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Analysis of negative cooperativity for glutamate dehydrogenase

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Abstract

The empirical equation, which describes negative cooperativity in the enzyme kinetics, has been proposed. The equation is obtained from the Michaelis–Menten equation where the Michaelis constant is replaced by the effective Michaelis constant, which is a linear function of the v/V_{max} ratio (v is the rate of the enzymatic reaction and V_{max} is the limiting value of v at saturating concentrations of substrate). The equation allows the limiting values of the Michaelis constant at $v/V_{max} \rightarrow 0$ and $v/V_{max} \rightarrow 1$ to be estimated, K_0 and K_{lim}, respectively. The K_{lim}/K_0 ratio is considered as a quantitative characteristic of negative cooperativity. The applicability of the equation has been demonstrated for the kinetic data obtained for glutamate dehydrogenases from various sources (negative kinetic cooperativity for coenzyme). The negative cooperativity for the functions of saturation of protein by ligand is also analyzed. The data on binding of spin-labeled NAD, NADH, and NADPH by beef liver glutamate dehydrogenase are used as examples.

Keywords: Enzyme kinetics; Allosteric enzymes; Negative cooperativity; Glutamate dehydrogenase

1. Introduction

Glutamate dehydrogenases catalyzing transformation of L-glutamate to 2-oxoglutarate or reverse reaction have been found in nearly every organism and have a pivotal role in nitrogen and carbon metabolism [1]. Mammalian glutamate dehydrogenase (EC 1.4.1.3) uses NAD and NADP as a coenzyme with comparable efficacy. The enzymatic properties and structure of glutamate dehydrogenase from beef liver mitochondria have been studied in detail. The enzyme molecule consists of six identical subunits with molecular mass of 56 000 Da each. One of the distinctive kinetic characteristics of beef liver glutamate dehydrogenase is a non-hyperbolic character of the depen-

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dence of the enzymatic reaction rate on NAD concentration: the dependence of the reciprocal value of the enzymatic reaction rate on 1/[NAD] is convex [2–8]. Analysis of such dependences using the Hill equation gives the values Hill coefficient less than unity.

Recently, Peterson and Smith [9] determined the atomic structure of bovine glutamate dehydrogenase in complex with NADH, glutamate, and the allosteric inhibitor GTP to 2.8 Å resolution. The molecule of glutamate dehydrogenase is a dimer of trimers stacked on top of each other with 32 symmetry. The antennae involving 23-residue helical segment and 25-residue random coil segment protrude from the body of each subunit. The antennae from each trimer are twisted counterclockwise along the threefold axis. It is suggested that the subunit interactions within the antenna region play an important role in the realization of the mechanism of allosteric regulation [9]. Two sites for NADH binding have been observed in each subunit: one NADH molecule is bound in the active site and other NADH molecule is bound in the allosteric site, the distance between the active and allosteric sites being ~ 20 Å.

Banerjee et al. [10] showed that each subunit of beef liver glutamate dehydrogenase contained two NAD-binding sites with equal affinity to NAD. However, in the presence of dicarboxylic acid coligands (2-oxoglutarate or glutarate) the affinity to one site (presumed to be the active site) is considerably increased.

Bacterial glutamate dehydrogenases, for example, dehydrogenase from *Clostridium symbiosum* also show non-michaelian kinetics. The three-dimensional structure of NAD^+-linked glutamate dehydrogenase from *Clostridium symbiosum* has been solved to 1.96 Å resolution [11]. The enzyme molecule is a hexamer with 32 symmetry composed from six identical subunits with molecular mass of 48 000 Da. Each subunit consists of two domains separated by a deep cleft. NAD is bound in an extended conformation with the nicotinamide moiety deep in the cleft between the two domains.

The goal of the present paper is to analyze negative cooperativity for mammalian and bacterial glutamate dehydrogenases.

2. New empirical equation for description of negative cooperativity

The deviations from the hyperbolic law for the dependences of the enzymatic reaction rate on the substrate concentration are of frequent occurrence in the enzyme kinetics [12]. They may be connected with the complicated mechanism of the enzymatic process (especially in the case of multisubstrate enzyme reactions), non-equivalency of the substrate-binding sites or interactions between substrate-binding sites in the molecule of oligomeric enzyme, the occurrence of the enzymic forms (including oligomeric forms) differing in their catalytic properties in the enzyme preparation ([13], pp. 32–54). In order to interpret the deviations from the michaelian kinetics, experimenters, apart from the different kinetic approaches, should use the physical and physico–chemical methods providing the control of conformational and oligomeric state of the enzyme at different stages of the enzymatic process. Experimenter has often great difficulty in choosing between the mechanisms, which are proposed for explanation of the kinetic anomalies under observation. Therefore, the empirical equations designed for description of the dependence of the enzymatic reaction rate (v) on the substrate concentration (S) remain popular. These equations are used for general characterization of the shape of the dependence of v on [S].

2.1. Hill equation

One of the empirical equations applied for description of anomalous enzyme kinetics is the Hill equation:

$$v = V_{\max}\frac{([S]/[S]_{0.5})^h}{1 + ([S]/[S]_{0.5})^h}, \quad (1)$$

where v is the steady-state rate of the enzymatic reaction, [S] is the initial concentration of the

substrate, V_{max} is the limiting value of v at saturating concentrations of the substrate (i.e. at [S] $\rightarrow \infty$), $[S]_{0.5}$ is the 'semisaturation' concentration, i.e. the substrate concentration at which $v = V_{max}/2$, and h is the Hill coefficient. The case when $h > 1$ corresponds to the S-shaped dependences of v on [S], whereas the case when $h < 1$ corresponds to the dependences of v on [S] with delayed approaching the limiting value of the enzymatic reaction rate (V_{max}) with increasing substrate concentration. Among three parameters of the Hill equation (V_{max}, $[S]_{0.5}$, and h), only parameter V_{max} has clear physical meaning. Another two parameters are used for characterization of the shape of the v vs. [S] curve: parameter $[S]_{0.5}$ characterizes the position of the point at which $v = V_{max}/2$ on the abscissa axis, whereas parameter h characterizes the slope of the straight line in coordinates $\{\lg[v/(V_{max} - v)]; \lg[S]\}$.

At $h = 1$ the Hill equation is transformed into the classic Michaelis–Menten equation:

$$v = V_{max}\frac{[S]/K_m}{1 + [S]/K_m}, \tag{2}$$

where K_m is the Michaelis constant.

In order to describe the deviations from the hyperbolic law, the Michaelis constant may be considered as an effective constant (K_m^{eff}), which is a function of the substrate concentration. A comparison of Eqs. (1) and (2) where K_m is replaced by K_m^{eff} shows that the Hill equation corresponds to the situation when K_m^{eff} is a power function of the substrate concentration:

$$K_m^{eff} = K[S]^m, \tag{3}$$

where K and m are constants. The parameters of Eqs. (1) and (3) are connected by the following relationships: $[S]_{0.5} = \sqrt[1-m]{K}$ and $h = (1 - m)$.

The chief disadvantage of the Hill equation is that the equation does not allow the values of the Michaelis constant at low and high substrate concentrations to be estimated. Actually, it is seen from Eq. (3) that at [S] $\rightarrow$ 0 the K_m^{eff} value approaches zero and at [S] $\rightarrow \infty$ the K_m^{eff} value tends to infinity. However, it is evident that the finite values of K_m should exist at [S] $\rightarrow$ 0 and [S] $\rightarrow \infty$. Thus, the Hill equation may be used only for description of the central region of the values of v/V_{max} (i.e. the values of v/V_{max} near 0.5).

2.2. New empirical equation

It is of special interest to go from the Hill equation to equations, which would allow us to make estimates of the limiting values of K_m at [S] $\rightarrow$ 0 and [S] $\rightarrow \infty$. Consider the situation when the deviations from the michaelian kinetics are due to interactions between substrate-binding sites in the molecule of oligomeric enzyme and are manifested as the case $h < 1$. For such a situation the effective Michaelis constant should increase with increasing the v/V_{max} ratio. Assume that K_m^{eff} and v/V_{max} are connected by a linear relationship:

$$K_m^{eff} = K_0 + (K_{lim} - K_0)(v/V_{max}), \tag{4}$$

where K_0 and K_{lim} are the values of K_m at $v/V_{max} \rightarrow 0$ and $v/V_{max} \rightarrow 1$, respectively. The applicability of this relationship in certain cases will be substantiated in the section 'Analysis of experimental data'.

If we accept the connection between K_m^{eff} and v/V_{max} given by Eq. (4), the dependence of v on [S] will acquire the following form:

$$v = V_{max}\frac{[S]/K_m^{eff}}{1 + [S]/K_m^{eff}} = V_{max}\frac{[S]/\{K_0 + (K_{lim} - K_0)(v/V_{max})\}}{1 + [S]/\{K_0 + (K_{lim} - K_0)(v/V_{max})\}}. \tag{5}$$

The equation may be transformed in such a way that its right part contains only the substrate concentration:

$$v = V_{max}\left\{\sqrt{(K_0 + [S])^2 + 4(K_{lim} - K_0)[S]} - K_0 - [S]\right\}/2(K_{lim} - K_0)$$

$$= V_{max}\frac{\sqrt{(1+[S]/K_0)^2+4(\alpha-1)[S]/K_0}-1-[S]/K_0}{2(\alpha-1)}$$

$$= V_{max}\frac{\sqrt{\{2+(1+\alpha)[S]/[S]_{0.5}\}^2+8(\alpha^2-1)[S]/[S]_{0.5}}-2-(1+\alpha)[S]/[S]_{0.5}}{4(\alpha-1)}, \quad (6)$$

where $[S]_{0.5}$ is the 'semisaturation' concentration: $[S]_{0.5}=(K_0+K_{lim})/2$; α refers to the K_{lim}/K_0 ratio.

Eq. (5) may be written as a function of $v/[S]$ of v:

$$\frac{v}{[S]}\frac{V_{max}-v}{K_m^{eff}}=\frac{V_{max}-v}{K_0+(K_{lim}-K_0)(v/V_{max})}, \quad (7)$$

If we use dimensionless magnitudes $[S]/[S]_{0.5}$ and v/V_{max}, the equation acquires the following form:

$$\frac{v/V_{max}}{[S]/[S]_{0.5}}=\frac{(1+\alpha)(1-v/V_{max})}{2\{1+(\alpha-1)(v/V_{max})\}}. \quad (8)$$

Thus, the experimental data may be analyzed in coordinates $\{v/[S];\ v\}$. These coordinates are equivalent to well-known Scatchard coordinates which are applied for analysis of binding of ligands by proteins: $\{r/[L];\ r\}$ (r is concentration of bound ligand divided by the protein concentration and [L] is equilibrium concentration of ligand).

When $v \to 0$ the curve in coordinates $\{v/[S];\ v\}$ approaches a linear asymptote:

$$\frac{v}{[S]}=\frac{V_{max}}{K_0}-\frac{K_{lim}}{K_0^2}\cdot v. \quad (9)$$

The intercept on the ordinate axis is V_{max}/K_0 and the slope of the asymptote is equal to $-K_{lim}/K_0^2$.

The linear asymptote at $v \to V_{max}$ has the following form:

$$\frac{v}{[S]}=\frac{V_{max}}{K_{lim}}-\frac{1}{K_{lim}}\cdot v. \quad (10)$$

Intercept on the abscissa axis gives the V_{max} value and the slope of the asymptote is equal to $-1/K_{lim}$. The value of v corresponding to the intersection point of two asymptotes is equal to $V_{max}/(1+\alpha)$.

Analysis of Eq. (5) in coordinates $\{v;\ v/[S]\}$ is given in Appendix A.

Fig. 1a shows the theoretical dependences of the relative enzymatic rate v/V_{max} on the dimensionless substrate concentration $[S]/[S]_{0.5}$ calculated from Eq. (6) at various values of the K_{lim}/K_0 ratio ($K_{lim}/K_0=\alpha$). At $\alpha=1$ the dependence of v/V_{max} on $[S]/[S]_{0.5}$ is evidently hyperbolic. At $\alpha>1$ the deviations from the michaelian kinetics are manifested in convex curves in reciprocal coordinates (Fig. 1b) or concave curves in coordinates $\{v/[S];\ v\}$ (Fig. 1c). Such curves correspond to the situation when the affinity to substrate decreases with the degree of saturation of substrate-binding sites in the enzyme oligomer (negative cooperativity). Hill plots have linear asymptotes with the slope equal to unity at sufficiently low and sufficiently high substrate concentrations; the slope of the curve at $[S]=[S]_{0.5}$ is less than unity (Fig. 1d).

It should be noted that if the experimental data allow the limiting value of v at $[S] \to \infty$ to be estimated, the value of V_{max} obtained by such a way may be directly used for calculation of the values of the effective Michaelis constant:

$$K_m^{eff}=[S](V_{max}/v-1). \quad (11)$$

The construction of the K_m^{eff} vs. v/V_{max} plot allows us to prove the assumption that the effective Michaelis constant linearly increases with increasing the v/V_{max} ratio.

2.3. The order of the enzymatic reaction rate with respect to substrate

If the dependence of v on [S] follows the expression (6), the variation of the order of the enzymatic reaction rate with respect to substrate

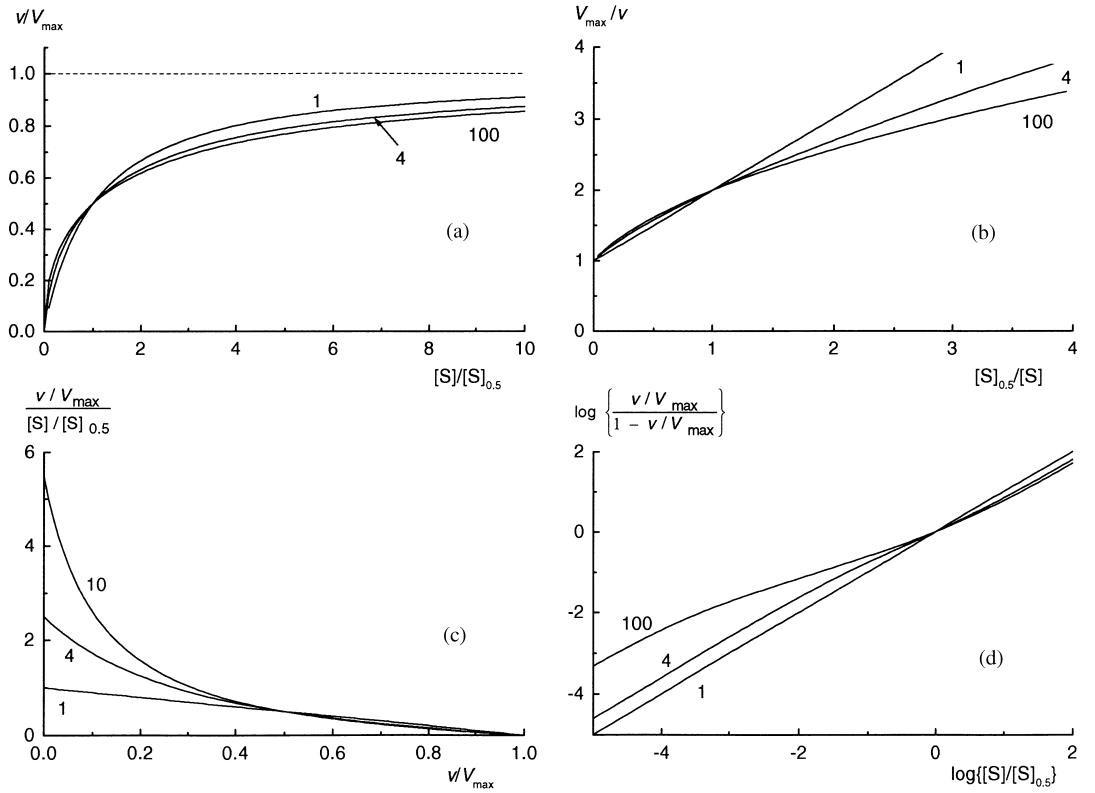

Fig. 1. Theoretical dependences of the rate of the enzymatic reaction on the substrate concentration calculated from Eq. (6) at various values of the K_{lim}/K_0 ratio ($K_{lim}/K_0 = \alpha$) in coordinates $\{v/V_{max}; [S]/[S]_{0.5}\}$ (a), in reciprocal coordinates (b), in coordinates $\{(v/V_{max})/([S]/[S]_{0.5}); v/V_{max}\}$ (c), and Hill coordinates (d). Numbers near the curves refer to the values of α.

$n_S = \mathrm{dlg}v/\mathrm{dlg}[S]$ with increasing the v/V_{max} ratio is described by the equation:

$$n_S = \frac{1 - v/V_{max}}{1 + \dfrac{(K_{lim} - K_0)(v/V_{max})(1 - v)/V_{max}}{K_0 + (K_{lim} - K_0)(v/V_{max})}}$$

$$= \frac{1 - v/V}{1 + \dfrac{(\alpha - 1)(v/V_{max})(1 - v/V_{max})}{1 + (\alpha - 1)(v/V_{max})}}. \qquad (12)$$

It should be noted that, when describing the kinetic anomalies by the Hill equation, negative cooperativity is characterized by the values of the Hill coefficient less than unity. When we use empirical Eq. (5), the degree of deviations from the hyperbolic law is characterized by the K_{lim}/K_0 ratio ($K_{lim}/K_0 = \alpha$). It is of interest to compare the values of the Hill coefficient with the α values for the dependences of v on [S] relating to the case of negative cooperativity. Taking into account that $h = \mathrm{dlg}[(v/V_{max})/(1 - v/V_{max})]/\mathrm{dlg}[S] = n_S/(1 - v/V_{max})$, we can get the following relationship between the value of h at a point of semisaturation (i.e. at $v/V_{max} = 0.5$) and the value of α: $h_{0.5} = 2(1 + \alpha)/(1 + 3\alpha)$. If $\alpha = 1$, the $h_{0.5}$ value is equal to 1. As the value of α increases, the value of $h_{0.5}$ decreases ($h_{0.5} \rightarrow 2/3 = 0.667$ at $\alpha \rightarrow \infty$). Thus, the value of $h_{0.5}$ for the dependence of v on [S] described by Eq. (6) may not be less than 0.667.

The use of n_S for linearization of Eq. (5) is discussed in Appendix B.

2.4. Functions of saturation of protein by ligand

When analyzing the deviations of the functions of saturation of protein by ligand from the hyperbolic law, we can use the equations equivalent to Eqs. (5)–(7):

$$r = r_{max}\frac{[L]/K^{eff}}{1+[L]/K^{eff}} = r_{max}\frac{[L]/\{K_0+(K_{lim}-K_0)(r/r_{max})\}}{1+[L]/\{K_0+(K_{lim}-K_0)(r/r_{max})\}}, \quad (13)$$

$$r = r_{max}\left\{\sqrt{(K_0+[L])^2+4(K_{lim}-K_0)[L]}-K_0-[L]\right\}/2(K_{lim}-K_0), \quad (14)$$

$$\frac{r}{[L]} = \frac{r_{max}-r}{K^{eff}} = \frac{r_{max}-r}{K_0+(K_{lim}-K_0)(r/r_{max})}, \quad (15)$$

where r is the number of ligand molecules bound by one protein molecule, r_{max} is the adsorption capacity of the protein (the limiting number of ligand molecules bound by one protein molecule) and [L] is the equilibrium concentration of ligand. In Eqs. (13) and (15) K^{eff} refers to the effective dissociation constant for the complex of protein with ligand, which is a linear function of the r/r_{max} ratio: $K^{eff} = K_0 + (K_{lim} - K_0)(r/r_{max})$, where K_0 and K_{lim} are the limiting values of K^{eff} at $r \rightarrow 0$ and $r \rightarrow r_{max}$, respectively.

At $r \rightarrow 0$ the curve in coordinates $\{r/[L]; r\}$ approaches a linear asymptote:

$$\frac{r}{[L]} = \frac{r_{max}}{K_0} - \frac{K_{lim}}{K_0^2}\cdot r. \quad (16)$$

The intercept on the ordinate axis is equal to r_{max}/K_0 and the slope of the asymptote is equal to $-K_{lim}/K_0^2$.

The linear asymptote at $r \rightarrow r_{max}$ has the following form:

$$\frac{r}{[L]} = \frac{r_{max}}{K_{lim}} - \frac{1}{K_{lim}}\cdot r. \quad (17)$$

The intercept on the abscissa axis is equal to r_{max} and the slope of the asymptote is equal to $-1/K_{lim}$. The value of v corresponding to the intersection point of two asymptotes is equal to $r_{max}/(1+\alpha)$.

3. Calculations

All the calculations in the present paper were carried out using the program Scientist (MicroMath, Inc., USA). In order to characterize the degree of agreement between the experimental data and calculated values, we used the determination coefficient R^2 (without considering the statistical weight of the results of measurements) [14]:

$$R^2 = \frac{\sum_{i=1}^{i=n}\left(Y_i^{obs}-\bar{Y}^{obs}\right)^2 - \sum_{i=1}^{i=n}\left(Y_i^{obs}-Y_i^{calc}\right)^2}{\sum_{i=1}^{i=n}\left(Y_i^{obs}-\bar{Y}^{obs}\right)^2}, \quad (18)$$

where $\bar{Y}^{obs} = 1/n\sum_{i=1}^{i=n} Y_i$ is the average of the experimental data (Y_i^{obs}), Y^{calc} is the theoretically calculated value of the function Y, and n is the number of measurements.

4. Analysis of experimental data

4.1. Dependence of the enzymatic reaction rate on coenzyme concentration

The dependence of the rate of the enzymatic reaction catalyzed by beef liver glutamate dehydrogenase on concentration of NAD in the wide range of the concentrations of coenzyme encom-

passing three orders of the magnitude was first obtained by Dalziel and Engel [2]. We have analyzed the dependence of such a type obtained in later work by Engel [7]. Fig. 2a shows the dependence of the enzymatic reaction rate on NAD concentration in coordinates $\{v/[\mathrm{NAD}];\ v\}$ (Na-phosphate buffer, pH 7.0, $I = 0.25$, 25°C, 50 mM glutamate). The v value refers to the specific enzymatic activity, i.e. the rate of the enzymatic reaction divided by the enzyme concentration. The measurements of the enzymatic activity were carried out in the range of NAD concentrations from 0.001 to 1 mM. Application of Eq. (7) for description of the dependence of $v/[\mathrm{NAD}]$ on v gives the following values of parameters: $V_{\max} = 97 \pm 7\ \mathrm{s}^{-1}$, $K_0 = 0.052 \pm 0.004$ mM, and $K_{\lim} = 0.58 \pm 0.11$ mM ($R^2 = 0.9970$). The $K_{\lim}/K_0$ is found to be 11 ± 3. The value of $[\mathrm{S}]_{0.5}$ calculated as $(K_{\lim} + K_0)/2$ is equal to 0.32 ± 0.11 mM. The straight lines 2 and 3 in Fig. 1a are asymptotes calculated by Eqs. (9) and (10) , respectively. Fig. 2b demonstrates the linear character of the dependence of the effective Michaelis constant K_m^{eff} calculated from Eq. (11) on the relative rate of the enzymatic reaction $v/V_{\max}$.

It is of interest to give the results of analysis of the dependence of the enzymatic reaction rate catalyzed by beef liver glutamate dehydrogenase on NAD concentration under discussion using the Hill equation: $V_{\max} = 105 \pm 6\ \mathrm{s}^{-1}$, $[\mathrm{S}]_{0.5} = 0.40 \pm 0.07$ mM, and $h = 0.65 \pm 0.02$ ($R^2 = 0.9993$).

The dependence of the order of the enzymatic reaction rate with respect to substrate n_{S} on the $v/V_{\max}$ ratio represented in Fig. 2c (curve 1) is calculated by Eq. (12). When the dependence of v on [S] follows the hyperbolic law, the n_{S} value linearly decreases with increasing the $v/V_{\max}$ ratio (straight line 2). The order of the enzymatic reaction rate with respect to substrate characterizes the sensitivity of the enzymatic reaction rate to variation of the substrate concentration. The comparison of the curves 1 and 2 directly demonstrates that the enzymatic reaction rate catalyzed by glutamate dehydrogenase is characterized by the lower sensitivity to variation of the coenzyme concentration than the rate of the enzymatic reaction following the michaelian kinetics. Therein, probably, lies the physiological significance of negative cooperativity for glutamate dehydrogenase ([15–18], and also [13], pp. 68–70).

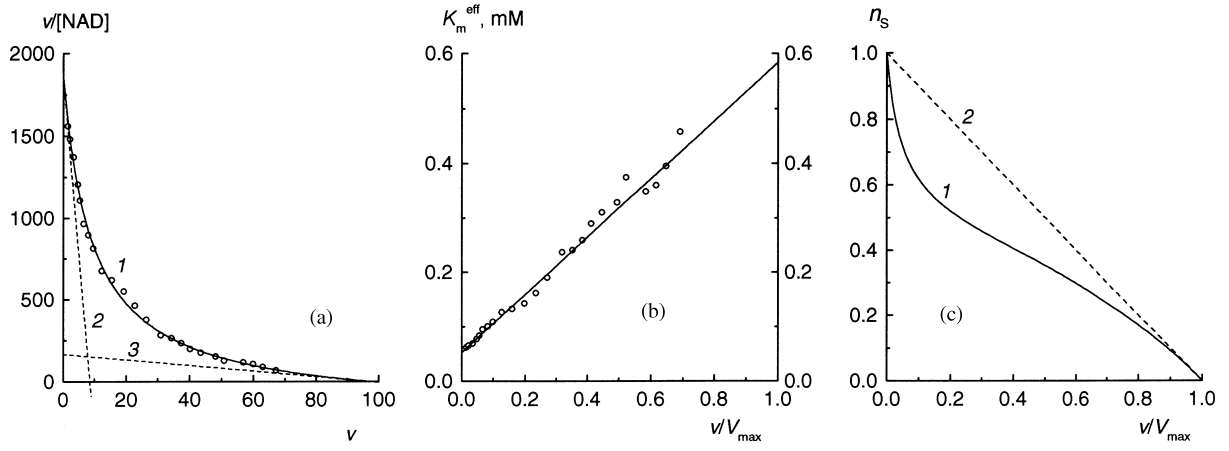

Fig. 2. Analysis of the dependence of the rate of the enzymatic reaction catalyzed by beef liver glutamate dehydrogenase on NAD concentration (Na-phosphate buffer, pH 7.0, $I = 0.25$, 25°C, 50 mM glutamate) [7]. (a) The kinetic data in coordinates $\{v/[\mathrm{NAD}];\ v\}$. Dimension of [NAD] is mM; dimension of v, specific enzymatic activity, is s^{-1}. Points are experimental data. Solid curve 1 is calculated from Eq. (7) at the values of parameters given in text. Straight lines 2 and 3 are asymptotes calculated from Eqs. (9) and (10), respectively. (b) The dependence of the effective Michaelis constant K_m^{eff} defined by Eq. (11) on the $v/V_{\max}$ ratio. (c) The dependence of the order of the enzymatic reaction rate with respect to substrate n_{S} on the $v/V_{\max}$ ratio (curve 1 corresponds to the experimental dependence of v on [NAD]; straight line 2 corresponds to the hyperbolic dependence of v on [S]).

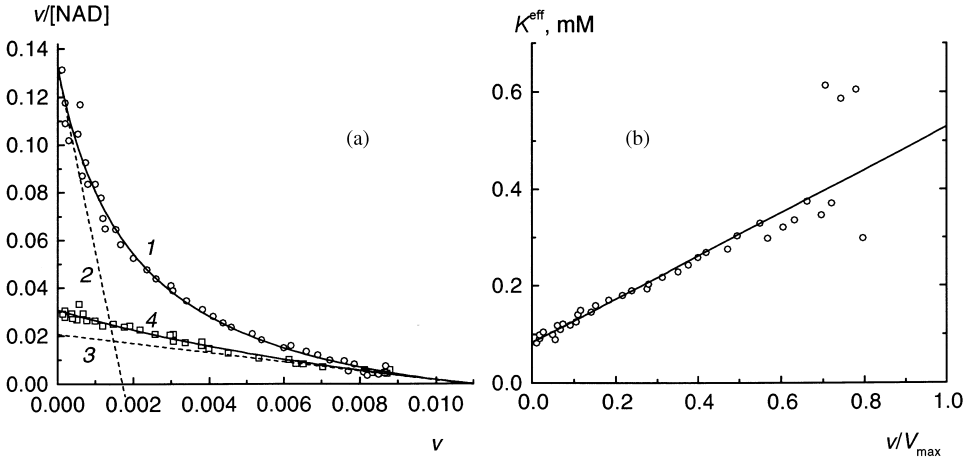

Fig. 3. Analysis of the dependence of the rate of the enzymatic reaction catalyzed by glutamate dehydrogenase from *Clostridium symbiosum* on NAD concentration (0.1 M K-phosphate buffer, pH 7.0, 25°C, 40 mM glutamate) [19]. (a) The kinetic data in coordinates $\{v/[\mathrm{NAD}]; v\}$. Dimension of [NAD] is mM. Open circles correspond to hexamer with six active subunits; open squares correspond to hexamer with one active subunit (enzyme concentrations were 2.35 and 0.35 μg/ml, respectively). Solid curve 1 is calculated from Eq. (7) at the values of parameters given in text. Straight lines 2 and 3 are asymptotes calculated from Eqs. (9) and (10), respectively. (b) The dependence of the effective Michaelis constant K_m^{eff} defined by Eq. (11) on the $v/V_{\max}$ ratio for hexamer with six active subunits.

In order to elucidate the mechanism of negative cooperativity for glutamate dehydrogenase from *Clostridium symbiosum*, Aghajanian and Engel studied hybridization of the wild-type enzyme and the mutant form of the enzyme, in which Cys320 was replaced by serine residue [19]. Modification of residue Cys320 in the wild-type enzyme by SH-reagents results in full inactivation of the enzyme. By contrast, C320S-mutant, which has the catalytic characteristics close to those of the wild-type enzyme, is insensitive to treatment of SH-reagents. Aghajanian and Engel obtained the hybrid form containing five subunits of the wild-type enzyme modified by 5,5′-dithiobis-(2-nitrobenzoate) and one subunit of C320S-mutant. In this hybrid form only subunit belonging to C320S-mutant is catalytically active. After treatment of this hybrid form by β-mercaptoethanol all six subunits of hexamer become active.

When studying the dependence of the enzymatic reaction rate on NAD concentration for hexamer with six active subunits, the clearly defined negative cooperativity is observed: the curves in coordinates $\{v/[\mathrm{NAD}]; v\}$ are concave (Fig. 3a, open circles; 0.1 M K-phosphate buffer, pH 7.0, 25°C, 40 mM glutamate). The application of Eq. (7) for description of the dependence of $v/[\mathrm{NAD}]$ on v gives the following values of parameters: $V_{\max} = 0.0109 \pm 0.0010$ units, $K_0 = 0.083 \pm 0.007$ mM, and $K_{\mathrm{lim}} = 0.53 \pm 0.12$ mM ($R^2 = 0.9839$). The K_{lim}/K_0 ratio is equal to 6.4 ± 1.9. The straight lines 2 and 3 in Fig. 3a are asymptotes calculated by Eqs. (9) and (10) , respectively. Fig. 3b demonstrates the linear character of the dependence of the effective Michaelis constant $K_{\mathrm{m}}^{\mathrm{eff}}$ calculated from Eq. (11) on the relative rate of the enzymatic reaction $v/V_{\max}$.

The experimental dependence of v on [NAD] has been analyzed also using the Hill equation: $V_{\max} = 0.0098 \pm 0.0003$ units, $[\mathrm{S}]_{0.5} = 0.21 \pm 0.02$ mM, and $h = 0.80 \pm 0.03$ ($R^2 = 0.9955$).

The catalytic activity of hexamer containing one active subunit of C320S-mutant was measured at the enzyme concentration, which was about 6 times lower than the concentration of fully active enzyme form, i.e. the hexameric form with six active subunits. As seen from Fig. 3a, there is no pronounced negative cooperativity for

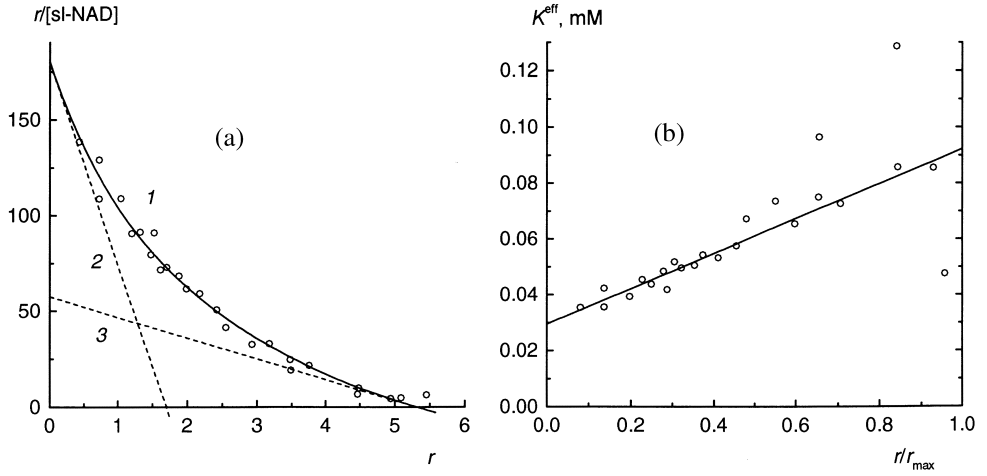

Fig. 4. Analysis of the data on binding of spin-labeled NAD (sl-NAD) by beef liver glutamate dehydrogenase in the presence of 80 mM 2-oxoglutarate (0.1-M Na-phosphate buffer, pH 7.4) [20]. (a) Binding curve in Scatchard coordinates. [sl-NAD] is equilibrium concentration of sl-NAD (mM). Points are experimental data. Solid curve 1 is calculated from Eq. (15) at the values of parameters given in text. Straight lines 2 and 3 are asymptotes corresponding to Eqs. (16) and (17). (b) The dependence of the effective dissociation constant K^{eff} defined by Eq. (19) on the $r/r_{\max}$ ratio.

the enzyme form with one active subunit in hexamer (open squares) and the dependence of $v/[\text{NAD}]$ on v is close to the limiting asymptote for hexamer with active subunits corresponding to the region $v \to V_{\max}$. Thus, data obtained may be considered as a direct evidence that the reason of kinetic anomalies for glutamate dehydrogenase from *Clostridium symbiosum* are interactions between NAD-binding sites in the hexameric enzyme molecule. It is believed that the formation of hexamer from subunits is accompanied by a marked decrease in the Michaelis constant for NAD (i.e. the increase in affinity to NAD) for each subunit. As the hexameric enzyme molecule is saturated by coenzyme, the influence of neighbor subunits is weakened and the Michaelis constant increases to the value corresponding to free subunit.

4.2. Binding of specific ligands to glutamate dehydrogenase

Fig. 4 shows the data on binding of spin-labeled analog of NAD (sl-NAD) by beef liver glutamate dehydrogenase in the presence of 2-oxoglutarate (0.1 M phosphate buffer, pH 7.4, 20°C; [20]). sl-NAD is capable of functioning as a coenzyme in the enzymatic reaction catalyzed by glutamate dehydrogenase. As seen from Fig. 4a, binding of sl-NAD is characterized by negative cooperativity. The experimental data may be satisfactorily described by Eq. (15) at the following values of parameters: $r_{\max} = 5.3 \pm 0.2$, $K_0 = 0.0295 \pm 0.0015$ mM, and $K_{\text{lim}} = 0.0924 \pm 0.013$ mM ($R^2 = 0.9841$). The K_{lim}/K_0 ratio is equal to 3.1 ± 0.6. Asymptotes at $r \to 0$ and $r \to r_{\max}$ are drawn in Fig. 4a by dashed lines. Fig. 4b shows the linear character of the dependence of the effective dissociation constant of the complex of protein with ligand calculated with the formula

$$K^{\text{eff}} = [\text{L}](r_{\max}/r - 1) \tag{19}$$

on the $r/r_{\max}$ ratio. Thus, as in the case of NAD [10], in the presence of 2-oxoglutarate only six coenzyme-binding sites in the molecule of glutamate dehydrogenase show sufficiently high affin-

ity to sl-NAD. The fact that binding of sl-NAD is characterized by negative cooperativity in combination with the results of the work by Dalziel and Egan [21] demonstrating negative cooperativity for binding of NAD by beef liver glutamate dehydrogenase in the presence glutarate allows us to suggest that negative cooperativity for coenzyme in the kinetic experiments is connected primarily with the change in the affinity of coenzyme but not the catalytic constant as the enzyme oligomer is saturated by coenzyme.

Binding of NADH by beef liver glutamate dehydrogenase is also characterized by negative cooperativity (0.067 M Na-phosphate buffer, pH 7.6; 20°C) [22]. The experimental data represented in Fig. 5a may be satisfactorily described by Eq. (15) at the following values of parameters: $r_{max} = 11.9 \pm 0.6$, $K_0 = 0.0169 \pm 0.0008$ mM, and $K_{lim} = 0.061 \pm 0.009$ mM ($R^2 = 0.9810$). The K_{lim}/K_0 ratio is equal to 3.6 ± 0.7. The straight lines 2 and 3 in Fig. 5a are asymptotes calculated with Eqs. (16) and (17), respectively. Fig. 5b demonstrates the linear character of the dependence of the effective dissociation constant of the complex of protein with ligand calculated with formula [Eq. (19)] on the r/r_{max} ratio. The value of r_{max} obtained ($r_{max} \approx 12$) means that each subunit of the hexameric molecule of glutamate dehydrogenase contains two NADH-binding sites.

When studying binding of NADH by beef liver glutamate dehydrogenate in the presence of allosteric inhibitor GTP, Malencik and Anderson [23] observed an increase in affinity of the enzyme to NADH and appearance of additional six NADH-binding sites (Fig. 6). Analysis of the binding data using Eq. (15) gave the following values of the parameters: $r_{max} = 19 \pm 1$, $K_0 = 0.0054 \pm 0.0008$ mM, and $K_{lim} = 0.014 \pm 0.003$ mM ($R^2 = 0.9499$). The K_{lim}/K_0 ratio is equal to 2.6 ± 1.2.

The study of binding of NADPH by beef liver glutamate dehydrogenase showed that only 6 from 12 coenzyme-binding sites have sufficiently high affinity to NADPH (Fig. 7a; [22]). This circumstance allowed us to apply Eq. (15) to the initial part of the isotherm of binding ($r \leq 4.6$). The following values of parameters have been obtained: $r_{max} = 5.8 \pm 0.3$, $K_0 = 0.0104 \pm 0.0004$ mM, and $K_{lim} = 0.021 \pm 0.004$ mM ($R^2 = 0.9841$).

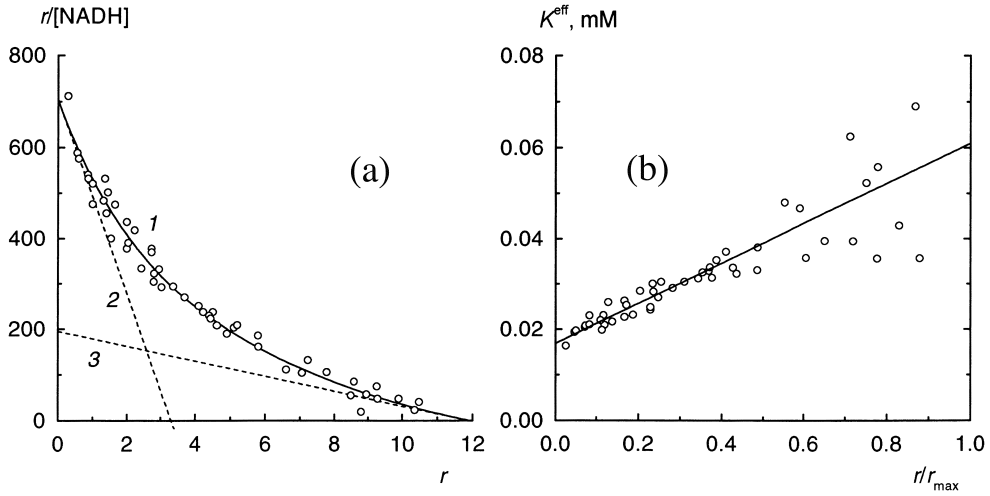

Fig. 5. Analysis of the data on binding of NADH by beef liver glutamate dehydrogenase (0.067 M Na-phosphate buffer, pH 7.6; 20°C) [22]. (a) Binding curve in Scatchard coordinates. [NADH] is equilibrium concentration of NADH (mM). Points are experimental data obtained by the sedimentation methods. Solid curve 1 is calculated from Eq. (15) at the values of parameters given in text. Straight lines 2 and 3 are asymptotes corresponding to Eqs. (16) and (17). (b) The dependence of the effective dissociation constant K^{eff} defined by Eq. (19) on the r/r_{max} ratio.

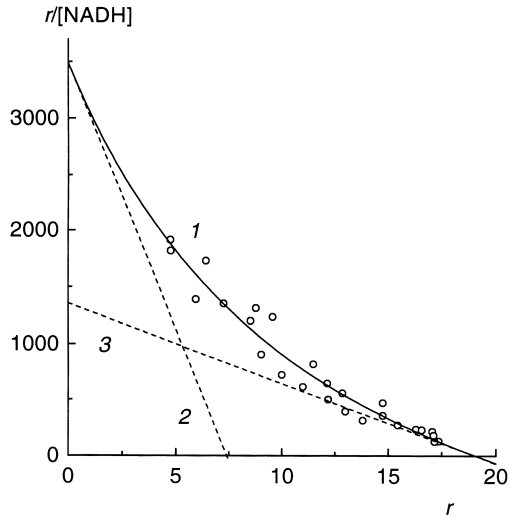

Fig. 6. Scatchard plot for binding of NADH by beef liver glutamate dehydrogenase in the presence on 300 μM GTP (0.05 M Na-phosphate buffer, pH 7.6; 20°C) [23]. [NADH] is equilibrium concentration of NADH (mM). Points are experimental data. Solid curve 1 is calculated from Eq. (15) at the values of parameters given in text. Straight lines 2 and 3 are asymptotes corresponding to Eqs. (16) and (17).

The K_{lim}/K_0 ratio is equal to 2.0 ± 0.5. The straight lines 2 and 3 in Fig. 7a are asymptotes calculated with Eqs. (16) and (17) , respectively. Earlier, Malencik and Anderson [23] also demonstrated the presence of six NADPH-binding sites in the molecule of beef liver glutamate dehydrogenase, the binding curve displaying negative cooperativity. These data are represented in Fig. 7b. When describing this binding curve with Eq. (15), the following values of parameters have been obtained: $r_{\max} = 5.8 \pm 0.4$, $K_0 = 0.0076 \pm 0.0006$ mM, and $K_{\mathrm{lim}} = 0.033 \pm 0.007$ mM ($R^2 = 0.9840$). The K_{lim}/K_0 ratio is equal to 4.2 ± 1.3.

5. Discussion

5.1. Physical meaning of parameters K_0 and K_{lim}

Consider the situation when deviations of the functions of protein saturation by ligand from hyperbolic law are connected with interactions between ligand-binding sites in the protein oligomer resulting in the change in the affinity to ligand as the ligand-binding sites in the protein molecule are occupied by ligand. In this case

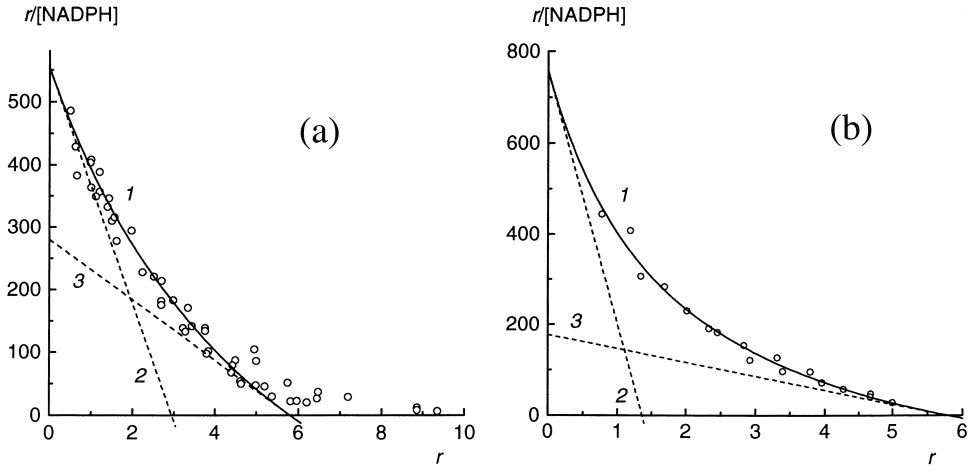

Fig. 7. Scatchard plots for binding of NADPH by beef liver glutamate dehydrogenase. (a) Data obtained by the sedimentation methods [22] (0.067 M Na-phosphate buffer, pH 7.6; 20°C). (b) Binding curve obtained in [23] (0.05 M Na-phosphate buffer, pH 7.6; 20°C). [NADPH] is equilibrium concentration of NADPH (mM). Points are experimental data. Solid curves 1 are calculated from Eq. (15) at the values of parameters given in text. Straight lines 2 and 3 are asymptotes corresponding to Eqs. (16) and (17).

parameters K_0 and K_{lim} are the limiting values of the dissociation constant corresponding to initial ($r/r_{max} \to 0$) and final ($r/r_{max} \to 1$) steps of saturation of protein by ligand.

It is of special interest to discuss what kind of interactions between ligand-binding sites in the protein molecule may result in the linear dependence of the effective dissociation constant on the r/r_{max} ratio. Assume that the protein molecule contains n equivalent ligand-binding sites interacting with each other. Let K_i be the microscopic dissociation constant for protein-ligand complex containing i molecules of ligand (P is the protein molecule):

$$PL_{i-1} + L \overset{K_i}{\leftrightarrows} PL_i \quad (K_i = [PL_{i-1}][L][PL_i]). \tag{20}$$

The function of saturation of protein by ligand has the following form ([13], p. 122):

$$\frac{r}{r_{max}} = \frac{\sum_{i=1}^{i=n} i \frac{n!}{(n-i)!i!} \cdot \frac{[L]^i}{K_1 K_2 \ldots K_i}}{n\left\{1 + \sum_{i=1}^{i=n} \frac{n!}{(n-i)!i!} \cdot \frac{[L]^i}{K_1 K_2 \ldots K_i}\right\}}. \tag{21}$$

Negative interactions between ligand-binding sites corresponds to the case when the K_i values increase with increasing i, whereas positive interactions between ligand-binding sites corresponds to the case when the K_i values decrease with increasing i.

Consider, for example, the situation when the molecule of a model protein contains six equivalent ligand-binding sites interacting with each other. Assume that the saturation function is described by empirical Eq. (5) and the K_{lim}/K_0 ratio is equal to 10 ($\alpha = 10$). Let $K_0 = K_1$ be 1 mM ($K_{lim} = K_6 = 10$ mM). We have constructed the theoretical dependence of r on [L] in the range of the values of [L] from 10^{-2} to 10^3 mM and selected 100 points uniformly distributed on the logarithmic scale in this interval. The model curve was described further by Eq. (21). The following values of K_i were obtained:

i	1	2	3	4	5	6
K_i, mM	1	5.3 ±0.2	3.1 ±0.2	8.7 ±0.4	7.3 ±0.2	10
K_i/K_{i-1}		5.3 ±0.2	0.58 ±0.06	2.8 ±0.3	0.84 ±0.06	1.37 ±0.04

Thus, the linear dependence of K^{eff} on the r/r_{max} ratio in the case under discussion results if negative ($K_2/K_1 > 1$, $K_4/K_3 > 1$, $K_6/K_5 > 1$) and positive ($K_3/K_2 < 1$, $K_5/K_4 < 1$) interactions between ligand-binding sites alternate with the degree of saturation of ligand-binding sites in the protein molecule by ligand.

Analysis of the experimental data on glutamate dehydrogenase allows us to suggest that non-hyperbolic character of the dependence of the enzymatic reaction rate on NAD concentration is due to interactions between six NAD-binding sites in hexameric enzyme molecule. If we assume further that interactions between the active sites result only in the change in the Michaelis constant (but not the catalytic constant), the calculated values of K_0 and K_{lim} give the estimations of K_m for NAD at $v/V_{max} \to 0$ and $v/V_{max} \to 1$, respectively.

What is the physical meaning of parameters K_{lim} and K_0 for the case when the ligand-binding sites in the protein molecule are non-identical? The saturation function for the protein containing n non-identical and non-interacting ligand-binding sites has the following form:

$$\frac{r}{r_{max}} = \frac{1}{n} \sum_{i=1}^{i=n} \frac{[L]/K_i}{1 + [L]/K_i}, \tag{22}$$

where K_i is the microscopic dissociation constant for binding of ligand in the site of i type. At $r \to 0$ we get the following expression for r/r_{max}:

$$\frac{r}{r_{max}} = \frac{[L]}{K^{eff}} = \frac{[L]}{n} \sum_{i=1}^{i=n} \frac{1}{K_i}, \tag{23}$$

and, consequently,

$$\lim_{r\to 0} K^{\text{eff}} \equiv K_0 = \frac{1}{\frac{1}{n}\sum_{i=1}^{i=n}\frac{1}{K_i}}. \quad (24)$$

At $r \to r_{\max}$ the expression

$$\frac{r}{r_{\max}} = 1 - \frac{K^{\text{eff}}}{[\text{L}]} = 1 - \frac{1}{n[\text{L}]}\sum_{i=1}^{i=n} K_i \quad (25)$$

holds for $r/r_{\max}$ and, consequently,

$$\lim_{r\to r_{\max}} K^{\text{eff}} \equiv K_{\lim} = \frac{1}{n}\sum_{i=1}^{i=n} K_i. \quad (26)$$

Thus, the calculation of $r_{\max}$ ($r_{\max} = n$), K_0, and $K_{\lim}$ allows the following combinations of constants K_i to be obtained: the sum of reciprocal values of the constants K_i and the sum of values of the constants K_i. It is worthy noting that $1/K_0$ is the mean of the reciprocal values of the constants K_i and $K_{\lim}$ is the mean of the values of the constants K_i.

Consider, for example, the situation when the molecule of a model protein contains six non-identical and non-interacting ligand-binding sites. Assume that the saturation function is described by empirical Eq. (5) and the $K_{\lim}/K_0$ ratio is equal to 10 ($\alpha = 10$). Let K_0 be 1 mM ($K_{\lim} = 10$ mM). The model dependence of r on [L] was described by us by Eq. (22) in the range of values of [L] from 10^{-2} to 10^{3} mM. The following values of K_i given in order in their increasing were obtained:

i	1	2	3	4	5	6
K_i, mM	0.19 ±0.02	6.2 ±0.2	6.2 ±0.2	6.2 ±0.2	6.2 ±0.8	35.0
K_i/K_{i-1}		27.7 ±3.6	~1	~1	~1	5.8 ±0.3

Thus, the linear dependence of K^{eff} on the $r/r_{\max}$ ratio in the case under discussion results if the constants K_i for main set of the ligand-binding sites are of the same value and there are a few number of sites with high affinity and a few number of sites with low affinity.

It seems likely that negative cooperativity observed for binding of NADH by beef liver glutamate dehydrogenase (Fig. 5) is due to non-equivalency of NADH-binding sites as well as to interactions between NADH-binding sites in the enzyme molecule. As for binding of NADPH, negative cooperativity for this ligand (for the initial part of the isotherm of binding) is due solely to interactions between NADPH-binding sites in the glutamate dehydrogenase molecule.

5.2. The K_{lim}/K_0 ratio as a quantitative characteristic of negative cooperativity

The empirical equation proposed may be used for the quantitative description of the deviations from the Michaelian kinetics, which manifest themselves as 'negative cooperativity' (convex curves in coordinates $\{1/v;\ 1/[\text{S}]\}$ or concave curves in coordinates $\{v/[\text{S}];\ v\}$ as well as for description of negative cooperativity for the functions of saturation of protein by ligand. The equation contains three parameters: $V_{\max}$ (or $r_{\max}$), K_0, and $K_{\lim}$. The $K_{\lim}/K_0$ ratio may be considered as a quantitative characteristic of the degree of deviations from the hyperbolic law. If the protein molecule contains (1) n equivalent ligand-binding sites interacting with each other or (2) n non-identical and non-interacting ligand-binding sites, parameters K_0 and $K_{\lim}$ have clear physical meaning. In the former case $K_0 = K_1$ and $K_{\lim} = K_n$ (K_1 and K_n are the microscopic dissociation constants for the complexes of protein with ligand containing one and n ligand molecules, respectively). The relationship between parameters K_0 and $K_{\lim}$ allows the free energy of intersubunit interaction to be estimated [24]: $\Delta F = -R\text{Tln}(K_1/K_n) = -R\text{Tln}(K_0/K_{\lim})$. In the latter case (case 2) parameters K_0 and $K_{\lim}$ are defined by Eqs. (24) and (26) ($1/K_0$ is the mean of the reciprocal values of the constants K_i and $K_{\lim}$ is the mean of the values of the constants K_i; K_i is the microscopic dissociation constant for binding of ligand in the site of i type).

The comparison of the Hill equation and the empirical equation where $K_{\text{m}}^{\text{eff}}$ is a linear function

of v/V_{max} has shown that the empirical equation results in the v vs. [S] curves (or r vs. [L] curves) with the Hill coefficient at semi-saturation falling in the interval from 0.667 to 1. Negative cooperativity for glutamate dehydrogenase is not beyond the region bounded by the values of the Hill coefficient $h = 0.667$ and $h = 1$. Since the variations of the Hill coefficient in the case of negative cooperativity are limited by the narrow interval (to our knowledge there are no experimental data contradictory to this prediction), it is reasonable to consider the K_{lim}/K_0 ratio as an appropriate parameter for characterization of negative cooperativity.

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Appendix A: Eadie–Hofstee plot

It is expedient to discuss what is the shape of the dependence of v on [S] following Eq. (5) in coordinates $\{v; v/[S]\}$ (Eadie–Hofstee plot). The v value may be presented as a function of $v/[S]$:

$$v = \frac{V_{max} - K_0(v/[S])}{1 + (K_{lim} - K_0)(v/[S])/V_{max}}. \quad (A1)$$

When $v/[S] \rightarrow 0$ the curve in coordinates $\{v; v/[S]\}$ approaches a linear asymptote:

$$v = V_{max} - K_{lim} \cdot \frac{v}{[S]}. \quad (A2)$$

The intercept on the ordinate axis is V_{max} and the slope of the asymptote is equal to $-K_{lim}$.

The linear asymptote at $v \rightarrow 0$ has the following form:

$$v = \frac{K_0 V_{max}}{K_{lim}} - \frac{K_0^2}{K_{lim}} \cdot \frac{v}{[S]}. \quad (A3)$$

Intercept on the abscissa axis gives the V_{max}/K_0 ratio and the slope of the asymptote is equal to $-K_0^2/K_{lim}$. The value of $v/[S]$ corresponding to the intersection point of two asymptotes is equal to $V_{max}/(K_0 + K_{lim})$.

Appendix B: Linearization of the empirical equation

If the V_{max} value is unknown, the Hill equation may be linearized using the order of the enzymatic reaction rate with respect to substrate (n_S): $n_S = h - hv/V_{max}$ ([13], p. 44). In other words, when the Hill equation holds, the n_S value linearly decreases with increasing the v/V_{max} ratio. Analogously, calculation of the order of the enzymatic reaction rate with respect to substrate allows Eq. (5) to be linearized. Differentiation of Eq. (7) gives

$$\frac{d(v/[S])}{dv} = -\frac{K_{lim}}{\{K_0 + (K_{lim} - K_0)v/V_{max}\}^2}. \quad (B1)$$

Further transformations of this equation allows us to get the following linear anamorphosis:

$$\frac{\sqrt{[S]n_S}}{1 - n_S} = \frac{K_0}{\sqrt{K_{lim}}} + \frac{(K_{lim} - K_0)}{V_{max}\sqrt{K_{lim}}} \cdot v. \quad (B2)$$

Thus, experimental data may be linearized in coordinates $\{\sqrt{[S]n_S/(1 - n_S)}; v\}$.

References

[1] R.C. Hudson, R.M. Daniel, l-Glutamate dehydrogenases: distribution, properties and mechanism, Comp. Biochem. Physiol. B 106 (1993) 767–792.
[2] K. Dalziel, P.C. Engel, Antagonistic homotropic interactions as a possible explanation of coenzyme activation of glutamate dehydrogenase, FEBS Lett. 1 (1969) 349.
[3] P.C. Engel, K. Dalziel, Kinetic studies of glutamate dehydrogenase with glutamate and norvaline as substrates. Coenzyme activation and negative homotropic interactions in allosteric enzymes, Biochem. J. 115 (1969) 621–631.
[4] B. Eisenkraft, C. Veeger, Effect of 2-oxoglutarate on the catalytic activity and stability of glutamate dehydrogenase, Biochim. Biophys. Acta 167 (1968) 227–238.

[5] J.S. Barton, J.R. Fisher, Nonlinear kinetics of glutamate dehydrogenase. Studies with substrates-glutamate and nicotinamide-adenine dinucleotide, Biochemistry 10 (1971) 577–585.
[6] K. Markau, J. Schneider, H. Sund, Kinetic studies on the mechanism of the action of ADP on the glutamate dehydrogenase reaction, FEBS Lett. 24 (1972) 32–36.
[7] P.C. Engel, W. Ferdinand, The significance of abrupt transitions in Lineweaver–Burke plots with particular reference to glutamate dehydrogenase, Biochem. J. 131 (1973) 97–105.
[8] A.H. Electricwala, F.M. Dickinson, Kinetic studies on dogfish liver glutamate dehydrogenase, Biochem. J. 177 (1979) 449–459.
[9] P.E. Peterson, T.J. Smith, The structure of bovine glutamate dehydrogenase provides insights into the mechanism of allostery, Structure 7 (1999) 769–782.
[10] A. Banerjee, H.R. Levy, G.C. Levy, C. LiMuti, B.M. Goldstein, J.E. Bell, A transfer nuclear Overhauser effect study of coenzyme binding to distinct sites in binary and ternary complexes in glutamate dehydrogenase, Biochemistry 26 (1987) 8443–8450.
[11] P.J. Baker, K.L. Britton, P.C. Engel, G.W. Farrants, K.S. Lilley, D.W. Rice, T.J. Stillman, Subunit assembly and active site location in the structure of glutamate dehydrogenase, Proteins: Struct. Funct. Genet. 12 (1992) 75–86.
[12] C.M. Hill, R. Waight, W.G. Bardsley, Does any enzyme follow the Michaelis–Menten equation? Mol. Cell. Biochemistry 15 (1977) 173–178.
[13] B.I. Kurganov, Allosteric enzymes. Kinetic behaviour, John Wiley and Sons, Chichester, 1982.
[14] Scientist for Experimental Data Fitting. Microsoft Windows Version 2.0 (MicroMath, Inc., Salt Lake City, 1995) p. 466.
[15] A. Cornish-Bowden, The physiological significance of negative co-operativity, J. Theor. Biol. 51 (1975) 233–235.
[16] A. Levitzki, Quantitative aspects of allosteric mechanisms, Springer-Verlag, Berlin, 1978, pp. 79–88.
[17] P. Friedrich, On the physiological significance of positive and negative cooperativity in enzymes, J. Theor. Biol. 81 (1979) 527–532.
[18] R. Ghosh, On the physiological significance of positive and negative co-operativity, J. Theor. Biol. 93 (1981) 395–401.
[19] S. Aghajanian, P.C. Engel, Use of protein engineering to explore subunit interactions in an allosteric enzyme: construction of inter-subunit hybrids in *Clostridium symbiosum* glutamate dehydrogenase, Protein Eng. 11 (1998) 569–575.
[20] A. Zantema, W.E. Trommer, H. Wenzel, G.T. Robillard, Binding studies of a spin-labelled oxidized coenzyme to bovine-liver glutamate dehydrogenase, Eur. J. Biochem. 72 (1977) 175–184.
[21] K. Dalziel, R.R. Egan, The binding of oxidized coenzymes by glutamate dehydrogenase and the effects of glutarate and purine nucleotides, Biochem. J. 126 (1972) 975–984.
[22] J. Krause, M. Büchner, H. Sund, Studies of glutamate dehydrogenase. The binding of NADH and NADPH to beef-liver glutamate dehydrogenase, Eur. J. Biochem. 41 (1974) 593–602.
[23] D.A. Malencik, S.R. Anderson, Reduced pyridine nucleotide binding to beef liver and dogfish liver glutamate dehydrogenases, Biochemistry 11 (1972) 2766–2771.
[24] J. Wyman, Linked functions and reciprocal effects in haemoglobin: A second look, Adv. Prot. Chem. 19 (1964) 223–286.