THE BINDING OF NICOTINAMIDE-ADENINE DINUCLEOTIDE TO
YEAST D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE:
TEMPERATURE-JUMP RELAXATION STUDIES ON THE MECHANISM
OF AN ALLOSTERIC ENZYME*

BY K. KIRSCHNER, M. EIGEN, R. BITTMAN, AND B. VOIGT

MAX-PLANCK-INSTITUT FÜR PHYSIKALISCHE CHEMIE, GÖTTINGEN, GERMANY

Communicated September 6, 1966

Enzymes exhibiting sigmoidal response of rate and binding to the concentration of substrates, activators, or inhibitors have been implied in a number of regulating mechanisms involving feedback control.¹⁻³ It has become clear that these cooperative interactions of ligand molecules are intimately related (1) to the subunit structure of the enzyme and (2) to conformational changes accompanying ligand binding. Several mechanisms have been proposed to account for sigmoidal saturation curves: For the classical case of oxygen binding to hemoglobin, Adair⁴ suggested a mechanism involving four consecutive, bimolecular steps with successively increasing affinity for the ligand. An alternative mechanism, the "allosteric model," has been put forward by Monod *et al.*⁵ It postulates the existence of an "allosteric" pre-equilibrium between two conformations of the oligomeric protein, each of which has a different affinity for the ligand. The allosteric equilibrium is shifted in the course of ligand binding. By introducing the concept of the "induced fit," Koshland *et al.*⁶ recently offered another physical explanation for the increase of affinity as the protein is saturated with ligand. In this mechanism, emphasis is put on sequential changes of subunit structure and ligand affinity.

In the same paper (*loc. cit.*, ref. 6) it is clearly shown that a careful fit of equilibrium data to the various possible mechanisms does not permit an unequivocal distinction between them. It is evident that additional information is needed for establishing a mechanism for cooperative ligand binding. Such information could be obtained from kinetics. However, in order to demonstrate the individual elementary steps of the reaction, the various intermediates must be present in detectable concentrations. This requires an increase of enzyme concentration to substrate levels leading to an acceleration of the bimolecular reactions. Relaxation methods are adequately suited for kinetic investigations under these conditions.

In the course of studies concerning the binding of nicotinamide-adenine dinucleotide (NAD) to glyceraldehyde-3-phosphate dehydrogenase from yeast, it was found that this particular case is an ideal system for the investigation of cooperative, "homotropic"⁵ ligand binding. The relaxation spectrum obtained by temperature-jump measurements is extremely simple. It consists of three relaxation processes which are well separated from each other on the time axis. This leads to simple, analytical expressions for the concentration dependence of each relaxation step. Therefore, the data can be analyzed directly from theory without taking recourse to numerical methods.

Materials and Methods.—(a) *Enzyme:* Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purified from baker's yeast following the procedure of Krebs.⁷ Treatment of the first crystals with charcoal was introduced to remove bound NAD.⁸ After two additional crystallizations, the enzyme had a ratio of OD 280:-

FIG. 1.—Difference spectrum of the GAPDH-NAD complex. Buffer: $5 \cdot 10^{-2} M$ Na-pyrophosphate, $5 \cdot 10^{-3} M$ Na-EDTA, pH 8.5; enzyme: $1.43 \cdot 10^{-4} M$ (20 mg protein/ml); NAD: $3 \cdot 10^{-3} M$; temperature = $20^\circ C$; ϵ is calculated per mole of binding sites, with four binding sites per mol wt of 145,000.⁹

FIG. 2.—Spectrophotometric titration of apoenzyme with NAD. The titration was carried out after the method of Stockell.¹⁰ Enzyme concentration and buffer as in Fig. 1. A micrometer syringe was used to add μ l increments of a 0.1 M solution of neutralized NAD to the enzyme in a cuvette ($d = 2.0$ cm). Readings were taken at 400 $m\mu$ after completion of the slow relaxation (2–3 min) and were corrected for blank absorption and turbidity.

OD 260 of 2.15 and was virtually free of NAD and protein impurities. For kinetic measurements crystals of enzyme were centrifuged down, and dissolved in and dialyzed against the appropriate buffer. β -NAD was purchased from Boehringer und Söhne, Mannheim. The enzyme assay closely followed the procedure recommended in the "Boehringer Information" on GAPDH. Under these conditions pure enzyme had a specific activity of 150 IU/mg ($= \mu$ moles NADH reacted per min and mg protein). Protein concentrations were determined from the OD at 280 $m\mu$ with $\epsilon_{280} = 0.86$ [ml/mg cm].⁸ The molecular weight of GAPDH was taken to be 145,000.⁹

(b) *Spectrophotometric titrations* were carried out after Stockell¹⁰ in 2-cm cuvettes with the Zeiss PMQ II spectrophotometer equipped with thermospacers.

(c) *Temperature-jump measurements* were made as previously described¹¹ using a cell with Pt electrodes. Solutions routinely contained 20 mg enzyme/ml ($= 1.43 \cdot 10^{-4} [M]$) and concentrations of coenzyme varying between 5 and 95 per cent saturation ($5 \cdot 10^{-5}$ to $2 \cdot 10^{-3} [M]$ NAD). Both the difference spectrum and enzyme activity were repeatedly checked before and after temperature-jump experiments. In general, no change was found even after 20–30 high-voltage pulses. Changes in concentrations were followed at 366 $m\mu$. The traces were evaluated by hand from photographs of the oscilloscope screen.

(d) *Stopped-flow kinetics* were done with an instrument from Durrum Instruments Corp., Palo Alto. In evaluating the third relaxation process, only the last 5 per cent of the reaction was evaluated.

Results.—(a) *Equilibrium measurements:* The difference spectrum of the enzyme-NAD complex is presented in Figure 1. It agrees with the spectrum reported by Stockell.¹⁰ Spectrophotometric titration of the apo-enzyme with NAD (Fig. 2) permits the evaluation of the apparent dissociation constant K and the number of binding sites n . In contrast to the value of $n = 2$ found by Stockell,¹⁰ we consis-

FIG. 3.—Evaluation of spectrophotometric titration data. For conditions see Fig. 2. $D_0/p = K/(E_0 - p) + n$; E_0 = total enzyme concentration; $p = \Delta E/\Delta E(\text{saturation}) \times E_0$.

FIG. 4.—Effect of temperature on the binding of NAD. For experimental details see Fig. 2.

tently determined values of 3.6–4 (Fig. 3).⁸ This result is in excellent agreement with chemical analysis⁹ of the enzyme which demonstrates that it is composed of four identical subunits. The temperature dependence of the dissociation constant at constant pH is marked: On raising the temperature from 20° to 50°C at pH 8.5, the saturation curve is shifted from hyperbolic to weakly sigmoidal (Fig. 4). The dependence of over-all initial velocity on the concentration of NAD is influenced in a qualitatively similar way by increasing the temperature from 20° to 40°C.⁸

(b) *Temperature-jump experiments:* Under conditions leading to sigmoidal titration curves, e.g., pH 8.5, 40°C (Fig. 4), the equilibration of a solution perturbed by a temperature jump proceeds in three discrete steps (Fig. 5). The time constants of the two rapid processes lie between $1/\tau_1 = 10^3$ to 10^4 and $1/\tau_2 = 10^2$ to 10^3 sec^{-1} . The third step with $1/\tau_3 = 0.1$ – 10 sec^{-1} is relatively slow. The three relaxation processes remain well separated from each other over the whole range of concentrations tested. All three processes depend on the concentration of NAD in a characteristic manner. This is shown in Figure 6a–c. With increasing concentration

FIG. 5.—Three relaxation processes at pH 8.5/40°C. $E_0 = 1.43 \cdot 10^{-4} M$ (20 ing protein/ml); $D_0 = 6 \cdot 10^{-4} M$; buffer as in Fig. 1.

of NAD the rates of the two rapid processes increase, whereas the rate of the slow process drops steeply to a plateau value. Therefore, the rapid steps can tentatively be identified with two discrete, bimolecular reactions probably involving two different classes of binding sites. However, the slow process is also independent of enzyme concentration from 0.25 to 25 mg protein/ml as shown in Figure 7. This strongly indicates an isomerization reaction of the enzyme.

Interpretation.—Using the well-documented fact^{9, 12} that glyceraldehyde-3-phosphate dehydrogenase is an oligomeric protein composed of four identical subunits, it turns out that the “allosteric” mechanism of Monod *et al.*⁵ (as adapted in scheme 1 below) is the simplest which accommodates the experimental data presented in Figures 6 and 7.

(k_R , k_D , k_R' , and k_D' are “intrinsic” rate constants; $i = 0, 1, 2, 3, 4$.)

The rate and equilibrium constants are defined as follows:

$$L_0 = T_0/R_0 = k_0/k'_0; \quad L_i = T_i/R_i = (c)^i \cdot L_0 = k_i/k'_i.$$

$$K = 4R_0 \cdot D/R_1 = 3R_1 \cdot D/2R_2 = 2R_2 \cdot D/3R_3 = R_3 \cdot D/4R_4 = k_D/k_R.$$

$$K' = K/c = 4T_0 \cdot D/T_1 = 3T_1 \cdot D/2T_2 = 2T_2 \cdot D/3T_3 = T_3 \cdot D/4T_4 = k_D'/k_R'.$$

The species symbols used in the definitions are taken from scheme 1 and denote species concentrations.

Applying the theory obtained for the case that the structural transitions are slow compared to the bimolecular reactions and that the latter processes are well separated from each other, the following analytical expressions are obtained for the three time constants:¹³

$$\frac{1}{\tau_1} = k_D + k_R (D + \Phi R), \quad (1)$$

$\Phi R = 4R_0 + 3R_1 + 2R_2 + R_3$ = sum of free binding sites of the R -state. D = concentration of free ligand (NAD),

$$\frac{1}{\tau_2} = k_D' + k_R' \left(D + \Phi T \left[\frac{K + D}{K + D + \Phi R} \right] \right), \quad (2)$$

$\Phi T = 4T_0 + 3T_1 + 2T_2 + T_3$ = sum of free binding sites in the T -state.

FIG. 6.—Dependence of relaxation times on NAD-concentration (pH 8.5/40°C). Experimental conditions as in Fig. 5. D_0 is varied as shown in the figures. Above 10^{-4} M D_0 , $1/\tau_3$ is obtained from stopped-flow experiments by evaluating the last 5% of the reaction (= close to equilibrium). The curve in (c) is calculated from theory with the values of the constants given in Table 1.

$$\frac{1}{\tau_3} = k_0' + k_0 \left[\frac{1 + \frac{cD}{K}}{1 + \frac{D}{K}} \right]^4 \quad (3)$$

The first expression is identical to the relation obtained for a one-step, bimolecular reaction.¹¹ The second equation pertains to the slower of two simple, bimolecular steps coupled by a common ligand (D). The expression for $1/\tau_3$ was obtained with the simplifying but justified assumptions¹³ that the concentration changes of free ligand during equilibration are negligible (D is quasi-buffered), and that all transitions from the T -state to R -state are characterized by identical rate constants (i.e., $k_0' = k_1' = k_2' = k_3' = k_4'$). The expression for the general case has been derived.¹³ It leads to a very similar concentration dependence of $1/\tau_3$.

FIG. 7.—Independence of the slow relaxation process on enzyme concentration (pH 8.5/40°C). Buffer as in Fig. 1; $D_0 = 5 \cdot 10^{-3}$ M.

TABLE 1

Rate constants	Equilibrium constants
$k_R = 1.9 \cdot 10^7 M^{-1} \text{ sec}^{-1}$	$K = 5.3 \cdot 10^{-5} M$
$k_D = 1 \cdot 10^3 \text{ sec}^{-1}$	
$k_R' = 1.37 \cdot 10^6 M^{-1} \text{ sec}^{-1}$	$K' = 1.5 \cdot 10^{-4} M$
$k_D' = 210 \text{ sec}^{-1}$	
$k_0 = 5.5 \text{ sec}^{-1}$	$c = K/K' = 0.35$
$k_0' = k_1' = k_2' = k_3' = k_4' = 0.18 \text{ sec}^{-1}$	$L_0 = 30.5$
$k_1 = c \cdot k_0 = 2.0 \text{ sec}^{-1}$	
$k_2 = c^2 \cdot k_0 = 0.7 \text{ sec}^{-1}$	
$k_3 = c^3 \cdot k_0 = 0.24 \text{ sec}^{-1}$	
$k_4 = c^4 \cdot k_0 = 0.08 \text{ sec}^{-1}$	

Values of rate and equilibrium constants obtained from kinetic data at pH 8.5, 40°C. Data in Fig. 6a-c. Notations as in scheme 1. A computer was used to obtain an optimal fit of the data to the theory. Errors in rate constants $\pm 20\%$ and equilibrium constants $\pm 40\%$.

By plotting the experimental values of $1/\tau_1$, $1/\tau_2$, and $1/\tau_3$ against the corresponding concentration terms (e.g., $1/\tau_1$ vs. $(D + \Phi R)$), it is possible to determine the values of all rate constants included in scheme 1 from extrapolated intercepts and slopes (Figs. 6a-c). The values obtained for pH 8.5, 40°C, are listed in Table 1. The theoretical curve for $1/\tau_3$ computed from the values in Table 1 has been included in Figure 6c. The fit is good except for $1/\tau_1$ at low concentrations of NAD. The deviation is probably due to the participation of protolytic groups in the binding process. A refined theory taking into account the pH-dependence of NAD binding is being developed.

Discussion.—Since a maximum of 9 relaxation times is to be expected for the general case of the allosteric model,¹³ the binding of NAD to GAPDH represents a case of extreme apparent degeneracy of the relaxation spectrum. Within the error limits of the method ($\pm 25\%$) one can therefore conclude that the apo-enzyme exists in two tetrameric conformations only. The dissociation constants of the various binding sites on each isomer are identical and independent of each other. Furthermore, the cooperativity between the subunits appears to be so high that the structural transition is an all-or-none process. This can be concluded from the lack of evidence for relaxation times of hybrid intermediates such as R_3T , and from the fourth power dependence of $1/\tau_3$ on $(1 + cD/K)/(1 + D/K)$. In other words, all of the specific postulates characterizing Monod's model⁵ for homotropic ligand interactions seem to be fulfilled within the limits of experimental error.

At present, the allosteric mechanism is the simplest one accommodating all kinetic and equilibrium data for the reaction in question. The mechanism implicated in Adair's hypothesis⁴ is too simple, since it does not include independent isomerization steps. However, several important questions regarding glyceraldehyde-3-phosphate dehydrogenase remain to be answered: e.g., the biological significance of the allostery of GAPDH; the possible role of additional, modifying ligands enhancing the cooperativity of NAD binding; the pH-dependence, possibly involving an effect similar to the Bohr effect of hemoglobin; the possible interaction of identical binding sites below the limits set by experimental error, etc. Therefore, more accurate kinetic measurements in the future may require an amplification of the "simple" allosteric model,⁵ for instance, the abandonment of the postulate of the independence of identical binding sites. On the other hand, a modification of the Koshland model⁶ would then lead to the same, more complicated mechanism. Such a modification could be brought about by permitting the ligand to bind to *both* conformations.

Finally, the binding of NAD and GAPDH represents an ideal test case for the power and versatility of relaxation methods. The advantage of the method lies primarily in the directness of approach: Intermediates become detectable by using substrate levels of enzyme; rapid reactions can be followed down to half lives in the microsecond range. Furthermore, the discreteness of elementary steps in biochemical reactions, together with the particular conditions of perturbation will permit the exact solution of the rate equations in many cases, leading to simple expressions relating the time constants to rate constants and equilibrium concentrations.

Although our kinetic data on glyceraldehyde-3-phosphate dehydrogenase fit well to the predictions derived from the allosteric model,⁵ a generalization from these data to other systems involving cooperative ligand binding would be premature. However, the successful application of relaxation methods to the elucidation of one particular problem suggests that similar studies conducted with other systems may lead to equally clear-cut answers. A survey of the information obtained from a number of allosteric enzymes may eventually demonstrate an interesting prevalence of one of the mechanisms under current discussion.

We wish to thank Dr. T. M. Schuster for the contribution of important ideas and for many stimulating discussions. Our gratitude is also expressed to Dr. G. Ilgenfritz for active participation in the development of the theory and, together with E. Menge, for writing the necessary computer programs.

* We gratefully acknowledge the financial support of this investigation by grants from the du Pont de Nemours International S.A. and from the Deutsche Forschungsgemeinschaft.

¹ Umbarger, H. E., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 26 (1961), p. 301.

² Wilson, A., and A. B. Pardee, *Comp. Biochemistry*, **6**, 73 (1964).

³ Gerhart, J. C., and H. K. Schachman, *Biochemistry*, **4**, 1054 (1965).

⁴ Adair, G. S., *J. Biol. Chem.*, **63**, 529 (1925).

⁵ Monod, J., J. Wyman, and J.-P. Changeux, *J. Mol. Biol.*, **12**, 88 (1965).

⁶ Koshland, D. E., Jr., G. Nemethy, and D. Filmer, *Biochemistry*, **5**, 365 (1966).

⁷ Krebs, E. G., *J. Biol. Chem.*, **200**, 471 (1952).

⁸ Kirschner, K., manuscript in preparation.

⁹ Perham, R. N., and J. I. Harris, *J. Mol. Biol.*, **13**, 876 (1965).

¹⁰ Stockell, A., *J. Biol. Chem.*, **234**, 1286 (1959).

¹¹ Eigen, M., and L. De Maeyer, in *Technique of Organic Chemistry*, ed. A. Weissberger (New York: J. Wiley and Sons, 1963), 2nd ed., vol. 8, part 2, p. 895 ff.

¹² Watson, H. C., and L. J. Banaszak, *Nature*, **204**, 918 (1964).

¹³ Eigen, M., G. Ilgenfritz, and K. Kirschner, manuscript in preparation.