Vol. 85, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 29, 1978 Pages 593-600

RELAXATION KINETIC STUDIES OF COENZYME BINDING TO GLUTAMATE DEHYDROGENASE FROM BEEF LIVER

M. A. KEMPFLE and R. F. MÜLLER, Physiologsich-chemisches Institut der Universität Bonn, Nußallee 11, D-5300 Bonn, Germany

H. A. WINKLER, Max-Planck-Institut für biophysikalische Chemie, Am Faßberg, D-3400 Göttingen-Nikolausberg, Germany

Dedicated to Prof. K.-O. Mosebach on the occasion of his 60th birthday.

Received September 27,1978

Summary: Fluorescence temperature-jump experiments were performed to study the binding of coenzyme to glutamate dehydrogenase from beef liver in 0.1 M sodium phosphate buffer, pH 7.4, T = 25.0° C.
1) NADPH shows a single second-order relaxation process, indicating coenzyme binding to one site per enzyme subunit. The dissociation constant of the reaction was calculated from the rate constants (k_{21} = 92 sec^{-1}, k_{12} = 2.13 .10^6 $M^{-1}sec^{-1}$) to be 43 µM. No deviation from a straight line was observed over the whole concentration range.
2) With NADH two well separated second-order processes are detected.
3) The observed concentration dependence of these two relaxation processes is consistent with a reaction scheme assuming two completely independent but non-equivalent NADH binding sites per enzyme subunit.
4) From the rate constants of the faster process (k_{21} = 235 sec^{-1}, k_{12} = 3.83 .10^6 $M^{-1}sec^{-1}$) and of the slower process (k_{32} = 4.2 sec^{-1}, k_{23} = 0.18 .10^6 $M^{-1}sec^{-1}$) the dissociation constants are calculated to be $K_{21} = k_{21}/k_{12}$ = 61 µM and $K_{32} = k_{32}/k_{23}$ = 23 µM, respectively.
5) Small amounts of ADP (<10 µM) abolish the slower relaxation process, while the faster (but weaker binding) process remains uninfluenced. Therefore, the weaker binding site must be the active site.

Introduction:

Considerable differences exist in the literature about the number and nature of glutamate dehydrogenase (EC. 1.4.1.3) coenzyme binding sites as well as the magnitude of the dissociation constants (1-5). As relaxation kinetic techniques offer an appropriate tool for resolving the overall binding process into its elementary steps, one should be able to elucidate the mechanism of even a rather complex reaction scheme. The formation of glutamate dehydrogenase-

0006-291X/78/0852-0593$01.00/0

coenzyme complex is accompanied by a fluorescence change of this complex as well as of the coenzyme itself. Therefore, the fluorescence temperature-jump method could be applied to investigate this system.

Material and Methods:

Crystalline glutamate dehydrogenase in 2 M ammonium sulphate suspension, nucleotides, and coenzymes were obtained from Boehringer, Mannheim, in the highest purity available; buffer material from Merck, Darmstadt.
Relaxation kinetic measurements were performed in a highly improved double-monochromator temperature-jump apparatus (6) equipped for fluorescence detection (7). The excitation wavelength was 340 nm; fluorescence changes due to nucleotide binding (λ_{em} = 456 nm) were measured at right angles, and KV 418 glass filters were inserted into the emission light beam to eliminate light of wavelengths shorter than 400 nm. The cell (1 ml volume) was thermostated at 22.0° C and the discharge of a 50 nF capacitor, charged to 20 kV, produced a temperature jump of 3.0° C, thus allowing the relaxation process to be observed at 25.0° C.
Compared to relaxation kinetic studies published earlier (2) our measurements were carried out in 0.1 M sodium phosphate buffer, pH 7.4, in order to avoid the disadvantages of Tris-buffer (additional pH-jump and necessity of high salt concentrations to stabilize the system). No significant loss of enzymatic activity was observed after about 20 temperature jumps.

Results and Discussion:

NADPH binding:

The system glutamate dehydrogenase:NADPH exhibits a single second-order relaxation process. The simplest reaction scheme for binding is

$$E + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EL$$

where k_{12} and k_{21} represent the rate constants for recombination and dissociation of the enzyme-ligand complex and hence k_{21}/k_{12} is the dissociation constant K_{21} of this complex.
Such a simple reaction scheme results in a second-order relationship with only one relaxation time, according to equation (1):

$$(1) \qquad 1/\tau = k_{21} + k_{12}([\bar{E}] + [\bar{L}])$$

where $[\bar{E}]$ and $[\bar{L}]$ represent the concentrations of free binding sites of the enzyme and unbound substrate, respectively, at equilibrium.
The data have been evaluated by two different methods which are explained in the following paragraph.

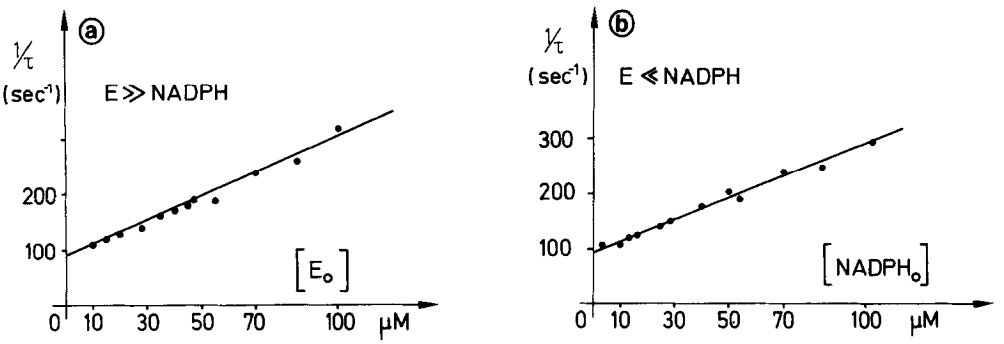

Fig. 1: Plot, according to (eq. 1), of fluorimetric data from temperature jump perturbations of binding between glutamate dehydrogenase and NADPH. Each point is the mean of at least six temperature jumps.

Conditions: T = 25.0° C, 0.1 sodium phosphate buffer, pH 7.4.

Fig. 1a: Plot, according to (eq. 1a). Total NADPH concentration 3 µM.

Fig. 1b: Plot, according to (eq. 1b). Total enzyme concentration 3 µM.

a) High excess of one of the reactants allows the replacement of ($[\bar{E}] + [\bar{L}]$) either by $[E_o]$ or by $[L_o]$. Thus eq. (1) simplifies to

(1a) $1/\tau = k_{21} + k_{12}[E_o]$ $\qquad [E_o] \gg [L_o]$

(1b) $1/\tau = k_{21} + k_{12}[L_o]$ $\qquad [L_o] \gg [E_o]$

The subscript "$_o$" indicates the total concentrations of the reactants.

b) Expressing the relaxation equations in total concentrations of enzyme binding sites $[E_o]$ or of ligand $[L_o]$ the following relation holds

(2) $(1/\tau)^2 = k_{21}^2 + 2k_{12}k_{21}([E_o] + [L_o]) + k_{12}^2([E_o] - [L_o])^2$

Provided $[E_o] = [L_o]$ eq. (2) becomes

(2a) $(1/\tau)^2 = k_{21}^2 + 2k_{12}k_{21}([E_o] + [L_o])$

A plot of $(1/\tau)^2$ versus $([E_o] + [L_o])$ yields in a straight line with an intercept of k_{21}^2 and a slope of $2k_{12}k_{21}$.

Both methods of evaluation result in almost identical rate and dissociation constants for NADPH binding (figures 1a, 1b, and 2):

$k_{21} = 92\ sec^{-1}$, $k_{12} = 2.13 \cdot 10^6\ M^{-1}sec^{-1}$; $K_{21} = k_{21}/k_{12} = 43\ \mu M$

As shown in figs. 1 and 2 there is (in contrast to a stopped-flow study

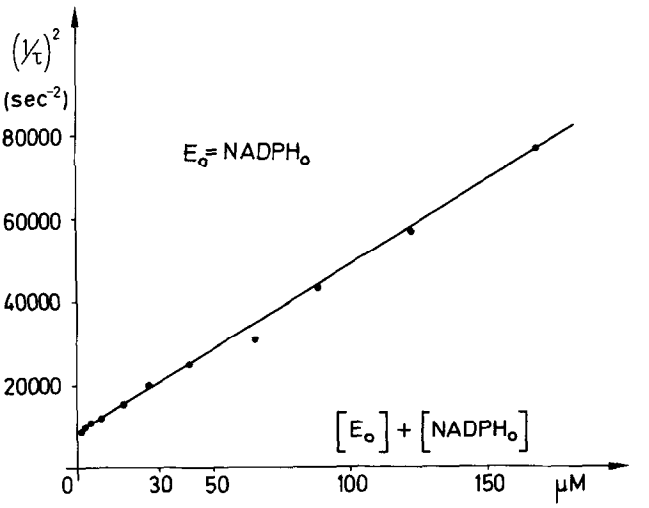

Fig. 2: Plot, according to (eq. 2). Conditions see figs. 1.

published recently (4)) no evidence for a deviation from a straight line. This and other findings (2) support the suggestion that there are no interactions between the subunits which are affected by the binding of NADPH.

NADH binding:

Our studies on NADH binding to glutamate dehydrogenase show two well separated second-order relaxation processes; τ_1, in the time range of a few milliseconds, and τ_2, in the loo millisecond range ($1/\tau_1 >> 1/\tau_2$). An extensive mathematical treatment of the three most feasible reaction mechanisms is presented elsewhere (8); all mechanisms result in two relaxation processes.

These three mechanisms are:

(I) The ligand binding process is accompanied by a conformational change of the protein-ligand complex:

$$E + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EL \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} EL^*$$

(II) The ligand L can be bound simultaneously to two non-equivalent but completely independent binding sites E_1 and E_2 located on the same subunit of the enzyme molecule:

$$E_1 + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E_1L \; ; \; E_2 + L \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} E_2L$$

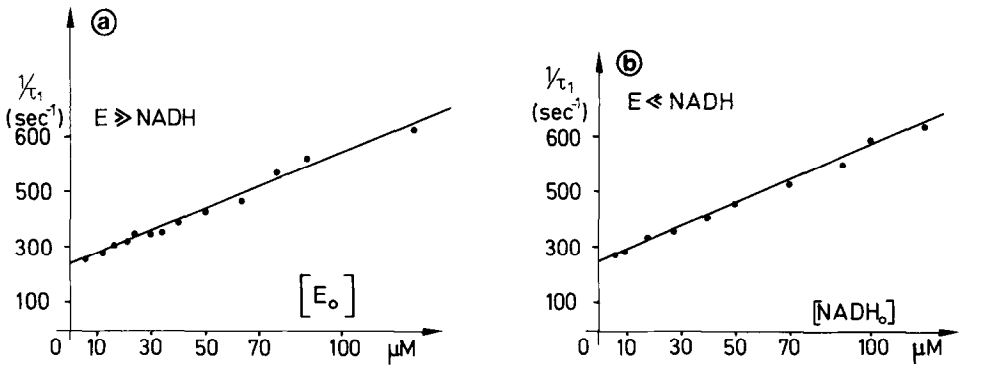

Fig. 3: Plot for the fast relaxation time τ_1, according to (eq. Ia = fig. 3a) and to (eq. Ib), of fluorimetric data from temperature jump perturbations of the binding between glutamate dehydrogenase and NADH.
Conditions see fig. 1.

Fig. 3a:Plot, according to (eq. Ia). Total NADH concentration 3 µM.

Fig. 3b:Plot, according to (eq. Ib). Total enzyme concentration 3 µM.

(III) Binding of the ligand proceeds in a consecutive manner; the second molecule can only be bound after the first one. Any kind of cooperativity might occur and has to be considered in this mechanism by different values of the rate constants k_{ij}:

$$E + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EL \; ; \; EL + L \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} EL_2$$

To derive the concentration dependence of the relaxation times of these three mechanisms we introduce the following simplifying assumption: the first of the two steps, characterized by k_{12} and k_{21}, should react much faster than the second one ($1/\tau_1 \gg 1/\tau_2$) and thus will be equilibrated before the slower one proceeds. Then the expressions for the reciprocal relaxation time $1/\tau_1$ become identical for all three mechanisms:

$$1/\tau_1 = k_{21} + k_{12}([\bar{E}] + [\bar{L}])$$

This is eq. (I) and therefore the evaluation of the data can be performed as described above and gives figs. 3a and b. Following constants are obtained:

$k_{21} = 235\ sec^{-1}$, $k_{12} = 3.83 \cdot 10^6\ M^{-1}sec^{-1}$; $K_{21} = k_{21}/k_{12} = 61\ \mu M$

Since the equations for $1/\tau_1$ are identical and show linear behaviour with

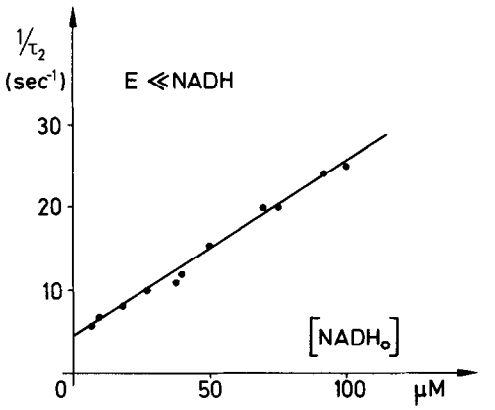

Fig. 4: Plot of the slow relaxation time τ_2, according to (eq. 4). Conditions see fig. 1. Total glutamate dehydrogenase concentration 3 μM.

respect to $[E_o]$ as well as $[L_o]$ we must inspect the concentration dependence of the second relaxation process in order to distinguish between the different mechanisms of ligand binding.

A clear cut decision can already be achieved by titration of small amounts of enzyme (< 10 μM) with increasing concentrations of NADH ($[L_o] >> [E_o]$).

This leads to following expressions:

Mechanism (I) (eq. 3) $$1/\tau_2 = k_{32} + k_{23} \cdot \frac{[L_o]}{K_{21} + [L_o]}$$

Mechanism (II) (eq. 4) $$1/\tau_2 = k_{32} + k_{23} \cdot [L_o]$$

Mechanism (III) (eq. 5) $$1/\tau_2 = k_{32} + k_{23} \cdot \frac{[L_o]^2}{K_{21} + [L_o]}$$

The different mechanisms are characterized by different plot of $1/\tau_2$ vs. $[L_o]$: Mech. (I) reaches a plateau at high concentrations of $[L_o]$ with a constant value of $1/\tau_2 = k_{32} + k_{23}$. Mech. (II) results in a straight line with an intercept k_{32} and a slope k_{23}. In Mech. (III), however, $1/\tau_2$ increases quadratically at low concentrations, but at high concentrations ($[L_o] > K_{21}$) the concentration dependence becomes linear with the limiting slope equal to k_{23}.

A plot of the experimentally determined reciprocal relaxation time $1/\tau_2$ vs. $[L_o]$ is given in Fig. 4 and results in a straight line. No deviation from linearity could be detected over the entire concentration range.

Therefore, we conclude that NADH binding to glutamate dehydrogenase proceeds according to mechanism (II), i.e. two completely independent but non-equivalent coenzyme binding sites per enzyme subunit.

The following rate and dissociation constants were evaluated:

$$k_{32} = 4.2\ sec^{-1},\quad k_{23} = 0.18 \cdot 10^{6}\ M^{-1}sec^{-1};\quad K_{32} = k_{32}/k_{23} = 23\ \mu M$$

Ligand binding to the weaker binding site is reflected by the faster relaxation process. The slower relaxation process on the other hand obviously represents coenzyme binding to a stronger site.

Now the question arises, which of these two binding sites is the catalytic one? Small amounts of ADP (< 10 µM) abolish the relaxation signal due to the stronger site, while the faster process is not influenced. Thus ADP competes for the stronger binding site. As the catalytic activity of the enzyme remains unchanged at that ADP concentration (9,10), we conclude that the weaker binding site must be the catalytic site.

At higher ADP concentrations (> 100 µM) a further slow relaxation process was observed and might be related to a conformational rearrangement of the protein and which is probably responsible for the known ADP activation of glutamate dehydrogenase (2,10).

Thus we see that relaxation kinetic methods are a powerful tool to determine the rate constants for any known reaction mechanism but also to distinguish among unknown reaction mechanisms.

Acknowledgements:

The authors would like to thank Prof. M. Eigen and Dr. P. Richter for stimulating discussions, and Mrs. Ingrid Botta for skillful technical assistance.

References:

(1) Yielding,K.L. and Holt,B. (1967) J.Biol.Chem. 242, 1079 - 1082.
(2) Malcolm,A.D.B. (1972) Eur. J. Biochem. 27, 453 - 461.
(3) Pantaloni,D. and Dessen,P. (1969) Eur. J. Biochem. 11, 510 - 519.
(4) Markau,K. and Weber,K. (1977) Pyridine Nucleotide-Dependent Dehydrogenases, pp. 426 - 443, Walter de Gruyter, Berlin, New York.
(5) Kempfle,M., Winkler,H., and Mosebach,K.-O. (1974) Abstract s2e4, 9th FEBS-Meeting Budapest.

(6) Eigen, M. and DeMaeyer,L.C.M. (1973) Techniques of Chemistry, Vol.VI, Part 2, pp. 63 - 146, Wiley, New York.
(7) Rigler,R., Rabl,C.-R., and Jovin,T.M. (1974) Rev.Sci.Instrum. 45, 58o - 584.
(8) Kempfle,M. (1977) Bull. Math. Biol. 39, 297 - 31o.
(9) Koberstein,R. and Sund,H. (1973) Eur. J. Biochem. 36, 545 - 552.
(1o) Colen,A.H., Cross,D.C., and Fisher,H.F. (1974) Biochemistry 13, 2341 - 2347.