Diagnostic Tests on the Mode of Ligand Binding to Proteins: Application to Zymomonas mobilis Strains

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The occurrence of biphasic responses when kinetic data, which describe the binding of ligands to proteins are outlined in Eadie - Hofstee plots are not uncommon as in several cases of glucose uptake of Zymomonas mobilis strain ATCC 10988 and its derivative CU1Rif2/clone. In this work the authors report two novel diagnostic tests, which can be used easily and routinely in distinguishing the two possible cases. In the first case, two proteinaceous components are involved having one binding site per protein molecule and in the second case a single proteinaceous component having two binding sites per protein molecule is involved. These tests are based on the evaluation of either the fractal dimension and/or the coefficients of a virial expansion, of the Michaelis-Menten equation.

Keywords: Zymomonas mobilis, diagnostic, fractal, virial,glucose uptake

Introduction

The ability of Zymomonas mobilis to grow on elevated glucose concentrations using the derivative CU1Rif2 of strain ATCC 10988 was investigated (Douka et al., 1999). Although, CU1Rif2 showed more than 20 hrs lag period when transferred from a 0.11 M to 0.55 M glucose liquid medium, however, it grew normally on elevated concentrations of other sugars. CU1Rif2 cells grew without any delay on 0.55 M glucose on which wild type cells had been incubated for 3 hrs and removed at the beginning of their exponential phase. At that time, the authors suggested that in Z. mobilis a diffusible heat-labile proteinaceous factor, transitionally not present in 0.55 M glucose of CU1Rif2 cultures, triggers growth on 0.55 M glucose. Biochemical analysis of glucose uptake and glycolytic enzymes implied that glucose assimilation was not directly involved in that phenomenon. However, in several cases glucose update data showed biphasic responses in Eadie-Hofstee plots.

It has been accepted that Z. mobilis transports glucose by means of a low-affinity, high-velocity glucose-facilitated diffusion system based on GLF protein (Parker et al., 1995; DiMarco & Romano, 1987; Parker et al., 1997). On the other hand, Walsh and coworkers (Walsh et al., 1994) based on the biphasic nature of Eadie-Hofstee plots obtained from uptake experiments using Saccharomyces cerevisiae, postulated that glucose transport could be composed of one or more similar transporter proteins. They suggested that poor data analysis may contribute to the lack of progress in the area of the elucidation of glucose transport.

In this paper the authors present new results helpful in the diagnosis of number of possible transporter proteins of the glucose transport in Z. mobilis. Consequently, they applied a conventional methodology, by using the strain ATCC 10988 as well as its CU1Rif2 mutant, and collected series of glucose uptake experimental data, which were analyzed kinetically by applying two novel diagnostic tests.

Materials and Methods

Strains and growth conditions, estimation of glucose concentrations, bacterial conjugation, and
DNA methods and sequencing have been described in detail (Douka et al, 1999). The glucose uptake assays protocol was as follows: cells were harvested at the mid-exponential phase, washed with phosphate buffer (100 mM, pH 6.5) and re-suspended in the same buffer essentially as described by others (Walsh et al, 1994). Glucose uptake was measured using D-[14C]glucose (Amersham, England, 291 mCi/mmol) at concentrations ranging from 0.25 to 50 mM. Z. mobilis cells (50 μl) and 5-fold-concentrated, radiolabelled glucose (12.5 μl) were preincubated separately at 20°C, mixed together to give the appropriate glucose concentration and vortexed immediately. The uptake was stopped by adding 10 ml phosphate cold buffer (100 mM, pH 7.5, -2.5°C) containing 500 mM unlabelled glucose. Cells were immediately filtered and washed with 10 ml of the same buffer. The uptake rate was expressed as nanomoles of glucose taken up per min per mg of total protein.

Results and Discussion

Table 1 depicts the whole series of glucose uptake data (Douka et al, 1999). The one kinetic component Eq. (1), analogous to the Michaelis-Menten one, failed to fit all collected series of glucose uptake experimental data by nonlinear regression. In all cases, in the second column of Table 1, glucose uptake data exhibited biphasic responses in Eadie-Hofstee plots, and thus additional parameters were determined by nonlinear curve fitting of Eqs. (2) and (3) to these data.

\[
v_{\text{uptake}} = \frac{V_{\text{max}} [S]}{K_m + [S]} \quad \ldots \quad (1)
\]

\[
v_{\text{uptake}} = \frac{V_{\text{max}_1} [S]}{K_{m_1} + [S]} + \frac{V_{\text{max}_2} [S]}{K_{m_2} + [S]} \quad \ldots \quad (2)
\]

\[
v_{\text{uptake}} = \frac{V_{\text{max}_1} [S]}{K_{m_1} + [S]} + \frac{V_{\text{max}_2} [S]}{K_{m_2} + [S]} \quad \ldots \quad (3)
\]

Eq. (2) is valid when one molecule of proteinaceous transporter comprises two binding sites one of low and another of high affinity, available for two glucose molecules, respectively. Correspondingly, Eq. (3) is valid when each molecule of two different proteinaceous transporters, comprises one binding site available only for one molecule of glucose. In Eqs (2) and (3), \(K_{m_1}\) and \(K_{m_2}\) are the apparent affinity constants, and \(V_{\text{max}_1}\) and \(V_{\text{max}_2}\) are the respective maximal rates of glucose uptake; \(S\) is the concentration of glucose. In all fitting procedures, the Least Squares criterion of convergence was used; in addition, robust weighting and four statistical criteria were applied (Theodorou et al, 2000) in order to decide which equation best fitted each series of experimental data. All uptake experimental data, appearing in the second column of Table 1, fitted equally in both the Eqs (2) and (3). Therefore, based on the results from these fitting procedures, one cannot distinguish whether one transporter having two binding sites (one of low and another of high affinity) exists or two glucose transporters each of them having one binding site exist.

To resolve this uncertainty, the authors developed two suitable diagnostic tests, which were applied to the above experimental data. As it has been proposed earlier (López-Quintela & Casado, 1989; Lymeropoulos et al, 1998) there are alternative ways to study kinetically the binding of ligands to proteins, although, both of the previous publications deal with
enzymic reactions. However, their results can be easily applied to any protein-ligand interactions. In the first publication (López-Quintela & Casado, 1989), authors proposed a fractal approach to the Michaelis-Menten equation of the form appeared in Eq. (4). Alternatively, another form that of Eq. (5) has been proposed (Lymperopoulos et al, 1998), which is based on the virial expansion of the Michaelis-Menten equation, and it can replace all previous equations dealing with enzyme kinetics.

\[
V(\text{any}) = \frac{V_{\text{eff}} [S](2-D)}{K_m^{\text{eff}} + [S]} \quad \ldots \quad (4)
\]

In Eq. (4), \(D\) is the fractal dimension, and \(V_{\text{eff}}\) and \(K_m^{\text{eff}}\) are the effective individual parameters describing the global character of the studied mechanism; in Eq. (5) \(A, A_j\) are the virial coefficients (Lymperopoulos et al, 1998; Laidler & Meiser, 1982).

Based on the above two formulae (4) and (5) the authors investigated the values of the fractal

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**Fig. 1—ATCC 10988 at 5% Glucose:** a. Best fit of uptake data by Eq. (2), where \(V_{\text{max}} = 0.05 \pm 0.03, V_{\text{max}} = 0.28 \pm 0.02, K_m = 0.70 \pm 0.84,\) and \(K_m = 16.82 \pm 7.43\) (at 95% confidence); b. Best fit of the same uptake data by Eq. (4), where \(D = 0.66 \pm 0.05\) (at 95% confidence); c. Best fit of the same uptake data by Eq. (5), having two virial coefficients, a first negative virial coefficient whose absolute value was estimated as \(30 \pm 1.8\) times larger (at 95% confidence) than that of the second positive one (at 95% confidence).
dimension D, and the number and the sign of the virial coefficients as well as the relevance of their magnitudes. Moreover, in order to confirm the proposed tests they performed appropriate computer simulations, by using appropriate software (Evmiridis & Papamichael, 1991), and also applying their experience on this matter. Finally, the authors found out a statistical difference at 95% level between Eqs, (2) and (3): (a) in the estimated fractal dimension D, and (b) in the relevance of the magnitude of the first virial coefficient relative to the second one.

The fractal dimension, which corresponds to Eq. (2) was estimated as $D = 0.75 \pm 0.16$ and that of Eq. (3) as $D = 0.50 \pm 0.09$. Accordingly, it was found that an equation similar to (5), having two virial coefficients, the first of which was estimated as negative and the second as positive, could replace Eqs, (2) and (3). The absolute value of first virial coefficient was estimated as $30 \pm 0.9$ times larger than that of the second one, in case of Eq. (2), and as $22952 \pm 100$ times larger than that of the second one, in case of Eq. (3).

By fitting Eq. (4) to whole series of experimental data of the second column of Table 1, a mean value of

![Graph](image-url)
Fig. 3—CU1R2/clone at 5% Glucose: A, Best fit of uptake data by Eq. (2), where \( V_{max_1} = 0.07 \pm 0.32, V_{max_2} = 0.38 \pm 0.23, K_m_{1} = 2.05 \pm 9.88, \) and \( K_m_{2} = 19.90 \pm 35.71 \) (at 95% confidence); B, Best fit of the same uptake data by Eq. (4), where \( D = 0.78 \pm 0.16 \) (at 95% confidence); C, Best fit of the same uptake data by Eq. (5), having two virial coefficients, a first negative virial coefficient whose absolute value was estimated as 81 ± 13 times larger (at 95% confidence) than that of the second positive one (at 95% confidence).

\[ D = 0.73 \pm 0.22 \] (at 95% confidence) for the fractal dimension was estimated. Additionally, by fitting Eq. (5) with two virial coefficients, to whole series of experimental data of the second column of Table 1, a negative first virial coefficient was estimated whose absolute value was 76 ± 12 (at 95% confidence) times larger that that of the second positive one. Hence, it is more likely to conclude that for the system under investigation there is only one proteinaceous factor, which functions as a glucose transporter having two binding sites; the binding of one glucose molecule onto the first site enhances the binding of a second glucose molecule. This is the first report on the diagnosis between two kinetic equations having the same number of parameters and giving the same biphasic plot when one tries to rearrange their nonlinear forms.

Figures 1-3 depict selected examples from the fitting of cases presented in Table 1, where Eq. (2) and Eq. (5) having two virial coefficients, fitted the
corresponding experimental data for glucose uptake. Fig. 4 depicts an example where simulated data were best fitted in Eq. (3) and Eq. (5) having two virial coefficients. In all figures, the estimated values of $V_{max}$ are given in nmol of glucose/mg/sec, the values of $K_m$ in mM, and the parameter D and the virial coefficients are dimensionless numbers.

References


