ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 3, pp. 288-292. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 3, pp. 355-361.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-231, December 14, 2008.

Metabolic Control Analysis of L-Cysteine Producing Strain TS1138 of *Pseudomonas* sp.

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Received July 15, 2008
Revision received August 27, 2008

Abstract—A kinetic model describing the biosynthesis of L-cysteine by *Pseudomonas* sp. TS1138 has been developed. The two enzymes catalyzing this pathway, L-cysteine synthetase (CS) and L-cysteine desulfhydrase (CD), follow Michaelis–Menten kinetics with noncompetitive inhibition of CS by L-cysteine. From measurements of intermediates and end products that were made during L-cysteine enzymatic synthesis, metabolic control analysis of the pathway was carried out using the kinetic model. The elasticity coefficients and the flux control coefficients were calculated, and the analysis revealed a shift in the flux control from CS to CD during the reaction. The findings further implicate potential targets and strategies for increasing L-cysteine production; for example, the strain TS1138 could be manipulated by site-directed mutagenesis to reduce CD activity.

DOI: 10.1134/S0006297909030079

Key words: *Pseudomonas* sp. TS1138, L-cysteine, kinetic model, metabolic control analysis, enzymatic synthesis

To optimize the flux through a metabolic pathway, in order that the production rate of a specific cellular metabolite is maximized, it is important to understand how the metabolic flux of the pathway is controlled by individual reactions. The rates of these individual steps are in turn dependent on the cultivation conditions, metabolic effectors, and the environment. Metabolic engineering, defined as the purposeful modification of intermediary metabolism by recombinant DNA techniques [1-3], enables one to design in a systematic fashion new and better strains with improved productivity of both traditional metabolites and novel compounds. Metabolic engineering is a multidisciplinary field that applies information and techniques from biochemistry, genetics, molecular biology, cell physiology, and chemical engineering. For production of metabolites such as amino acids and antibiotics, the key issues in process improvement are productivity, titer, and product yield from the carbon source. Therefore, one of the most important aspects in new strain design is the control of cellular fluxes. Before recombinant DNA technology can be applied to enhance the rate of production of the desired metabolite [1, 4, 5], it is essential to identify the best targets for specific genetic manipulation and to obtain a quantitative idea of the extent to which the identified pathway steps control the overall flux through the pathway [6-8].

Metabolic control analysis (MCA) provides a means of quantifying control structures through a mathematical formulation based on two sets of coefficients that define the properties of a system at steady state [9, 10]. One of these coefficient sets, elasticity coefficients, establishes how the pool level of an individual metabolite in a metabolic pathway influences the rate of a specific enzymatic step. Thus, elasticity coefficients represent the local properties of the metabolic network. Meanwhile, the second coefficient set, the flux control coefficients, determines how the overall flux of a metabolic network is influenced by a particular reaction. They represent systemic properties of the network. These two sets of coefficients are related by the connectivity and summation theorems. Thus, the elasticity coefficients can be used to calculate a

Abbreviations: CD, L-cysteine desulfhydrase; CS, L-cysteine synthetase; DL-ATC, DL-2-amino-Δ^2-thiazoline-4-carboxylic acid; MCA, metabolic control analysis.

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flux control coefficients of a pathway [11-13]. The degree of control exerted by each step of the network, and also the overall flux through the network, is given by the numerical magnitude of these coefficients.

Simulation by computer modeling of a metabolic pathway, or of particular aspects of cellular metabolism, provides advantages over an experimental protocol for estimating flux control coefficients. Simulation of the flux through a pathway using a mathematical model allows for perturbation on the pathway and observation of the resulting effect. This can substantially help in establishing an effective experimental strategy to determine the flux control coefficients [14]. Several reports describing the use of models in metabolic studies for improvement of final yields by recombinant DNA manipulation and regulation of metabolic pathways have been published. For example, Gallazo and Bailey used a detailed kinetic model to study the effect of alginate entrapment on glycolytic regulation in suspended and immobilized *Saccharomyces cerevisiae* cultures [15]. Nielsen and Pissara applied MCA to the penicillin biosynthetic pathway in a high-yielding strain of *Penicillium chrysogenum* [7, 16].

The aim of the present study was to identify the potential metabolic rate-limiting steps and enhance the production of L-cysteine produced by *Pseudomonas* sp. TS1138 from DL-2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC). To achieve this aim, MCA theory was employed to develop a kinetic model that accurately describes L-cysteine biosynthesis. Elasticity coefficients were derived from the kinetic model, and the flux control coefficients for the individual steps in the L-cysteine metabolic network were subsequently determined. These studies could provide important clues on how each individual step controls the overall flux through the pathway. This research could also be used to establish a rational genetic strategy for L-cysteine overproduction in *Pseudomonas* sp. TS1138.

MATERIALS AND METHODS

Strain and medium. *Pseudomonas* sp. TS1138 was kindly provided by Prof. Wenbo Yang (NanKai University, China). The organism was maintained on yeast malt agar slants at 4°C in a refrigerator and was subcultured on a monthly basis. The seed medium contained 3% glucose, 0.3% DL-ATC·$3H_2O$, 1% yeast extract, 1% peptone, 0.3% urea, 0.1% $MnSO_4 \cdot 5H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.15% NaCl, and 0.3% K_2HPO_4. The medium used for enzyme production, kept in a shake flask or a bioreactor, contained 3% glucose, 0.3% DL-ATC·$3H_2O$, 0.3% corn steep liquor, 0.3% urea, 0.1% $MnSO_4 \cdot 5H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.15% NaCl, and 0.3% K_2HPO_4.

Inoculum preparation and cultivation. An inoculum from a loop full of cells from a slant was transferred into a 500-ml flask containing 30 ml of seed medium and was allowed to grow for 12 h at 29°C at 190 rpm in a rotary shaker. To produce enzyme, the inoculum seed (3 ml) was transferred to a 500-ml flask containing 30 ml of production medium and was cultivated at 29°C at 190 rpm for 22 h.

Microbial transformation. The whole culture was centrifuged for 10 min at 4000*g* at 4°C. The cells were washed twice with 30 ml of 0.2 M phosphate buffer (pH 7.4), resuspended in 30 ml of 0.2 M phosphate buffer (pH 7.4), and stored at 4°C to be used for subsequent experiments. To produce L-cysteine, the resuspended cells (30 ml) were transferred to a 250-ml centrifuge tube containing 60 ml of 0.9% DL-ATC·$3H_2O$ and 0.9% K_2HPO_4, and they were maintained at 42°C in a water bath for 3 h.

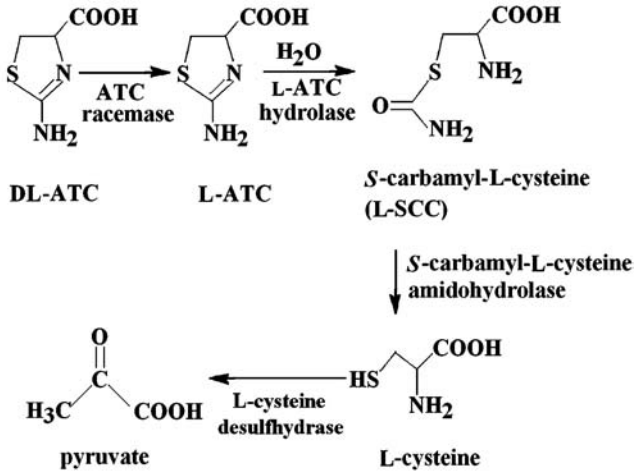
Dry mass determination. The cell culture was centrifuged for 10 min at 5000*g*. The pellet was washed twice with deionized water and dried at 80°C. The mass of the pellet was determined after drying. The procedure was continued until a constant mass was obtained.

L-Cysteine concentration in the enzymatic reaction was quantitatively determined by acidic ninhydrin solution methods [17].

DL-ATC concentration in the enzymatic reaction was quantitatively determined by HPLC. An Agilent Technology 1100 Series HPLC system (Wilmington, DE, USA) equipped with a quaternary pump, degasser, sampling injector, and UV detector system was used for identification of DL-ATC. DL-ATC was separated using a Spherisorb ODS(2) column, 5 μm, 250 × 4.6 mm, with a C18 guard column (Waters, USA). The column was kept at room temperature (about 25°C). The mobile phase was a mixture of MeCN–H_2O with a volume ratio of 1 : 1 at a flow rate of 0.8 ml/min. The injection volume was 20 μl. The detector was set at 217 nm.

Metabolic pathway of microbial enzymatic conversion of DL-ATC to L-cysteine. It is known that bacteria convert DL-ATC to L-cysteine through two pathways—the N-metabolic pathway [18, 19] and the S-metabolic pathway [20]. Both N-carbamyl-L-cysteine (L-NCC) and S-carbamyl-L-cysteine (L-SCC) are intermediates that can be hydrolyzed to form L-cysteine by its corresponding enzyme. As Yu et al. reported in 2006 [21], *Pseudomonas* sp. TS1138 converts DL-ATC to L-cysteine through the S-metabolic pathway (the Scheme).

There are three enzymes with concerted catalysis in the S-metabolic pathway—ATC racemase (EC 5.1.1.10), L-ATC hydrolase (EC 3.5.2.7), and S-carbamyl-L-cysteine amidohydrolase (EC 3.5.1.87). ATC racemase converts DL-ATC to L-ATC, and L-ATC hydrolase hydrolyzes the C=N double bond of the ATC thiazole ring to produce S-carbamyl-L-cysteine as an intermediate. Then, S-carbamyl-L-cysteine amidohydrolase hydrolyzes the intermediate to produce L-cysteine. L-Cysteine desulfhydrase (CD, EC 4.4.1.1) is also involved



Metabolic pathway of microbial enzymatic conversion of DL-ATC to L-cysteine in *Pseudomonas* sp. TS1138. The three enzymes—ATC racemase, L-ATC hydrolase, and S-carbamyl-L-cysteine amidohydrolase—were regarded as one total enzyme called L-cysteine synthetase (CS) in this study

in the studied cells (including the S-metabolic pathway and the N-metabolic pathway) and hydrolyzes L-cysteine to give pyruvate, hydrogen sulfide, and ammonia [22]. Because the concentration of S-carbamyl-L-cysteine is very low, it could not be determined easily in the enzymatic reaction system, so the three enzymes above, ATC racemase, L-ATC hydrolase, and S-carbamyl-L-cysteine amidohydrolase, can be regarded as one total enzyme called L-cysteine synthetase (CS). Therefore, the conversion of DL-ATC to pyruvate can be seen as a two-step reaction, the conversion of DL-ATC to L-cysteine by CS, followed by the hydrolysis of L-cysteine to pyruvate by CD.

RESULTS AND DISCUSSION

Kinetics of CS. The CS reaction is based on Michaelis–Menten kinetics, with noncompetitive inhibition of CS by L-cysteine. The overall kinetics for the conversion of DL-ATC to L-cysteine is as follows:

$$r_{Cys} = \frac{V_{mCS}}{(1 + K_{mCS} C_{ATC}^{-1})(1 + C_{Cys} K_{Cys}^{-1})}, \quad (1)$$

where r_{Cys} is the rate of L-cysteine synthesis by CS, V_{mCS} is the maximum velocity of L-cysteine synthesis, K_{mCS} is the Michaelis constant of CS, C_{ATC} is the concentration of DL-ATC, C_{Cys} is the concentration of L-cysteine, and K_{Cys} is the inhibition constant of CS for L-cysteine. The values of V_{mCS} and K_{mCS} were determined by the Lineweaver–Burk method (Fig. 1). The value of V_{mCS} was 0.024 mmol/liter per min, and the value of K_{mCS} was 7.06 mM. During the process of the enzymatic reaction, the concentrations of DL-ATC and L-cysteine were measured (the table). By fitting the calculated rate to r_{Cys} (in Eq. (1)) using the experimental data from the table, K_{Cys} was estimated to be 3.60 mM.

CD kinetics. The CD reaction is also based on Michaelis–Menten kinetics, and the kinetics for the conversion of L-cysteine to pyruvate is as follows:

$$r_{pyr} = \frac{V_{mCD}}{1 + K_{mCD} C_{Cys}^{-1}}, \quad (2)$$

where r_{pyr} is the rate of pyruvate synthesis by CD, V_{mCD} is the maximum velocity of pyruvate synthesis, and K_{mCD} is the Michaelis constant of CD. The value of K_{mCD} is 21.9 mM, as reported by Pae [23].

Elasticity coefficients. The formulation of the pathway reactions in terms of a mathematical model enabled a quantitative description of the pathway dynamics. Thus, the rate expressions for the individual steps in the pathway were used to derive expressions for the elasticity coefficients. For a linear pathway consisting of L enzymatic steps, the elasticity coefficient for the ith reaction with respect to the jth intermediate is given by:

$$\varepsilon_i^j = \frac{C_j}{r_i} \frac{\partial r_i}{\partial C_j},$$
$$i = 1, ..., L, \quad j = 1, ..., L-1, \quad (3)$$

where C_j is the intracellular concentration of the jth pathway intermediate, and r_i is the rate of the ith pathway reaction.

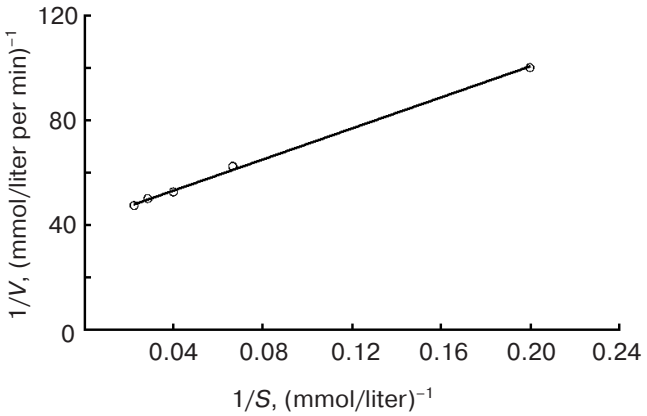


Fig. 1. Lineweaver–Burk plot for calculation of kinetic parameters for CS. The plot of $1/V$ versus $1/S$ gives a straight line (y = 293.57x + 41.562) with a slope of K_{mCS}/V_{mCS} and a y-intercept of $1/V_{mCS}$. Experimental data (open circles) and linear approximation (solid line) are shown.

Concentrations of DL-ATC and L-cysteine in the enzymatic reaction

Reaction time, min	[L-Cysteine], mM	[DL-ATC], mM
10	0.0903	20.0
20	1.4763	12.7
30	3.9392	6.9
40	5.5415	6.4
50	8.2563	3.8
60	10.4953	3.6
70	11.3349	3.0
80	12.4544	3.0
90	14.9732	2.6
100	17.7720	2.7
110	24.1251	2.4
120	23.6493	1.8
130	22.8937	1.9
140	22.8097	2.0
150	24.3490	1.8
160	18.3317	1.7
170	17.9119	1.7
180	17.7160	1.5

Next, the elasticity coefficients for CS and CD were derived from the kinetic expressions (Eqs. (1) and (2)):

$$\varepsilon_{CS}^{Cys} = -\frac{C_{Cys}K_{Cys}^{-1}}{1+C_{Cys}K_{Cys}^{-1}}, \quad (4)$$

$$\varepsilon_{CD}^{Cys} = \frac{K_{mCD}}{K_{mCD}+C_{Cys}}, \quad (5)$$

where ε_{CS}^{Cys} is the elasticity coefficient for CS and ε_{CD}^{Cys} is the elasticity coefficient for CD. Substitution of K_{Cys} = 3.60 mM and K_{mCD} = 21.9 mM in Eqs. (4) and (5) gives the following equations:

$$\varepsilon_{CS}^{Cys} = -\frac{C_{Cys}}{C_{Cys}+3.60}, \quad (6)$$

$$\varepsilon_{CD}^{Cys} = \frac{21.87}{C_{Cys}+21.87}. \quad (7)$$

From the measurements of L-cysteine concentrations (the table), the elasticity coefficients were calculated using Eqs. (6) and (7) during the microbial enzymatic conversion (Fig. 2). It was observed that the elasticity coefficient for CS was negative, i.e. an increasing concentration of L-cysteine diminished the rate of L-cysteine synthesis by CS, and the numerical value of the elasticity coefficient increased throughout the enzymatic conversion because of the increasing L-cysteine concentration. The elasticity coefficient for CD was positive, i.e. the effect of increasing L-cysteine concentration was positive, but its value decreased throughout the enzymatic conversion due to CS becoming saturated with L-cysteine.

Flux control coefficients. Flux control coefficients can be determined experimentally, but this is very laborious because it requires independent variation of the activity *in vivo* of all the enzymes within the pathway. Therefore, MCA is often based on kinetic information of the individual reactions from which the elasticity coefficients are derived, and thereafter the flux control coefficients can be calculated using the summation and connectivity theorems.

For a linear pathway consisting of L enzymatic steps, the flux control coefficient is defined by:

$$C_i^J = \frac{E_i}{J}\cdot\frac{\partial J}{\partial E_i}, \quad i = 1, ..., L, \quad (8)$$

where J is the steady state flux, and E_i is the activity of the ith enzyme (which is assumed to be proportional to the net rate r_i of the ith pathway reaction). As a consequence of the normalization of the flux control coefficients, they all sum to one. Furthermore, the flux control coefficients

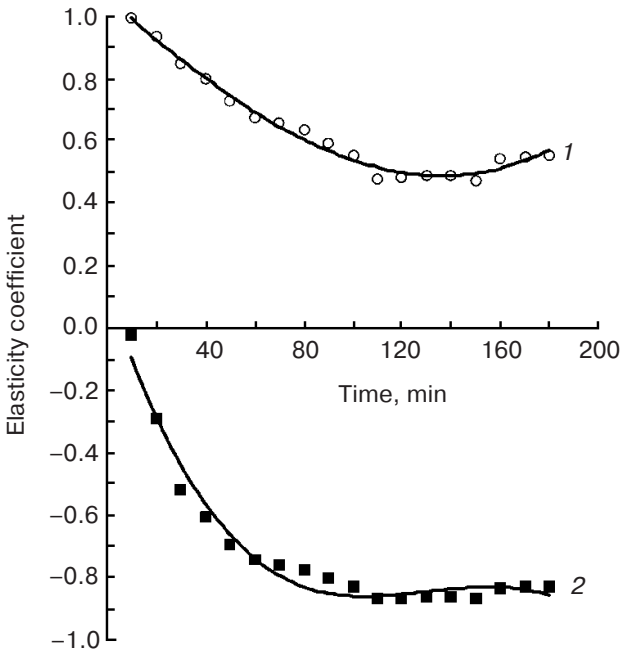


Fig. 2. Elasticity coefficients for the two enzymes CS and CD during the enzymatic conversion. The elasticity coefficient for CD (*1*) is positive, and the elasticity coefficient for CS (*2*) is negative. The solid line is a linear approximation of the experimental data.

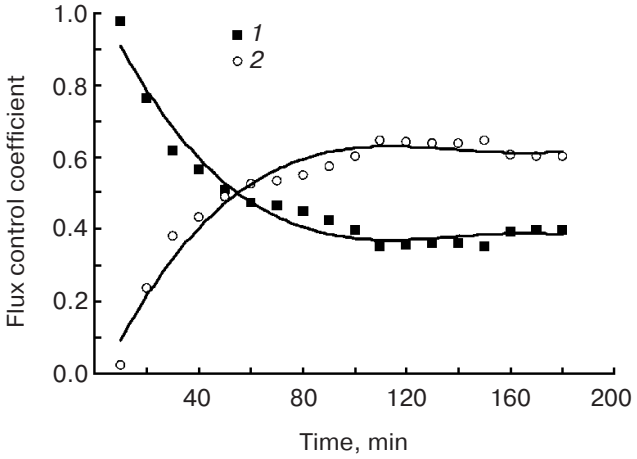


Fig. 3. Flux control coefficients for the two enzymes CS (*1*) and CD (*2*) during the enzymatic conversion. The flux control coefficients for CS are shown by filled squares, and the flux control coefficients for CD are shown by open circles. The solid line is a linear approximation of the experimental data. The flux control is shifted from CS to CD at about 50th min.

and the elasticity coefficients are related through the so-called flux control connectivity theorem [12]. In this study, the pathway was a two-step reaction; therefore, these constraints can be summarized as:

$$\begin{pmatrix} 1 & 1 \\ \varepsilon_{CS}^{Cys} & \varepsilon_{CD}^{Cys} \end{pmatrix} \begin{pmatrix} C_{CS}^{J} \\ C_{CD}^{J} \end{pmatrix} = \begin{pmatrix} 1 \\ 0 \end{pmatrix}, \qquad (9)$$

where C_{CS}^{J} is the flux control coefficient for CS, and C_{CD}^{J} is the flux control coefficient for CD. Then, the solutions of Eq. (9) are as follows:

$$C_{CS}^{J} = \frac{\varepsilon_{CD}^{Cys}}{\varepsilon_{CD}^{Cys} - \varepsilon_{CS}^{Cys}}, \qquad (10)$$

$$C_{CD}^{J} = -\frac{\varepsilon_{CS}^{Cys}}{\varepsilon_{CD}^{Cys} - \varepsilon_{CS}^{Cys}}. \qquad (11)$$

From the elasticity coefficients, the flux control coefficients were calculated using Eqs. (10) and (11), and the results are shown in Fig. 3.

Initially, as shown in Fig. 3, the flux control coefficient for CS was high (close to 1) since there was very little inhibition from the intermediate L-cysteine. However, when the L-cysteine concentration increased, the flux control was shifted from CS to CD, and after about 50 min, the flux was mainly controlled by CD. With the shift in flux control from CS to CD, neither of the two enzymes could be identified as the rate-limiting step. *Pseudomonas* sp. TS1138 was isolated from the DL-ATC production workshop, and the environment lacked sufficient carbon and nitrogen sources for growth and metabolism. Therefore, DL-ATC became the source of carbon and nitrogen. L-Cysteine is produced from DL-ATC, and L-cysteine is converted to pyruvate, hydrogen sulfide, and ammonia by L-cysteine desulfhydrase. Pyruvate is the key intermediate for the main metabolism of a strain, which can be converted to some amino acids and glucides for cell construction and growth. So, the L-cysteine desulfhydrase gene could not be knocked out to increase the production of L-cysteine during the enzymatic conversion by *Pseudomonas* sp. TS1138. Thus, the best strategy to enhance L-cysteine production is to reduce the activity of CD by site-directed mutagenesis.

This work was financially supported by the Foundation for Application and Basic Research of the Scientific Committee of Tianjin, China (grant 05YFJZJC00901).

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