Mechanism of Nicotinamide–Adenine Dinucleotide Binding to Rabbit Muscle Glyceraldehyde 3-Phosphate Dehydrogenase*

Gordon G. Hammes,† Peter J. Lillford,‡ and Jon Simplicio§

ABSTRACT: The kinetics of NAD binding to glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle have been studied by the temperature-jump method with native and charcoal-treated enzyme in both 0.05 M pyrophosphate and 0.1 M Tris-HCl buffers at pH 8.5. No relaxation processes could be detected with charcoal-treated enzyme in either buffer until NAD was bound to the first subunit. On binding to the second subunit, a single relaxation time was observed which showed no dependence on coenzyme concentration and is interpreted as a conformational change in the ES_2 species. With NAD:enzyme ratios greater than two, the observed kinetic behavior was dependent on the buffer used. In Tris-HCl, with native enzyme, a complex spectrum of relaxation times was observed. Although all of the individual processes could not be studied in detail, the results were analyzed in terms of two rapid bimolecular processes, associated with binding of coenzyme to the third and fourth sites, and an intramolecular process, associated with a conformational change in the enzyme species with three NAD molecules bound. With charcoal-treated enzyme, similar behavior was observed but with slightly modified kinetic parameters. In pyrophosphate buffer, with native enzyme, two bimolecular processes were observed, which could be assigned to NAD binding to the third and fourth sites. Qualitatively similar behavior was observed at both 25 and 35°. With charcoal-treated protein, only a single relaxation process was observed. Two mechanisms are suggested, both involving a conformational change of the ES_2 species followed by further binding through a bimolecular process. The results of this study and those of rapid mixing experiments on the same system performed elsewhere can be accounted for by a mechanism involving sequential conformational changes of the enzyme accompanying the binding of NAD.

The enzyme D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) catalyzes the reaction

$$\text{3-phosphoglyceraldehyde} + \text{phosphate} + \text{NAD} \rightleftharpoons \text{1,3-diphosphoglycerate} + \text{NADH} + \text{H}^+ \quad (1)$$

Enzyme isolated from yeast, rabbit, pig, and lobster show many similarities (Perham and Harris, 1963; Harris *et al.*, 1963; Davidson *et al.*, 1967). Each enzyme has a molecular weight of approximately 145,000 and consists of four similar subunits. Furthermore, the 18-residue peptide surrounding the active site cysteine is identical for all subunits from all sources. The enzyme binds NAD to form a complex with a characteristic ultraviolet absorption maximum at 360 nm (Racker *et al.*, 1959; Allison and Kaplan, 1964). The stoichiometry of NAD binding to the tetrameric enzymes, yeast, rabbit, and lobster, has been extensively studied by spectral titration (Chance and Park, 1967), equilibrium dialysis (Conway and Koshland, 1968; De Vijlder *et al.*, 1969; Cook and Koshland, 1970), and ultracentrifugation (De Vijlder and Slater, 1968). The more recent studies indicate that 4 moles of NAD are bound per mole of enzyme tetramer. However, close investigation has shown that the binding mechanism is quite complex. For the yeast enzyme, ultracentrifugation experiments indicate that the dissociation constant for the NAD–enzyme complex decreases with increasing coenzyme concentration (Velick, 1953); however recent binding experiments suggest the situation is more complex than this (Cook and Koshland, 1970). Spectrophotometric titrations display a sigmoidal binding curve at 40° and the cooperative subunit interactions implied by this behavior were investigated by relaxation kinetics (Kirschner *et al.*, 1966). The results were interpreted in terms of the allosteric mechanism of Monod *et al.* (1965).

In contrast, the binding of coenzyme to rabbit (Conway and Koshland, 1968; De Vijlder and Slater, 1968) and lobster muscle (De Vijlder *et al.*, 1969) enzyme exhibits the opposite effect; the dissociation constant of the complex increases as successive subunits are bound. This "negative cooperativity" cannot be explained in terms of the concerted allosteric model, but a sequential mechanism of substrate binding allows both positive and negative cooperativity (Adair, 1925; Koshland *et al.*, 1966), and this model has been applied in detail to coenzyme binding with the rabbit muscle enzyme (Conway and Koshland, 1968). Rapid-mixing kinetic studies of this reaction show complex behavior (De Vijlder and Slater, 1968; Bloch, 1970). At least two phases are detectible, one portion of the reaction being complete within the mixing time of the stopped-flow apparatus (~3 msec), the other being of up to 1-sec duration. The amplitude of both phases shows a marked dependence on the presence of phosphate and pyrophosphate, the most dramatic effect being the complete obliteration of the slower phase. Since phosphate is a substrate of the catalytic reaction and pyrophosphate is a substrate analog, an explanation involving specific binding of the anion to the enzyme subunits has been suggested (Bloch, 1970). In solutions of noninteracting buffers the slow phase is seen only when more than 2 equiv of NAD are rapidly mixed with the apoenzyme, suggesting that the effect is connected with the binding of the third coenzyme molecule. However, the amplitude of this effect

* From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received April 30, 1971.* Supported by a grant from the National Institutes of Health (GM 13292).

† To whom correspondence should be addressed.

‡ Present address: Cardiovascular Research Institute, University of California Medical School, San Francisco, Calif. 94122.

§ Present address: Department of Chemistry, University of Miami, Coral Gables, Fla. 33124.

decreases linearly with preincubation of the apoenzyme with NAD, and only 2 equiv is required to eliminate it completely. A satisfactory explanation of this behavior has not yet been presented.

The application of the temperature-jump technique to this system permits a more rigorous investigation of the elementary steps since reactions of very much shorter time scale may be studied. We report here a study of the binding of NAD to the rabbit muscle enzyme at 25° in pyrophosphate and Tris buffers. An additional investigation in pyrophosphate buffer at 35° was also performed, to provide a more direct comparison to the temperature-jump study of the yeast enzyme (Kirschner *et al.*, 1966).

Experimental Section

Materials. The β-diphosphopyridine nucleotide (β-DPN, β-NAD Grade III), dithiothreitol, and Tris (Trizma, reagent grade) were obtained from Sigma Chemical Co. and were used without further purification. The DL-glyceraldehyde-3-phosphoric acid was obtained as the diethyl acetal monobarium salt from Sigma Chemical Co. The free acid was prepared by ion exchange with Dowex 50X4-200R resin, and the resultant concentration of D isomer assayed enzymatically (Velick, 1957). Samples were stored frozen and remained stable for several weeks. Frozen rabbit muscle (type I) was obtained from Pel-Freez Biologicals. Charcoal (Darco-activated carbon) was washed extensively with acid, alkali, and distilled water. All other reagents were the best available commercial grade. Solutions were made up in distilled, deionized water.

Preparation of Holoenzyme. The enzyme was isolated by the method of Cori *et al.* (1948), with the modifications of Amelunxen and Carr (1967). Four recrystallizations were usually sufficient to remove hemoprotein impurities. Solutions of the purified enzyme gave an $A_{280}:A_{260}$ ratio between 1.2 and 1.3, corresponding to 2–3 moles of NAD bound per mole of enzyme (De Vijlder and Slater, 1968).

Preparation of Apoenzyme. The enzyme was isolated from frozen tissue by the method of Ferdinand (1964). The product of this extraction has been identified as the acyl-enzyme (Bloch, 1970). To obtain free enzyme, the acyl-enzyme was further treated according to the method of Amelunxen and Carr (1967). The final product contains adenosine diphosphate ribose in place of NAD (Bloch, 1970). This nucleotide binds much more weakly than the coenzyme so that enzyme, completely free of NAD, was more easily prepared from this product rather than that of the previously described extraction procedure. The adenosine diphosphate ribose was removed from the product either by chromatography on a column of carboxymethylcellulose (Bloch, 1970) or by charcoal treatment (De Vijlder and Slater, 1968). The apoenzyme preparations showed the $A_{280}:A_{260}$ ratio expected for complete removal of NAD. However, to confirm the absence of NAD, samples of the protein were digested with pepsin and the liberated coenzyme assayed enzymatically (Ferdinand, 1964). Less than 0.1 mole of coenzyme/mole of enzyme was found in all cases.

The protein obtained from both extraction procedures was stored as a suspension in saturated ammonium sulfate solution, pH 8.5 at 2°. For kinetic experiments, a sample of the enzyme, suspended in ammonium sulfate was spun down and redissolved in the appropriate buffer. If apoenzyme was required, the solution was stirred with charcoal for 30 min at 2° and recentrifuged. The resultant solution of holo- or apoenzyme was dialyzed for 12 hr at 2° against two changes of 50-fold excess of the required buffer. Slight denaturation occurred and a final high-speed centrifugation was performed prior to using the enzyme. Protein concentrations were determined spectrophotometrically (Fox and Dandliker, 1956), and diluted to give $3–6 \times 10^{-5}$ M enzyme in temperature-jump experiments. (The concentration of NAD binding sites is four times the molar concentration of enzyme.)

A stock solution of NAD was made up in distilled water and the concentration determined spectrophotometrically using a molar extinction coefficient at 260 nm of 1.8×10^4 cm^{-1} M^{-1}. Small aliquots of NAD were added by micropipet to the protein solution to give the required coenzyme concentration for kinetic experiments. No more than four successive additions of NAD were performed with any enzyme solution. In the case of apoenzyme in pyrophosphate buffer, only two additions per protein sample were performed, and apoenzyme in Tris buffer was so sensitive to mechanical agitation that fresh protein was required for each NAD concentration studied.

Assay of Enzymic Activity. Holoenzyme and apoenzyme were assayed by the method of Velick (1957), except that a Tris-acetate buffer (0.03 M Tris with 0.003 M EDTA, pH 8.5) was substituted for pyrophosphate and no preincubation of the enzyme with cysteine was required. Enzyme from both extraction procedures had a specific activity of 120–160 μM DPN reduced per min per mg. Charcoal treatment appeared to have no effect on catalytic activity. Enzyme was routinely assayed before and after temperature-jump experiments. No deterioration of holoenzyme solutions occurred over a 12-hr period at 25°. Apoenzyme reconstituted with NAD was less stable. In pyrophosphate buffer, solutions were stable for a few hours, and in Tris-HCl buffer, fresh solution was required for each experiment.

Kinetic Measurements. The temperature-jump apparatus has been described previously (Faeder, 1970). The final temperature in the cell, after the 7.5° jump, was 25°, except in one study of the holoenzyme in pyrophosphate buffer where a final temperature of 35° was employed.

Concentrations of enzyme from 3.65×10^{-5} to 11.7×10^{-5} M and of NAD from 0 to 5.67×10^{-3} M were used. The kinetic changes were monitored at 360 nm, and the chemical relaxation was displayed on a Tektronix storage oscilloscope and photographed. The photographs were analyzed by use of a curve tracer linked to a PDP-9 computer, which performed a weighted least-squares fit of the logarithm of signal amplitude *vs.* time. In several cases the reciprocal relaxation time obtained by this method was compared to the value obtained by direct measurement of the photographs and plotting. Provided the curves exhibited only a single relaxation time, the correlation was within the experimental uncertainty (±10%). Between five and eight traces were photographed and analyzed for each experimental point reported.

Results and Treatment of Data

Holoenzyme. At 25° in 0.05 M pyrophosphate–0.005 M EDTA (pH 8.5) a single relaxation process was observed up to an NAD:enzyme ratio of about 3.0. Between a ratio of about 3.0 to 3.8, two relaxation processes were observed although the individual relaxation times could not be precisely determined. In this concentration range the faster effect rapidly diminished in amplitude, while that associated with the slower process increased as NAD was added. At NAD:enzyme ratios greater than about 3.8 the faster process disappeared and only the chemical relaxation corresponding to the slower process could

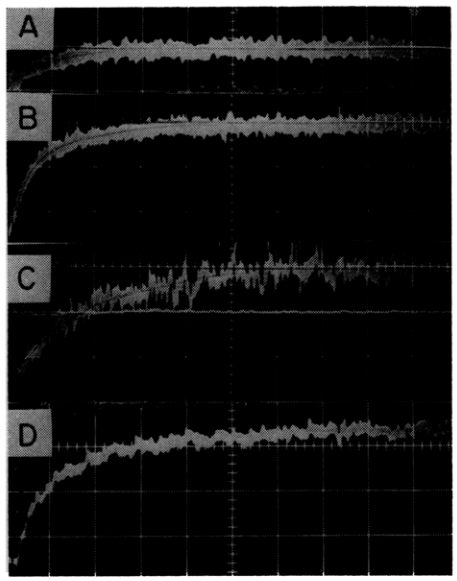

FIGURE 1: Some typical oscillograph traces illustrating the relaxation spectra of the NAD–glyceraldehyde 3-phosphate dehydrogenase (holoenzyme) interaction. The vertical scale is approximately 0.003 A unit/large division except for C where the sensitivity is a factor of two greater. A, B, and C: 0.05 M pyrophosphate–0.005 M EDTA, pH 8.5, 25°. (A) $ES_2 = 7.43 \times 10^{-5}$ M, NAD $= 3.32 \times 10^{-5}$ M, horizontal scale of 1 msec/large division, $\tau = 1.18$ msec. (B) $ES_2 = 6.45 \times 10^{-5}$ M, NAD $= 10.6 \times 10^{-5}$ M, horizontal scale of 2 msec/large division, $\tau_1 \sim 0.8$ msec, $\tau_2 \sim 3$ msec. (C) $ES_2 = 7.43 \times 10^{-5}$ M, NAD $= 24.6 \times 10^{-5}$ M, horizontal scale of 0.5 msec/large division, $\tau = 0.68$ msec. (D) 0.1 M Tris-HCl–0.005 M EDTA, pH 8.5, 25°. $ES_2 = 4.15 \times 10^{-5}$ M, NAD $= 3.2 \times 10^{-5}$ M, horizontal scale of 0.5 msec/large division, $\tau_1 \sim 0.2$ msec, $\tau_2 \sim 0.8$ msec. A more precise evaluation of the longest relaxation time can be made by utilizing a different oscilloscope sweep rate for the photograph. In fact, a detailed analysis of the absorbancy–time curves under these conditions suggests that three relaxation processes may be occurring, but the experimental precision is not sufficient to obtain all three relaxation times.

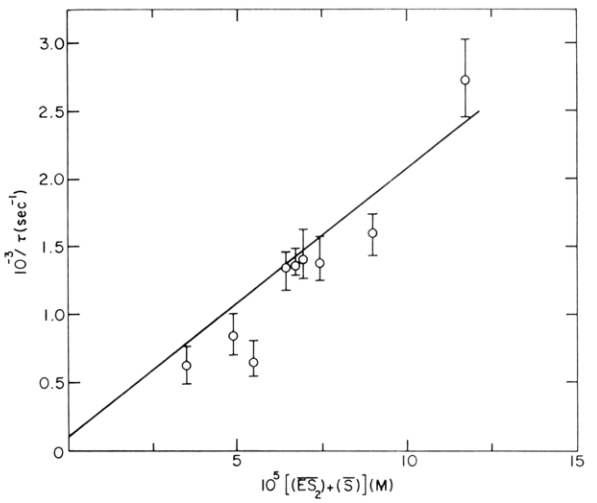

FIGURE 2: Plot of the reciprocal relaxation time, $1/\tau_1$, *vs.* the sum of the equilibrium concentrations of ES_2 and S with holoenzyme in 0.05 M pyrophosphate–0.005 M EDTA, pH 8.5, 25°. The straight line was calculated using the parameters in Table I and eq 4, with the parameters being obtained using the trial and error procedure described in the text.

be detected. The amplitude of this process went through a maximum and gradually decreased with increasing NAD concentration. Some typical oscillograph traces illustrating the general behavior described above are shown in Figures 1A–C. In Figure 1A a curve which can be described by a single exponential function is shown at a low NAD:enzyme ratio; in Figure 1B a single exponential function no longer describes the curves obtained at an intermediate NAD:enzyme ratio; finally in Figure 1C at a high NAD:enzyme ratio the amplitude of the relaxation process observed in Figure 1A has become undetectable and a single exponential function again characterizes the curve shown. The relaxation times for both processes showed a marked dependence on NAD concentration suggesting the occurrence of coupled bimolecular reactions. Since two of the NAD molecules are stoichiometrically bound (Conway and Koshland, 1968; De Vijlder and Slater, 1968), a reasonable mechanism is the successive addition of the third and fourth molecules to the enzyme:

$$ES_2 + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_3 \qquad (2)$$

$$ES_3 + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_4 \qquad (3)$$

In general this system would be characterized by two coupled relaxation times. However, when the NAD:enzyme ratio is less than 3, eq 3 can be neglected to a good approximation, while when this ratio is greater than 3.8, eq 2 can be neglected to a good approximation (*i.e.*, the third site is essentially fully occupied). Then in the former case the reciprocal relaxation time, $1/\tau_1$, is

$$1/\tau_1 = k_1[(\overline{ES_2}) + (\overline{S})] + k_{-1} \qquad (4)$$

and in the latter case

$$1/\tau_2 = k_2[(\overline{ES_3}) + (\overline{S})] + k_{-2} \qquad (5)$$

where the overbars designate equilibrium concentrations. Unfortunately the binding constants for the third and fourth NAD molecules are not known under the appropriate experimental conditions so that the equilibrium concentrations cannot be calculated. A computer program was used which calculated the equilibrium concentrations of eq 4 and 5, given the stoichiometric enzyme and NAD concentrations $[(ES_2)_0$ and $(S)_0$, respectively] and the equilibrium dissociation constants K_3 and K_4 (eq 2 and 3, respectively). An initial estimate of K_4 was obtained from the kinetic data by a plot of $1/\tau$ *vs.* $[(S)_0 - (ES_2)_0]$ (a first approximation of $(\overline{ES_3}) + (\overline{S})$ for an NAD:enzyme ratio greater than 3.8). The value of K_3 was initially arbitrarily taken as $K_4/10$ (Conway and Koshland, 1968). The values of K_3 and K_4 obtained were then used to replot the data, and this process was continued until the initial and final dissociation constants were identical. Figures 2 and 3 show the final plots of the reciprocal relaxation time vs. $[(\overline{ES_2}) + (\overline{S})]$ and $[(\overline{ES_3}) + (\overline{S})]$, respectively, where the NAD:enzyme ratio is less than 3.0 in the former case and greater than 3.8 in the latter case. The straight lines shown are consistent with both the assumed equilibrium constants and the kinetic data. The values of the rate constants and equilibrium dissociation constants are summarized in Table I. The fit of the experimental data in Figure 3 is quite good. Since the relaxation times in Figure 2 could only be determined with great difficulty over a very narrow range of concentrations, the fit

TABLE I: Equilibrium and Kinetic Parameters.

Holoenzyme			Apoenzyme	
0.05 M pyrophsophate, pH 8.5			0.05 M pyrophosphate, pH 8.5, 25°	
K_3 (μM)	5.0	(25°)	K_3 (μM)	40
	28	(35°)	K_4 (μM)	800
K_4 (μM)	66	(25°)	k_6 (sec^{-1})	250
	145	(35°)	k_{-6} (sec^{-1})	250
k_1 (M^{-1} sec^{-1})	2.0×10^7	(25°)	k_7 (M^{-1} sec^{-1})	1.85×10^7
k_{-1} (sec^{-1})	100	(25°)	k_{-7} (sec^{-1})	370
k_2 (M^{-1} sec^{-1})	5.9×10^6	(25°)		
	6.0×10^6	(35°)		
k_{-2} (sec^{-1})	390	(25°)		
	870	(35°)		
0.1 M Tris, pH 8.5, 25°			0.1 M Tris, pH 8.5, 25°	
K_3 (μM)	1.5		K_3 (μM)[a]	4
K_4 (μM)	10		K_4 (μM)[a]	35
$k_3 + k_{-3}$ (sec^{-1})	300		$k_8 + k_{-8}$ (sec^{-1})	550
k_4 (M^{-1} sec^{-1})	4.4×10^7		k_9 (M^{-1} sec^{-1})	1.4×10^7
k_{-4} (sec^{-1})	400		k_{-9} (sec^{-1})	490

[a] De Vijlder and Slater (1968).

of the experimental data is satisfactory. In principle the kinetic coupling of the two binding steps should be explicitly taken into account. The exact relaxation times were calculated for the mechanism of eq 2 and 3 using the rate constants in Table I and are in reasonable agreement with the measured values. The precision of the data is not sufficient to warrant an extensive analysis in terms of the exact expressions for the relaxation times.

At 35°, similar behavior was observed except that only a single relaxation process was detected and the binding constants for the third and fourth molecules appeared to be much weaker than at 25°. The data could be analyzed as at 25° if the measured relaxation times were assumed to be associated with the binding of the fourth NAD molecule. The kinetic and equilibrium parameters are included in Table I, and the kinetic data are plotted in Figure 3.

At this point it is worth noting that the reported rate constants are not necessarily those of the elementary steps. For example, if the vacant sites are all equivalent, the rate constant for the formation of ES_n is $(5 - n)$ times the rate constant for the elementary step and if the occupied sites are all equivalent, the dissociation rate constant is n times that for the elementary step.

At 25° in 0.1 M Tris–0.005 M EDTA (pH 8.5), very different behavior was observed. With a ratio of NAD to enzyme between 2 and 3, at least two relaxation effects could be detected. A typical oscilloscope trace showing a minimum of two relaxation processes is presented in Figure 1D. The fastest process ($1/\tau \sim 5 \times 10^3$ sec^{-1}) had a maximum amplitude at an NAD:enzyme ratio of about 2.5; however the amplitude was too small to permit a quantitative study of this process. The slowest effect, which was analyzed in detail, increased in amplitude as NAD was added but exhibited little dependence on coenzyme concentration until sufficient NAD was added to saturate the third binding site. At higher concentrations of NAD the reciprocal relaxation time showed a linear dependence on the added concentration of NAD, as

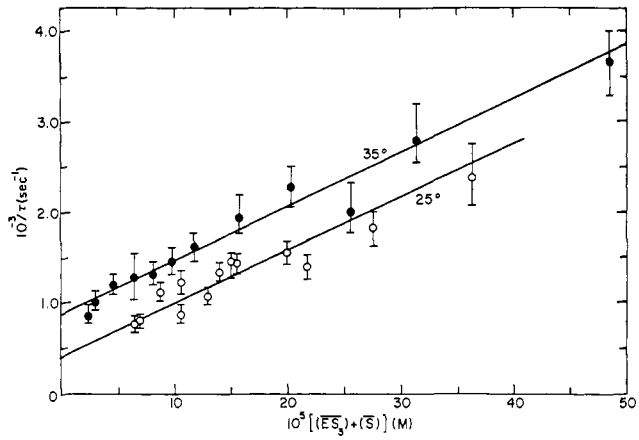

FIGURE 3: Plot of the reciprocal relaxation time, $1/\tau_2$, *vs.* the sum of the equilibrium concentrations of ES_3 and S with holoenzyme in 0.05 M pyrophosphate–0.005 M EDTA, pH 8.5 at 25° (○) and 35° (●). The straight lines were calculated using the parameters in Table 1 and eq 5, with the parameters being determined by the trial and error procedure described in the text.

shown in Figure 4. The amplitude of this process went through a maximum and eventually decreased at high NAD concentrations. A possible mechanism consistent with these findings is

$$ES_2 + S \underset{}{\overset{K_3'}{\rightleftharpoons}} ES_3 \quad (6)$$

$$ES_3 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES_3' \quad (7)$$

$$ES_3' + S \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} ES_4 \quad (8)$$

Normally three relaxation times would be expected for the above mechanism, whereas only two well-defined time constants could be obtained (Eigen and De Maeyer, 1963; Hammes and Schimmel, 1970). However, a possible explanation of the experimental observations is as follows. The faster observed relaxation time can be associated with eq 6 and the

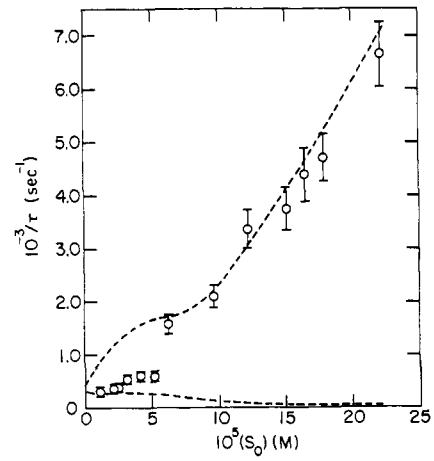

FIGURE 4: Plot of the reciprocal relaxation time, $1/\tau$, *vs.* the total concentration of NAD, (S_0), with holoenzyme (4.15×10^{-5} M) in 0.1 M Tris-HCl–0.005 M EDTA, pH 8.5 at 25°. The dashed lines are two of the relaxation times (eq 9) associated with the mechanism of eq 6–8, calculated using the parameters in Table I.

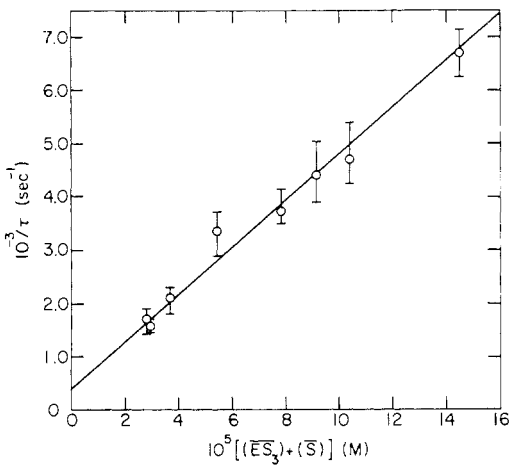

FIGURE 5: Plot of the reciprocal relaxation time, $1/\tau$, *vs.* the sum of the equilibrium concentrations of ES_3 and S. These data have been taken from Figure 4 at high NAD concentrations. The straight line has been drawn using eq 11 and the parameters in Table I.

slower relaxation time can be associated with eq 7 and 8. In principle, two relaxation times are associated with eq 7 and 8, and if eq 6 is considered to be a preequilibrium they can be written to a good approximation as

$$1/\tau = \frac{1}{2}\left[(a_{11} + a_{22}) \pm \sqrt{(a_{11} + a_{22})^2 - 4(a_{11}a_{22} - a_{12}a_{21})}\right] \quad (9)$$

where

$$a_{11} = \frac{k_3}{1 + K_3'/[(\bar{S}) + (\overline{ES}_2)]} + k_{-3}$$

$$a_{12} = k_3$$

$$a_{21} = k_4\left[(\bar{S}) - \frac{(\overline{ES}_3')}{1 + [(\bar{S}) + (\overline{ES}_2)]/K_3'}\right]$$

$$a_{22} = k_{-4} + k_4[(\overline{ES}_3') + (\bar{S})]$$

Two possible interpretations of the observed slow relaxation time are possible. First it can be interpreted as representing one of the roots of the square root above. The relaxation time utilizing the positive square root has qualitatively the correct form: at low NAD concentrations with the enzyme concentrations employed $a_{22} \ll a_{11}$ and

$$1/\tau \approx k_3 + k_{-3} \quad (10)$$

at high NAD concentrations $a_{22} \gg a_{11}$ and

$$1/\tau \approx k_4[(\overline{ES}_3') + (\bar{S})] + k_{-4} \quad (11)$$

Although this relaxation time has qualitatively the correct concentration dependence, it was not possible to fit the data quantitatively in the transition region, where $a_{11} \approx a_{22}$ utilizing reasonable equilibrium dissociation constants. A second interpretation of the data is that eq 10 and 11 are correct in the limits of low and high NAD concentrations, but that in the transition region the two relaxation times defined by eq 9 are being observed, but cannot be resolved experimentally. This is not unreasonable since the relaxation effects have fairly small amplitudes. An analysis of the data was carried out assuming eq 10 to be valid at low NAD concentrations (actually as (NAD) $\rightarrow$ 0) and eq 11 to be valid at high NAD concentrations. The equilibrium dissociation constants are not known for the holoenzyme under the experimental condition employed, but reasonable values could be obtained utilizing the trial and error procedure described above on the data at high NAD concentrations in Figure 4. The linear plot obtained is shown in Figure 5 and the kinetic and equilibrium parameters obtained are included in Table I. These parameters were then used to calculate the two relaxation times according to eq 9. These relaxation times are shown as dashed lines in Figure 4 for the enzyme concentration employed in most of the experiments (4.15×10^{-5} M). It can be seen that one of the roots of the relaxation time describes the data well at low NAD concentrations, while the other fits the data at high NAD concentrations. The experimental points for the transition region fall between the two calculated curves, as expected from the assumption underlying the analysis. In this transition region both the amplitudes and time constants should be comparable; although some indication of three relaxation processes could be observed in the absorbancy–time curves, the time constants could not be resolved. On either side of the transition region, the amplitude of one of the relaxation processes should predominate so that the observation of a single relaxation time is not surprising.

Although only semiquantitative significance can be attributed to the rate parameters obtained, the analysis used is reasonably consistent with all of the data. The ratio k_{-3}/k_3 could not be obtained so that the overall dissociation constants K_3 and K_4 were assumed to be equal to K_3' and k_{-4}/k_4, whereas actually they are equal to $K_3'/(1 + k_3/k_{-3})$ and $(k_{-4}/k_4)(1 + k_{-3}/k_3)$, respectively. A more exact analysis cannot be carried out at this time, but in any event would not markedly alter the concentration dependence of the relaxation times. Finally it should be mentioned that a mechanism, where ES_3, rather than ES_3', reacts with the fourth NAD molecule, is also consistent with the data.

Apoenzyme. With apoenzyme in 0.05 M pyrophosphate buffer, the relaxation spectrum was studied with NAD:apoenzyme ratios from 0 to 5.1. No effects were detectable up to a ratio of $\sim$0.9. Between 0.9 and 2 a single relaxation process was observed with no apparent coenzyme concentration dependence. With more than two equivalents of NAD a single relaxation process was still observed, but the reciprocal relaxation time increased as coenzyme was added. At no point in the concentration range was more than a single relaxation process resolved. The results obtained are shown in Figure 6.

A simple mechanism consistent with these observations is

$$ES + S \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} ES_2 \quad (12)$$

$$ES_2 \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} ES_2' \quad (13)$$

$$ES_2' + S \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} ES_3 \quad (14)$$

where the binding of the fourth NAD molecule is assumed to be negligible at the concentrations employed. Unfortunately binding studies have not been carried out under exactly the same experimental conditions, but equilibrium dialysis experiments under somewhat different conditions (Conway and

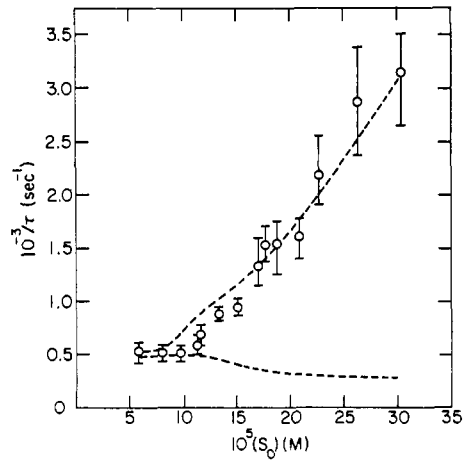

FIGURE 6: Plot of the reciprocal relaxation time, $1/\tau$, *vs.* the total concentration of NAD for apoenzyme (5.60×10^{-5} to 5.95×10^{-5} M) in 0.05 M pyrophosphate–0.005 M EDTA, pH 8.5 at 25°. The dashed lines are two of the relaxation times (eq 9) associated with the mechanism of eq 12–14, calculated using the parameters in Table I.

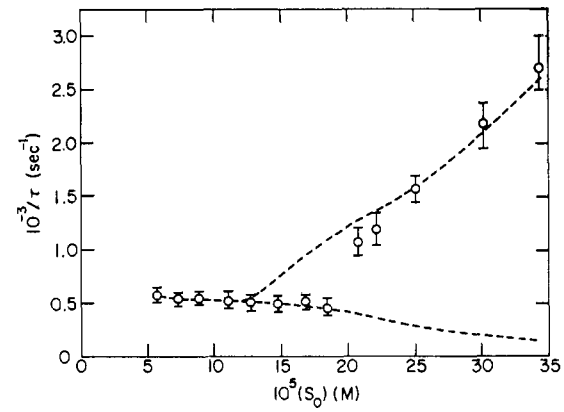

FIGURE 7: Plot of the reciprocal relaxation time, $1/\tau$, *vs.* the total concentration of NAD for apoenzyme (6.38×10^{-5} M) in 0.1 M Tris-HCl–0.005 M EDTA, pH 8.5 at 25°. The dashed lines are two of the relaxation times (eq 9) associated with the mechanism of eq 15–19, calculated using the parameters in Table I.

Koshland, 1968) indicate the dissociation constants for NAD binding to the charcoal-treated enzyme are greatly increased, although negative cooperativity is retained. In the above mechanism the binding of the first and second molecules of NAD to the enzyme is assumed to be too rapid to be observed, or alternatively the associated relaxation amplitude is too small. Exactly as in the mechanism just discussed, the observed relaxation time is attributed to a coupling of eq 13 and 14, and the expected relaxation times are given by eq 9 where the a_{ij}'s can be obtained by analogy of eq 12–14 to eq 6–8. An approximate value of K_3, the dissociation constant of NAD from the third site, was obtained from a plot of $[(S)_0 - 2(E)_0]$ *vs.* $1/\tau$ at high NAD concentrations. A value of K_4, the dissociation constant for the fourth site was chosen to give minimal binding under the conditions employed. The kinetic and equilibrium parameters were obtained by the iterative procedure previously described. The final values of the parameters are given in Table I, and the two calculated relaxation times are shown in Figure 6. Again in the intermediate concentration region, the observed relaxation time appears to be a mean of the two calculated relaxation times.

An alternative mechanism is to assume that the binding site for the third and fourth NAD molecules are equivalent. Such a possibility has been suggested (Bloch, 1970) and is consistent with the kinetic data with an apparent dissociation constant of 100 μM. Formally this mechanism is the same as eq 12–14, except that the species ES_2' has two equivalent binding sites so that its effective concentration is $2(ES_2')$.

Because of the instability of the apoenzyme in 0.1 M Tris buffer, a less complete study of the concentration dependence of the relaxation spectrum was obtained. Nevertheless, the general features of the concentration dependence of the relaxation spectrum are apparent. No relaxation processes were detectable with an NAD:apoenzyme ratio less than ~0.8. When this ratio was between ~0.8 and 2, a single relaxation process was observed which exhibited no concentration dependence. Between a ratio of 2 and 3, a slow process was also observed, but an additional very fast process ($1/\tau > 3 \times 10^3$ sec^{-1}) was in evidence. Above an NAD:enzyme ratio of 3, only a single process was observed, with a relaxation time which decreased with increasing coenzyme concentration.

Unfortunately only the slowest relaxation time could be determined over an appreciable concentration region, and it is shown in Figure 7 as a function of the total NAD concentration. A mechanism consistent with these observations is

$$ES + S \rightleftharpoons ES_2 \tag{15}$$

$$ES_2 \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} ES_2' \tag{16}$$

$$ES_2' + S \rightleftharpoons ES_3 \tag{17}$$

$$ES_3 \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} ES_3' \tag{18}$$

$$ES_3' + S \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} ES_4 \tag{19}$$

The complete relaxation spectrum of the above scheme is very complex; however the data can be rationalized in terms of this mechanism as follows. The bimolecular binding of the second NAD molecule (eq 15) cannot be seen, either because the associated relaxation time is too fast, or the relaxation amplitude is too small. The bimolecular binding of the third NAD molecule (eq 17) is characterized by the fast relaxation process which could not be well characterized. The slow relaxation time is then due to a conformational change of ES_2 and ES_3 (eq 16 and 18) and the binding of the fourth NAD molecule. The rate of the conformational change is apparently independent of whether two or three NAD molecules are bound to the enzyme. The problem now is identical to that discussed previously for the binding of NAD to holoenzyme in tris buffer, and an approximate solution was obtained in the same manner. In this case, values of the equilibrium dissociation constants are available (De Vijlder and Slater, 1968). The calculated values of the two relaxation times for this system, using the parameters in Table I, are shown in Figure 7. These parameters were obtained by a trial and error fitting of the data. Again this appears to be an example where the relaxation spectrum cannot be resolved in the concentration region where coupling is very important, but the data can be fit well at very low and very high NAD concentrations. Considering both the experimental and theoretical difficulties and approximations, the fit of the data is not unreasonable.

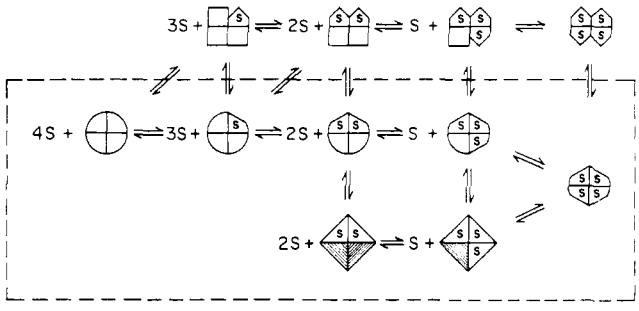

FIGURE 8: Schematic mechanism for the binding of NAD to D-glyceraldehyde 3-phosphate dehydrogenase. The different geometrical shapes represent different conformations of the enzyme. As explained in the text, the entire mechanism is required to explain the stopped-flow and temperature-jump results, while the portion enclosed in the dashed lines suffices for the temperature-jump results.

Discussion

The results obtained indicate that the relaxation spectrum for this system is very complex, and in fact the individual relaxation times cannot be well resolved experimentally. In a complex system such as this, the experimental difficulty of resolving the relaxation spectra should be emphasized; relaxation times which differ by less than a factor of two usually cannot be distinguished if the relaxation processes have equal amplitudes. In the present case, the large difference in binding constants for successive addition of NAD molecules causes a dramatic change in relaxation amplitudes over the concentration ranges employed, and this factor is what makes it possible to resolve many of the individual steps in the binding mechanism. This point is best illustrated by the data obtained for the holoenzyme in pyrophosphate: the two relaxation times can be resolved because the amplitude of one of the relaxation processes is much smaller than the other except for a very small concentration range. In general, the behavior of the relaxation amplitudes corresponds to that predicted on the basis of the proposed mechanisms.

What has been obtained is a semiquantitative determination and analysis of the relaxation spectrum. Although the rate and binding constants can only be regarded as rough estimates, it is readily apparent that a number of binding steps and conformational transitions are occurring. Equilibrium binding studies have not been carried out under all of the exact conditions used in these experiments; however the binding constants obtained from the kinetics are quite consistent with those obtained in equilibrium studies under similar, but not identical, conditions (Conway and Koshland, 1968; De Vijlder and Slater, 1968).

Some general assessment of the binding mechanism can be made by detailed consideration of the results. First the results obtained in 0.1 M Tris-HCl, pH 8.5, are considered. Although the enzyme is quite unstable in this media so that it is difficult to obtain extensive data, Tris buffer is believed not to interact with the enzyme, unlike pyrophosphate. No relaxation effects were detectable for the binding of the first molecule of NAD to the enzyme. This binding process cannot be slow since rapid-mixing studies (Bloch, 1970; De Vilder and Slater, 1968) indicate it is complete within 3 msec after mixing. Therefore, either this process is too fast ($\tau = <10\ \mu$ sec) or more likely the relaxation amplitude is too small to be detected by our instrument. As the second molecule of NAD binds, a relaxation process is observed which can be associated with a conformational change of the ES_2 species. With the native enzyme dialyzed extensively against buffer so that only 2 moles of NAD remain bound, this process could not be detected suggesting that a single conformation has been stabilized in the holoenzyme. The effect of charcoal treatment on the enzyme is not well understood and it may be that two conformations are artificially created by charcoal. However, similar behavior was observed in pyrophosphate with apoenzyme prepared by the much milder procedure described in the Experimental Section. The bimolecular reaction leading to the formation of ES_2 could not be detected, again presumably due either to its rapidity or to an undetectable relaxation amplitude.

The binding of the third and fourth NAD molecules is qualitatively similar for both native and charcoal-treated enzyme. Two distinct relaxation processes were observed. The faster process can be associated with the binding of the third NAD molecule although a detailed concentration dependence could not be determined, and the slow process is related to a conformational change of the ES_3 species and the binding of the fourth NAD molecule. The reciprocal relaxation time associated with the conformational change is slightly larger for the charcoal-treated enzyme (Table I), but the difference is not great and the conformational transition is undoubtedly similar in both cases. Moreover, the conformational change in the ES_2 species of the charcoal-treated enzyme has the same relaxation time as the transition in the ES_3 species and therefore also is presumably similar in nature. The mechanism for the binding of the fourth NAD molecule is the same for both native and charcoal-treated enzyme: a simple bimolecular reaction. Considering the approximate treatment of the data, the rate constants and equilibrium dissociation constants obtained are remarkably similar in both cases (Table I). The simplest mechanism for the binding process consistent with all of the data is shown schematically as the portion of Figure 8 enclosed within the dashed lines. The different geometrical shapes designate different conformational states of the enzyme.

The results obtained with the apoenzyme in 0.05 M pyrophosphate are slightly different from those found in 0.1 M Tris buffer: a conformational change is observed in the ES_2 species, but not in the ES_3 species. However, the sum of the rate constants associated with the conformational change is essentially identical in both cases (5.5×10^2 sec^{-1}). The apoenzyme prepared by column chromatography gave a slightly different value for this sum, which was identical with that found for the holoenzyme in Tris buffer ($\sim 3 \times 10^2$ sec^{-1}). The question as to whether the third and fourth molecules bind to equivalent sites or cooperative effects occur cannot be resolved by this work as both possibilities are consistent with the data. However, in any event, a conformational transition in the ES_2 species followed by a bimolecular binding step is suggested by the data. With native enzyme, the binding of both the third and fourth NAD molecules could be resolved. The fact that a conformational change is not observed in the ES_3 species with either native or charcoal-treated enzyme, suggests that pyrophosphate stabilizes a single conformation state. Therefore, the overall binding process also can be depicted as the enclosed portion of Figure 8, except that only one of the ES_3 species is present in significant amounts.

The mechanism of the enclosed part of Figure 8 provides an explanation of all the results obtained in this study; however rapid-mixing studies have also been carried out of the NAD–enzyme interaction (De Vijlder and Slater, 1968; Bloch, 1970). Upon mixing apoenzyme and NAD, a biphasic process is observed, one portion of the reaction being complete within

the mixing time of the apparatus, the other process having a relatively long relaxation time ($1/\tau \sim 0.3$ sec^{-1}). This slower process has been analyzed in terms of conformational change of ES_2, ES_3, and ES_4 species (Bloch, 1970). A clue to a possible explanation for the difference in the results obtained with temperature-jump and rapid-mixing techniques is given by the effects of preincubation of apoenzyme with coenzyme prior to rapid-mixing experiments. Slow binding effects are only observed when more than 2 mole equiv of NAD is mixed with apoenzyme; moreover preincubation with NAD causes a linear decrease in the amplitude of the observed effect until it completely disappears when incubation is with 2 or more mole equiv of NAD. Preincubation always occurs in temperature-jump experiments since solutions are made up at least 1 min before measurements are made. Therefore, it is not surprising that slow processes are not observed with the temperature-jump method, and it must be concluded that metastable states are observed in the rapid mixing studies.

A possible mechanism consistent with all of the findings is shown in Figure 8. In this mechanism, the top row of conformations of ES_2, ES_3, and ES_4 species are metastable species, and the rapid-mixing experiments measure the rates of the vertical conformational transitions of the first row. The states below the first row then represent the stable system formed by preincubation, which is of importance in the temperature-jump experiments. The nature of the various conformational states cannot be specified, and could involve intra- or intersubunit changes.

Although some apology must be made for the necessarily approximate nature of the data analysis, a complex stepwise mechanism is indicated for the addition of NAD to the rabbit muscle enzyme. This is in contrast to the conclusions reached for the yeast enzyme, where the concerted mechanism of Monod *et al.* (1965) has been postulated to be operative (Kirschner *et al.*, 1966). This mechanism predicts three relaxation times should be observed and that one of the relaxation times should reach a limiting value as the NAD concentration increases and the enzyme approaches saturation. This behavior is clearly not seen in the relaxation spectra associated with the rabbit muscle enzyme. The apparent difference in binding mechanism for two very structurally similar enzymes is puzzling and merits further investigation.

Acknowledgment

The authors are indebted to Dr. S. Bernard for supplying us with a copy of the Ph.D. thesis of W. A. Bloch, Jr.

References

Adair, G. S. (1925), *J. Biol. Chem. 63*, 529.
Allison, W. S., and Kaplan, N. O. (1964), *J. Biol. Chem. 239*, 2140.
Amelunxen, R. E., and Carr, D. O. (1967), *Biochim. Biophys. Acta 132*, 256.
Bloch, W. A., Jr. (1970), Ph.D. Thesis, University of Oregon.
Chance, B., and Park, J. H. (1967), *J. Biol. Chem. 242*, 5093.
Conway, A., and Koshland, D. E., Jr. (1968), *Biochemistry 7*, 4011.
Cook, R. A., and Koshland, D. E., Jr. (1970), *Biochemistry 9*, 3337.
Cori, G. T., Stein, M., and Cori, C. F. (1948), *J. Biol. Chem. 173*, 605.
Davidson, B. E., Sajgo, M., Noller, H. F., and Harris, J. I. (1967), *Nature (London) 216*, 1181.
De Vijlder, J. J. M., Boers, W., and Slater, E. C. (1969), *Biochim. Biophys. Acta 191*, 214.
De Vijlder, J. J. M., and Slater, E. C. (1968), *Biochim. Biophys. Acta 167*, 23.
Eigen, M., and De Maeyer, L. (1963), *Tech. Org. Chem. 8* (2), 895.
Faeder, E. (1970), Ph.D. Thesis, Cornell University, Ithaca, N. Y.
Ferdinand, W. (1964), *Biochem. J. 92*, 578.
Fox, J. B., Jr., and Dandliker, W. B. (1956), *J. Biol. Chem. 221*, 1005.
Hammes, G. G., and Schimmel, P. R. (1970), *Enzymes 2*, 67.
Harris, J. I., Meriwether, B. P., and Park, J. H. (1963), *Nature (London) 198*, 154.
Kirschner, K., Eigen, M., Bittman, R., and Voight, B. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1661.
Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.
Monod, J., Wyman, J., and Changeaux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Perham, R. N., and Harris, J. I. (1963), *J. Mol. Biol. 7*, 316.
Racker, E., Klybas, V., and Schramm, H. (1959), *J. Biol. Chem. 234*, 2510.
Velick, S. F. (1953), *J. Biol. Chem. 203*, 563.
Velick, S. F. (1957), *Methods Enzymol. 1*, 401.