ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 5 (1998) 249–253

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Feasibility of the thermodynamically controlled synthesis of amoxicillin

M.B. Diender *, A.J.J. Straathof, L.A.M. van der Wielen, C. Ras, J.J. Heijnen

Delft University of Technology, Kluyver Laboratory for Biotechnology, Julianalaan 67, NL-2628 BC Delft, The Netherlands

Received 26 September 1997; accepted 1 December 1997

Abstract

The enzymatic, thermodynamically controlled synthesis of amoxicillin in aqueous solution was measured in order to study the feasibility of a 'solid-to-solid' conversion. In aqueous solution, however, the synthetic yield of amoxicillin remains lower than the amoxicillin solubility. Therefore, a 'solid-to-solid' synthesis of amoxicillin in aqueous solution is not feasible. Synthetic yields in enzymatic condensation reactions can often be improved by adding organic solvents in monophasic systems. Addition of cosolvents is calculated to improve the apparent equilibrium constant of amoxicillin synthesis considerably, but probably not the synthetic yield, due to solubility restrictions of the reactants.

Keywords: Amoxicillin; Solid-to-solid conversion; Thermodynamically controlled synthesis

1. Introduction

Amoxicillin is one of the major β-lactam antibiotics, with sales of US$2200 million as a bulk-formulated drug in 1994 [1]. Traditionally, semisynthetic β-lactam antibiotics are produced by chemical modification of the basic penicillin structure. However, enzymatic procedures may offer important advantages over the conventional chemical transformations. For example, it can be performed in a one-step reaction with high yield and high specificity, under mild reaction conditions [2–5].

The most frequently applied strategy for enzymatic synthesis is a kinetically-controlled reaction, in which an activated acyl donor is coupled to an antibiotic nucleus. High yields on nucleus are achievable, but part of the activated acyl donor hydrolyses is not coupled to the nucleus, so recycling and reactivation of the acyl donor is necessary, leading to costly extra process steps. In addition, hydrolysis of the product may occur [3]. A simpler strategy for enzymatic synthesis would be a direct condensation of antibiotic nuclei and non-activated acyl donors in a thermodynamically-controlled reaction, which avoids these processes of recycling and reactivation. This strategy can only be applied successfully if conditions for a favorable equilibrium position can be found. The enzyme

* Corresponding author.

1381-1177/98/$19.00
PII: S1381-1177(98)00044-7

does not influence the position of the equilibrium, but only the rate at which the equilibrium is established. In water, however, hydrolysis of the antibiotic may be favored to synthesis, because of unfavorable thermodynamics. To shift the reaction equilibrium towards synthesis one may add moderate to high concentrations of water-miscible organic solvents [6,7] and/or increase the substrate concentration up to the solubility level or even higher, which gives a suspension of substrate. In favorable cases the concentration of the product that is formed exceeds its solubility. Then a suspension of solid substrates can be converted into a suspension of solid products. This situation, to which we refer as the 'solid-to-solid' process, has been achieved for several enzymatic reactions [2,8].

The purpose of this paper is to describe the thermodynamics of the synthesis of amoxicillin in order to study the feasibility of the 'solid-to-solid' concept. The thermodynamic condition for a 'solid-to-solid' conversion to occur is that the ratio of dissolved concentrations (i.e., the apparent equilibrium constant, K_{app}) is larger than the ratio of solubilities (R_S):

$$K_{app} = \frac{c_{Amox}}{c_{APA}c_{HPG}} > \frac{S_{Amox}}{S_{APA}S_{HPG}} = R_S \qquad (1)$$

where c_i is the actual concentration of species i and S_i its solubility.

2. Materials and methods

2.1. Materials

Immobilized penicillin G acylase (EC 3.5.1.11) from *Escherichia coli*, 6-aminopenicillanic acid (6-APA, 98.5% pure) and amoxicillin trihydrate (Amox, 98.9% pure) were donated by Gist-brocades (Delft, Netherlands). D-(*p*-)Hydroxyphenylglycine (HPG) was a kind gift of DSM (Geleen, Netherlands).

2.2. Solubility measurements

Solubility measurements of Amox, 6-APA and HPG were performed as described by Gude et al. [9].

2.3. Reaction equilibrium measurements in water

Equilibrium measurements of the synthesis and hydrolysis of amoxicillin were performed by approaching equilibrium from both directions of reaction at pH 5.0, 5.6 and 6.0 using a pH-stat (Impulsomat 614, Dosimat 665, and pH meter 691 of Metrohm). For each experiment a solution was prepared in 0.1 mol l^{-1} potassium phosphate buffer (pH 5.65) with an excess of APA and HPG to obtain saturation, and 0 mol Amox l^{-1} (hydrolysis experiments), or 0.6×10^{-3} mol Amox l^{-1} (synthesis experiments at pH 5.6 and 6) or 2.3×10^{-3} mol Amox l^{-1} (synthesis experiments at pH 5.0). Equilibration and filtration were performed as described for the solubility experiments. The pH was adjusted using 1.0 mol l^{-1} aqueous NaOH or HCl.

Reactions were performed in a thermojacketed reaction vessel at 25°C by addition of 5.0 ml of 0.11 g ml^{-1} enzyme stock solution (in 0.1 mol l^{-1} potassium phosphate buffer, pH 5.65). Generally, reactions were carried out for approximately 20 to 44 h. For calculation of K_{app} the ratios of concentrations were averaged for the flat region of the Amox curve in the synthesis experiments and for the corresponding region in the hydrolysis experiments.

2.4. Analysis

APA, HPG and Amox were identified and analyzed by HPLC using a Waters System with a Zorbax SB-C_{18} 4.6×75 mm 3.5-μm column, thermostated at 33°C, and a Waters 996 PDA detector at 230 nm, a Waters 910 Wisp 10-μl injector, and using a Waters 590 pump with a flow of 1 ml min^{-1}. Prior to analysis, the

samples were diluted with eluens. The eluens was composed of 8 mmol l^{-1} tetrabutylammonium bromide, 10 mmol l^{-1} Na_2HPO_4 and 15% (v/v) acetonitrile. It was brought to pH 6.60 with H_3PO_4.

3. Results and discussion

3.1. Reaction equilibria in water as a function of pH

Precise determination of the equilibrium constant for the synthesis of Amox from APA and HPG is difficult because of chemical degradation of these compounds during equilibration and because of the low equilibrium concentration of Amox. We carried out the reactions in duplicate or triplicate, starting from compositions close to either side of the equilibrium. In order to minimize chemical degradation, the amounts of enzyme were maximized. Yet, degradation was not completely excluded during these experiments and an equilibrium was not reached. For the Amox concentration profile this was hardly visible in some examples, and a reasonable correspondence between the concentration in hydrolysis and synthesis experiments was obtained (Fig. 1), but for APA the degradation amounted up to 15% (data not shown). Still, in this dynamic state the equilibrium constant could be determined from the ratio of concentrations, because the pseudo-first order rate constants for the enzymatic reaction were much higher than for the chemical degradation according to blanks and literature data [10].

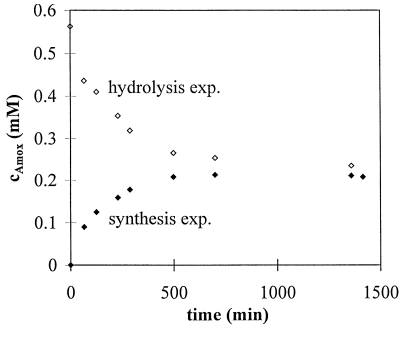

Fig. 1. Equilibrium measurement of amoxicillin at pH 6.0 and 25°C. Equilibrium was achieved from both directions of reaction. Concentrations of APA and HPG are not displayed.

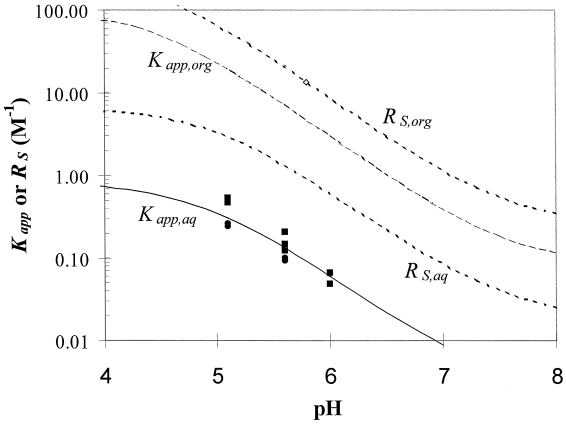

Fig. 2. Apparent equilibrium constants for the synthesis of amoxicillin in water and a 50% (w/w) DMF mixture as a function of pH at 25°C. Experimental values for synthesis (●) and hydrolysis (■), and predicted values of $K_{app,aq}$ (————) and $K_{app,org} \times 10^{-2}$ in 50% (w/w) DMF mixture (——). K_{app} = [Amox]/[APA][HPG]. Calculated ratio of solubilities $R_{S,aq}$ (· · · ·) and $R_{S,org}$ (----). Experimental value of $R_{S,org}$ (△).

As shown in Fig. 2, there is a reasonable correspondence between equilibria achieved from either synthesis or hydrolysis experiments, except for pH 5.1. Nevertheless, results from all experiments were used for further calculations. The apparent equilibrium constant can be modeled as a function of the dissociation of the reactants and the pH-independent equilibrium constant of a reference reaction (K_{ref}) [11]. We have used a reference reaction for the synthesis of amoxicillin, which involves cationic APA, anionic HPG and zwitterionic Amox:

$$APA^+ + HPG^- \rightleftharpoons Amox^{+-} + H_2O$$

Using the dissociation constants of the reactants (Table 1), the experimental values of K_{app} (see Fig. 2) the average value of K_{ref} is 623 l mol^{-1}.

Using this value of K_{ref}, K_{app} is calculated as a function of pH (see Fig. 2, model line) [12]. The measured values support the model, because experimental K_{app}-values decrease with increasing pH according to the prediction. How-

Table 1
Effect of DMF on the apparent pK_a-values of APA, HPG and Amox at 25°C

Compound	$pK_{a,app}$	Solvent	
		Water	50% (w/w) DMF
APA	$pK_{a,APA1}$	2.5	3.9
	$pK_{a,APA2}$	4.9	4.8
HPG	$pK_{a,HPG1}$	2.2	3.6
	$pK_{a,HPG2}$	9.2	9.2
Amox	$pK_{a,Amox1}$	2.9	4.5
	$pK_{a,Amox2}$	7.4	7.5

Experimental results were measured by Diender et al. [12].

ever, the K_{app}-values observed in this work are an order of magnitude smaller than the values for ampicillin hydrolysis reported by Svedas et al. (1980) [11] and Blinkovsky and Markaryan (1993) [13]. They gave values of K_{app} of 158 l mol^{-1} at pH = 4.5, 78 l mol^{-1} at pH = 5.0 [11] and 5.5 l mol^{-1} at pH = 5.5 [13], all at 25°C. Ampicillin is an antibiotic very similar in structure to amoxicillin. We had expected the apparent equilibrium constant of amoxicillin and ampicillin to be approximately equal. The low value of K_{app} observed for amoxicillin has important implications for the feasibility of the 'solid-to-solid' reaction, as will be shown below.

3.2. Solid-to-solid process

In order to study the feasibility of a 'solid-to-solid' conversion of amoxicillin in water, the solubilities in water of the reactants and product were determined as a function of pH. Results of these solubility measurements are represented in Fig. 3.

A study of the feasibility of the 'solid-to-solid' conversion of amoxicillin based on the equilibrium constant reported for ampicillin [13] and the solubilities of Fig. 3 may suggest that 'solid-to-solid' conversion could be successful. However, our experimental equilibrium concentrations of amoxicillin in water (e.g., Fig. 1) are an order of magnitude lower than its solubility (Fig. 3), so the 'solid-to-solid' conversion should not occur. Also, preliminary results by others indeed showed no 'solid-to-solid' conversion in water (L.M. van Langen and R. Sheldon, personal communication). This conclusion seems valid in the whole pH range from 4 to 8, because the calculated value of R_S is always larger than K_{app} (Fig. 2). Schroën et al. [14] describe similar results for cephalexin.

As mentioned in Section 1, one may add moderate to high concentrations of water-miscible organic solvents to shift the reaction equilibrium towards synthesis. The addition of organic cosolvents lowers the water activity, and stabilizes the neutral forms of dissociating groups of the substrates (mainly carboxylic acid groups) [15]. To predict enzymatic reaction equilibria in monophasic water-organic solvent mixtures we recently developed a model that uses reaction equilibria in water and apparent pK_a-values of the reactants in different water–cosolvent mixtures as input parameters [12]. This model confirms the high yields that have been found for thermodynamically controlled synthesis of penicillin G from APA and phenylacetic acid in water–dimethylformamide (DMF) mixtures, and can also be used to predict the synthetic yield of amoxicillin in this water–cosolvent mixture. In Table 1 the apparent dissociation constants of the amoxicillin-related reactants in water and a 50% (w/w) DMF mixture are presented, which

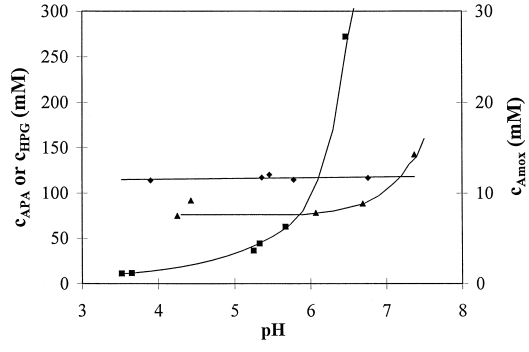

Fig. 3. Experimental solubilities of Amox (▲), APA (■) and HPG (◆) in water as a function of pH at 25°C. The full curves are fits according to the model of Gude et al. [9].

we used to calculate $K_{app,org}$ using the model [11] and $K_{ref} = 623\ l\ mol^{-1}$. It is calculated that K_{app} increases nearly a factor 100 in 50% (w/w) DMF mixture compared to water (Fig. 2). However, this does not mean that 'solid-to-solid' conversion will occur, because the solubilities of the substrates and the product will also change in the water-organic solvent mixtures. In a mixture with DMF, the solubilities of Amox, APA, HPG are much lower than in water, overall leading to an increase of R_S in Eq. (1), that counteracts the increase of K_{app} (see Fig. 2). Thus a 'solid-to-solid' conversion of amoxicillin is expected not to be feasible in a water-cosolvent mixture, but this can be tested only if an enzyme is available that is and remains sufficiently active under such conditions.

4. Conclusions

In aqueous solution the equilibrium constant of the enzymatic synthesis of amoxicillin from APA and HPG is much lower than was expected from extrapolated literature data on ampicillin synthesis. Therefore, a 'solid-to-solid' conversion in water is not feasible. The presence of an organic cosolvent, e.g., 50% (w/w) DMF mixture is calculated to improve the equilibrium constant considerably by a factor of nearly 100. However, it is expected that, due to solubility restrictions in the DMF–water mixture, also here the 'solid-to-solid' concept is not feasible.

Acknowledgements

We thank Gist-brocades (Delft, The Netherlands) and DSM (Geleen, Netherlands) for their generous gifts of amoxicillin, 6-aminopenicillanic acid, D-(*p*-)hydroxyphenylglycine, and Penicillin G acylase. This work is supported by Chemferm and the Dutch Ministry of Economic Affairs (through Senter). We are also very grateful to H.H.J. Meuwissen for the solubility measurements.

References

[1] R.A. Sheldon, J. Chem. Technol. Biotechnol. 67 (1996) 1.
[2] V. Kasche, Enzyme Microb. Technol. 8 (1986) 4.
[3] R. Fernandez-Lafuente, C.M. Rosell, B. Piatkowska, J.M. Guisan, Enzyme Microb. Technol. 19 (1996) 9.
[4] W.G. Choi, B. Lee, D.D.Y. Ryu, Biotechnol. Bioeng. 23 (1981) 361.
[5] V. Kasche, U. Haufler, L. Riechmann, Meth. Enzymol. 136 (1986) 280.
[6] M.G. Kim, S.B. Lee, J. Mol. Catal. B: Enzyme 1 (1996) 201.
[7] Y.B. Tewari, M.M. Schantz, P.C. Pandey, M.V. Rekharsky, R.N. Goldberg, J. Phys. Chem. 99 (1995) 1594.
[8] P.J. Halling, U. Eichhorn, P. Kuhl, H.D. Jakubke, Enzyme Microb. Technol. 17 (1995) 601.
[9] M.T. Gude, L.A.M. van der Wielen, K.Ch.A.M. Luyben, Ind. Eng. Chem. Res. 35 (1996) 4700.
[10] A. Tsuji, E. Nakashima, A. Hamano, T. Yamana, J. Pharm. Sci. 67 (1978) 1059.
[11] V.K. Svedas, A.L. Margolin, I.V. Berezin, Enzyme Microb. Technol. 2 (1980) 138.
[12] M.B. Diender, A.J.J. Straathof, J.J. Heijnen, submitted.
[13] A.M. Blinkovsky, A.N. Markaryan, Enzyme Microb. Technol. 15 (1993) 965.
[14] C.G.P.H. Schroën, V.A. Nierstrasz, P.J. Kroon, R. Bosma, A.E.M. Janssen, H.H. Beeftink, J. Tramper, submitted.
[15] R. Fernandez-Lafuente, C.M. Rosell, J.M. Guisán, Enzyme Microb. Technol. 13 (1991) 898.