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Kinetic modelling of coupled redox enzymatic systems for in situ regeneration of NADPH

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Abstract

In the past few years, we have been working in a complete comparative kinetic study involving three different NADPH recycling reactions having the reduction of 6-methyl-5-hepten-2-one (sulcatone) catalysed by alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH) and producing *S*-(+)-6-methyl-5-hepten-2-ol (sulcatol) as the main reaction. A fundamental step in this study involves the elucidation of the kinetic mechanism of the main reaction, which must be the rate-limiting step of the overall process in all systems studied. In the present work, we demonstrated by initial velocity and product inhibition studies that sulcatone/sulcatol reduction reaction follows a Theorell–Chance BiBi mechanism. Using the kinetic parameters hereby and previous determined, it was possible to simulate time-course curves for coupled enzymatic systems used. Considering the good correlation between the adjusted curves and experimental data it is possible to affirm that coupled enzymatic reaction systems can be kinetically described by evaluating just the kinetic behaviour of the main reaction.

Keywords: Alcohol dehydrogenase; Sulcatone; Coenzyme regeneration; Kinetic study; Theorell–Chance BiBi mechanism

1. Introduction

Chiral technology has progressed a long way. It has found applications in the production of biochemicals, pesticides, flavour chemicals, pigments, liquids crystals, non-linear optical materials and polymers. This is justified because enantiomers are often readily distinguished by biological systems, and may have different pharmacological or toxicological effects [1–5].

Oxidoreductions, especially the reduction of carbonyl compounds, are very important organic reactions in asymmetric synthesis allowing the transformation of prochiral substrates into valuable chiral building blocks of pharmaceutical interest [2–4].

Alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH), a NAD(P)-linked enzyme, is being increasingly used as catalyst in chiral synthesis because of its high enantiospecificity, its broad substrate specificity and its extraordinary tolerance towards high concentrations of organic solvents such as 2-propanol 20% (v/v). Moreover, TBADH presents a remarkable capacity to withstand up to 85 °C. All these features attracted much technological interest [6–10].

However, preparative scale reductions using NAD(P)-dependent dehydrogenases can only be

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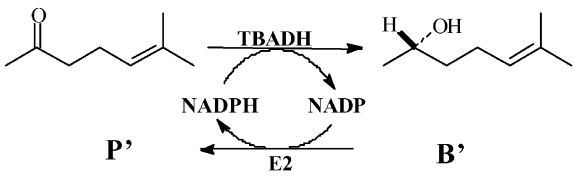


Scheme 1. General representation for the three coupled enzymatic systems studied by Bastos et al. [10]. Enzyme-coupled regeneration systems: B′, glucose or glucose-6-phosphate (G6P); and E2, glucose dehydrogenase from *Bacillus megaterium* (GDH) or glucose-6-phosphate dehydrogenase from *Saccharomyces cerevisiae* (G6PDH). Substrate-coupled regeneration system: B′, 2-propanol 20% (v/v); and E2, TBADH.

achieved if an efficient in situ regeneration system, to return the coenzyme to its original redox form, is used [3,6–15]. There are two types of coupled enzymatic systems:

- Those with only one enzyme acting in both the main and recycling reactions also called substrate-coupled regeneration systems and those with two different enzymes called enzyme-coupled regeneration systems (Scheme 1).

A comparative kinetic study between three different coupled enzymatic systems for in situ regeneration of NADPH was performed by Bastos et al. [10]. All three systems are represented in Scheme 1.

Steady-state kinetic studies are a very powerful tool for predicting the behaviour of biocatalytic processes and for enantioselective production of chiral building blocks.

Although kinetic studies with TBADH have been performed [16–19], the kinetic mechanism of the reduction of sulcatone catalysed by this enzyme was unknown and a fundamental step in kinetic modelling of coupled enzymatic reactions involves the elucidation of the kinetic mechanism of the main reaction, that must be the rate limiting step of the overall process in all systems studied [10,15].

In the present work we demonstrate that the reduction of sulcatone to *S*-(+)-sulcatol catalysed by TBADH follows a Theorell–Chance BiBi kinetic mechanism. Based on this kinetic model and by using the parameter estimates of the corresponding rate equation a complete kinetic modelling of the three different coupled enzymatic reactions described by Bastos et al. was also performed [10].

2. Experimental

2.1. Materials

Alcohol dehydrogenase from *T. brockii* (EC 1.1.1.2), glucose dehydrogenase from *Bacillus megaterium* (EC 1.1.1.47), glucose-6-P-dehydrogenase from baker's yeast (EC 1.1.1.49), glucose-6-phosphate, Trizma base, bovine serum albumine (BSA, fraction V) and NADPH were purchased from Sigma Chemical Co., 6-methyl-5-hepten-2-one, (±)-6-methyl-5-hepten-2-ol, 2-propanol and 2-butanol were products from Aldrich. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Enzyme activity

TBADH activity was measured in the direction of 2-butanol oxidation by following NADPH absorption at 340 nm essentially as described by Pereira et al. [16]. Protein concentration was determined as described by Hartree using BSA as standard [20].

2.2.2. Kinetic mechanism

The determination of the kinetic mechanism of reduction of sulcatone catalysed by TBADH was done by initial velocity and product inhibition studies with NADPH 0.5 mM, 20 μl of the enzyme stock solution (3 mg/ml in Tris–HCl buffer 0.1 M pH 7.8 with 0.1% BSA), *rac*-sulcatol (0.05–0.8 mM) as product inhibitor and sulcatone (0.05–0.5 mM) as variable substrate. All the experiments were performed in Tris–HCl buffer 0.1 M pH 7.8 at 37 °C by following NADPH concentration decrease at 340 nm.

2.2.3. Data processing

The values (symbols) appearing in the Figs. 1 and 2 are the mean of experimentally determined initial velocities (triplicates), and were used in determining the kinetic parameters. Estimates of kinetic parameters (K_B, V_m, K_i and K_{ii}), and of their standard error (S.E.) were obtained by fitting the appropriate rate equation to a set of 40 experimental initial velocity data obtained as described above using a non-linear least-squares computer program, developed in our laboratory [21]. Eqs. (1) and (2), which correspond to the linear mixed-type inhibition model and to

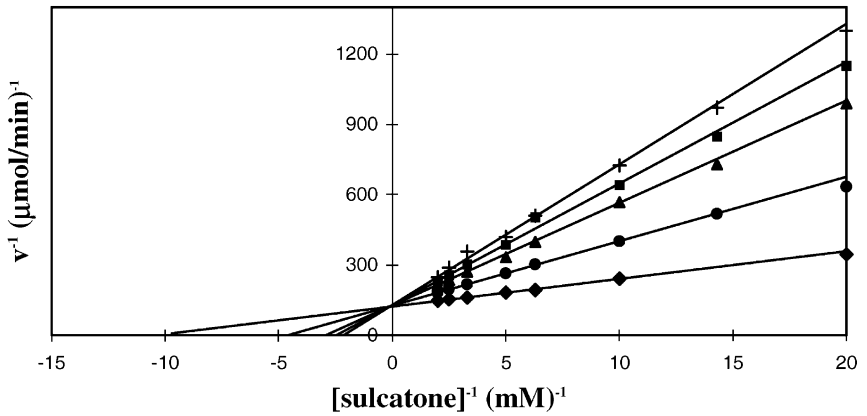


Fig. 1. Fitting of the mixed-type rate equation to experimental data. Concentration of *rac*-sulcatol: (◆) 0 mM; (●) 0.2 mM; (▲) 0.4 mM; (■) 0.5 mM; (+) 0.6 mM. The concentration of NADPH was constant and equal to 0.5 mM and the concentration of sulcatone varied from 0.05 to 0.5 mM. The lines represent the best-fit curves obtained by fitting Eq. (3) to the experimental data.

linear competitive inhibition rate equation, respectively, were fitted to these experimental data.

$$v_i = \frac{V_m[B]}{K_B(1 + ([I]/K_i)) + [B](1 + ([I]/K_{ii}))} \quad (1)$$

$$v_i = \frac{V_m[B]}{K_B(1 + [I]/K_i) + [B]} \quad (2)$$

where v_i is the initial velocity, V_m the maximum velocity; [B] the concentration of sulcatone, K_B the Michaelis constant for sulcatone, K_i the inhibition constant for *rac*-sulcatol, K_{ii} the apparent value of the dissociation constant of enzyme-varied substrate–product inhibitor complex, [*I*] is the concentration of sulcatol.

2.2.4. Recycling systems

The three coupled enzymatic recycling systems used for the kinetic modelling were set up as described by Bastos et al. [10].

2.2.5. Kinetic modelling

Coupled enzymatic recycling systems can be represented by the general scheme showed in Scheme 2.

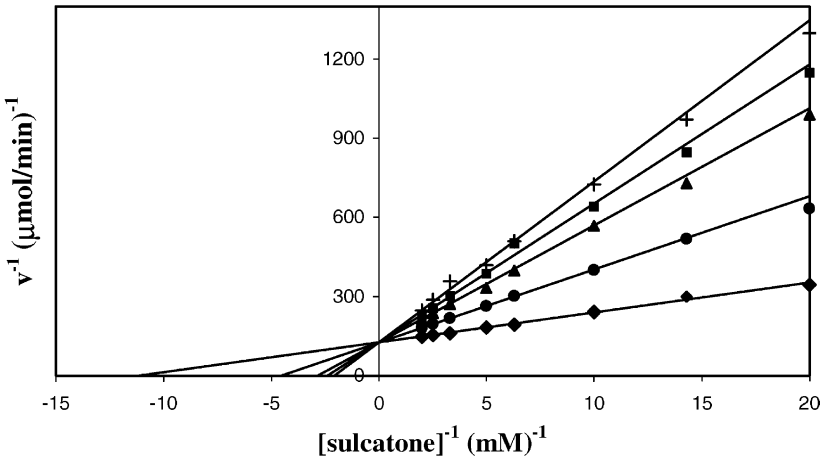
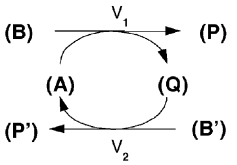


Fig. 2. Fitting of the competitive rate equation to experimental data. All experimental conditions were described in the caption of Fig. 1.



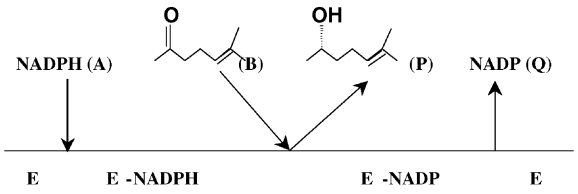
Scheme 2. General representation of enzymatic coupled enzymatic systems: v_1, main initial reaction velocity; v_2, recycling initial reaction velocity; A, NADPH; Q, NADP; B, sulcatone; P, sulcatol; B′, recycling substrate; P′, recycling product; v_p, velocity of formation of P; $v_{p'}$, velocity of formation of P′; v_Q, velocity of formation of Q; $v_{B'}$, velocity of formation of B′. The following equations system was introduced into the program: (1) $B = B_0 + P_0 - P$; (2) $A = A_0 + Q_0 - Q$; (3) $v_p = v_1$; (4) $v_{p'} = v_2$; (5) $v_{B'} = -v_{p'}$; (6) $v_Q = v_p - v_{p'}$.

Therefore, based on this general scheme and on the kinetic mechanism proposed in the present work, Eq. (3) represents the rate equation for the Theorell–Chance BiBi mechanism (Scheme 3) in the presence of both the products of the main (P) and recycling reactions (P′) and considering the inhibition produced by excess of *B*:

$$v = \frac{V_{m_1} B}{K_B(1 + (K_{ia}/A))(1 + (P/K_{ip}))(1 + (P'/K_{ip'})) + B(1 + (K_A/A) + (B/K_{ib}))} \quad (3)$$

where V_{m_1} is the maximum forward velocity, K_B the Michaelis constant for sulcatone, K_{ia} the dissociation constant of the E–NADPH complex, $K_{ip'}$ the inhibition constant for the recycling product reaction, K_{ip} the inhibition constant for the product of the main reaction, K_A the Michaelis constant for NADPH and K_{ib} is the inhibition constant for excess of sulcatone.

Time-course curves simulations were performed with a computer program developed in our laboratory named Runge–Kutta (RK)-RECTD written in QUICKBASIC and based on the method of Runge–Kutta of fourth order for numeric integration of differential equations. This program simulates a coupled enzymatic system where the main reaction follows a Theorell–Chance BiBi mechanism (Eq. (3)) (Scheme 3).



Scheme 3. Theorell–Chance BiBi kinetic mechanism for the reduction of sulcatone catalysed by TBADH.

Table 1
Values of the parameters used for simulations of time-course curves experiments

Parameters[a]	Value
V_{m_1} (mM/h)	6.19
V_{m_2} (mM/h)[b]	51.0
K_{ib} (mM)	9.38
K_B (mM)	0.088
K_{ip} (mM)	0.013
K_Q[b] (mM)	0.013
K_A[b] (mM)	0.00141
K_{ia}[b] (mM)	0.03
K_{iq}[b] (mM)	0.013
$K_{ip'}$ (mM)[c]	10,000

[a] All the parameters are referred to main reaction except V_{m_2} that is related to the recycling reactions.
[b] Data from [16].
[c] Value chosen in order to overcome kinetic contribution of the recycling reaction.

The kinetic parameters used in the simulation are shown in Table 1.

The boundary conditions utilised in the simulations were the same used to obtain the experimental data and are described in Table 2.

It must be emphasised that the values of $K_{ip'}$ and the initial concentration of B′ were chosen in order to overcome the kinetic and thermodynamic contributions of the recycling reaction. This could be done because all three recycling systems described by Bastos et al. [10] were irreversible.

Table 2
Boundary conditions used in the simulations

Parameters	Value
Time (h)	0
Concentration of P_0 (mM)	0
Recycling reaction product P'_0 (mM)	0
Recycling reaction substrate B'_0[a] (mM)	3000
NADP, Q_0 (mM)	0
NADPH, A_0 (mM)	0.2
Main reaction substrate, B_0 (mM)	25

[a] Value used to overcome kinetic contribution of the recycling reaction.

2.2.6. Enzymes and coenzyme stability

The kinetic of TBADH denaturation was determined by following, in independent experiments, its activity for 96 h when submitted to the same reactional conditions of the coupled systems. The kinetics of NADPH denaturation was determined by measuring its concentration changes at 340 nm during 96 h in Tris–HCl buffer 0.1 M pH 7.8 at 25 °C. Both processes were demonstrated to follow a kinetic of pseudo-first order with the equations shown below. The value appearing in the exponential terms represent the pseudo-first order rate constant, h^{-1}.

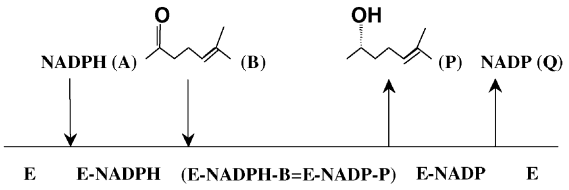
For substrate-coupled regeneration approach: $[\text{NADPH}] = [\text{NADPH}]_0\, e^{(-0.0067t(h))}$; $[\text{TBADH}] = [\text{TBADH}]_0\, e^{(-0.0093t(h))}$.

For two enzyme-coupled regeneration systems: $[\text{TBADH}] = [\text{TBADH}]_0\, e^{(-0.0249t(h))}$; $[\text{NADPH}] = [\text{NADPH}]_0\, e^{(-0.0102t(h))}$ when GDH is the recycling enzyme; $[\text{NADPH}] = [\text{NADPH}]_0\, e^{(-0.0565t(h))}$ when G6PDH is the recycling enzyme.

These equations were introduced in the simulation program used in this work to take into account these deleterious side reactions.

3. Results and discussion

Discrimination between the two possible steady-state kinetic mechanisms for the reduction of sulcatone catalysed by TBADH, Theorell–Chance BiBi (Scheme 3) and sequential ordered Theorell–Chance BiBi (Scheme 4) [17,22] was achieved by analysing the inhibition produced by *rac*-sulcatol, with respect to sulcatone acting as varied substrate. Since the reduction of sulcatone to sulcatol catalysed by TBADH has been reported to be absolutely enantioselective, producing (*S*)-(+)-sulcatol with an enantiomeric excess higher than 99% [6–8], the use of *rac*-sulcatol instead of its (*S*)-enantiomer, can be considered kinetically acceptable. The identification of this inhibition pattern is essential to evaluate the kinetic significance of the ternary complexes of the main reaction [17,22].



Scheme 4. Sequential ordered BiBi kinetic mechanism for the reduction of sulcatone catalysed by TBADH.

In the case of the sequential ordered Theorell–Chance BiBi model (Scheme 4), as a consequence of the existence of ternary central complexes the inhibition referred to is linear mixed-type. However, if the steady state concentration of the central complex is not kinetically significant, i.e. Theorell–Chance BiBi model (Scheme 3), a linear competitive inhibition pattern must be obtained.

For this purpose, a set of experimental data obtained as described under Experimental, was used to fit Eqs. (1) and (2). Although the data displayed in Figs. 1 and 2 apparently conform to a linear competitive inhibition pattern as well as to the mixed-type one, the data contained in Table 3 show that the K_{ii} value is at least two orders of magnitude higher than K_{ip}. Moreover as shown in Table 3, the asymptotic standard error of K_{ii} is indicative of poor precision in the determination of this parameter, strongly suggesting that this kinetic parameter is redundant. According to this analysis, the $[I]/K_{ii}$ term of the Eq. (1) can be eliminated and, in this way Eq. (1) is transformed into the linear competitive rate equation (Eq. (2)).

Optimal experimental conditions for coupled enzymatic systems are those that allow the maximum conversion of the substrate in the shortest period of time. In this way, the complete conversion of the substrate of the main reaction (sulcatone) will be favoured under irreversible conditions of the coupled system where only product inhibition will limit this conversion.

To obtain these ideal experimental conditions, it must be assured that the main reaction is the rate-limiting step of the coupled system, and therefore,

Table 3
Comparison of kinetic parameters estimates for both mechanisms

Parameters	Value ± S.E.	
	Sequential ordered BiBi	Theorell–Chance BiBi
V_m ± S.E. (μmol/min)	0.008 ± 0.00008	0.008 ± 0.00008
K_B ± S.E. (mM)	0.088 ± 0.0299	0.089 ± 0.0031
K_i ± S.E. (mM)	0.132 ± 0.00392	0.138 ± 0.00441
K_{ii} ± S.E. (mM)	16.374 ± 5.4676	–
Syx	7.05×10^{-5}	7.02×10^{-5}

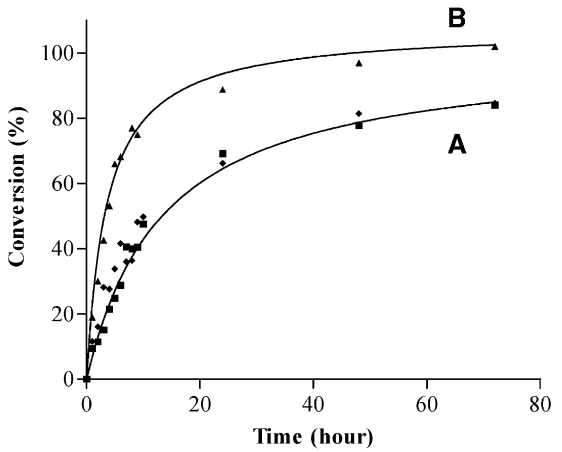


Fig. 3. Time-course curves of the reduction of sulcatone catalysed by TBADH. (A) Enzyme-coupled regeneration systems: (■) GDH as recycling enzyme; sulcatone (25 mM), 2.9 international units of TBADH, glucose (100 mM) and GDH were diluted in Tris–HCl buffer 0.1 M pH 7.8 in 3 ml. The reaction was started with NADPH (0.2 mM) and the mixture was incubated at 25 °C for 72 h (◆) G6PDH as recycling enzyme. Sulcatone (25 mM), 2.9 international units of TBADH, G6P (30 mM) and G6PDH were diluted in Tris–HCl buffer 0.1 M pH 7.8 in 3 ml. The reaction was started with NADPH (0.2 mM) and the mixture was incubated at 25 °C for 72 h. (B) Substrate-coupled regeneration approach (▲). Sulcatone (25 mM) and 2.9 international units of TBADH were added to a solution of 2-propanol (20% (v/v)) in Tris–HCl buffer 0.1 M pH 7.8 in 3 ml. The reaction was started with NADPH (0.2 mM) and the mixture was incubated at 25 °C for 72 h. The lines represent the best-fit curves obtained by computer simulation.

the rate of the whole system must be kinetically independent of the nature of the recycling reaction.

Bastos et al. [10] in order to make sure that the main reaction was, in fact, the rate-limiting step, chose, for substrate-coupled regeneration approach, the strategy of using saturating concentration of the substrate of the recycling reaction (B′), 2-propanol 20% (v/v) (Scheme 1). For enzyme-coupled regeneration systems besides saturation with the recycling substrate, the authors increased the recycling enzyme concentration.

Fig. 3 shows the time-course curves obtained for two enzyme-coupled regeneration systems and for the substrate-coupled regeneration approach. As depicted in Fig. 3, an excellent correlation between the experimental data (symbols) and the simulated curve was obtained for both enzyme-coupled regeneration systems tested by using the estimates of kinetic parameters obtained from initial rate experiments in the absence or presence of sulcatone (Table 1). This result is clearly suggestive that the reaction rate of whole system only depends upon the rate of the main reaction. As shown in Fig. 3 (curve A), with this kind of recycling system, the reaction attained 84% of conversion in 72 h.

On the other hand, when the substrate-coupled regeneration was used, the experimental data showed >99% of conversion also in 72 h (Fig. 3, curve B). This difference as can be seen in Fig. 3 is due to a higher reaction rate in curve B that can be attributed to the overcoming of the inhibition of the main reaction produced by sulcatol (P) in the presence of 2-propanol 20% (v/v) (that corresponds to 3 M 2-propanol). Furthermore, the driving force arising from the substrate-coupled regeneration system must be higher than that of the two enzyme-coupled regeneration systems (Fig. 3, curve A).

In order to find the value of K_{ip} when substrate-coupled regeneration system was used, all the parameter estimates summarised in Table 1 were maintained, except K_{ip}. The value of K_{ip} was varied systematically and the Syx value of the time-course curve generated was computed. This procedure was repeated several times, a graph of Syx versus K_{ip} was drawn and the value of K_{ip} corresponding to the minimum of the resulting curve was considered as the actual value of this kinetic parameter (results not shown). A value of 1.14 mM was estimated in this way for K_{ip}. This value is approximately hundred times higher than the K_{ip} value estimated when the two enzyme-coupled regeneration system were used. The difference found in K_{ip} values estimated as described is indicative of overcoming of inhibition produced by sulcatol (P) and this is clearly explained by a Theorell–Chance BiBi mechanism (Scheme 3). Substituting the value of K_{ip} by 1.14 mM in the set of parameters shown in Table 1 and on performing the simulation as described under Experimental an excellent correlation between the simulated curve and the experimental data was obtained (Fig. 3, curve B).

Acknowledgements

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