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Mathematical model of the MenD-catalyzed 1,4-addition (Stetter reaction) of α-ketoglutaric acid to acrylonitrile

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ABSTRACT

The Stetter reaction, a conjugate umpolung reaction, is well known for cyanide-catalyzed transformations of mostly aromatic aldehydes. Enzymatic Stetter reactions, however, have been largely unexplored, especially with respect to preparative transformations. We have investigated the kinetics of the MenD-catalyzed 1,4-addition of α-ketoglutaric acid to acrylonitrile which has shown that acrylonitrile, while an interesting candidate, is a poor substrate for MenD due to low affinity of the enzyme for this substrate. The kinetic model of the reaction was simplified to double substrate Michaelis–Menten kinetics where the reaction rate linearly depends on acrylonitrile concentration. Experiments at different initial concentrations of acrylonitrile under batch, repetitive batch, and fed-batch reactor conditions were carried out to validate the developed mathematical model. Thiamine diphosphate dependent MenD proved to be quite a robust enzyme; nevertheless, enzyme operational stability decay occurs in the reactor. The spontaneous reactivity of acrylonitrile towards polymerization was also taken into account during mathematical modeling. Almost quantitative conversion of acrylonitrile was achieved in all batch reactor experiments, while the yield of the desired product was dependent on initial acrylonitrile concentration (i.e., the concentration of the stabilizer additive). Using the optimized reactor parameters, it was possible to synthesize the product, 6-cyano-4-oxohexanoic acid, in a concentration of 250 mM. The highest concentration of product was achieved in a repetitive batch reactor experiment. A fed-batch reactor experiment also delivered promising results, especially regarding the short reaction time needed to achieve a 200 mM concentration of product. Hence, the enzymatic Stetter reaction with a highly reactive acceptor substrate can be performed on a preparative scale, which should enable similar transformations with acrylate, methacrylate, and methyl vinyl ketone.

1. Introduction

The thiamine diphosphate (ThDP) dependent enzyme 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (MenD, EC 2.2.1.9) from *Escherichia coli* catalyzes the decarboxylation of α-ketoglutaric acid (α-KG) and concomitant addition of succinyl-ThDP to isochorismate (Jiang et al., 2007; Kurutsch et al., 2009; Westphal et al., 2013). Being involved in the synthesis of menaquinones, this enzyme is crucial for the survival of *E. coli* and other bacteria (Dosselaere and Vanderleyden, 2001; Jiang et al., 2007). MenD accepts different aldehydes as acceptor substrates, producing chiral 2-hydroxy ketones (Beigi et al., 2014; Bongaerts et al., 2011). It catalyzes nonphysiological C−C bond-forming reactions (Kurutsch et al., 2009) and shows unexpected activity towards short-chain carboxylic acids and several α,β-unsaturated open-chain Michael acceptors. This opens up novel biocatalytic pathways for the synthesis of products different from those currently accessible by ThDP-dependent enzyme catalysis (Beigi et al., 2014; Beigi et al., 2016). Unlike nonenzymatic transformations, biocatalytic C−C bond formations are carried out under physiological conditions without any need for protecting groups (Bernacchia et al., 2015; Müller, 2012). Thus, the study of C−C bond-forming enzymes and new substrates they can accept is of great importance for synthetic applications and implementation in biotechnological processes (Resch et al., 2011).

To reach biotechnological application, enzyme reaction systems need to be kinetically characterized. Enzyme operational stability also has to be evaluated due to the harsh conditions of technological processes (Johannes et al., 2006; Ringborg and Woodley, 2016). Reaction engineering plays an important role in the development of biocatalytic processes, while kinetic modeling combined with reactor modeling

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Nomenclature

List of symbols and abbreviations

c	Molar concentration, mM
f	Coefficient that quantifies the effect of ThDP and Mg^{2+} concentration (Eq. (3))
k_1	Kinetic constant of the first order for the rate of AN polymerization, min^{-1}
k	Kinetic constant of the first order, min^{-1} (Eqs. (4), (5), (7))
k_d	Operational stability decay rate constant, h^{-1}
K_i	Inhibition constant, mM
K_m	Michaelis constant, mM
q	Volume flow rate, mL min^{-1}
q_1	Volume flow rate of AN in the fed-batch experiment, mL min^{-1}
q_2	Volume flow rate of the second feed in the fed-batch experiment containing ThDP, α-KG and Mg^{2+} salt, mL min^{-1}
Q_P	Volume productivity, g L^{-1} d^{-1}
r	Reaction rate of enzymatic reaction, mM min^{-1}
r_1	Reaction rate of AN polymerization, mM min^{-1}
S.A.	Specific activity, U mg^{-1}
t	Reaction time, min
V_{enz}	Enzyme volume, mL
V_m	Maximum reaction rate, U mg^{-1}
V	Reactor volume, mL
Y_P	Product yield, %
γ	Mass concentration, mg mL^{-1}
AN	Acrylonitrile
2,3-CHD	(2*S*,3*S*)-2,3-Dihydroxy-2,3-dihydrobenzoate
$BnONH_2$·HCl	*O*-benzylhydroxylamine hydrochloride
HPLC	High-performance liquid chromatography
inhibitor	*p*-Methoxyphenol
α-KG	α-Ketoglutaric acid
LC–MS	Liquid chromatography–mass spectrometry
MenD	2-Succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase
P	6-Cyano-4-oxohexanoic acid, product of the reaction
TEA	Triethanolamine
TFA	Trifluoroacetic acid
ThDP	Thiamine diphosphate

provides the basis for the choice of reactor mode and design (Findrik et al., 2005; Vasić-Rački et al., 2011) and, moreover, is very useful in establishing optimal operating conditions. Modeling increases our knowledge of the process and leads to a better understanding of the effect of different variables on its outcome (Zimmermann et al., 2007). Different approaches to derive a kinetic model are reported in the literature (Al-Haque et al., 2012; Franceschini and Macchietto, 2008; Zavrel et al., 2008).

Herein, we describe the kinetic characterization of the MenD-catalyzed conjugate addition (Stetter reaction) of α-KG to acrylonitrile (AN) (Fig. 1). This reaction process was introduced in 2014 and the structure of the resulting product, 6-cyano-4-oxohexanoic acid, has been confirmed by ^{1}H and ^{13}C NMR spectroscopy (Beigi et al., 2014). The reaction kinetics and scope of such enzymatic Stetter-type reactions have remained unexplored; nevertheless, it is known that the products are valuable building blocks: compounds containing a nitrile group, for example, can be easily transformed into diverse other chemicals (Zou et al., 2015). For its physiological reaction, MenD follows a one-site ping-pong bi-bi kinetic mechanism (Fang et al., 2011). We aimed to develop a robust kinetic model applicable for process development of a conjugate addition with a highly reactive acceptor substrate.

2. Materials and methods

2.1. Chemicals

Acrylonitrile, disodium α-ketoglutarate hydrate, triethanolamine (TEA), trifluoroacetic acid (TFA), formic acid, sodium hydrogen phosphate, potassium dihydrogen phosphate, ThDP, acetonitrile, and methanol were purchased from Sigma Aldrich (Germany). Magnesium chloride, *O*-benzylhydroxylamine hydrochloride ($BnONH_2$·HCl), pyridine, and ethyl acetate were purchased from Acros Organics (Belgium). (2*S*,3*S*)-2,3-Dihydroxy-2,3-dihydrobenzoate (2,3-CHD) was synthesized at the Institute of Pharmaceutical Sciences, University of Freiburg (Germany). MenD was kindly provided by Prozomix Ltd (United Kingdom) as a 2.274 mg mL^{-1} pure protein suspension in 3.2 M $(NH_4)_2SO_4$ solution.

2.2. HPLC analysis

Reactants and product were analyzed on a Prominence HPLC system (Shimadzu) with UV detection at 195 nm (substrates) or 215 nm (product).

α-KG and AN were analyzed on a HP Hypersil APS C18 column (5 µm, 4.6 × 200 mm). Conditions: isocratic [water/TFA (0.1% v/v)], flow rate 1 mL min^{-1}, column temperature 30 °C. Retention times for AN and α-KG were 3.95 and 6.70 min, respectively. Before analysis, samples were diluted with water to fit the linear range of the calibration curve and filtered through a 0.2 µm PTFE hydrophilic filter. The linear range of calibration curve for AN and α-KG was 2 and 1 mM, respectively.

6-Cyano-4-oxohexanoic acid was analyzed using a Phenomenex LiChrospher C18 column (5 µm, 4 × 250 mm). Conditions: gradient (from 10% to 70% B within 25 min), flow rate 1.2 mL min^{-1}, column temperature 30 °C. Mobile phase A: water/TFA (0.1% v/v); mobile phase B: acetonitrile/water/TFA, 80:20:0.095 v/v. Samples (5 µL) were derivatized before analysis with a solution (50 µL) containing $BnONH_2$·HCl (130 mM in pyridine/methanol/water, 33:15:2) for 20 min at 25 °C and 1000 rpm (Garrabou et al., 2009). Then, 450 µL of methanol was added, which was followed by centrifugation at 14000 rpm for 2 min. The enzyme precipitated, and the upper phase was used for analysis. The retention time of the derivatized product was 19.5 min. Its identity was confirmed by LC–MS analysis. Samples were diluted to fit the linear range of calibration curve for the product which was up to 50 mM.

2.3. LC–MS analysis of the derivatized product

The column and the conditions for LC–MS analysis were the same as for HPLC, except formic acid was used instead of TFA. HPLC with DAD and MS detection (Shimadzu LCMS-2020 single quadrupole instrument) was used. The selected ion monitoring method was used to confirm the

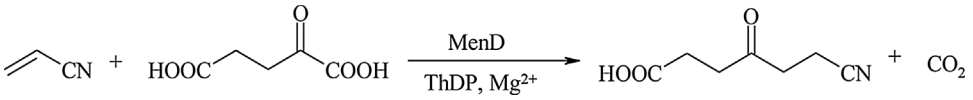

Fig. 1. Reaction scheme of the 1,4-addition of α-KG to AN catalyzed by MenD.