, and Assays. Dehydrogenase was extracted from the hind leg and back muscles of freshly killed New Zealand White rabbit does, and partially purified by the procedure of Ferdinand (1964). These and subsequent purification steps were conducted in a cold room (2°). For preparation of the holoenzyme, NAD was added to the Ferdinand preparation at about a four molar ratio to enzyme concentration to displace enzyme-bound ADP-ribose and stabilize the enzyme, which was then further purified on a carboxymethylcellulose column following closely the method of Bloch *et al.* (1971). In the following procedure, a slightly higher pH for the EDTA buffers was used to avoid enzyme instability at pH <6.5; deacylation of dehydrogenase was achieved at pH 8 to avoid use of arsenate, and fractions from the column were caught in imidazole–dithiothreitol buffer to enhance enzyme stability. Extraneous proteins were washed from the column with one bed volume of 5 mM EDTA, pH 6.65, before eluting dehydrogenase with a solution of 5 mM EDTA and 0.1 M KCl at pH 6.65. Fractions (5 ml) were collected into tubes containing 0.5 ml of 0.25 M imidazole–5 mM EDTA–5 mM dithiothreitol (pH 7.5). The dilute enzyme in the pooled fractions was concentrated and precipitated by dialysis against saturated $(NH_4)_2SO_4$ and 5 mM EDTA, adjusted to pH 8.0 (pH paper) with concentrated NH_4OH. The enzyme was stored in this $(NH_4)_2SO_4$–EDTA solution. Keeping enzyme in this solution for at least a day deacylated it, as was verified by analysis for 3-phosphoglycerate (Czok and Eckert, 1963) released from enzyme after incubation in buffer with 20 mM arsenate and 0.3 mM NAD. Enzymatic analysis for NAD (Klingenberg, 1963) from this dehydrogenase, redialyzed into imidazole buffer and precipitated with perchloric acid, gave about 3 moles of NAD per mole of enzyme. This NAD content is in agreement with the values predicted by the $A_{280}:A_{260}$ ratios of Fox and Dandliker (1956).

Apoenzyme was prepared by placing the Ferdinand preparation, without added NAD, on the carboxymethylcellulose column, and then washing (~3 bed volumes) with 5 mM EDTA at pH 6.65 prior to eluting with the KCl–EDTA buffer used for eluting holoenzyme. Depending on the extent of washing, apoenzyme had variable $A_{280}:A_{260}$ ratios. Enzymes with the lowest $A_{280}:A_{260}$ ratio had less than 0.01 mole of NAD per mole of dehydrogenase as measured by enzymatic analysis. The most extensively washed apoenzyme with $A_{280}:A_{260} = 1.89 \pm 0.02$ was used for the spectrophotometric titrations.

For each experiment, enzyme suspended in ammonium sulfate was collected by centrifugation and dialyzed for a total of 16 hr with four changes of tenfold excess of the chosen buffer, or in the case of apoenzyme, with three changes of 50 volumes each. Traces of denatured protein were centrifuged down at 12,000*g* prior to spectrophotometric determination of protein concentration from absorbance measurements at 280 and 260 nm using the absorptivity values of Fox and Dandliker (1956). Enzyme taken out of ammonium sulfate was used within 36 hr. Commercial, crystalline rabbit muscle dehydrogenase, used in one experiment (curve 1 of Figure 1) was purchased from Boehringer, Mannheim. The molecular weight of dehydrogenase was assumed to be 145,000 (Harris and Perham, 1965; Harrington and Karr, 1965). (One mole of enzyme binds 4 moles of substrate.) Specific activities were measured according to the procedure of Ferdinand (1964), except that the correction factor of 1.07 for nonsaturation with respect to phosphate was not used.

Spectrophotometric Titrations. Specific activities of dehydrogenase in the dialyzed apoenzyme stock solutions used for spectrophotometric titrations held within 10% of specific activities of the original apoenzyme eluted from the carboxymethyl cellulose column. Dehydrogenase solution (20–45 μM in 2 ml) and buffer in separate 1-cm cuvets were placed in the sample and reference beams, respectively, of a Coleman 124 spectrophotometer equipped with a Coleman No. 0319 thermostatted cell holder, No. 801 scale expander and No. 165 recorder set for 0.1 or 0.2 absorbance unit full scale. Temperature was maintained at 3 ± 2° and dry N_2 gas was passed

[1] Abbreviation used is: G3P, glyceraldehyde 3-phosphate.

TABLE I: Equilibrium and Rate Constants for Dehydration of D-G3P Diol in Different Buffers and pH at 25°.

Buffer pH[a]	Dehydrogenase (μM)	NAD (mM)	Total D-G3P (μM)	10^2K^c Ald/Diol	10^3k^d (sec^{-1})
8.6[b]	1.0–3.0	0.48	530	3.4	87
8.6	1.5–3.0	0.48	456	3.8 ± 0.4[e]	88 ± 3[e]
7.4	3.6–18	1.0	7.5	22.0 ± 3	42 ± 4
7.0	34.0–67	1.4	95.2	53.0 ± 6	63 ± 4

[a] Buffers: pH 8.6, 200 mM triethanolamine–1 mM EDTA–30 mM AsO_4^{-3}; pH 7.4, 100 mM imidazole–1 mM EDTA–5 mM dithiothreitol–0.5 or 5 mM AsO_4^{-3}; pH 7.0, 50 mM imidazole–100 mM KCl–1 mM EDTA–5 mM dithiothreitol–10 mM AsO_4^{-3}. [b] Data at 21° (Trentham *et al.*, 1969). [c] Aldehyde/diol. [d] Rate constant for dehydration. [e] Means ± 0.5 of the differences between two determinations.

through the cell chamber to prevent condensation of moisture. Equal additions of coenzyme, from 2–8 mM stock solutions, were made to both cuvets for each titration point by means of micropipets and solutions were gently stirred with plastic plumpers (CalBiochem) prior to measuring absorbance differences, ΔA. All ΔA values were corrected for volume changes caused by the additions. When titrations were made with many additions to the same protein solution (as in Figure 2, for example), corrections, explained in the next paragraphs, were also required for absorbance changes caused by stirring of enzyme solutions in the cuvet (~10–20% corrections), and also changes caused by dehydrogenase-catalyzed destruction of NADH (Hilvers *et al.*, 1966) (~10% corrections), in the case of these titrations. The accuracy of the latter two corrections and the overall titration curve was evaluated by making several separate and more rapid titrations with larger coenzyme aliquots per addition (about six additions per titration, for example), in which these corrections were a smaller percentage of ΔA. Measurements also were made with but one or two additions of coenzyme to achieve the maximum coenzyme/enzyme molar ratios used (5 to 11). Slopes and maximum ΔA values of titration curves were reproducible within a 15% range at most, from five titrations with NADH and four titrations with NAD.

Simple stirring of the enzyme solution in the cuvet (dummy additions) caused an increase in ΔA proportional to the length of stirring. Three gentle stirring strokes caused a reasonably constant $\Delta A = 0.004$, independent of the presence or absence of coenzymes. This is ascribed to aggregation of enzyme by stirring, causing increased light scattering to appear, as was noted by Bloch (1970). The specific activity of aggregated apoenzyme stirred more extensively than in the titrations remains the same as unstirred apoenzyme, suggesting that the aggregated protein is not denatured, or that it represents but a small per cent of total enzyme. Unstirred solutions of apoenzyme maintain stable absorbancies for hours.

In the titrations with NADH, slow destruction of coenzyme and resulting absorbance changes were considered proportional to the dehydrogenase–NADH complex concentrations. Since stoichiometric binding of coenzyme was achieved in the affected titrations, corrections to measured ΔA values at each point were calculated as rate of absorbance loss with saturating NADH ($\sim 0.27 \times 10^{-4}$ ΔA/min μM of dehydrogenase) × time between additions × ($\Delta A/\Delta A$ at saturation) × enzyme concentration. Boers *et al.* (1971) considered NADH titration of dehydrogenase to be unfeasible, but we do not believe the per cent error in the derived absorptivity is appreciably greater than in the NAD titrations. This may be the result of more favorable buffer conditions in our measurements, in which ions that stimulate the destruction of NADH by dehydrogenase (Hilvers *et al.*, 1966) are absent.

Kinetic Measurements, Instrument Calibration, and Computer Methods. The rapid reaction were followed on a Durrum-Gibson stopped-flow spectrometer using a 2.34-mm path-length cell and wavelength of 340 nm with 1-mm slits for all measurements. Temperature was maintained at 25.0 ± 0.1°, except for one experiment at 4°. At least four traces were photographed for each measurement. The mixing "dead time" of the instrument, ascertained by mixing 1 mM $K_3Fe(CN)_6$ with 25, 50, 75, and 100 mM ascorbate at pH 7.5 (Hammes and Haslam, 1968), was 2.6 ± 0.5 msec. Instrument wavelengths were calibrated with a Beckman Instrument, No. 181150 mercury vapor lamp.

On mixing high concentrations of dehydrogenase–NAD and D-G3P, there was a large burst phase within the instrument dead time. Absorbances at zero time were calculated as the sum of the absorbances of the solutions in each of the two drive syringes divided by 2. Care was taken to flush the previous solution exhaustively before measuring absorbances. Independent measurements of initial absorbances on a Coleman 124 spectrophotometer were within 3% of the stopped-flow spectrometer values. Absorbance of a 30 μM dehydrogenase–14.0 mM NAD solution in a 0.2-cm cuvet was monitored on the Coleman spectrophotometer for 20 min at 25°. No change in absorbance could be observed, in contrast to the results of Bloch (1970), which he ascribed to unidentified contaminants. Calibrations to estimate stray light under conditions of our experiment gave values of from 5 to 14 mV on a 100% transmittance scale of 800 mV. Over the 25–100% transmittance range used in the stopped-flow measurements, concentrations calculated with a stray light correction of either 5 or 14 mV differ at most by 2.6%. Absorbances on the Perkin-Elmer Coleman spectrophotometer were calibrated with standard potassium chromate in 0.05 N KOH (Haupt, 1952), and the wavelength scale was calibrated as on the stopped-flow spectrometer.

All experiments, except those noted in Table I, were made with enzyme in 0.05 M imidazole buffer containing 0.1 M KCl, 1 mM EDTA, 5 mM dithiothreitol, and HCl required to adjust this solution to pH 7. Least-squares computer fits to eq 3 were obtained with the algorithm of Marquardt (1963).

Results

Equilibrium Concentrations of the Enzymatically Active Form of D-G3P. The aldehyde form of D-G3P is in equilibrium with the enzymatically inactive hydrate, a gem diol, and at pH 8.6 in 0.2 M triethanolamine·HCl buffer, Trentham *et al.*

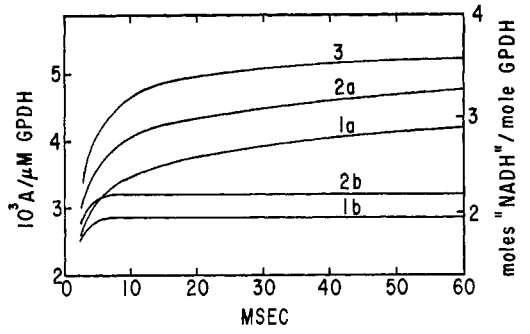

FIGURE 1: Progress curves of the reaction[2] [dehydrogenase + NAD] + [6 mM D-G3P] in 50 mM imidazole–100 mM KCl–1 mM EDTA–5 mM dithiothreitol (pH 7.0) at 25°, for various enzyme preparations and conditions. Left ordinate is 10^3 times the absorbance, A, recorded from a 0.234-cm path-length cell at 340 nm, divided by the concentration of enzyme (mol wt = 145,000). Right ordinate is the apparent molar ratio of NADH/dehydrogenase assuming mM "NADH" = $A/(0.234 \times 6.22)$; it equals the true molar ratio when all NADH is displaced from the enzyme, assuming enzyme acylation has no effect on the absorptivity[3] of dehydrogenase-NAD. NAD and enzyme concentrations after mixing are: curves 1a and b, 14 and 0.113 mM NAD, respectively, with 28 μM dehydrogenase of specific activity (sp act.) = 121 IU/mg; curves 2a and b, 7.0 and 0.117 mM NAD, respectively, with 29 μM dehydrogenase of sp act. = 145 IU/mg; curve 3, 7.0 mM NAD with 30 dehydrogenase of sp act. = 159 IU/mg. Enzyme used for curves 1a and b was from Boehringer Mannheim, while fresh enzyme prepared in our lab was used for other data.

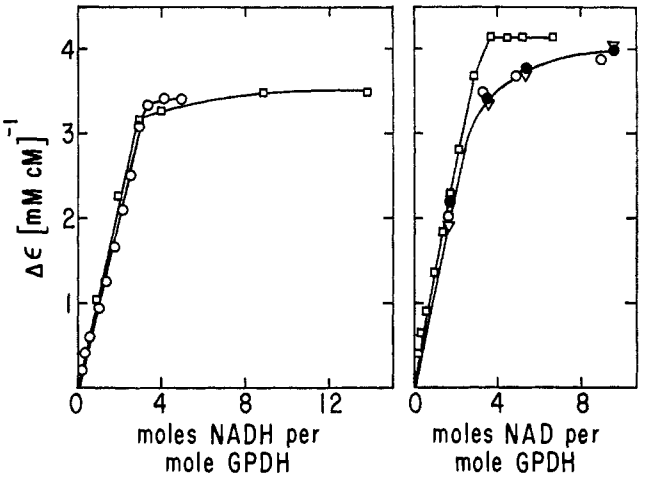

FIGURE 2: Difference absorptivities at 340 nm *vs.* molar ratios of coenzymes to dehydrogenase, in imidazole buffer as in Figure 1, but at 3 ± 2°. (a, right) □, 45 μM dehydrogenase of specific activity (sp act.) = 160; ○, ●, and ▽, 20 μM dehydrogenase, sp act. = 145. (b, left) ○, 45 μM dehydrogenase, sp act. = 160; □, 20 μM dehydrogenase, sp act. = 145.

(1969) found only 3.4% of D-G3P as the aldehyde. At high concentrations of dehydrogenase the enzymatic progress curves measure the rate and equilibrium constants for the diol–aldehyde conversion. Table I shows that reducing the pH to 7.0 in 0.05 M imidazole buffer containing 0.1 M KCl, 1 mM EDTA, and 5 mM dithiothreitol increases the aldehyde concentrations to 34.4% of the total D-G3P concentration.

Enzymatic Oxidation of D-*G3P.* Stopped-flow experiments of [dehydrogenase + NAD] + [D-G3P] were performed[2] to measure the rates and extents of D-G3P oxidation by NAD with dehydrogenase in the imidazole–KCl buffer at pH 7.0, under conditions where primarily only a single turnover of D-G3P oxidation would occur. Due, presumably, to small P_i impurities in the D-G3P, a much slower reaction did proceed to a small extent beyond a single turnover, when excess NAD was present, but this was easily resolved from the initial redox reaction, as described below. Typical progress curves are shown in Figure 1 for three different enzyme preparations with specific activities of 122, 145, and 159 IU per mg, respectively. The data, normalized for enzyme concentration, are plotted as absorbance changes (in 0.234-cm path-length cell) on the left ordinate, since substantial changes in molar absorptivities occur throughout the transient phases. Each curve possesses a fast phase, which is nearly complete within the apparatus dead time (2.6 msec). When NAD concentrations exceed four times the dehydrogenase concentration, a slower phase, nearing completion in 30 msec occurs, followed by a still slower phase lasting until about 400 msec. With a given enzyme preparation all curves with substrate concentrations equal or in excess of 3.0 mM D-G3P and 7 mM NAD (concentrations after mixing) were coincident within experimental error, whereas with the lower substrate concentrations examined, reaction curves fell below the saturation ones. The following reactions are expected under conditions of these experiments

$$\mathrm{Co} + \mathrm{G} + \mathrm{E}_{\mathrm{Co}} \rightleftharpoons \mathrm{E}_{\mathrm{CoH}}^{\mathrm{AcP}} \overset{\mathrm{CoH}}{\rightleftharpoons} \mathrm{E}^{\mathrm{AcP}} \overset{\mathrm{Co}}{\rightleftharpoons} \mathrm{E}_{\mathrm{Co}}^{\mathrm{AcP}} \quad (1)$$

where Co(H), G, E, and AcP represent NAD(H), D-G3P, dehydrogenase, and the enzyme thioester of D-G3P, respectively. These reactions will be driven to completion at high NAD concentrations by virtue of the last reaction. The fastest transient can be assigned to the reduction of coenzyme (first reaction of eq 1). The slower transient within 30 msec is considered to be the release of NADH from the acylated enzyme, which leads to increased absorptivity at 340 nm for NADH. The still slower phase lasting to about 0.4 sec is attributed to phosphorolysis from phosphate impurity in the D-G3P. Evidence for the latter two assignments, and evaluations of their absorbance contributions to the data are considered in the next three sections, prior to calculating the number of fast catalytic sites of dehydrogenase.

Spectrophotometric Titrations. ABSORPTIVITIES OF ENZYME–COENZYME COMPLEXES. Absorbance differences between free and enzyme-bound coenzymes are plotted in Figure 2 against the molar ratio of total concentrations, NAD(H)/dehydrogenase, in the imidazole buffer at 3° and pH 7.0. Due to the instability of the apoenzyme (Hammes *et al.*, 1971), titrations at 25° have not been attempted as yet. The titration curves are linear through all experimental points below a NAD/dehydrogenase ratio of 2. Below this point, the coenzymes are, therefore, stoichiometrically bound to the enzyme in these experiments and the absorptivity differences per coenzyme binding site, $\Delta\epsilon$, may be directly calculated from the slopes in Figure 2 and similar graphs. The corresponding absorptivities per mole of bound coenzyme at 340 nm are 1.22 ± 0.11 (cm mM)$^{-1}$ (4 determinations) for dehydrogenase–NAD, and 5.12 ± 0.14 (cm mM)$^{-1}$ (4 determinations) for dehydrogenase–NADH, assuming the absorptivity of free NADH to be 6.22 (cm mM)$^{-1}$. Velick (1953) had measured an absorptivity of 1.02 (cm mM)$^{-1}$ for dehydrogenase–NAD (when

[2] A stopped-flow experiment is described as [A + B] + [C] when reactants A and B in one syringe are mixed against reactant C in the other drive syringe (Trentham, 1971a,b).

corrected to a dehydrogenase molecular weight of 145,000), and we calculate a value of 0.90 from Bloch's (1970) data, both at 340 nm and 25°. The errors involved in our titrations were examined as described in the Experimental Section.

With the freshest enzyme used in titration experiments (sp act. = 160 IU/mg), linear changes in absorbance were obtained for binding to about 3.0–3.4 enzyme sites, while significant deviations occur near this point and above, in NAD titrations with the same enzyme preparation, which had aged in the ammonium sulfate suspension for 12 days or longer (sp act. = 145 IU/mg). In NADH titrations, enzyme concentrations used were too high to allow accurate absorbance measurements above an abscissa of about 5 ($A > 1.5$), except in one experiment with lower specific activity enzyme.

Rates of enzyme–NADH dissociation. The rates of NADH dissociation from enzyme were measured at 25 and 4° by following absorbance changes at 340 nm during the reactions of [NAD] + [dehydrogenase + NADH]. At high NAD concentrations the reactions

$$\text{E·NADH} \overset{\text{NADH}}{\rightleftharpoons} \text{E} \overset{\text{NAD}}{\rightleftharpoons} \text{E·NAD} \qquad (2)$$

become irreversible first order, and rate limited by the NADH dissociation. NADH/dehydrogenase molar ratios of 0.7, 2.0, 4.0, and 8.0 were used at 25°, while a ratio of 8.0 was used for data at 4°. All data with NADH/dehydrogenase molar ratios of 2 and above are described well by first-order plots (Figure 3) with the same rate constant of 98 ± 9.4 sec^{-1} (5 measurements) at 25°. This rate constant was also independent of NAD concentrations used (0.06 to 14 mM). At a molar ratio of 0.7 NADH/dehydrogenase, a faster, transient increase of absorbance occurs during the first 5 msec (Figure 3), while the remaining reaction falls on a first-order plot with the same rate constant as found with high NADH/dehydrogenase ratios. Since both reactions in eq 2 cause absorbance increases at 340 nm, the faster reaction with 0.7 NADH/dehydrogenase is most likely due to the changes in Racker band absorbance following the association of NAD with the unoccupied enzyme binding sites, as observed by Bloch (1970) and DeVijlder *et al.* (1969) in stopped-flow measurements of [dehydrogenase] + [NAD]. The slower, first-order reaction in our measurements is considered to be the dissociation of enzyme-bound NADH. The absence of the faster transient at 2 NADH/dehydrogenase is analogous to the abolished transients observed with NAD binding to apoenzyme when 2 moles of NAD (NADH in our case) are incubated with 1 mole of enzyme (Bloch, 1970; De Vijlder *et al.*, 1969). The rate constant for dissociation of enzyme-bound NADH decreases from 98 sec^{-1} at 25° to 13.1 sec^{-1} at 4°. The quantity of NADH bound per mole of enzyme can be calculated from the ordinate intercepts of the first-order plots at high NAD concentrations and the absorptivities of enzyme-bound NADH and NAD. The moles of NADH bound per mole of dehydrogenase, calculated in this way, increase with total NADH concentrations used to 3.2 at 25° and are 2.8 at 4°. The absorptivities, ϵ, of enzyme-bound NAD and NADH used in these calculations were both measured at 3° only, and our value for dehydrogenase–NAD is 20–35% higher than published determinations at 25°. These differences may be largely due to error and not temperature, but either published ϵ value gives us close to 4 moles of bound NADH per mole of enzyme. In any event, we believe that at least 3 moles of NADH were bound per mole of enzyme at

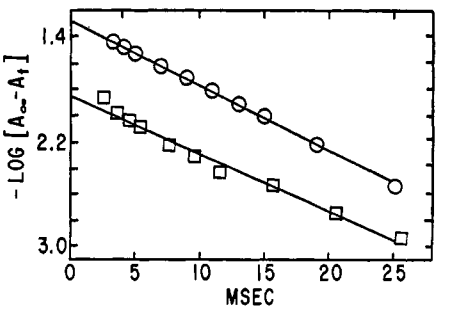

FIGURE 3: First-order plots of absorbance changes at 340 nm *vs.* time in the reaction[2] [60 μM dehydrogenase + NADH] + [28 mM NAD] in buffer of Figure 1 at 25°. NADH concentrations after mixing are 120 μM in top curve and 21 μM in bottom curve. Enzyme specific activity was 165 IU/mg.

25°. A higher value would be expected at 4° if the specific activity of the enzyme used (115 IU/mg) had been comparable to the specific activity of enzyme used at 25° (165 IU/mg).

The reaction [60 μM dehydrogenase + 120 μM NADH] + [6.0 mM D-G3P] gave a first-order rate of absorbance increase with a rate constant of 196 ± 7 sec^{-1} at 25°, and an amplitude equivalent to the release of all NADH from the enzyme, assuming an absorptivity change of 1.10 (cm mM)$^{-1}$ for this reaction as determined from the titration experiments. Alternatively, we must assume that formation of the dehydrogenase–NADH–G3P complex abolishes the absorptivity difference between free and enzyme-bound NADH.

Slow Turnover. Phosphorolysis from phosphate impurities. The slow absorbance increase seen in Figure 1 after 30 msec is ascribed to phosphorolysis due to inorganic phosphate impurity in the D-G3P for the following reasons. (1) Reactions recorded to equilibrium (~10 sec) with lower (1.8 μM) dehydrogenase and 0.7, 1.4, 2.8, or 5.6 mM D-G3P proceeded to an extent proportional to the D-G3P, which was approximately 0.7% of D-G3P. This quantity of NADH represents nearly ten turnovers at 5.6 mM D-G3P, which requires an acyl acceptor. Since the hydrolysis rates, obtained from longer time records on the same experiment, were at least 10^3 smaller than the phosphorolysis rates, the turnover prior to 10 sec cannot be due to hydrolysis. Trentham (1971b) also claimed phosphate impurity in his D-G3P solutions were the cause of several turnovers of lobster dehydrogenase. (2) Reactions run with stoichiometric amounts of NAD (curves 1b and 2b of Figure 1, for example) were completed within 6 msec with no further turnover. (3) It is highly unlikely that the redox reaction occurs at any of the four binding sites during this slow "phosphorolysis" phase, as it is concluded below that the reduction of all four NAD per dehydrogenase molecule occurs within the fastest phase (<6 msec). (4) Ion-exchange chromatography of DL-G3P samples gave partially resolved G3P and P_i peaks, as determined by chemical analyses for both. A P_i impurity of about 2 mole % of D-G3P was indicated. This must be considered a rough estimate due to incomplete resolution of DL-G3P and P_i peaks and the possibility of some G3P hydrolysis under conditions of the Fiske–Subbarow P_i test, but this agreement with the extent of the enzymatic oxidation of D-G3P is good, when the equilibrium of the phosphorolysis reaction is considered (Cori *et al.*, 1950).

The concentrations of enzyme intermediates are maintained essentially constant during phosphorolysis due to their more rapid regeneration by reactions of eq 1, providing

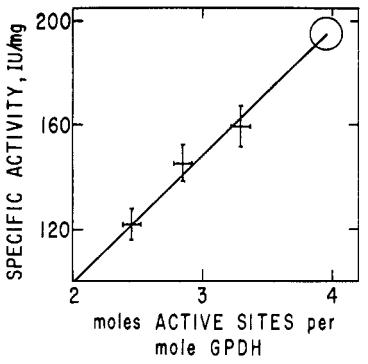

FIGURE 4: Correlation between enzyme specific activity and number of catalytically active sites. Moles of active sites were determined by extrapolation to zero time of the essentially linear portions after 30 msec of curves 1a, 2a, and 3 of Figure 1. The line through the three experimental points, +, is extrapolated to a specific activity of 195 IU/mg, the highest enzyme specific activity obtained in this and other laboratories.

first-order rates of phosphorolysis after 30 msec. The rates and extents of phosphorolysis were determined from such first-order plots (slopes and intercepts) and are summarized in Table II along with other rate constants as described below.

Number of Rapidly Reacting Catalytic Sites of Dehydrogenase. At high NAD concentrations where the reactions of eq 1 are saturated, NAD completely replaces NADH from 30 μM enzyme within 30 msec. Absorbance values thereafter are assumed to be a direct measure of free NADH concentrations with an absorptivity of 6.22 (mM cm)$^{-1}$, since the amount of E_{NAD} complex is the same as at zero time, and the absorbances of E_{NAD} and E_{NAD}^{Acyl} of eq 1 are assumed to be the same.[3] The free NADH concentration minus that which results from phosphorolysis should then equal the concentration of active sites. At high NAD concentrations and 25°, phosphorolysis is well separated from the preceding phase, permitting a linear extrapolation to zero time from the limited data beyond 30 msec in Figure 1. This simple extrapolation is also in good agreement with that obtained from the intercept of first-order plots of more complete phosphorolysis data, differing no more than 5 μM or 0.17 of an enzyme site. Though phosphorolysis would not be expected to reach a steady state until approximately 5 msec after mixing, an uncertainty of even 10 msec corresponds to an error of only 0.06 of an enzyme site, at most. The number of active sites determined as described above are plotted in Figure 4 against enzyme specific activities. The highest specific activity (159 IU/mg) used in our stopped-flow measurements corresponds to 3.3 sites, and the curve may be extrapolated to 195 IU/mg, which is the highest specific activity obtained in this and other laboratories (Bloch *et al.*, 1971). A value of 3.95 sites per dehydrogenase molecule is obtained at this intercept which is considered to represent native enzyme.

With a stoichiometric ratio of 4 NAD/dehydrogenase (curves 1b and 2b of Figure 1), it appears that only 2.2 sites (curve 2b) are occupied, but calculations taking into account the changes in molar absorptivities, indicate that the same number of sites are active with either 117 μM or 7 mM NAD. These changes in absorptivity arise because at 117 μM NAD there is no excess NAD to displace enzyme bound NADH, thus the absorptivity, ϵ, of E_{NAD} is lost on the reactions of eq 1; corrections are also needed for the change in ϵ of E_{NADH}^{AcylP} relative to free NADH, and for any displacement of NADH from enzyme by the excess D-G3P (see above). The NAD correction, which is most accurately known since the effect of enzyme acylation on the ϵ of dehydrogenase–NADH is not known, suffices to raise the number of active sites in curve 2b to 2.8, in excellent agreement with curve 2a (2.8 sites also). If also enzyme acylation has no effect on ϵ of dehydrogenase–NADH, 3.5 active sites are obtained from curve 2b, suggesting that either acylation abolishes the $\Delta\epsilon$ of NADH–(dehydrogenase–NADH), or that excess D-G3P induces dissociation of NADH from acylated enzyme, as it appears to do from nonacylated enzyme (see above). The latter possibility seems most likely since, if enzyme acylation destroys the Racker band of bound NAD as Bloch claims (1970)[3] and also abolishes the $\Delta\epsilon$ of enzyme bound NADH there would be no absorbance change for the second reaction of eq 1. Though these latter uncertainties exist, they do not alter the conclusion that at least as many catalytic sites rapidly react with a stoichiometric equivalent of NAD as with a large excess.

Rate Constants of D-G3P Oxidation and NADH Dissociation in the Complete Reaction. Progress curves for high NAD concentrations should follow the kinetics of two consecutive pseudo-first-order reactions, to which the slow phosphorolysis reaction may be added. Absorbance changes in time, Ab, per path length, l, are given by

$$\frac{Ab}{l} = \left\{\left[\frac{(\epsilon_d + \epsilon_c - \epsilon_a)}{\epsilon_d} \times \frac{k_2}{(k_1 - k_2)} - \frac{(\epsilon_b - \epsilon_a)}{\epsilon_d} \times \frac{k_1}{(k_1 - k_2)}\right] A_0 - P_0(1 - e^{-k_3 t})\right\} e^{-k_1 t} - \left[\frac{(\epsilon_c + \epsilon_d - \epsilon_b)}{\epsilon_d} \times \frac{k_1}{(k_1 - k_2)} A_0\right] e^{-k_2 t} + \frac{(\epsilon_d + \epsilon_c - \epsilon_a)}{\epsilon_d} A_0 + P_0(1 - e^{-k_3 t}) \quad (3)$$

where A_0 and P_0 are initial concentrations of dehydrogenase–NAD and free phosphate respectively, k_1, k_2, and k_3 are rate constants for the redox, NADH release, and phosphorolysis reactions, respectively, and the absorptivities, ϵ, refer to the subscript species, as derived in the Appendix. Analysis was made of eq 3 to test the validity of our assumptions, the internal consistency of our data, and, in addition, to provide estimates of the rate constants. The phosphorolysis constants k_3 and P_0 were evaluated from first-order plots of data beyond 30 msec, as described earlier, in order to reduce the number of adjustable parameters in seeking the least-squares fit of eq 3 to our data (Marquardt, 1963). Best fit values of the remaining constants, A_0, k_1, and k_2 are summarized in Table II along with the phosphorolysis constants used. The concentrations of enzyme sites, A_0, determined in this way are in good agreement with the values obtained by graphical procedures used above. If enzyme acylation has no effect on ϵ of dehydrogenase–NAD; *i.e.*, $\epsilon_c = \epsilon_a$, the rate constant, k_2, for NADH release from acyl-enzyme is found to be twice that calculated for apoenzyme, but nearly the same as found for the D-G3P displacement of NADH from apoenzyme as presented above.

[3] Bloch (1970) concludes from titration data that NAD bound to acylated subunits of dehydrogenase has no Racker band absorbance. This conclusion is questionable since acylation of dehydrogenase is thought to increase the dissociation constant of NAD from enzyme (Trentham, 1971a). Hence during the titration, little NAD may bind to the acylated subunits until the acyl groups hydrolyze. But if the absorptivity of acylated dehydrogenase–NAD is zero, the concentration of active enzyme sites will be increased 24% from those calculated assuming acylation has no effect on this absorptivity.

TABLE II: Rate Constants, k, Catalytic Site Concentrations, A_0, and Number of Active Sites, n, per Enzyme Molecule, from Eq 3.[a]

Sp Act.[b] (IU/mg)	P_0[c] (μM)	k_3[c] (sec^{-1})	σ[d] (μM)	k_1[e] (sec^{-1})	k_2[e] (sec^{-1})	A_0 (μM)	n
159	11.6	12.0	1.5	754 ± 52	208 ± 18	105.4 ± 0.5	3.5
159[f]	11.6	12.0	1.4	542 ± 22[f]	148 ± 20[f]	131.3 ± 0.7[f]	4.4[f]
145	18.4	16.7	0.8	1000 ± 140	210 ± 23	82.2 ± 0.5	2.8
122	23.4	14.0	0.45	1400 ± 400	260 ± 40	67.4 ± 0.5	2.4

[a] Data of Figure 1. [b] Specific activity, sp act. [c] Quantities of P_i impurity, P_0, and phosphorolysis rate constants, k_3, were determined by first-order plots of data from 40 to 400 msec as explained under Results. [d] Standard deviation of points about best fit curve. [e] Rate constants ± standard deviations. [f] Same data as in first column, but fitted to eq 3 assuming the absorptivity of acylated-dehydrogenase–NAD is zero.

The values of k_1, however, should be considered more tentative as they are based on only the last portion of the redox reaction. Reaction data at lower temperatures should allow a more accurate evaluation of this reaction. Computer fits were obtained assuming various absorptivities to assess their effect on best fit rate constants and A_0. If ϵ_c is assumed equal to ϵ_a, only the difference in absorptivities between dehydrogenase–NAD and dehydrogenase–NADH, $\epsilon_b - \epsilon_a$, appear in eq 3, aside from that of free NADH. A 10% in-increase in $\epsilon_b - \epsilon_a$ causes a 16% decrease in k_1, an 18% decrease in k_2, and negligible change (<0.1%) in A_0, the concentration of catalytically active sites. Similarly, even a 100% simultaneous increase in both the amplitude and rate constant of phosphorolysis decrease A_0 only 10% and affect k_1 negligibly, although k_2 is increased 100%. Such a large error in the phosphorolysis terms is not likely, as is also indicated by the statistics of the latter fit, which show it to be a decidedly poorer one than those obtained with the phosphorolysis constants in Table II. Computer fits were also obtained to eq 3 modified by the assumption that acylation of enzyme abolishes the absorptivity of dehydrogenase–NAD.[3] This increased A_0 by 24% as expected and lowered k_1 and k_2, as shown in Table II. There is not a sufficient difference in the statistics of the fits to evaluate the validity of this assumption, but when independent measurements of the absorptivities of acylated enzyme–coenzyme complexes become available, better estimates of the rate constant are expected.

Discussion

Hydration of D-*G3P.* The large dependence of the D-G3P diol to aldehyde equilibrium on buffer conditions was an unexpected, but favorable result. This change in equilibrium constant is probably related to the pH and ionization of the phosphate with a pK near 6.8 (Johnson, 1959), rather than a consequence of ionic strength or specific ion effects. Our data are too limited to be sure about these points, but they demonstrate the errors in assuming an equilibrium independent of buffer. This equilibrium constant is important in judging the displacement of the aldolase and triose phosphate isomerase reactions from equilibrium *in vivo* (Achs *et al.*, 1971).

Coenzyme Titrations of Dehydrogenase. Constant slopes are observed in coenzyme titration curves of Figure 2 throughout the region of stoichiometric binding; *i.e.*, up to 3.4 moles of NAD or NADH per mole of enzyme with highest specific activity. We did not use high enough coenzyme concentrations to insure complete saturation of the fourth enzyme site. But Bloch's (1970) extensive NAD titration data demonstrate equivalent absorptivity changes for saturation of all enzyme sites. The equivalence in spectral response of all NAD binding sites has also been recently shown with optical rotatory dispersion (ORD) (Bloch, 1970) and protein fluorescence quenching (Price and Radda, 1971), in contrast to earlier conclusions. Bloch suggested that partial alteration of enzyme-binding properties occurred in earlier studies, where older methods of enzyme purification were used, while Price and Radda believe that failure to saturate the fourth enzyme binding site explains the earlier fluorescence data (Velick, 1958). Previous absorption titration data with NADH have not been published to our knowledge and thus a constant absorptivity decrease in going from 3.4 to 4.0 moles of bound NADH per mole of enzyme is not experimentally demonstrated, though certainly expected, both in principle and by analogy to all the enzyme–NAD spectral properties. Actually, the absorptivity of the fourth NADH molecule bound has no effect on our calculations of the number of catalytic sites from experiments illustrated in Figure 1, since in the only experiment with greater than 3 moles of NADH produced per mole of dehydrogenase, the excess NAD displaces enzyme-bound NADH. Negligible changes occur in rate constants calculated from our data even if the fourth binding site contributes no absorbance alterations. NAD titrations with aged enzyme (lower curve in Figure 2a) suggest that the K_d of the fourth site had decreased with age. Bloch (1970), however, found no correlation between the number of NAD binding sites on the enzyme and its specific activity, and data in Figure 2 are too limited for us to be certain of any correlation with K_d at this time. In view of the possible significance of negative cooperativity, and the correlation shown in Figure 4, this possibility deserves further examination.

Rates of NADH Dissociation from Dehydrogenase. The kinetic data on the dissociation rates of dehydrogenase–NADH demonstrate that the first three bound coenzymes dissociate with the same rate constant (Figure 3). The least-squares fits to eq 3 with only 1.1% average absolute deviation indicates a single dissociation rate constant for the acylated dehydrogenase–NADH complex also. Further analysis demonstrates that the reactions are well enough resolved in time and amplitudes to permit a good determination of the NADH dissociation rate constant from eq 3, at least when the absorptivities of acylated enzyme–coenzyme complexes are measured.[3] Similar information is contained in the cor-

relation coefficients among k_1, k_2, and A_0, none of which are greater than 0.7 in the least-squares fit. If the absorptivity of acylated dehydrogenase–NAD is assumed to be zero, however, the ΔA of the NADH dissociation reaction (eq 1) is forced to a smaller amplitude consistent with A_0, which is affected to a much smaller extent (Table II). This displaces to the right the portions of the curve in Figure 1 to which k_1 and k_2 are fitted, thus decreasing their values. This illustrates the benefit of an overall fit, which reveals the related effects of an assumption in an internally consistent way.

Since the calculated rate constants for NADH dissociation from either the acylated enzyme or the aldehyde complex, dehydrogenase–D-G3P–NADH, are the same, but twice that for NADH dissociation from apoenzyme, it is possible that binding of D-G3P to dehydrogenase produces some effects in common with acylation of enzyme. But more data are needed to rule out coincidence and justify this correlation. Our rate constant for NADH dissociation from acylated rabbit muscle dehydrogenase (150–210 sec^{-1}) is in good agreement with the value for lobster enzyme (150 sec^{-1}) determined by Trentham (1971b).

The Number and Symmetry of Rapidly Reacting Catalytic Sites. The redox reactions of rabbit muscle dehydrogenase at 25° are too rapid to decide whether they occur simultaneously or not, but it is clear that with high D-G3P levels, oxidation at all four enzyme sites is completed before significant turnover occurs. The only reasonable alternative estimates of absorptivities[3] raise the calculated catalytic site concentrations even closer to 4 times dehydrogenase concentrations (4.1 to 4.4 for curve 3 of Figure 1, for example). Furthermore, subsequent NADH release occurs at the same rate from all four sites. These conclusions appear valid for all concentrations of NAD equal to, or in excess of enzyme catalytic site concentrations, which is thought to be the prevailing condition in muscle sarcoplasm (Bloch, 1970). Thus the asymmetries in enzyme–coenzyme equilibrium constants are not apparent in the rate constants of D-G3P oxidation under conditions of our experiments. Teipel and Koshland (1970) reached similar conclusions from measurements with pseudosubstrates and Trentham (1971b) found no asymmetry in rate constants of lobster dehydrogenase in either reaction direction, although this enzyme is also reported to bind NAD with negative cooperativity (De Vijlder *et al.*, 1969).

MacQuarrie and Bernhard (1971), however, found that only two of the four enzyme sites can be easily acylated with 1,3-diphosphoglycerate. But Bloch (1970, pp 185ff) describes more recent experiments like those of MacQuarrie and Bernhard, where all four dehydrogenase sites are rapidly acylated and suggests that the earlier results were predisposed by the approximately 2 molar ratio of NAD/dehydrogenase that existed in the earlier experimental conditions. A correlation between NAD content of holoenzyme and the number of acylatable sites was found, in support of this view.

We do not think that the large coenzyme inhibition constants, K_i, rule out catalytic activity of the first three NAD binding sites of enzyme as suggested by Boers *et al.* (1971). The extent of competitive inhibition is determined by the ratio of Michaelis constants, K_m, to K_i, and the K_m's for the first three catalytic sites should be much lower than for the fourth site. In dilute enzyme solutions with excess coenzyme, the first three sites were most likely saturated or very close to saturation throughout the coenzyme concentration range investigated in published reports (1–150 μM NAD), by virtue of the low dissociation constants ($<10^{-5}$, $<10^{-3}$, and 0.3 μM for the first three NAD sites, respectively; Conway and Koshland, 1968). Thus the K_m's of NAD at the first three enzymes sites are not likely to be revealed by measurements with dilute enzyme solutions.

Orsi and Cleland (1972) have concluded that rabbit muscle dehydrogenase has an ordered ter-bi mechanism at pH 8.6 in which NADH cannot dissociate from enzyme until after it is deacylated. This is not in agreement with out conclusions from reactions at pH 7.0 in buffer where little deacylation occurs. We suspect that, as with lobster dehydrogenase (Trentham, 1971b) the deacylation rate increases rapidly with pH to a point where it exceeds the NADH dissociation rate constant. This occurs at pH 8 and above with lobster enzyme, in which the NADH dissociation rate is nearly independent of pH. If this is also the case with rabbit muscle enzyme, the release of products from the enzyme will be random, with the favored pathway shifting from one to the other as pH is increased.

Bloch (1970) discusses evidence against the theory that negative cooperativity in dehydrogenase equilibrium constants of NAD binding serves to buffer *in vivo* NAD levels. Our results indicate that asymmetry is not reflected in the overall rates of D-G3P oxidation. The significance then of negative cooperativity in dehydrogenase remains unclear.

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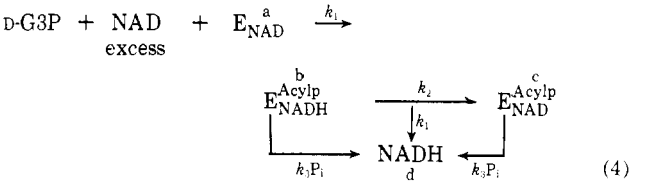
Appendix

Derivation of Eq 3. At saturating substrate concentrations, reactions of eq 1 can be represented by the following first-order reactions



(4)

where the bimolecular additions of D-G3P and NAD are much faster than the reactions shown. Absorbance changes during the reaction are

$$Ab = Ab_a - Ab_a(t = 0) + Ab_b + Ab_c + Ab_d \quad (5)$$

where subscripts denote chemical species identified in eq 4 and t is time. Therefore

$$\frac{Ab}{l} = \epsilon_a([A] - A_0) + \epsilon_b[B] + \epsilon_c[C] + \epsilon_d[D] \quad (6)$$

where ϵ and l are absorptivities and cell path length, respectively; [A], [B], [C], and [D] are concentrations of a, b, c, and d, and A_0 is the concentration of a (E_{NAD}) at zero time. The rate law for two consecutive first-order reactions (Amdur and Hammes, 1966) is used to give concentrations [A], [B], [C], and [D], the latter quantity representing the NADH rapidly generated by dissociation with rate constant, k_2. Processes k_1 and k_2 are assumed to be in rapid equilibrium with the slow phosphorolysis reactions, k_3P_i, which provide further

NADH through turnover. The rate of phosphorolysis is considered proportional to P_i since the concentrations of acylated enzyme intermediates remain constant after the transient period ($1 - e^{-k_1 t}$). Thus eq 6 becomes

$$\frac{Ab}{l} = \epsilon_a A_0(e^{-k_1 t} - 1) + \frac{\epsilon_b A_0 k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) + (\epsilon_c + \epsilon_d)A_0\left[1 + \frac{1}{k_1 - k_2}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right] + \epsilon_d P_0(1 - e^{-k_1 t})(1 - e^{-k_3 t}) \quad (7)$$

The last term, representing phosphorolysis from an initial P_i concentration of P_0, makes negligible absorbance contribution until the transient is nearly complete, hence more precise representation of it is not needed. Collection of exponential coefficients gives eq 3 in the text.

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