Oestreicher, E.G., Pereira, D.A. & Pinto, G.F. Steady-state kinetic mechanism of Thermoanaerobacter brockii alcohol dehydrogenase: a study discrimination between alternative kineti...

ARTICLE *in* JOURNAL OF BIOTECHNOLOGY · APRIL 1996

Impact Factor: 2.87 · DOI: 10.1016/0168-1656(95)00171-9

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Journal of Biotechnology 46 (1996) 23–31

JOURNAL OF Biotechnology

Steady-state kinetic mechanism of *Thermoanaerobium brockii* alcohol dehydrogenase: A study of discrimination between alternative kinetic models

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Received 18 June 1995; revised 31 October 1995; accepted 7 November 1995

Abstract

Two steady-state kinetic mechanisms have been proposed for the oxidation of 2-propanol catalyzed by *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH): the sequential ordered BiBi mechanism and the Theorell–Chance BiBi mechanism. Discrimination between these kinetic mechanisms was achieved by analyzing the inhibition produced by acetone, the first product of the reaction sequence in relation to the second substrate of the enzyme (2-propanol). Two types of experimental data were used: a data set combining initial rate experiments in the absence of products with data involving inhibition by acetone in relation to 2-propanol as variable substrate; and, data sets just containing product inhibition experiments by acetone with 2-propanol as variable substrate. The results obtained strongly suggest that the kinetic mechanism of TBADH is described by the Theorell–Chance BiBi kinetic model.

Keywords: *Thermoanaerobium brockii*; Alcohol dehydrogenase; Discrimination of kinetic model; Product inhibition; Enzyme kinetics

1. Introduction

Alcohol dehydrogenase from *Thermoanaerobium brockii* (EC 1.1.1.2) is a NADP-linked enzyme which is commercially available in highly purified form. By the fact of being remarkably stable at elevated temperatures

Abbreviations: TBADH, *Thermoanaerobium brockii* alcohol dehydrogenase; A, NADP; B, 2-propanol; P, acetone; Q, NADPH; E, enzyme; I, inhibitor; K_a, Michaelis constant of NADP; K_b, Michaelis constant of 2-propanol; K_p, Michaelis constant of acetone; K_q, Michaelis constant of NADPH; K_{ia}, dissociation constant of TBADH–NADP complex; K_{ip} inhibition constant of acetone; K_{iq}, dissociation constant of TBADH–NADPH complex; K_{is}, apparent value of the dissociation constant of enzyme–inhibitor complex; K_{ii}, apparent value of the dissociation constant of enzyme-varied substrate–inhibitor complex; K_m, apparent value of the Michaelis constant of 2-propanol; S, varied substrate; V_m, apparent value of the maximum velocity; V_1, maximum velocity; v, initial velocity; Syx, residual standard error; v_i ($i = 1,...,N$), the initial velocity of the ith experimental point; $\bar{v}_i$ ($i = 1,...,N$), the best-fitted initial velocity of the ith experimental point; N, the number of experimental points; P, the number of kinetic parameters of the fitted rate equation; SE_j ($j = 1,...,P$), the asymptotic standard error of the jth kinetic parameter; C_{jj}, the jth element of the main diagonal of the variance–covariance matrix.

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SSDI 0168-1656(95)00171-9

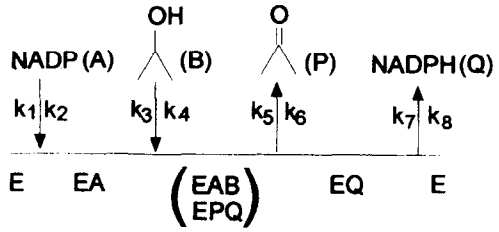

Fig. 1. Sequential ordered BiBi mechanism for the oxidation of 2-propanol catalyzed by TBADH.

and towards high concentrations of organic solvents (Lamed and Zeikus, 1981; Keinan et al., 1986, 1987), and also because it presents a broad substrate specificity (Keinan et al., 1987), TBADH has been increasingly used for the preparation of mono- or bifunctional chirons for the total synthesis of optically active compounds (Keinan et al., 1986, 1987; Belan et al., 1987; Röthing et al., 1990).

The elucidation of the kinetic mechanism of an enzyme, a procedure that involves the estimation of its kinetic parameters, represents an important step in the optimization, control and scale-up of preparative processes in which the enzyme is involved as a catalyst. For instance, the productivity of batch reactions could be improved by knowing the existence of inhibition (by substrates and/or products) or activation phenomena and the value of the different kinetic parameters of the overall model. Moreover, the productivity of reactors with coenzyme recycling systems, which are essential in preparative applications of dehydrogenases (Ward and Young, 1990; Wong, 1989; Bradshaw et al., 1992), could be optimized by the utilization of the appropriate steady-state equations and estimatives of their kinetic parameters directly as reaction models.

The kinetic mechanism of the oxidation of 2-propanol catalyzed by TBADH in the presence of NADP has been described by Pereira et al. (1994) and Al-Kassim and Tsai (1990). These groups concluded that it was of the sequential ordered type with the coenzymes binding to the free form of the enzyme, followed by the binding of the hydroxylated/ketonic substrate. However, differences exist between these two mechanistic proposals. Al-Kassim and Tsai (1990) concluded that the kinetic mechanism was the sequential ordered BiBi with formation of central ternary complexes (Fig. 1). On the other hand, Pereira et al. (1994) described the kinetic mechanism of TBADH as being the Theorell–Chance BiBi, i.e., the central ternary complexes do not exist or they are not kinetically significant (Cleland, 1963; Fig. 2).

The simplest and more direct way to discriminate between these two alternative kinetic mechanisms is the determination of the type of inhibition produced by the first product of the reaction (acetone) with respect to the second substrate of the enzyme (2-propanol). For this reason in the present work these product inhibition data, obtained by Pereira et al. (1994), were confronted with the corresponding ones extracted from the paper of Al-Kassim and Tsai (1990) with the aid curve fitting microcomputer programs. The conclusion arising from this study was that the reaction of oxidation of 2-propanol catalyzed by TBADH is best described by the Theorell–Chance BiBi mechanism.

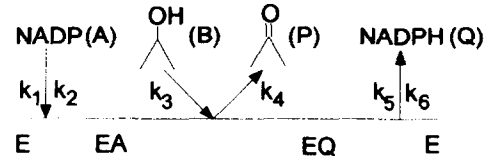

Fig. 2. Theorell–Chance BiBi mechanism for the oxidation of 2-propanol catalyzed by TBADH.

2. Methods

2.1. Kinetic data

Eq. 1 represents the rate equation for the sequential ordered BiBi mechanism in the presence of product P (acetone) and Eq. 2, is the equivalent one for the Theorell–Chance BiBi mechanism:

$$v = \frac{V_1[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_bK_q[P]}{K_pK_{iq}} + \frac{K_bK_q[A][P]}{K_pK_{iq}} + \frac{[A][B][P]}{K_{ip}}} \quad (1)$$

$$v = \frac{V_1[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_bK_q[P]}{K_pK_{iq}} + \frac{K_bK_q[A][P]}{K_pK_{iq}}} \quad (2)$$

The following expression relating parameters K_p, K_{iq} and K_p in Eq. 1 can be written:

$$K_1 = \frac{K_pK_{iq}}{K_q} \quad (3)$$

Substitution of Eq. 3 into Eq. 1 gives:

$$v = \frac{V_1[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_b[P]}{K_1} + \frac{K_b[A][P]}{K_1} + \frac{[A][B][P]}{K_{ip}}} \quad (4)$$

On the other hand, from the Haldane equations established for the Theorell–Chance BiBi kinetic mechanism, the following relationship:

$$K_{ip} = \frac{K_pK_{iq}}{K_q} \quad (5)$$

can be introduced into Eq. 2, which is then transformed into Eq. 6:

$$v = \frac{V_1[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_b[P]}{K_{ip}} + \frac{K_b[A][P]}{K_{ip}}} \quad (6)$$

Since the Haldane equations from which Eq. 5 was derived do not exist for the sequential ordered BiBi model (Segel, 1972; Plowman, 1972), parameter K_1 of Eq. 3 is intrinsically different from the kinetic parameter K_{ip} appearing in Eq. 6, and was just used to eliminate the hyperparametrization of Eq. 1.

Eqs. 4 and 6 were fitted to one experimental data set obtained in our laboratory with a purified TBADH enzyme preparation purchased from Sigma Chemical Company (Pereira et al., 1994). These data were formed by combining two different groups of experiments. The first one consisted of 80 experimental points of initial rate measurements in the absence of products, obtained with NADP as the variable substrate (eight different concentrations in the range of 10 to 100 μM) at five constant concentrations of 2-propanol (0.3, 0.45, 0.75, 1.2, and 3.0 mM) and with 2-propanol as the variable substrate (eight different concentrations in the range of 0.33 to 3.3 mM) at five fixed concentrations of NADP (20, 30, 50, 100 and 500 μM). The second group, consisting of 40 experimental points, corresponded to product inhibition data obtained with five fixed concentrations of acetone as product inhibitor (0, 0.05, 0.1, 0.15 and 0.2 mM) in the presence of 0.1 mM NADP and 2-propanol

as the variable substrate (eight different concentrations in the range of 0.33 to 3.3 mM). This experimental data set thus performed a total of 120 mean experimental points (triplicate experiments).

Al-Kassim and Tsai (1990) data describing the inhibition of TBADH by acetone in relation to 2-propanol were reproduced by computer simulation as described before (Pinto and Oestreicher, 1984) using the experimental conditions defined by these authors: $V_1/[\text{TBADH}] = 0.833\ s^{-1}$; $K_{ia} = 52.5\ \mu M$; $K_a = 76.3\ \mu M$; $K_b = 0.337$ mM; $K_{is} = 20.4\ \mu M$; $K_{ii} = 0.45$ mM; 2-propanol concentration (0.05–0.2 mM), NADP 0.12 mM, TBADH 0.94 μM, and the following acetone concentrations 0, 0.025, 0.05, 0.075, 0.1 and 0.2 mM (Al-Kassim and Tsai, 1990). The apparent values of V_1 (V_m) and K_b (K_m) were calculated from the following relationships:

$$V_m = \frac{V_1 \times 60}{1 + K_a/A} = 28.7\,\mu\text{M}\,\text{min}^{-1}$$

$$K_m = K_b \frac{(1 + K_{ia}/A)}{(1 + K_a/A)} = 0.296\,\text{mM}$$

A set of 'perfect' (i.e., error-free) data was formed by setting the values of V_m, K_m, K_{is}, K_{ii} and the concentrations of acetone (I) described above, as well as the following concentrations of 2-propanol (S): 0.05, 0.065, 0.10, 0.12, 0.15, 0.2 mM; and then calculating v from the rate equation for the linear mixed-type inhibition (Eq. 7). Sets of 'experimental' data (i.e., containing error in v) for each concentration of S and I were constructed from the perfect set by multiplying each value of v by a series of normally distributed pseudo-random numbers with mean of 1 and standard deviation of 0.05. A series of five such numbers was computed for each concentration of S and I, originating five sets of 'experimental' data with normally distributed error of constant relative magnitude (coefficient of variation equals to 5%). The mean values of data thus obtained (36 mean values) were considered to represent experimentally the data set obtained by Al-Kassim and Tsai (1990), since an error of the magnitude considered is likely in practice (Singer, 1974).

2.2. Data processing

Three computer programs were used for the statistical analysis of discrimination between the alternative kinetic models postulated for TBADH (Al-Kassim and Tsai, 1990; Pereira et al., 1994). Eqs. 4 and 6 were fitted to the data set combining initial rate experiments with product inhibition experiments obtained by Pereira et al. (1994) with a computer program in which initial estimates of parameters were obtained with the Hooke–Jeeves method of direct search with acceleration in distance (Hooke and Jeeves, 1961) developed in our laboratory (Paiva et al., 1991). Final estimates of parameters of Eqs. 4 and 6 as well as their asymptotic standard errors were obtained with the software ESTIMA based on the re-iterative Gauss-Newton method (Pinto et al., 1991). Eqs. 7 and 8 which correspond to the linear mixed-type inhibition model and to the linear competitive inhibition rate equation, respectively, were fitted to the simulated 'experimental' data set, obtained as described under Kinetic data as well as to the product inhibition data obtained by Pereira et al. (1994), with the computer program already referred to (Paiva et al., 1991) and also with a computer program based on non-linear least-squares regression (Oestreicher and Pinto, 1987).

$$v = \frac{V_m[\text{S}]}{K_m(1 + [\text{I}]/K_{is}) + [\text{S}](1 + [\text{I}]/K_{ii})} \tag{7}$$

$$v = \frac{V_m[\text{S}]}{K_m(1 + [\text{I}]/K_{is}) + [S]} \tag{8}$$

The quality of fittings was evaluated by the determination of two statistical parameters: the residual standard

error and the asymptotic standard error of the estimate of parameters, that are defined by Eqs. 9 and 10, respectively (Cleland, 1967; Metzler, 1981):

$$\mathrm{Syx} = \sqrt{\sum_{i=1}^{N} \frac{(v_i - \bar{v}_i)^2}{N - P}} \tag{9}$$

$$\mathrm{SE}_j = \mathrm{Syx}\sqrt{C_{jj}} \tag{10}$$

3. Results and discussion

Al-Kassim and Tsai (1990), based on initial velocity experiments in the absence of products and product inhibition studies, concluded that the kinetic mechanism of the oxidation of 2-propanol by NADP catalyzed by TBADH was the sequential ordered BiBi kinetic mechanism (Fig. 1). On the other hand, Pereira et al. (1994) using the same techniques and also inhibition experiments with pyrazole, a competitive dead-end inhibitor in relation to 2-propanol, concluded that the kinetic mechanism of this reaction was the Theorell–Chance BiBi model (Fig. 2).

The only difference between the product inhibition patterns displayed by these two alternative kinetic models postulated for TBADH is the inhibition of acetone, the first product to be released from the enzyme, with respect to 2-propanol, the second substrate to combine with the enzyme. In the case of the sequential ordered BiBi model, as a consequence of the existence of ternary central complexes (EAB and EPQ), the inhibition referred to is of the linear mixed type. However, if the steady-state concentration of the central complexes is zero or if they are not kinetically significant (Theorell–Chance BiBi model), a linear competitive inhibition pattern is characteristic of this kinetic model (Segel, 1972; Plowman, 1972).

Discrimination between these two kinetic mechanisms can then be achieved by evaluating the kinetic importance of the central ternary complexes and also by determining with statistical confidence, the type of inhibition produced by acetone in relation to 2-propanol as the variable substrate. For the first purpose, the set of 120 experimental points obtained in our laboratory (Pereira et al., 1994) and described under Kinetic data was used to fit the rate equation for the sequential ordered model in the presence of acetone (Eq. 4) and the corresponding one for the Theorell–Chance kinetic mechanism (Eq. 6). The result of such analysis is summarized in Table 1. As shown in Table 1 both kinetic models are well fitted to these data as suggested by the small value of the residual standard error (Syx) and by the fact that this statistical parameter attained almost

Table 1
Comparison of kinetic parameter estimates for the sequential ordered BiBi and the Theorell–Chance BiBi kinetic mechanisms postulated for TBADH

Parameter	Sequential ordered	Theorell–Chance
V_1 [a]	14.273 ± 0.0643	14.257 ± 0.0605
K_{ia} (mM)	0.0130 ± 0.0067	0.0131 ± 0.0067
K_b (mM)	0.342 ± 0.0053	0.341 ± 0.0050
K_a (mM)	0.0126 ± 0.0003	0.0126 ± 0.0003
K_1 (mM)	14.459 ± 0.2983	–
K_{ip} (mM)	17.570 ± 19.2873	0.0683 ± 0.0009
Syx [a]	0.09114	0.09099

Eqs. 4 and 6, respectively, were fitted with a computer program which is described under Data processing, to one experimental data set formed by 80 experimental points obtained under initial rate conditions in the absence of added products and 40 experimental points corresponding to product inhibition by five fixed concentrations of acetone (Pereira et al., 1994) with respect to variable concentrations of 2-propanol. Other experimental details are given under Methods.

[a] μmol min^{-1} mg^{-1}.

Table 2
Inhibition of TBADH by acetone with 2-propanol as the variable substrate

Parameter	Linear competitive inhibition		Linear mixed-type inhibition	
	non-linear regression	Hooke–Jeeves method	non-linear regression	Hooke–Jeeves method
V_{mt} [a]	12.604 ± 0.012 [b]	12.602	12.612 ± 0.021 [b]	12.651
K_{mt} (mM)	0.338 ± 0.002	0.338	0.339 ± 0.002	0.343
K_{is} (mM)	0.068 ± 0.001	0.068	0.068 ± 0.001	0.069
K_{ii} (mM)	–	–	85.376 ± 70.705	17.640
Syx [a]	0.02779	0.02779	0.02808	0.02850

Kinetic parameter estimates were obtained on fitting different linear inhibition models with different fitting procedures. Estimates of kinetic parameters derived from acetone inhibition studies with 2-propanol as the variable substrate. Eqs. 7 and 8, respectively, were fitted to the product inhibition data set described under Kinetic data (40 experimental points; Pereira et al., 1994). Fittings were accomplished with two different curve fitting computer programs which are described under Data processing.

[a] μmol min^{-1} mg^{-1}.

[b] Asymptotic standard error.

the same value on fitting both rate equations. However, it can be observed in Table 1 that although the estimates of the kinetic parameters that are common to both rate equations are very similar, the estimate of K_{ip} of Eq. 4 is more than 2-orders of magnitude higher than that of Eq. 6. In addition, its asymptotic standard error has a magnitude that is higher than the parameter estimate itself, this fact that is illustrative of a very poor precision of this estimate, indicates that this parameter appearing in the term accounting for central ternary complex ($[A][B][P]/K_{ip}$) in Eq. 4, can assume different values without significant effect upon the value of Syx. In other words, K_{ip} in the non hyperparametrized rate Eq. 4, is a redundant parameter (Mannervick, 1981), and as such, the term accounting for the central ternary complex must be dropped out from Eq. 4, which in this condition is transformed into the Theorell–Chance rate equation (Eq. 6) with K_1 being equal to K_{ip}.

As stated earlier, the other way to discriminate between the two alternative kinetic models proposed for TBADH is to analyze the product inhibition data obtained with acetone as the product inhibitor in relation to 2-propanol as the variable substrate. Table 2 shows that when the product inhibition data (second data group obtained by Pereira et al., 1994; see also Kinetic data) were analyzed with two fitting procedures they conform to a linear competitive inhibition pattern as well as to the mixed-type one, as suggested by the similarity between the Syx values. However, on fitting Eq. 7 it was found that the K_{ii} value was at least 2-orders of magnitude higher than the K_{is} value and then, the $[I]/K_{ii}$ term of Eq. 7 can be eliminated. In this condition the rate equation is transformed into the linear competitive rate equation (Eq. 8). Table 2 also shows that on comparing the fitting of the mixed-type inhibition rate equation obtained with both fitting techniques, it is evident that although the estimates of V_m, K_m and K_{is} are very similar for both inhibition models, the estimates of K_{ii} are entirely different. In addition, as revealed by the non-linear least-squares regression computer program, the asymptotic standard error of K_{ii} has almost the same magnitude as the estimate of K_{ii}, this fact is indicative of a poor precision of this parameter estimate. Namely, K_{ii} can assume different values without significant effect upon the value of Syx. Table 3 shows that, in fact, K_{ii} can assume values ranging from 10 to 380 mM with the residual standard error of the fitting remaining constant. However, if the values attributed to K_{ii} are smaller than 10 mM, the quality of the fitting of Eq. 7 is drastically loosened. The results shown in Tables 2 and 3 then eliminate the mixed-type inhibition as the model describing the product inhibition data analyzed supporting the competitive inhibition pattern. The same conclusion can be drawn with the simulated data reproducing the experimental data of Al-Kassim and Tsai (1990) (Table 4 and Figs. 3 and 4). As shown in Table 4, both rival inhibition models, the mixed-type inhibition and the competitive inhibition rate equations can be well fitted to these data. However, although the values of the residual standard errors resulting from both fittings were very similar, the estimate of K_{ii} arising from the former inhibition model has a poor precision as

Table 3
Fitting of the linear mixed-type inhibition model: effect of K_{ii} value upon the quality of the fitting

K_{ii} (mM)	V_m [a]	K_m (mM)	K_{is} (mM)	Syx [a]
1	13.318	0.402	0.0935	0.1823
2	12.955	0.369	0.0797	0.0963
3	12.837	0.359	0.0757	0.0673
5	12.742	0.350	0.0725	0.0457
10	12.673	0.344	0.0702	0.0324
12	12.664	0.343	0.0698	0.0308
18	12.651	0.343	0.0691	0.0295
54	12.619	0.340	0.0684	0.0281
73	12.614	0.339	0.0683	0.0281
85	12.612	0.339	0.0682	0.0281
290	12.605	0.338	0.0683	0.0281
380	12.605	0.338	0.0682	0.0281

The product inhibition data (40 experimental points) described under Kinetic data obtained by Pereira et al. (1994) were used to fit Eq. 7 with the computer program based on the Hooke–Jeeves method (Paiva et al., 1991). Each fitting was performed by using a different fixed value of K_{ii} as indicated.
[a] μmol min^{-1} mg^{-1}.

indicated by the magnitude of its asymptotic standard error. This analysis suggests that K_{ii} is a redundant parameter, i.e., the rate equation that better describes the acetone inhibition data is the linear competitive inhibition model (Eq. 8). In fact, Fig. 3 shows that on fitting Eq. 7 to these data, the family of reciprocal plots at different fixed acetone concentrations intersects to the left of the $1/v$-axis, but the common intersection points is located almost on this axis, strongly resembling the linear competitive inhibition pattern. As shown in Fig. 4, fitting of this latter inhibition model to the simulated 'experimental' data of Al-Kassim and Tsai (1990) confirm this statement.

Peters and Kula (1991), analyzing the inhibition of TBADH by 2-butanone with respect to variable concentrations of 2-butanol, described this inhibition as being of the linear competitive type. This data of the literature supports our results.

The results of the present work strongly suggest that the oxidation of 2-propanol catalyzed by TBADH is best described by the Theorell–Chance BiBi model than by the sequential ordered BiBi kinetic mechanism and show

Table 4
Estimates of kinetic parameters of Eqs. 7 and 8 using a simulated data set reproducing the experimental data of Al-Kassim and Tsai (1990)

Parameter	Mixed-type inhibition		Competitive inhibition
	true value [a]	estimate ± SEe [b]	estimate ± SE [b]
V_m (μM min^{-1})	28.732	28.062 ± 0.988	27.934 ± 0.968
K_m (mM)	0.296	0.282 ± 0.015	0.283 ± 0.015
K_{is} (mM)	0.020	0.020 ± 0.001	0.019 ± 0.001
K_{ii} (mM)	0.450	0.680 ± 0.405	–
Syx [c]	–	0.08607	0.08504

Eqs. 7 and 8 were fitted with a non-linear least-squares regression computer program (Oestreicher and Pinto, 1987) to the simulated product inhibition data set computed as described under Kinetic data.
[a] Values calculated according to Al-Kassim and Tsai (1990); see Kinetic data.
[b] Asymptotic standard error.
[c] μM min^{-1}.

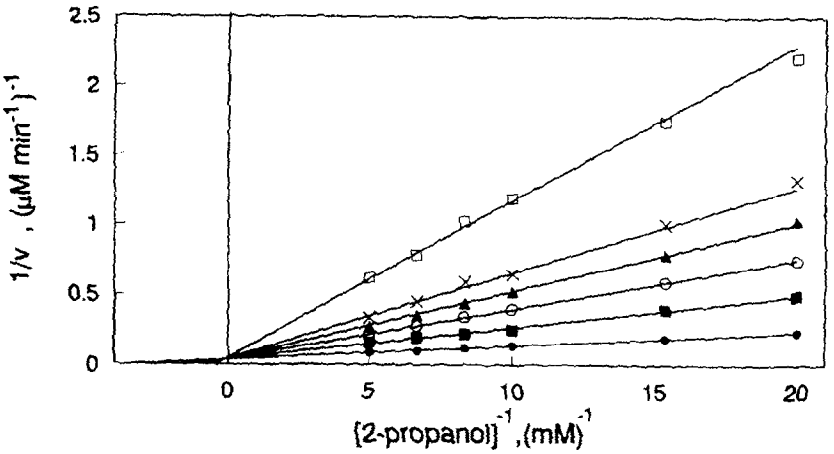

Fig. 3. Fitting of the mixed-type inhibition rate equation to simulated 'experimental' data reproducing Al-Kassim and Tsai (1990) experimental conditions. The concentrations of acetone were: ●, 0; ■, 0.025 mM; ○, 0.050 mM; ▲, 0.075 mM; ×, 0.10 mM; □, 0.20 mM. Other experimental conditions are given under Methods.

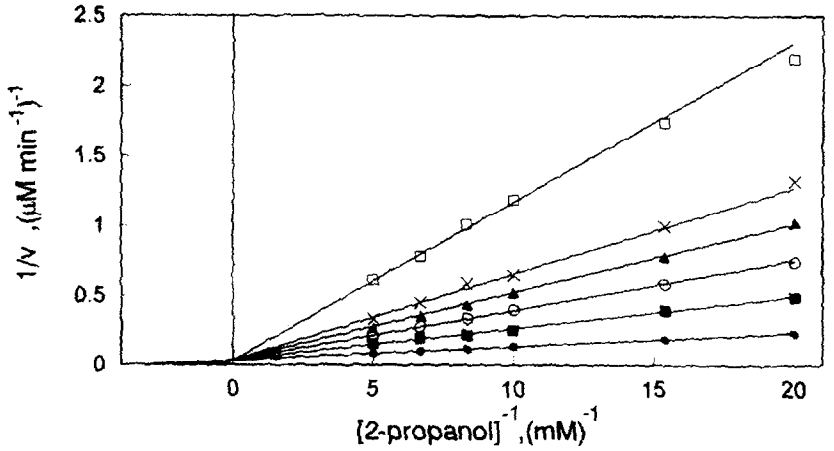

Fig. 4. Fitting of the competitive inhibition rate equation to simulated 'experimental' data reproducing Al-Kassim and Tsai (1990) experimental conditions. The concentrations of acetone were: ●, 0; ■, 0.025 mM; ○, 0.050 mM; ▲, 0.075 mM; ×, 0.10 mM; □, 0.20 mM. Other experimental conditions are given under Methods.

that on postulating a kinetic mechanism, a study of model discrimination between rival kinetic models as the one reported in this work is of fundamental importance.

Acknowledgements

This work was supported by grants from PADCT/CNPq.

References

Al-Kassim, L.S. and Tsai, C.S. (1990) Studies of $NADP^+$-preferred secondary alcohol dehydrogenase from *Thermoanaerobium brockii*. Biochem. Cell. Biol. 68, 907–913.

Belan, A., Bolte, J., Fauve, A., Gourcy, J.G. and Veschambre, H. (1987) Use of biological systems for the preparation of chiral molecules. 3. An application in pheromone synthesis: Preparation of Sulcatol enantiomers. J. Org. Chem. 52, 256–260.

Bradshaw, C.W., Hummel, W. and Wong, C.H. (1992) *Lactobacillus kefir* alcohol dehydrogenase: a useful catalyst for synthesis. J. Org. Chem. 57, 1532–1536.

Cleland, W.W. (1963) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. Biochim. Biophys. Acta 67, 104–137.

Cleland, W.W. (1967) The statistical analysis of enzyme kinetic data. In: North, F.F. (Ed.), Advances in Enzymology, Vol. 29, Interscience Publishers, New York, pp. 1–32.

Hooke, R. and Jeeves, T.A. (1961) Direct search solution of numerical and statistical problems. J. Assoc. Comput. Mach. 8, 212–219.

Keinan, E., Hafeli, J.G., Seth, K.K. and Lamed, R.J. (1986) Thermostable enzymes in organic synthesis. 2. Asymmetric reduction of ketones with alcohol dehydrogenase from *Thermoanaerobium brockii*. J. Am. Chem. Soc. 108, 162–169.

Keinan, E., Seth, K.K. and Lamed, R.J. (1987) Synthetic applications of alcohol dehydrogenase from *Thermoanaerobium brockii*. Ann. NY Acad. Sci. 501, 130–144.

Lamed, R.J. and Zeikus, J.G. (1981) Novel NADP-linked alcohol-aldehyde/ketone oxidoreductase in thermophilic ethanologenic bacteria. Biochem. J. 195, 183–190.

Mannervick, B. (1981) Design and analysis of kinetic experiments for discrimination between rival models. In: Endrenyi, L. (Ed.), Kinetic Data Analysis, Plenum Press, New York, pp. 235–270.

Metzler, C.M. (1981) Statistical properties of kinetic estimates. In: Endrenyi, L. (Ed.), Kinetic Data Analysis, Plenum Press, New York, pp. 25–37.

Oestreicher, E.G. and Pinto, G.F. (1987) A microcomputer program for fitting enzyme inhibition rate equations. Comput. Biol. Med. 17, 53–68.

Paiva, L.M.C., Pinto, G.F. and Oestreicher, E.G. (1991) Inhibition of horse liver alcohol dehydrogenase and rabbit muscle lactate dehydrogenase by phenylhydrazine. Biomed. Biochim. Acta 50, 25–29.

Pereira, D.A., Pinto, G.F. and Oestreicher, E.G. (1994) Kinetic mechanism of the oxidation of 2-propanol catalyzed by *Thermoanaerobium brockii* alcohol dehydrogenase. J. Biotechnol. 34, 43–50.

Peters, J. and Kula, M.R. (1991) Continuous production of NADPH with thermostable secondary alcohol dehydrogenase in an enzyme reactor. Biotechnol. Appl. Biochem. 13, 363–370.

Pinto, G.F. and Oestreicher, E.G. (1984) Pocket computer program for fitting the Hill equation. Comput. Biol. Med. 14, 507–511.

Pinto, J.C., Lobão, M.W. and Monteiro, J.L. (1991) Sequential experimental design for parameter estimation: Analysis of relative deviations. Chem. Eng. Sci. 46, 3129–3138.

Plowman, K.M. (1972) Enzyme Kinetics, McGraw-Hill Book Company, New York.

Röthing, T.R., Kulbe, K.D., Bückmann, F. and Carrea, G. (1990) Continuos coenzyme dependent stereoselective synthesis of sulcatol by alcohol dehydrogenase. Biotechnol. Lett. 12, 353–356.

Segel, I.H. (1972) Enzyme Kinetics, John Wiley & Sons, New York.

Singer, T.P. (1974) Determination of the activity of succinate, NADH, choline and α-glycero phosphate dehydrogenases. In: Glick, D. (Ed.), Methods of Biochemical Analysis, Vol. 22, John Wiley & Sons, New York, pp. 123–175.

Ward, O.P. and Young, C.S. (1990) Reductive biotransformations of organic compounds by cells or enzymes of yeast. Enzyme Microb. Technol. 12, 482–493.

Wong, C.H. (1989) Enzymatic catalyst in organic synthesis. Science 244, 1145–1152.