ESTIMATION OF DISSOCIATION CONSTANT OF ENZYME–LIGAND COMPLEX FROM FLUOROMETRIC DATA BY "DIFFERENCE" METHOD

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1. Introduction

In the previous paper [1] we suggested a "difference" method for determination of parameters of three parametric kinetic equations in enzyme kinetics. This method is based on analysis of differences of enzyme reaction rate values (or values of the saturation degree of the protein by ligand) corresponding to substrate (or ligand) concentrations $[S]_0$ and $[S]_0/\kappa$ (κ is a constant multiplier). Now we show that the similar difference method may be used for the determination of dissociation constants of enzyme–ligand complexes from fluorescence titration data. The interaction of L-α-glycerophosphate dehydrogenase (α-GPD; EC 1.1.99.5) with NADH is considered as an example. It has been shown [2] that NADH quenches the α-GPD fluorescence. It allows the observation of NADH binding by the fluorometric method.

2. Materials and methods

Rabbit muscle α-GDP was obtained in an ammonium sulphate suspension from Reanal (Hungary). This preparation was subjected to chromatography on a column of DEAE-cellulose as described by Telegdi [3]. NADH and Tris were purchased from C.F. Boehringer GmbH and E. Merck (West Germany), respectively. Spectrofluorometric measurements were carried out with a Hitachi MPF-2A commercial instrument. Fluorescence titration of α-GPD with NADH was performed in 0.03 M Tris-HCl buffer, pH 7.6, at 20°. The calibrated capillary was used for titration. The total change of volume after addition of NADH solution does not exceed 2%. Extinction coefficient of 0.7 $(mg/ml)^{-1} \cdot cm^{-1}$ at 280 nm was used to determine concentration of α-GPD [4].

3. Results and discussion

In fig. 1a the relative intensity of α-GDP fluorescence at 340 nm is plotted as a function of total NADH concentration. This figure demonstrates that NADH strongly quenches the α-GDP fluorescence. NADH does not fluoresce at the studied conditions. Therefore the fluorescence intensity (I) is composed of two terms, one of which is proportional to the free binding sites (e) concentration and the other is proportional to eL complex concentration (L is a ligand):

$$I = \alpha[e] + \beta[eL] \quad (1)$$

Since the total concentration of binding sites $[e]_0$ is equal to ($[e] + [eL]$) and the fluorescence intensity in the absence of ligand (I_0) is equal to $\alpha[e]_0$ we obtain

$$I_0 - I = (\alpha-\beta)[eL] \text{ or } 1 - \frac{I}{I_0} = \frac{(\alpha-\beta)}{I_0}[eL] \quad (2)$$

Let us denote by I_∞/I_0 the limiting value of relative fluorescence intensity at $[L]_0 \to \infty$ ($[L]_0$ is the total ligand concentration). It is evident that

$$1 - \frac{I_\infty}{I_0} = \frac{(\alpha-\beta)}{I_0}[e]_0 \quad (3)$$

The concentration of eL complex depends on I/I_0 in the following manner:

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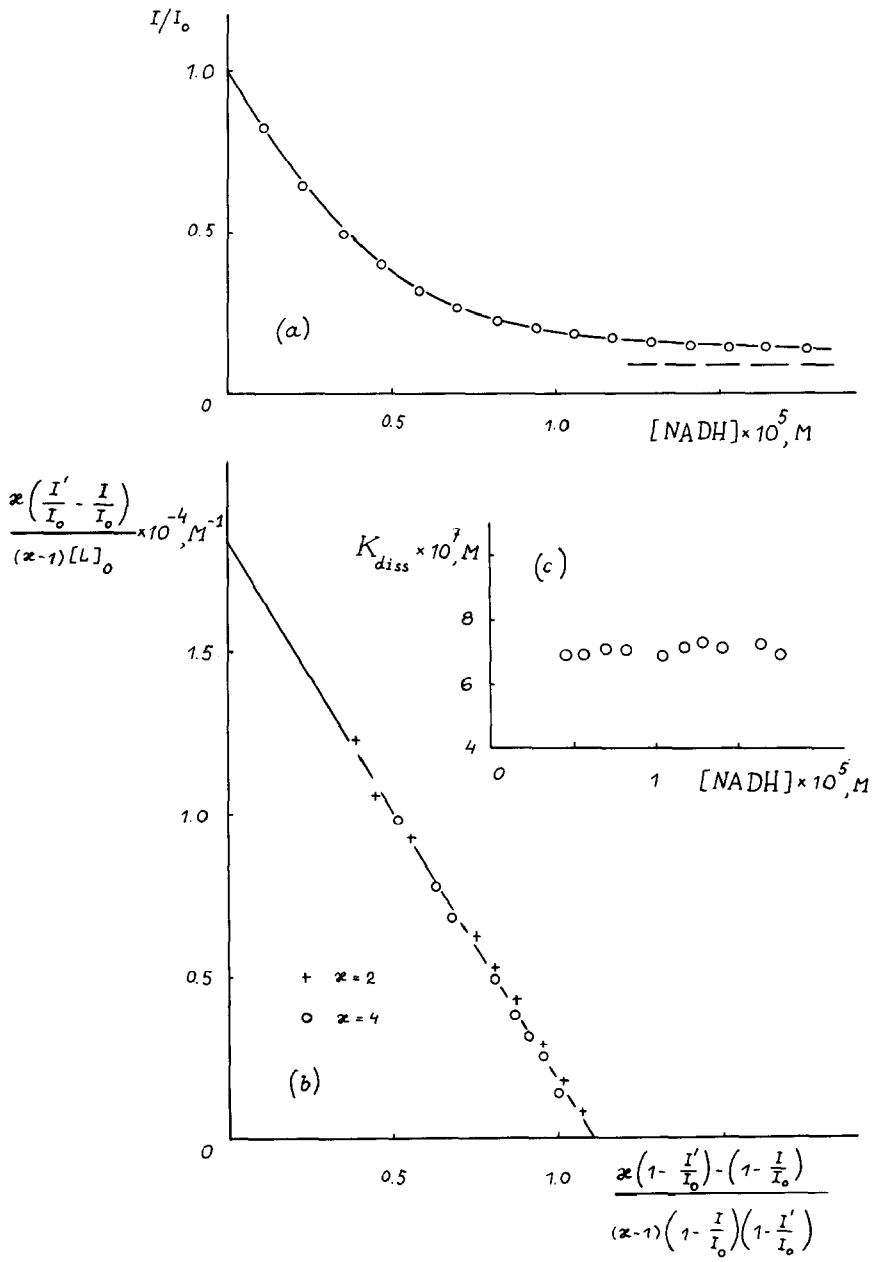

Fig. 1. The fluorescence titration of L-α-glycerophosphate dehydrogenase with NADH. The α-GPD concentration was 0.14 mg/ml. Emission was measured at 340 nm with excitation at 290 nm. (a) The dependence of α-GPD relative fluorescence intensity (I/I_0) on total NADH concentration. Points give experimental data; the solid line is the theoretical curve plotted at $K_{diss} = 7.1 \times 10^{-7}$ M, $[e]_0 = 5.0 \times 10^{-5}$ M and $I_\infty/I_0 = 0.09$. The dotted line is the limiting value of I_∞/I_0. (b) The linear transformation of titration curve. (c) Values of K_{diss} calculated according to formula (6) at various NADH concentrations.

$$[eL] = \frac{1-\frac{I}{I_0}}{1-\frac{I_\infty}{I_0}}[e]_0 \quad (4)$$

The dissociation constant (K_{diss}) of the eL complex is determined by the following expression:

$$K_{diss} = \frac{[e][L]}{[eL]} = \frac{([e]_0-[eL])([L]_0-[eL])}{[eL]} \quad (5)$$

where [L] is an equilibrium concentration of free ligand. Substitution of expression 4 for eL concentration in equation 5 yields, after simplification:

$$K_{diss} = \frac{\left(\frac{I}{I_0}-\frac{I_\infty}{I_0}\right)\left[\left(1-\frac{I_\infty}{I_0}\right)[L]_0-\left(1-\frac{I}{I_0}\right)[e]_0\right]}{\left(1-\frac{I}{I_0}\right)\left(1-\frac{I_\infty}{I_0}\right)} \quad (6)$$

Accordingly to our approach [1] we select on curve of I/I_0-vs-$[L]_0$ two points with abscissa $[L]_0$ and $[L]'_0 = [L]_0/\kappa$ (κ is a constant multiplier). Let us denote by I/I_0 and I'/I_0 ordinates of these points, respectively. The expression (6) is valid for the point with coordinates $\{I/I_0; [L]_0\}$ and an analogous expression may be written for the point with coordinates $\{I'/I_0; [L]'_0\}$:

$$K_{diss} = \frac{\left(\frac{I'}{I_0}-\frac{I_\infty}{I_0}\right)\left[\left(1-\frac{I_\infty}{I_0}\right)[L]_0-\kappa\left(1-\frac{I'}{I_0}\right)[e]_0\right]}{\kappa\left(1-\frac{I'}{I_0}\right)\left(1-\frac{I_\infty}{I_0}\right)} \quad (7)$$

By eliminating K_{diss} from expressions (6) and (7) we obtain the following linear relationship:

$$\frac{\kappa\left(\frac{I'}{I_0}-\frac{I}{I_0}\right)}{(\kappa-1)[L]_0} = \frac{1-\frac{I_\infty}{I_0}}{[e]_0} - \frac{\left(1-\frac{I_\infty}{I_0}\right)^2}{[e]_0} \times \frac{\left[\kappa\left(1-\frac{I'}{I_0}\right)-\left(1-\frac{I}{I_0}\right)\right]}{(\kappa-1)\left(1-\frac{I}{I_0}\right)\left(1-\frac{I'}{I_0}\right)} \quad (8)$$

The linear plot of $\kappa\left(\frac{I'}{I_0}-\frac{I}{I_0}\right)\Big/(\kappa-1)[L]_0$ vs $\left[\kappa\left(1-\frac{I'}{I_0}\right)-\left(1-\frac{I}{I_0}\right)\right]\Big/(\kappa-1)\left(1-\frac{I}{I_0}\right)\left(1-\frac{I'}{I_0}\right)$ may be constructed at various magnitudes of $[L]_0$ and κ and allows the estimation of the value of $(1-I_\infty/I_0)$, which is equal to the reciprocal intercept on the abscissa and the value of $[e]_0$, which is equal to the reciprocal value of the product of the intercepts on both axes.

The dependence of $\kappa\left(\frac{I'}{I_0}-\frac{I}{I_0}\right)\Big/(\kappa-1)[L]_0$ on $\left[\kappa\left(1-\frac{I'}{I_0}\right)-\left(1-\frac{I}{I_0}\right)\right]\Big/(\kappa-1)\left(1-\frac{I}{I_0}\right)\left(1-\frac{I'}{I_0}\right)$ which is constructed by us on the basis of fluorescence titrations of α-GPD with NADH at $\kappa = 2$ and $\kappa = 4$ is represented in fig. 1b. The value of $(1-I_\infty/I_0)$ appears to be equal to 0.91 and $[e]_0$ is equal to 5.0×10^{-6} M. The obtained value of $[e]_0$ is near to the value of 4.7×10^{-6} M calculated assuming the molecular weight of the α-GPD subunit is equal to 30,000 [4]. The values of K_{diss} calculated by means of the formula (6) at various NADH concentrations are represented in fig. 1c. The mean value of K_{diss} is equal to $7.1 \pm 0.2 \times 10^{-7}$ M. This value of K_{diss} of the κ-GPD–NADH complex is near to the corresponding value of K_{diss} (1.70×10^{-6}–1.88×10^{-6} M) calculated in [5] on the basis of measurements of NADH fluorescence increase in the presence of α-GPD (Tris-HCl buffer, pH 7.85, 25°).

The above proposed difference method of K_{diss} determination allows a virtually error-free estimation of I_∞/I_0 and does not require a preliminary evaluation of binding sites concentration.

References

[1] B.I. Kurganov and V.A. Yakovlev, Mol. Biol. USSR 5 (1970) 781.
[2] M. Telegdi and T. Keleti, Acta Biochim. Biophys. Acad. Sci. Hung. 3 (1968) 131.
[3] M. Telegdi, Acta Physiol. Hung. 25 (1964) 177.
[4] T.P. Fondy, C.R. Ross and S.J. Sollohub, J. Biol. Chem. 244 (1969) 1631.
[5] S.J. Kim and B.M. Anderson, J. Biol. Chem. 244 (1969) 1547.