Biocatalysis and Biotransformation, September–December 2010; 28(5–6): 348–356

informa
healthcare

ORIGINAL ARTICLE

Kinetic studies of the asymmetric Henry reaction catalyzed by hydroxynitrile lyase from *Hevea brasiliensis*

RUSLAN YURYEV[1], THOMAS PURKARTHOFER[2], MANDANA GRUBER[2], HERFRIED GRIENGL[2] & ANDREAS LIESE[1]

[1]*Institute of Technical Biocatalysis, Hamburg University of Technology, Hamburg, Germany and* [2]*Research Centre Applied Biocatalysis, Graz, Austria*

Abstract

The asymmetric Henry reaction catalyzed by hydroxynitrile lyase from *Hevea brasiliensis* is an example of enzymatic catalytic promiscuity. It could be an attractive method to produce optically active β-nitroalcohols, but unfortunately the enzyme has very low activity in this unnatural reaction. To get an insight into the reaction mechanism, the enzyme kinetics of this promiscuous biotransformation were studied using the cleavage and synthesis of 2-nitro-1-phenylethanol as a model system. The results indicate that the kinetic behavior of the enzyme in the Henry reaction fits the classical Rapid Equilibrium Random Bi Uni/Uni Bi mechanistic model with independent substrate binding. The measured kinetic parameters imply that the bottleneck for this biotransformation is the very low turnover number of the enzyme, not the binding of the substrates.

Keywords: *Enzyme kinetics, hydroxynitrile lyase, catalytic promiscuity, asymmetric Henry reaction*

Introduction

The phenomenon of enzyme promiscuity opens new opportunities in the field of organic biotransformations (Bornscheuer & Kazlauskas 2004). It is very attractive to use enzymes as a multifunctional 'Swiss knife' tool, which can be easily 'adjusted' to different applications, but this is still practically unrealizable. The main challenge here is usually the very low activity of enzymes in the promiscuous reactions compared with that in the native reactions. Over recent years many efforts have been made to overcome this problem by applying enzyme, reaction or substrate engineering. In some cases positive results were reported (Kazlauskas 2005), especially when the alterations were based on mechanistic information about the enzyme activity.

One of these promiscuous enzymes is hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL). Its natural catalytic function is to cleave acetone cyanohydrin, released from a cyanogenic glycoside linamarin, into acetone and HCN (Wajant & Förster 1996; Selmar et al. 1987b). However, the enzyme is more attractive in the reverse process called the cyanohydrin reaction (Figure 1(a)), when HCN adds to aldehydes or ketones yielding cyanohydrins – valuable precursors for various fine chemicals (Fechter & Griengl 2004). In this biotransformation *Hb*HNL shows high enantioselectivity and has a broad substrate range that makes it appealing for industrial exploitation. The multi-ton DSM process for production of the chiral cyanohydrin of (*S*)-3-phenoxybenzaldehyde, which is used as an intermediate in the synthesis of pyrethroids (Poechlauer et al. 2004), shows the high commercial potential of this biocatalyst.

The catalytic promiscuity of *Hb*HNL is revealed in the Henry reaction, where nitroalkanes are used instead of HCN (Figure 1(b)) (Purkarthofer et al. 2006). The products of this reaction – optically active β-nitroalcohols – similarly to the cyanohydrins have broad synthetic utility, but they are more stable and less toxic at the same time. For example, they can be

Correspondence: A. Liese, Institute of Technical Biocatalysis, Hamburg University of Technology, Denickestrasse 15, 21073 Hamburg, Germany. Tel: +49-40-42878-3218. Fax: +49-40-42878-2127. E-mail: biocatalysis@tuhh.de

(Received 30 May 2010; revised 6 September 2010; accepted 6 October 2010)

ISSN 1024-2422 print/ISSN 1029-2446 online © 2010 Informa UK, Ltd.
DOI: 10.3109/10242422.2010.530661

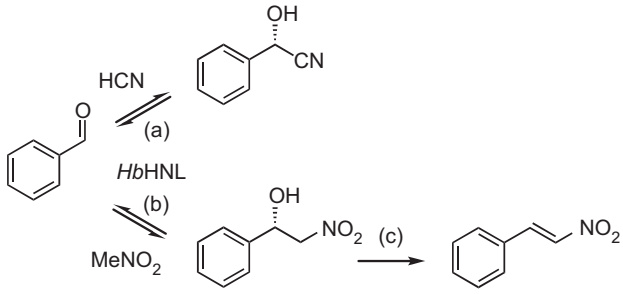

Figure 1. The cyanohydrin (a) and the Henry (b) reactions catalyzed by *Hb*HNL; (c) the dehydration of NPE to 1-nitro-2-phenylethylene.

easily converted to the chiral β-aminoalcohols, which are valuable chiral building blocks for a number of pharmaceuticals (Luzzio 2001; Ono 2001). Beside this the *Hb*HNL-catalyzed synthesis of chiral β-nitroalcohols has a major advantage over the biocatalytic cyanohydrin reaction in avoiding manipulations with dangerous HCN. Therefore, the catalytic promiscuity of *Hb*HNL raises the enzyme's practical value as a biocatalyst and establishes new perspectives for its industrial use. However, there is one serious limitation: the activity of *Hb*HNL in the nitroaldol condensation is too low for a feasible application (Gruber-Khadjawi et al. 2007).

In order to debottleneck the biocatalytic Henry reaction information about its mechanism is useful. Although it is believed that *Hb*HNL acts similarly in both promiscuous and native reactions, there is not much experimental evidence to support this. Since no systematic data on the kinetics of the biocatalytic Henry reaction are available, it remains unclear whether the extremely low specific activity of *Hb*HNL in the promiscuous biotransformation is due to very weak binding of nitromethane and β-nitroalcohols to the active site of the enzyme or to a very low turnover number. The present study aimed to provide this missing information.

Experimental

Materials

The chemicals used in this work were commercially available except (*rac*)-2-nitro-1-phenylethanol (NPE), which was synthesized according to the following procedure. Benzaldehyde (10 mmol), nitromethane (100 mmol) and triethylamine (10 mmol) were mixed and incubated overnight at 4°C. Then the mixture was dried *in vacuo* (20 mbar, 40°C), the yellow residue was purified by column chromatography on silica gel using cyclohexane–thylacetate mixture (16:1 v/v) as eluent. After solvent evaporation NPE was isolated as colorless oil in 73% yield. ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 2.86 (br, 1H, –OH); 4.49 (dd, 1H, $2\mathcal{J}$=13.2 Hz, $3\mathcal{J}$=2.9 Hz, –CHHNO$_2$); 4.58 (dd, 1H, $2\mathcal{J}$=13.2 Hz, $3\mathcal{J}$=9.8 Hz, –CHHNO$_2$); 5.43 (dd, IH, $3\mathcal{J}$=9.8 Hz, $3\mathcal{J}$=2.9 Hz, –CHOH–); 7.33–7.39 (m, 5H). ^{13}C-NMR (125 MHz, $CDCl_3$), δ (ppm): 71.2, 81.4, 126.2, 129.2, 129.3, 138.3.

The enzyme *Hb*HNL was kindly provided by DSM (Heerlen, the Netherlands) in the form of a solution with the following specifications: 99% purity, 73 mg mL^{-1}, specific activity 62 U mg^{-1}, stabilized by 30 ppm sodium azide (here, one unit of activity is defined as the amount of enzyme that cleaves one μmole of mandelonitrile per minute). The enzyme was stored at 4°C over the course of this work without any loss of activity, measured by the spectrophotometric assay as described (Bauer et al. 1999a). In the biocatalytic Henry reaction, one unit of activity is defined as the amount of enzyme that cleaves one μmole of NPE per minute.

Methods

The reactions were carried out in 1.5-mL glass vials thermostated at 25°C. Liquid handling was automated by using a Gilson 241–402 pipette robot (Middleton, WI, USA). In a typical procedure for NPE cleavage followed for 20 min, the reaction was started by injection of 100 μL of 0.5 M phosphate buffer pH 6.0 and 100 μL of *Hb*HNL solution to 800 μL of NPE solution in 1 mM HCl. After specified periods of time, 50 μL of the reaction mixture were withdrawn and quenched in 950 μL of 2 M HCl. The precipitated protein was separated by centrifugation at 6000 rpm for 20 min and the supernatant was analyzed by HPLC. Removal of the enzyme from the reaction mixture by quenching prior to HPLC analysis was necessary to extend the lifetime of the HPLC column. To monitor the reactions within a 1 min interval a modified procedure was applied; the reaction was started by rapid injection of 80 μL of the substrate solution in 1 mM HCl (NPE or benzaldehyde–nitromethane mixture), 10 μL of 0.5 M phosphate buffer pH 6.0 and 10 μL of *Hb*HNL solution into a 400-μL glass vessel thermostated at 25°C. After specified times the mixture was quenched by rapid injection of 350 μL of 2 M HCl, centrifuged and analyzed by HPLC. For convenience, 1 mM HCl was used to prepare stock solutions of the substrates (NPE or benzaldehyde–nitromethane mixture), because it eliminated the non-specific Henry reaction and, therefore, allowed use of the solutions for several hours.

Chromatography was performed on a Knauer Smartline chromatographic system (Berlin, Germany) equipped with a Merck RP-8 column (Darmstadt, Germany) thermostated at 30°C using a 67 mM KH_2PO_4

buffer–methanol mixture (65:35, v/v) as eluent. The flow rate was 2 mL min^{-1}; peaks were detected by monitoring the optical absorption of the eluate at 225 nm. The chromatograms were recorded and analyzed with ChromStar version 6 (SCPA, Stuhr, Germany) software.

Fitting of experimental data to algebraic equations was done by using the non-linear least-square Marquardt–Levenberg algorithm in GNUPLOT version 4.3 (freeware). The progress curve analysis and concentration profile simulation, which required the solution of differential equations, were implemented in MATLAB release 2007a (The MathWorks Inc., Natick, MA, USA) utilizing lsqnonlin and ode45 routines.

Results and discussion

Selection of model system and methods

Since the Henry reaction is reversible, it can be used either to synthesize or to cleave β-nitroalcohols. To distinguish each reaction direction, the cleavage of β-nitroalcohols is sometimes described as the retro-Henry reaction. This term is also adopted in the present work and used wherever the differentiation between both reaction directions is required.

The mechanism of *Hb*HNL in the native cyanohydrin reaction has already been elucidated by studying the kinetics of cleavage and synthesis of mandelonitrile (Figure 1(a)) (Bauer et al. 1999a). For kinetic investigations of the biocatalytic Henry reaction a similar model system was chosen, in which nitromethane was used instead of HCN and 2-nitro-1-phenylethanol (NPE) is the condensation product (Figure 1(b)). In this system two possible side reactions have to be considered besides the enantioselective enzymatic reaction: the unspecific spontaneous nitroaldol condensation, which runs parallel with the biocatalytic reaction; and the dehydration of NPE with formation of 1-nitro-2-phenylethylene (Figure 1(c)). The spontaneous Henry reaction could be suppressed by performing the process at pH 6. The chosen pH value also hindered the dehydration of NPE; in all performed experiments the concentration of nitrostyrene was negligible, and therefore was not considered in the analysis of kinetic data. By contrast, to diminish the chemical background reaction in the *Hb*HNL-catalyzed cyanohydrin condensation significantly lower pH values (around 4.5–5.0) are necessary (Selmar et al. 1987b).

The main problem during the selection of an appropriate method to monitor the progress of the biocatalytic Henry reaction was the high concentration of the enzyme required to make the enzymatic condensation significantly faster than the spontaneous reaction. The convenient on-line UV spectroscopic method, which was successfully applied to follow the cyanohydrin reaction by measuring the benzaldehyde concentration at 280 nm (Bauer et al. 1999a), was unsuitable in this case since the absorption of benzaldehyde was masked by *Hb*HNL. The problem was overcome by using off-line HPLC analysis, which enabled simultaneous determination of the concentrations of NPE, benzaldehyde and 1-nitro-2-phenylethylene in the reaction mixture at selected time points.

Before starting mechanistic studies of *Hb*HNL, it was important to estimate the equilibrium constant K_{eq} for the model reaction under the selected reaction conditions:

$$K_{eq} = \frac{k_1}{k_{-1}} = \frac{[\mathrm{NPE}]}{[\mathrm{BA}][\mathrm{MeNO_2}]}$$

where k_1 and k_{-1} are rate constants of the forward and backward reactions, respectively; [NPE], [BA] and [$MeNO_2$] are the equilibrium concentrations of the reactants (BA, benzaldehyde; $MeNO_2$, nitromethane). This constant determines the position of equilibrium in the system, where rates of NPE cleavage and synthesis are equal. To assess K_{eq} one may either measure the concentrations of reactants when the system has reached equilibrium or determine the rate constants k_1 and k_{-1}. The first approach is not very useful in this particular case because of the side dehydration reaction, which constantly removes NPE from the reaction mixture and thus causes a permanent shift of the equilibrium position. Therefore, the second approach was used. By measuring initial reaction rates of spontaneous NPE cleavage and synthesis at various starting concentrations of NPE, benzaldehyde and nitromethane under selected reaction conditions (data not shown), K_{eq}=6 M^{-1} was assessed. It is three orders of magnitude lower than the equilibrium constant for the addition of HCN to benzaldehyde (K_{eq}=4 mM^{-1}; Ching & Kallen 1978) indicating that, in comparison to the cyanohydrin reaction, the equilibrium position in the Henry reaction is shifted to the side of the carbonyl compound.

Using the estimated K_{eq} value it was possible to predict the conversion at the equilibrium position X_{eq} (if the dehydration of NPE to 1-nitro-2-phenylethylene is neglected) as a function of the concentration of starting materials: either NPE for the retro-Henry reaction or benzaldehyde and nitromethane for the Henry reaction (Figure 2). The prediction was done for the concentration range limited by the solubility of each substance in water: ~0.1 M for NPE and

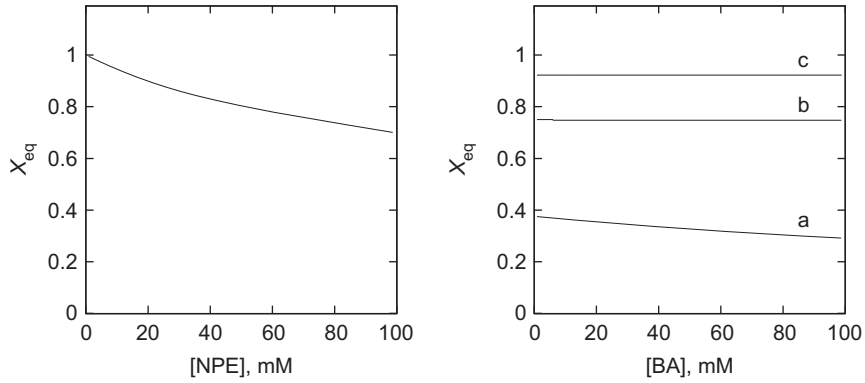

Figure 2. Equilibrium conversion, predicted for K_{eq}=6 M^{-1}, for the cleavage (left) and the synthesis (right) of NPE at 25°C in 50 mM phosphate buffer as a function of the concentration of the starting materials. Concentration of nitromethane: (a) 0.1 M, (b) 0.5 M, (c) 2 M.

benzaldehyde, ~2 M for nitromethane. The calculations revealed that, at low starting concentrations of NPE (≤10 mM), the retro-Henry reaction can be treated as irreversible (X_{eq}≈1). The same is true for the Henry reaction if the starting concentration of nitromethane is close to its solubility limit (~2 M).

Cleavage of NPE

Since *Hb*HNL is (*S*)-enantioselective in the Henry reaction, in the selected model reaction system the enzyme catalyzes only the synthesis and the cleavage of (*S*)-NPE. Therefore, to study the kinetics of the retro-Henry reaction, it is logical to use enantiopure (*S*)-NPE as a substrate. However, this chiral substance is commercially unavailable and cannot be easily synthesized in sufficient quantity. To overcome this problem, instead of the pure (*S*)-enantiomer, the racemic mixture of (*S*)- and (*R*)-enantiomers was used (Figure 3). While (*rac*)-NPE also cannot be purchased commercially, it can be easily obtained on a gram scale by chemical synthesis, in contrast to (*S*)-NPE. This approach was also applied in the work of Bauer et al. (1999a), who used racemic rather than (*S*)-mandelonitrile for the kinetic studies of *Hb*HNL in the cyanohydrin reaction under the assumption that the (*R*)-enantiomer has a negligible influence on the enzyme's kinetics in the studied concentration range. In the present work the same assumption regarding (*R*)-NPE was made.

The concentration of (*rac*)-NPE was varied in the range 5–106 mM (up to the solubility limit under the reaction conditions), while *Hb*HNL concentration was kept constant at 0.82 mg mL^{-1}. Under the applied conditions the rate of the spontaneous retro-Henry reaction was at least tenfold lower than that of the biocatalytic one. The cleavage of NPE was monitored over 1 min, where the concentration profile for benzaldehyde was still linear (Figure 4). The calculated initial rates (mM min^{-1}) were corrected for the spontaneous reaction, normalized by the enzyme concentration [*Hb*HNL] and plotted against the concentration of NPE (Figure 5). The graph obtained shows the saturation kinetics of NPE cleavage, which fits the simple one-substrate Michaelis–Menten model:

$$\frac{v}{[Hb\,\mathrm{HNL}]} = \frac{V_{\max,r}[\mathrm{NPE}]}{2K_{\mathrm{M},(S)\text{-NPE}} + [\mathrm{NPE}]}$$

where [NPE] is the concentration of (*rac*)-NPE; [*Hb*HNL]=0.82 mg mL^{-1}; $V_{max,r}$ is the maximum reaction rate of the biocatalytic cleavage of NPE, U mg^{-1}; and $K_{M,(S)\text{-}NPE}$ is the respective Michaelis

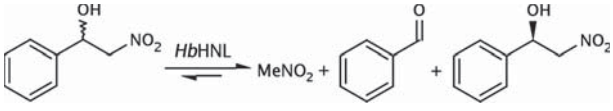

Figure 3. Cleavage of (*rac*)-NPE as a model system to study the kinetics of the biocatalytic retro-Henry reaction.

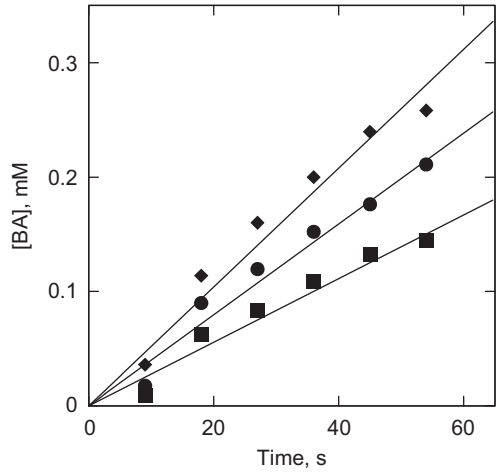

Figure 4. Initial conversion rates observed during NPE cleavage catalyzed by *Hb*HNL (0.82 mg mL^{-1}) in 50 mM phosphate buffer pH 6.0 at 25°C. Initial concentration of NPE: (▯) 14 mM, (•) 41 mM, (◆) 82 mM.

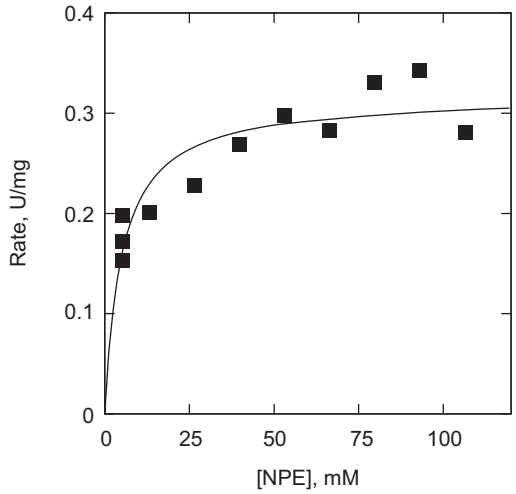

Figure 5. The Michaelis–Menten plot for NPE cleavage catalyzed by *Hb*HNL (0.82 mg mL^{-1}) in 50 mM phosphate buffer pH 6.0 at 25°C.

constant for (*S*)-NPE. The kinetic parameters determined by non-linear fitting are presented in Table I.

The Michaelis constant for (*S*)-NPE is of the same order of magnitude as the one for (*S*)-mandelonitrile in the cyanohydrin reaction (K_M=1.55 mM) reported by Bauer et al. (1999a), showing that *Hb*HNL has relatively good affinity to this β-nitroalcohol. Unfortunately, for the NPE cleavage it was not possible to determine precisely the enzyme's turnover number k_{cat}, which would show how many molecules of NPE are converted by one molecule of *Hb*HNL per second. The reason was that there is currently no suitable active site titration protocol which would allow determination of the exact molar concentration of *Hb*HNL active sites in a solution. However, assuming that 100% of the enzyme was present in its active form, i.e. there was no denatured or misfolded enzyme, the k_{cat} value could be approximated from $V_{max,r}$ as follows:

$$k_{cat} \approx V_{max,r} \times \frac{M_w}{60} = 0.16\ s^{-1}$$

where M_w=30 kg mol^{-1} is the molecular weight of *Hb*HNL. Thus, the enzyme needs around 6 s to split one molecule of NPE in the active site, compared with only 0.02 s for mandelonitrile.

Product inhibition during NPE cleavage

Since *Hb*HNL catalyzes the cleavage and synthesis of NPE, the enzyme should be able to bind both substances, NPE and benzaldehyde, in its active site. Hence benzaldehyde, formed as a product in the course of NPE cleavage, would compete with the β-nitroalcohol for the active site of *Hb*HNL and thereby inhibit the reaction. In the cleavage of mandelonitrile, *Hb*HNL is strongly inhibited by the formation of benzaldehyde, for which the enzyme has a high affinity ($K_{i,BA}$=1.2 mM; Bauer et al. 1999a). The same product inhibition effect was also observed in the cleavage of NPE; the rate of the enzymatic reaction decreased when benzaldehyde was initially present in the reaction mixture (Figure 6).

The empirical reaction progress curves fit the following kinetic model, which assumes that benzaldehyde is a competitive inhibitor:

$$\frac{d[BA]}{dt} = \upsilon + k_b[NPE]$$

$$\frac{d[NPE]}{dt} = -\frac{d[BA]}{dt}$$

$$\frac{d[(S)\text{-NPE}]}{dt} = -\upsilon - k_b[(S)\text{-NPE}]$$

$$\frac{\upsilon}{[Hb\,HNL]} = \frac{V_{max,r}[(S)\text{-NPE}]}{K_{M,(S)\text{-NPE}}\left(1 + ([BA]/K_{i,BA})\right) + [(S)\text{-NPE}]}$$

where [BA], [NPE] and [(*S*)-NPE] are molar concentrations of benzaldehyde, (*rac*)-NPE and (*S*)-NPE, respectively; k_b is the reaction rate constant for the spontaneous cleava ge of NPE, $k_b=5.3\times10^{-4}$ min^{-1} (data not shown); *V* is the reaction rate of the biocatalytic reaction, mM min^{-1}; $K_{M,(S)\text{-}NPE}$ is the Michaelis constant for (*S*)-NPE, $K_{M,(S)\text{-}NPE}$=2.55 mM; $K_{i,BA}$

Table I. Estimated kinetic parameters for cleavage and synthesis of NPE catalyzed by *Hb*HNL[a].

Cleavage			Synthesis		
Parameter	Value	Unit	Parameter	Value	Unit
$V_{max,r}$	0.32±0.02[b]	U mg^{-1}	$V_{max,f}$	0.027±0.003	U mg^{-1}
k_{cat}	0.16	s^{-1}	k_{cat}	0.013	s^{-1}
$K_{M,(S)\text{-NPE}}$	2.55±0.65	mM			
$K_{i,BA}$	0.37±0.04	mM	$K_{M,BA}$	0.33±0.01	mM
$K_{i,MeNO2}$	103±7	mM	$K_{M,MeNO2}$	64±21	mM

[a]Reaction conditions: 50 mM phosphate buffer pH 6.0, 25°C.
[b]95% confidence intervals were estimated from numerical fitting of experimental data to kinetic equations.

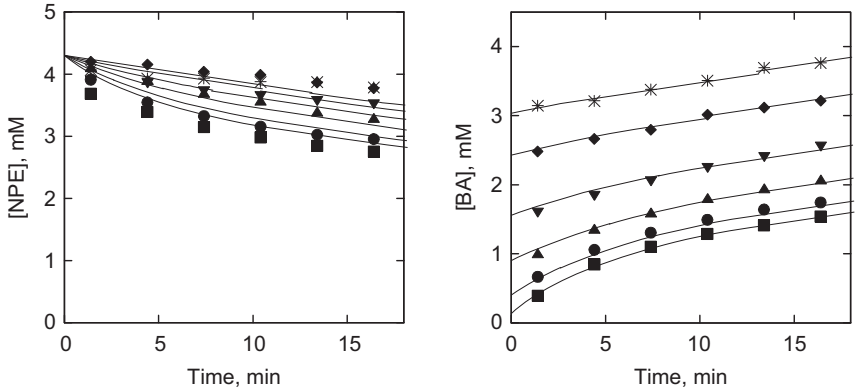

Figure 6. Inhibition of *Hb*HNL (2.1 mg mL^{-1}) by benzaldehyde in the cleavage of NPE in 50 mM phosphate buffer pH 6.0 at 25°C. Initial concentration of benzaldehyde: (■) 0 mM, (•) 0.5 mM, (▲) 1.0 mM, (▼) 1.5 mM, (◆) 2.5 mM, (*) 3.0 mM. Solid lines correspond to numerical simulation of reaction progresses.

is the inhibition constant for benzaldehyde; $V_{max,r}$ is the maximum reaction rate of the biocatalytic cleavage of NPE, $V_{max,r}$=0.32 U mg^{-1}; and [*Hb*HNL] is the applied *Hb*HNL concentration, [*Hb*HNL] = 2.1 mg mL^{-1}. This model also implies that nitromethane has negligible influence on the enzyme in the studied concentration range.

From this, $K_{i,BA}$ remained the only unknown parameter to be estimated. By non-linear fitting of the concentration profile to the model it was found that $K_{i,BA}$=0.37 ± 0.04 mM. This value is within the same order of magnitude as the inhibition constant for benzaldehyde determined by in the cleavage of mandelonitrile (Bauer et al. 1999a). Such coincidence supports the hypothesis that both the Henry and the cyanohydrin reactions take place in the same active site of *Hb*HNL and was expected a priori.

The concentration profile shown on Figure 6 was also fitted to the models assuming other types of inhibition for benzaldehyde (uncompetitive and non-competitive), but the corresponding $K_{i,BA}$ values, obtained from the non-linear fitting, were either physically meaningless or the numerical procedure did not converge.

The second product formed during the NPE cleavage – nitromethane – also inhibited the enzyme, albeit not as strongly as benzaldehyde (Figure 7). Its inhibition effect was assessed by measuring initial rates of cleavage of 5.2 and 52 mM NPE in the presence of 0.1–0.4 M $MeNO_2$. The inhibition was significant only when the concentration of nitromethane [$MeNO_2$] was higher than 0.1 M. In this case the progress curve analysis, which was used to determine the inhibition constant for benzaldehyde, was rather inappropriate, because the presence of nitromethane at such a high concentration, by analogy to HCN (Bauer et al. 1999b), could cause denaturation of the enzyme. Furthermore, at a high concentration of nitromethane the rate of the backward (*S*)-NPE synthesis should be considered as well.

The observed patterns for the relative inhibition v/v_0 of *Hb*HNL by nitromethane fit the following equation, which implies that nitromethane acts as a competitive inhibitor:

$$\frac{v}{v_0} = \frac{1 + \left([\mathrm{NPE}]_0 / 2K_{\mathrm{M},(S)\text{-NPE}}\right)}{1 + \left([\mathrm{NPE}]_0 / 2K_{\mathrm{M},(S)\text{-NPE}}\right) + \left([\mathrm{MeNO_2}] / K_{\mathrm{i,MeNO2}}\right)}$$

where v is the observed initial reaction rate corrected for the spontaneous reaction; v_0 is the corrected initial rate in the absence of nitromethane; $[NPE]_0$ is the initial molar concentration of (*rac*)-NPE, $[NPE]_0$=5.2 or 52 mM; and $K_{M,(S)\text{-}NPE}$=2.55 mM is the Michaelis constant for (*S*)-NPE, as determined from the NPE cleavage experiments. The inhibition constant for nitromethane $K_{i,MeNO2}$ found by non-linear fitting was equal to 103±7 mM. The attempt to fit the inhibition patterns to equations corresponding to other inhibitor types (uncompetitive, non-competitive or mixed) failed since the

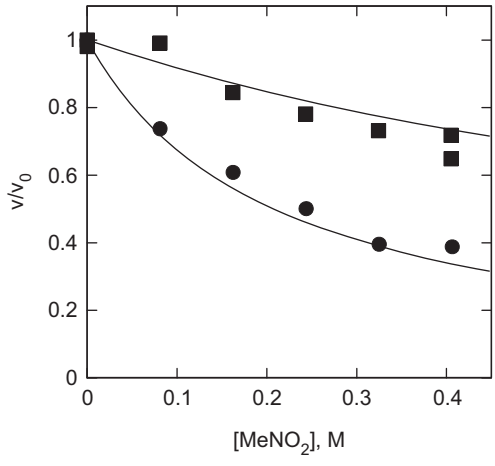

Figure 7. Relative inhibition pattern for nitromethane in NPE cleavage catalyzed by *Hb*HNL (1.4 mg/mL) in 50 mM phosphate buffer pH 6.0 at 25°C. Initial concentration of NPE: (■) 52 mM, (•) 5.2 mM.

numerical procedure did not converge. The relatively large value of $K_{i,MeNO2}$ implies that binding of nitromethane to *Hb*HNL is weak, but comparable with HCN in the cleavage of mandelonitrile ($K_{i,HCN}$=150 mM; Bauer et al. 1999a).

Remarkably, in the cyanohydrin reaction, HCN shows a mixed-type inhibition while benzaldehyde acts as a competitive inhibitor (Bauer et al. 1999a) that is consistent with the Ordered Bi Uni mechanism proposed for this reaction. In contrast, in the biocatalytic Henry reaction both nitromethane and benzaldehyde appeared to be competitive inhibitors. This indicates that, in the promiscuous biotransformation, the enzyme follows a Rapid Equilibrium Random Bi Uni mechanism.

Synthesis of NPE

The kinetics of NPE synthesis were studied using the initial rate method in the concentration range 0.3–3 mM for benzaldehyde and 80–320 mM for nitromethane. In all of these experiments the concentration of *Hb*HNL was kept constant at 4.0 mg mL^{-1}. The initial rates were determined from the linear fit of the conversion rates within 1 min (Figure 8). Under the reaction conditions chosen no spontaneous formation of NPE could be detected, thus no rate corrections were necessary.

The corresponding Michaelis–Menten plot (Figure 9), which is based on the measured initial rates V (mM min^{-1}) normalized by the enzyme concentration [*Hb*HNL] (4.0 mg mL^{-1}), shows that the synthesis of NPE also follows saturation kinetics meaning that *Hb*HNL can be saturated by both substrates – benzaldehyde and nitromethane. The empirical kinetic data fit the Rapid Equilibrium Random Bi Uni mechanism with independent substrate binding (Figure 10), the rate equation for which has the following form:

$$\frac{v}{[Hb\,\mathrm{HNL}]} = \frac{V_{max,f}[\mathrm{BA}][\mathrm{MeNO_2}]}{\left(K_{M,BA}+[\mathrm{BA}]\right)\left(K_{M,MeNO2}+[\mathrm{MeNO_2}]\right)}$$

where $V_{max,f}$ is the maximum reaction rate of the biocatalytic synthesis of NPE; [BA] and [$MeNO_2$] are molar concentrations of benzaldehyde and nitromethane, respectively; and $K_{M,BA}$ and $K_{M,MeNO2}$ are the corresponding Michaelis constants. The kinetic parameters, determined from non-linear fitting of the data to the model equation, are presented in Table I. Attempts to fit the data to the Ordered Bi Uni mechanism were not successful, since the numerical procedures did not converge.

The turnover number for NPE synthesis k_{cat}≈0.013 s^{-1}, which was estimated from $V_{max,f}$ in the same way as for NPE cleavage, reveals the kinetic preference of *Hb*HNL for the retro-Henry reaction: the enzyme requires approximately 1 min to synthesize one molecule of the nitroalcohol, but only around 6 s to cleave it.

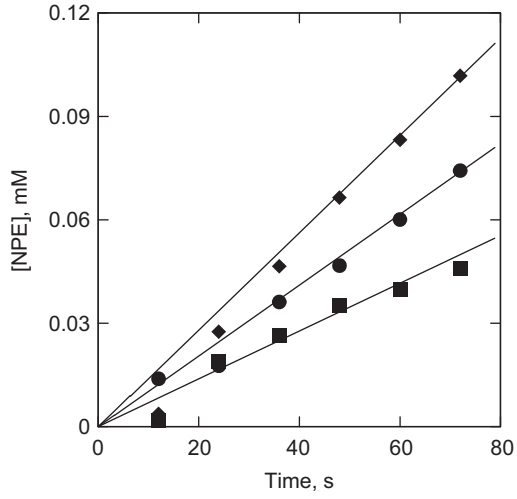

Figure 8. Initial conversion rates observed during synthesis of (*S*)-NPE catalyzed by *Hb*HNL (4.0 mg/mL) in the presence of 160 mM $MeNO_2$ in 50 mM phosphate buffer pH 6.0 at 25°C. Initial concentration of benzaldehyde: (■) 0.4 mM, (●) 1.2 mM, (◆) 2.0 mM.

The Random mechanism assumes that all binding (k_2 to k_4) and dissociation (k_{-2} to k_{-4}) steps are very rapid compared with the catalytic step (k_1 and k_{-1}), which seems to be true for the biocatalytic Henry reaction, and that the binding of one substrate does not influence the affinity of the enzyme for the other substrate (Segel 1975). The model also predicts that both benzaldehyde and nitromethane should be com-

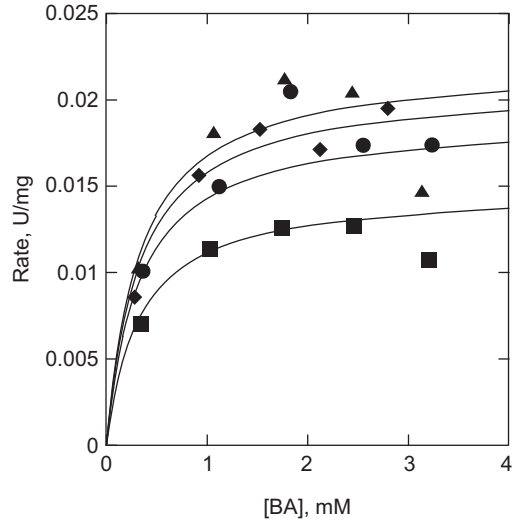

Figure 9. The Michaelis–Menten plot for (*S*)-NPE synthesis catalyzed by *Hb*HNL (4.0 mg/mL) in 50 mM phosphate buffer pH 6.0 at 25°C. Initial concentration of nitromethane: (■) 80 mM, (●) 160 mM, (▲) 240 mM, (◆) 320 mM.

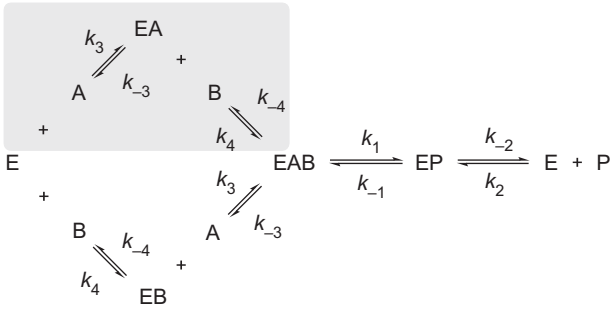

Figure 10. Rapid Equilibrium Random Bi Uni mechanism (including gray box) proposed in this work for the biocatalytic Henry reaction and Ordered Bi Uni mechanism (excluding gray box) suggested by Bauer et al. (1999a) for the biocatalytic cyanohydrin reaction. E is *Hb*HNL; B is benzaldehyde; A is either nitromethane or HCN; P is either NPE or mandelonitrile; EA, EB, EAB and EP denote the respective enzyme complexes.

petitive inhibitors for *Hb*HNL in the NPE cleavage reaction, which was confirmed by the inhibition studies. Moreover, according to this model, the inhibition constants for benzaldehyde and nitromethane should be equal to the respective Michaelis constants, because in this particular case the inhibition and the Michaelis constants for each substrate represent the substrate's true equilibrium binding constant ($K_A=k_{-3}/k_3$ or $K_B=k_{-4}/k_4$, Figure 10). This expected equivalence was obeyed here (Table I).

The consistency of the determined kinetic constants was checked by using the Haldane relationship (Segel 1975), which expresses the equilibrium constant for the Henry reaction in terms of the enzyme kinetic constants:

$$K_{eq} = \frac{V_{max,f} K_{(S)\text{-NPE}}}{V_{max,r} K_{BA} K_{MeNO2}} \cong 9\ M^{-1}$$

where $V_{max,r}$ and $V_{max,f}$ are the maximum reaction rates for cleavage and synthesis of NPE, respectively; K_{BA}, K_{MeNO2} and $K_{(S)\text{-}NPE}$ are the true equilibrium binding constants for benzaldehyde, nitromethane and (*S*)-NPE, which in this particular case are equal to the respective Michaelis or inhibition constants. On the other hand, $K_{eq}=6\ M^{-1}$ was calculated as a ratio of the rate constants for the unspecific NPE cleavage and synthesis. Both values are of the same order of magnitude, indicating that the estimated kinetic constants are consistent.

Interestingly, according to the Ordered Bi Uni mechanism proposed by Bauer et al. (1999a) for the *HbHNL*-catalyzed cyanohydrin reaction, the enzyme binds benzaldehyde first and then HCN. Based on the suggestion that *HbHNL* acts similarly in both the Henry and the cyanohydrin reactions (Purkarthofer et al. 2006), one would expect *HbHNL* to follow the same mechanism in the Henry reaction. The observed differences in kinetic behavior might indicate that, in the promiscuous reaction, *HbHNL* reacts differently from the native reaction. However, there is a plausible explanation which rationalizes the observed differences in kinetic behavior of *HbHNL* in the two reactions, even if they take place in the same active site of the enzyme.

The Ordered Bi Uni mechanism can be derived from the Random Bi Uni mechanism, when: (a) substrate B binds to the enzyme E much more strongly and much faster than substrate A ($K_B>>K_A$, $k_4 >> k_3$); (b) the dissociation rate of the enzyme complex with substrate B is much slower than the rate of the catalytic step ($k_1 >> k_{-4}$). In this case the upper pathway in the random mechanism via the EA complex would be non-operational (Figure 10). Both conditions might be true for the biocatalytic cyanohydrin reaction, and as a result *Hb*HNL follows the Ordered Bi Uni mechanism in this biotransformation. In contrast, in the biocatalytic Henry reaction it has been shown that the rate of the catalytic step (k_1 and k_{-1}) is very low in comparison to the cyanohydrin reaction. Therefore, the condition (b) could be unsatisfied, and as a consequence the upper pathway via the EA complex would be operational, resulting in the Rapid Equilibrium Random Bi Uni mechanism.

Conclusions

The kinetics of the *Hb*HNL-catalyzed promiscuous Henry reaction fits the classical mechanistic model – Rapid Equilibrium Random Bi Uni with independent substrate binding – which implies that the bottleneck for this biotransformation is a very low turnover number of the enzyme, not the binding of the substrates. This agrees with the hypothesis of Purkarthofer et al. (2006) based on molecular docking simulations, according to which NPE and mandelonitrile bind to the active site of *Hb*HNL in a similar manner. Unfortunately, the answer to the question why the enzyme shows such a low turnover number is beyond enzyme kinetics. In order to debottleneck the biocatalytic Henry reaction one needs a deeper insight into the mechanism of this transformation at a molecular level. In this respect the employment of other techniques, like X-ray structural analysis combined with molecular modeling, should be considered.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Bauer M, Griengl H, Steiner W. 1999a. Kinetic studies on the enzyme (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* using initial rate methods and progress curve analysis. Biotechnol Bioeng 62:20–29.

Bauer M, Grieng H, Steiner W. 1999b. Parameters influencing stability and activity of a *S*-hydroxynitrile lyase from *Hevea brasiliensis*. Enzyme Microb Technol 24:514–522.

Bornscheuer UT, Kazlauskas RJ. 2004. Catalytic promiscuity in biocatalysis: using old enzymes to form new bonds and follow new pathways. Angew Chem Int Ed 43:6032–6040.

Ching WM, Kallen RG. 1978. Mechanism of carbanion addition to carbonyl compounds. Equilibria and kinetics of substituted cyanohydrin cleavage and formation in aqueous solution. Substituted cyanohydrin proton dissociation constants. J Am Chem Soc 100:6119–6124.

Fechter MH, Griengl H. 2004. Hydroxynitrile lyases: biological sources and application as biocatalysts. Food Technol Biotechnol 42:287–294.

Gruber-Khadjawi M, Purkarthofer T, Skranc W, Griengl H. 2007. Hydroxynitrile lyase-catalyzed enzymatic nitroaldol (Henry) reaction. Adv Synth Catal 349:1445–1450.

Kazlauskas RJ. 2005. Enhancing catalytic promiscuity for biocatalysis. Curr Opin Chem Biol 9:195–201.

Luzzio FA. 2001. The Henry reaction: recent examples. Tetrahedron 57:915–945.

Ono N. 2001. The nitro-aldol (Henry) reaction. In: The nitro group in organic synthesis. Wiley-VCH New York. p 30.

Poechlauer P, Skranc W, Wubbolts M. 2004. The large-scale biocatalytic synthesis of enantiopure cyanohydrins. In: Schmidt E, Blaser H-U, editors. Asymmetric catalysis on industrial scale. Wiley-VCH Verlag GmbH & Co. KGaA Weinheim. p 149.

Purkarthofer T, Gruber K, Gruber-Khadjawi M, Waich K, Skranc W, Mink K, Griengl H. 2006. A biocatalytic Henry reaction – the hydroxynitrile lyase from *Hevea brasiliensis* also catalyzes nitroaldol reactions. Angew Chem Int Ed 45: 3454–3456.

Segel IH. 1975. Enzyme kinetics. New York: Wiley.

Selmar D, Carvalho FJP, Eric E. Conn EE. 1987a. A colorimetric assay for α-hydroxynitrile lyase. Anal Biochem 166: 208–211.

Selmar D, Lieberei R, Biehl B, Voigt J. 1987b. *Hevea* linamarase – a nonspecific β-glycosidase. Plant Physiol 83:557–563.

Wajant H, Förster S. 1996. Purification and characterization of hydroxynitrile lyase from *Hevea brasiliensis*. Plant Sci 115:25–31.