Site–site interaction in the lipid activation of β-hydroxybutyrate dehydrogenase

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Kinetic data for the activation of the β-hydroxybutyrate dehydrogenase by long-chain lecithins [(1979) Biochemistry 18, 2420–2429; (1983) J. Biol. Chem. 258, 208–214] are analyzed. A previous kinetic model [(1982) Biochemistry 21, 3899–3908] is shown not to apply. Instead, the use of a two-site Adair equation points to a strongly cooperative interaction between the lecithin binding sites (ΔG, −2.8 kcal/mol).

β-Hydroxybutyrate dehydrogenase *Phosphatidylcholine* *Lipid activation* *Cooperativity* *Adair equation*

1. INTRODUCTION

Cooperativity and signal amplification by membrane enzymes and receptor proteins are likely to be involved in information transfer within and across biological membranes. Early studies concentrated on the possible role of membrane lattice structures [1,2]. In general, however, membrane components show rather high lateral and rotational mobilities and one may ask whether short-lived lipid–protein interactions can lead to cooperativity. This question has been examined in the general terms of multiple binding site kinetics [3]. One major result was that the kinetic cooperativity of lipid-dependent enzymes can be a consequence of non-cooperative lipid-protein binding steps. The highly cooperative lipid activation of $(Na^+ + K^+)$-ATPase [4] could in fact be generated from non-cooperative binding events involving the known number of 60 lipid binding sites per enzyme dimer [5]. The cooperative activation of BDH by short-chain lecithins has also been attributed to non-cooperative lipid binding, in this case to two sites per functional unit [6,7]. Non-cooperativity of lipid binding is furthermore strongly supported by physical studies. In particular, extensive lipid binding data obtained by ESR [8,9] and fluorescence quenching spectroscopy [10,11] have been successfully analyzed in terms of multiple non-interacting lipid binding sites.

In contrast to the results above, evidence for a strong cooperative interaction between the lipid activator sites of a membrane protein is presented. This study was initiated because the proposed kinetic model for BDH [6,7] appeared to be inconsistent with most of the extensive previous activation data for the same enzyme. The kinetic model of [6,7] is characterized by a cooperativity index, $[L_{90}]/[L_{10}]$, of 40 (cf. [3]). However, previous lipid activation curves had cooperativity indices between 10 and 35 [12–15], so that activation proceeded with much higher kinetic cooperativity than possible in the kinetic model. Two particularly well documented activation curves obtained with long-

Abbreviations: BDH, β-hydroxybutyrate dehydrogenase; PC, phosphatidylcholine

Definition: $[L_{10}]$, $[L_{20}]$, $[L_{50}]$, $[L_{90}]$, lipid concentrations required to reach 10, 20, 50 and 90%, respectively, of maximal activation (lipid concentrations are expressed as molecules PC per BDH monomer because these units were used in the original reports [15,16])

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chain lecithins [15,16] have therefore been chosen for a detailed re-investigation.

2. RESULTS

2.1. *Extent of kinetic cooperativity*

The activation curves in [15,16] were obtained by adding various long-chain lecithins to purified BDH apoprotein in the presence of non-activating phospholipids. The main effect of the latter was to increase the efficiency of the lecithins. A cooperativity index, $[L_{90}]/[L_{10}]$, of 14 was obtained by graphical analysis of the published curves. The combined experimental data points were then examined in a Hill plot (fig.1A). The slope of the regression line led to a Hill coefficient, n_H, of 1.83. A phenomenological lipid dissociation constant, K_{coop}, of 7.05 was calculated from the intercept on the ordinate. The latter value was identical to $[L_{50}]$, as required by theory (cf. [3]). A close fit to the experimental data points was generated when the values, $n_H = 1.83$ and $K_{coop} = 7$, were used in the non-linearized Hill equation (fig.1B).

2.2. *Analysis assuming no site–site interaction*

In the previous model for BDH [6,7], kinetic cooperativity was generated because two non-interacting lecithin binding sites had to be simultaneously occupied. In the present case, it was, however, not possible to describe the experimental data by a single form of the basic eq.3 of [6]. The experimental points up to $[L_{10}]$ could be approximated using a microscopic lipid dissociation constant, K_l, of 3 molecules PC per BDH monomer. A fit to the data points at $\geqslant [L_{20}]$ required a K_l value of about 1 molecule PC per BDH monomer. This decrease pointed to a transition from lower to higher affinity, i.e., positive cooperativity. The failure of eq.3 of [6] is attributed to its limitation to a cooperativity index of 40. The general kinetic model [3] indicates that the smallest non-interacting oligomer with the present cooperativity index of about 14 is a hexamer with one tolerated vacant site. Use of the corresponding rate equation with $K_l = 1.8$ molecules PC per BDH monomer led in fact to a reasonable fit to the experimental data (not shown). However, a hexameric structure of BDH is not supported by available structural results, which rather indicate a

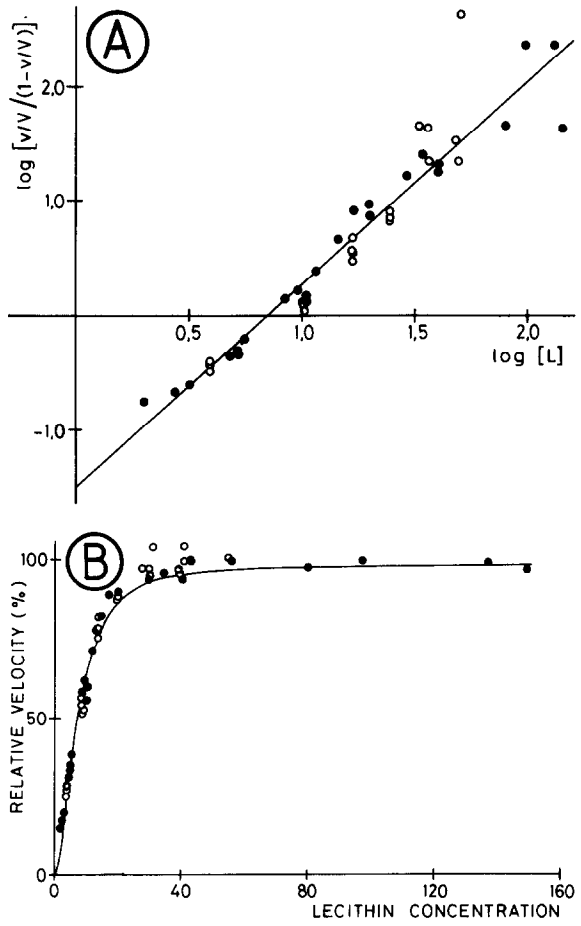

Fig.1. Activation of β-hydroxybutyrate dehydrogenase by various long-chain lecithins. The experimental points were taken from fig.2A of [15] (○) and from fig.1C of [16] (●). (A) Hill plot: $\log[(v/V)/(1 - v/V)]$ is plotted *vs* log[L]. The slope of the regression line is equal to the Hill coefficient ($n_H = 1.83$). The intercept on the ordinate is equal to $(-n_H \log K_{coop}$ (cf. [3]). A value of K_{coop} of 7.05 is obtained. (B) Activation curves: Relative velocity (v/V; % of maximal velocity) is plotted *vs* lipid concentration (molecules of PC per BDH monomer). The curve shown was calculated from the Hill equation (eq.5 of [3]) with $n = 1.83$ and $K_{coop} = 7$, and from the present eq.1, using $K_{l(1)} = 75$ and $K_{l(2)} = 0.65$. Both curves appear to superimpose but were not completely identical.

dimeric [6,17] or tetrameric [18] structure. The further analysis was based on a number of two lecithin binding sites per functional unit, as done in the previous kinetic model [6,7]. It should be noted that the Hill coefficient of 1.83 represents the minimum number of interacting binding sites.

2.3. *Analysis assuming site–site interaction*

To analyze site–site interaction quantitatively without invoking some specific mechanism for cooperativity, the well-known Adair equation for hemoglobin [19] is adapted to a two-site system as follows.

A microscopic lipid dissociation constant, $K_{l(1)}$, is assigned to the binding of the first lecithin molecule, L, to either site of the enzyme, E. The resulting complex, EL_1, is assumed to react with a second molecule L to give EL_2. A microscopic dissociation constant, $K_{l(2)}$, is assigned to the second binding step. The previous formalism [3] leads to the following expressions for the concentrations of EL_1 and EL_2:

$$[EL_1] = \frac{2[E][L]}{K_{l(1)}}$$

$$[EL_2] = \frac{[E][L]^2}{K_{l(1)}K_{l(2)}}$$

The ratio of the actual catalytic velocity, v, over the maximal velocity, V, is equal to the ratio of the number of bound lecithin molecules ($[EL_1] + 2[EL_2]$) over the total number of lecithin binding sites, $2([E] + [EL_1] + [EL_2])$. Introducing the above expressions for EL_1 and EL_2 and rearranging, one obtains:

$$\frac{v}{V} = \frac{1 + \left(\frac{[L]}{K_{l(2)}}\right)}{2 + \left(\frac{[L]}{K_{l(2)}}\right) + \left(\frac{K_{l(1)}}{[L]}\right)} \qquad (1)$$

This equation can be exactly solved by use of the following additional relationships [20]:

$$[L_{50}] = \sqrt{K_{l(1)}K_{l(2)}}$$

and

$$n_H = \frac{2}{1 + \sqrt{\frac{K_{l(2)}}{K_{l(1)}}}}$$

The present values of $[L_{50}] = 7$ molecules PC per BDH monomer, and $n_H = 1.83$ lead to $K_{l(1)} = 75$ and $K_{l(2)} = 0.65$ molecules PC per BDH monomer. These microscopic lecithin dissociation constants in fact result in a close fit to the experimental data (fig.1B). The free energy of site–site interaction is obtained from:

$$\Delta G = -RT\ln\frac{K_{l(1)}}{K_{l(2)}}$$

A value of -2.8 kcal/mol is obtained.

3. DISCUSSION

The free energy value derived above is valid only for a two-site system. Besides data pointing to a dimeric nature of BDH [6,17] there is also evidence for a tetramer [18]. It is, however, not possible to derive exact analytical equations for a four-site system [20]. A non-interacting tetramer can at best achieve a cooperativity index of 21 [21], so that the present value of 14 again requires site–site interaction. Assuming that the interaction is the same in each of the individual binding steps leading to EL_4, one obtains a free interaction energy of -1.4 kcal/mol from the corresponding Adair equation. The present analysis thus leads to a strong site–site interaction for both the dimer and the monomer, although definite proof requires direct lipid-protein binding experiments. With this proviso, it appears that membrane lipids may function as allosteric effector molecules in addition to their role in protein solvation [21,22]. Allosteric lipid activator sites are of particular interest as potential target sites for lipophilic drugs.

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