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Precursor and Cofactor as a Check Valve for Cephamycin Biosynthesis in *Streptomyces clavuligerus*

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The biosynthesis of secondary metabolites is closely linked to primary metabolism via the supply of precursors, cofactors, and cellular energy. The availability of these precursors and cofactors can potentially be rate-limiting for secondary metabolism. A combined experimental and kinetic modeling approach was used to examine the regulation of flux in the cephamycin biosynthetic pathway in *Streptomyces clavuligerus*. The kinetic parameters of lysine 6-aminotransferase (LAT), the first enzyme leading to cephamycin biosynthesis and one which was previously identified as being a rate-limiting enzyme, were characterized. LAT converts lysine to α-aminoadipic acid using α-ketoglutarate as a cosubstrate. The K_m values for lysine and α-ketoglutarate were substantially higher than those for their intracellular concentrations, suggesting that lysine and α-ketoglutarate may play a key role in regulating the flux of cephamycin biosynthesis. The important role of this precursor/cosubstrate was supported by simulated results using a kinetic model. When the intracellular concentrations and high K_m values were taken into account, the predicted intermediate concentration was similar to the experimental measurements. The results demonstrate the controlling roles that precursors and cofactors may play in the biosynthesis of secondary metabolites.

Introduction

Secondary metabolites produced by microorganisms and plants constitute some of the most important pharmaceutical drugs. Most secondary metabolite producing organisms synthesize them at relatively very low productivity and yields. To enhance their production efficiency by metabolic engineering, it is critical to decipher the regulatory mechanism controlling the biosynthesis of secondary metabolites. The biosynthesis of a secondary metabolite can be regulated at least at four different levels. Highest in the hierarchy of regulation is the influence of global and specific regulators of secondary biosynthetic gene expression. These genetic elements, although poorly understood, are capable of influencing biosynthetic rates significantly in microorganisms that produce secondary metabolites (Champness and Chater, 1994; Chater and Bibb, 1997). At the second level are the structural genes and their protein products that specify the biosynthetic pathway of a particular secondary metabolite. At this level, the activities of the enzymes constituting the pathway influence the rate of secondary metabolite production. At the interface between secondary and primary metabolism are the cofactors and precursors for secondary metabolite biosynthesis, whose supply rate represents a third level of cellular control. Finally, at the fourth level, the rate an individual cell can effectively transport the metabolites out is yet another controlling point on the production of secondary metabolites.

β-Lactams are among the most important secondary metabolites produced on a commercial scale. Notable examples of *β*-lactam antibiotics are penicillin, cephalosporin, and their derivatives. Their producers include both the fungi and procaryotic microorganisms (Aharonowitz et al., 1992; Martin, 1998). Over the years, productivity and yields of these antibiotics have increased by orders of magnitude, mainly by strain and process improvement. In the past, this strain improvement has largely been achieved by random mutagenesis (Vournakis and Elander, 1983). Over the past two decades, the biosynthetic pathways, the enzymes involved, and the associated genetic structures have been elucidated in many producing microorganisms (Aharonowitz et al., 1992; Martin, 1998). This has made a more rational approach to engineering the production of *β*-lactam antibiotics feasible. We have been investigating the metabolic control operative in the cephamycin biosynthetic pathway in *Streptomyces clavuligerus*.

Lysine, cysteine, and valine are the precursors for cephamycin C biosynthesis in *Streptomyces clavuligerus*

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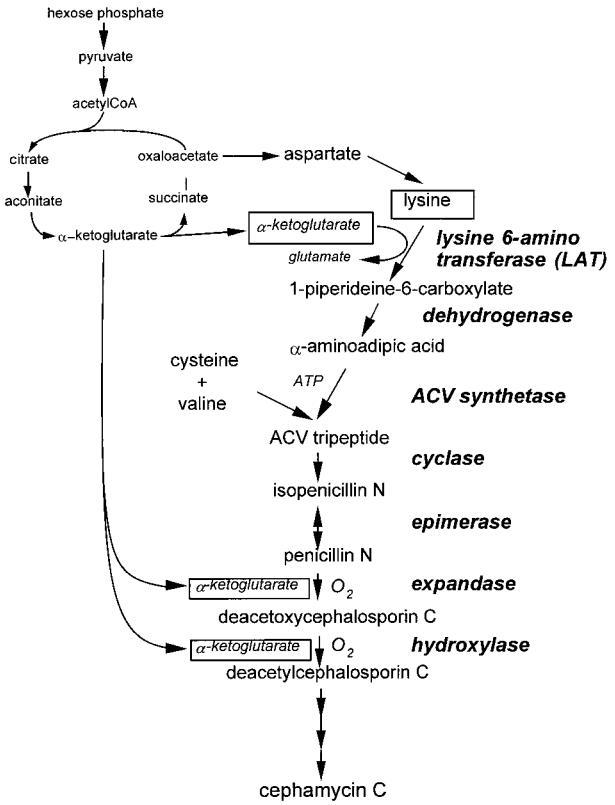

Figure 1. Cephamycin C biosynthetic pathway in *S. clavuligerus*. Lysine, cysteine, and valine are the three primary precursors for cephamycin C biosynthesis. α-Ketoglutarate is used as a cofactor by four enzymes in the pathway, three of which are shown.

(Figure 1). In the first steps in the cephamycin C biosynthesis, lysine is converted to α-amino adipic acid (α-AAA) through a two step conversion. The α-AAA then undergoes a condensation reaction with two amino acids, cysteine and valine, in a reaction catalyzed by a multifunctional ACV synthetase, to form tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). A cyclase converts the ACV tripeptide to yield a lactam ring-containing compound, isopenicillin N. This intermediate is then converted to cephamycin C by a series of reactions mediated by six other enzymes (Figure 1).

The deamination of lysine by lysine-6-aminotransferase (LAT) to form 1-piperidiene-6-carboxylate marks the first committed step of cephamycin biosynthesis in *Streptomyces clavuligerus*. LAT uses α-ketoglutarate as a cosubstrate and pyridoxyl phosphate as a cofactor (Kern et al., 1980). The second step is mediated by piperideine-6-carboxylate dehydrogenase (PCD), which converts 1-piperideine-6-carboxylate to α-aminoadipic acid and requires NAD as a cofactor (de La Fuente et al., 1997). The genes for LAT and PCD are clustered with the genes for the rest of the cephamycin biosynthesis enzymes in *S. clavuligerus* (Madduri et al., 1991). The introduction of an additional copy of the LAT gene resulted in an enhancement of LAT activity and a 2−5-fold increase of cephamycin titer (Malmberg et al., 1993; Khetan et al., 1996). The addition of lysine to culture medium has also been shown to lead to enhanced fluxes (Mendelovitz and Aharonowitz, 1982). The results, along with a theoretical analysis (Malmberg and Hu, 1991), suggest that LAT-catalyzed conversion of lysine to α-AAA is a rate-limiting step in cephamycin biosynthesis.

Combining kinetic analysis and experimentation, we investigated the roles that lysine and α-ketoglutarate played in the regulation of cephamycin C biosynthesis. The results are presented in this report.

Materials and Methods

Microorganisms, Medium, and Culture Conditions. *Streptomyces clavuligerus* NRRL 3585 was used in this study. The chemically defined medium of Aharonowitz and Demain was used for all experiments (Aharonowitz and Demain, 1978). Batch cultures were carried out at 29 °C and 250 rpm in baffled Erlenmeyer shake flasks as described previously (Malmberg et al., 1995; Zhang et al., 1989). Spores (10^8) were innoculated into 50 mL of seed medium in a 250 mL Erlenmeyer flask. The seed medium consisted of the defined medium supplemented with 1 g/L Bacto Yeast Extract and 1 g/L NH_4Cl. A 2% innoculum from the seed culture at its late exponential growth was used to initiate the fermentation. The defined medium which consisted of the following (per liter) was used in fermentation: glycerol, 10.0 g; L-asparagine, 2.0 g; K_2HPO_4, 3.5 g; $MgSO_4 \cdot 7H_2O$, 1.23 g; 4-morpholinepropanesulfonic acid (MOPS), 20.9 g; and trace salts solution, 1 mL (0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MnC1_2 \cdot 4H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, and 0.1 g of $CaCl_2$ per 100 mL of water).

Samples were taken periodically and centrifuged at 3000*g* for 10 min. The pelleted biomass was washed twice with water and dried at 80 °C overnight for dry cell weight measurement. The supernatant was used to measure the cephamycin C titer by the agar plate diffusion assay (Hu et al., 1984) using *Escherichia coli* ESS, a gift of A. L. Demain (Massachusetts Institute of Technology, Cambridge, MA).

LAT Activity. Mycelia were pelleted by centrifugation at 3000*g* for 10 min and washed with 8.5 g/L NaCl solution. After resuspending in 0.2 M potassium phosphate buffer (pH 7.5), the cell suspension was sonicated in an ice/water bath with a Biosonik sonicator (Bronwil Scientific, NJ) at 30% power for 1 min. LAT activity was measured by a modification of the method previously reported (Kern et al., 1980). Cell debris was removed by centrifugation at 14000*g* for 20 min and 4 °C. The reaction mixture for LAT assay consisted of 500 μL of cell extract (approximately 2 mg of total protein/mL), 40 μL of 1.0 M L-lysine, 80 μL of 1.0 M disodium α-ketoglutarate, 20 μL of 3.75 mM pyridoxal phosphate, and 360 μL of 0.2 M potassium phosphate buffer (pH 7.5) in a final volume of 1.0 mL. The reaction mixture was incubated at 30 °C for 60 min. The reaction was terminated by the addition of 0.5 mL of a 1:10 mixture of 50 g/L trichloroacetic acid and absolute ethanol. Precipitated protein was removed by centrifugation at 14000*g* for 20 min at 4 °C. The supernatant (0.5 mL) was mixed with 0.75 mL of 4.0 mM *o*-aminobenzaldehyde in 0.2 M phosphate buffer (pH 8) and incubated at 37 °C for 1 h. The derivatized product was quantified by measuring the absorbance at 465 nm using an extinction coefficient of 2800 L mol^{-1} cm^{-1} (Fothergill and Guest, 1977). Total protein in the cell extract was quantitated by the Bradford assay (Bradford, 1976) using Bovine Serum Albumin as the standard.

Cell Extraction for Amino Acid and α-Ketoglutarate Analysis. Trichloroacetic acid (TCA) was used for extraction of intracellular amino acids as described earlier (Malmberg et al., 1995). α-ketoglutarate extraction was performed according to the procedure of Burlina (Burlina, 1983). Twenty milliliters of cell broth was filtered and washed three times with 0.1 M KCl (in less than 2 min). The mycelial mass was collected and

immediately resuspended in 500 μL of 1.0 M perchloric acid and placed in an ice−water bath for 1 h. The mixture was precipitated after centrifugation at 12000*g* for 15 min at 4 °C. The supernatant was carefully separated from the pellets, which were saved at −20 °C for total protein assay. Five-hundred microliters of 1.0 M potassium phosphate buffer (pH 7.5) was added to the supernatant to neutralize the solution. After being kept in an ice bath for 15 min, the mixture was centrifuged at 12000*g* for 15 min at 4 °C to remove any precipitates. The supernatant was carefully removed and stored at −20 °C until assay. For the total protein assay, the saved pellets were resuspended in 5 mL of 1.0 M NaOH and heated to 100 °C for 15 min. The dissolved protein concentration was determined by the method of Bradford (Bradford, 1976) using BSA as standard and later used to normalize the intracellular content of α-ketoglutarate.

Analysis of Intracellular Amino Acids and α-Ketoglutarate. The amino acid concentration in the deproteinated cell extract was determined by HPLC after derivatization with *o*-pthaldialdehyde as described previously (Malmberg et al., 1995). A protein content of 0.55 mg of protein/mg of dry cell weight and a specific cell volume of 2 μL/mg dry cell weight was assumed (Malmberg and Hu, 1991), to convert the amino acid measurement to an intracellular concentration.

α-Ketoglutarate concentration was measured by an end-point fluorometric assay (Williamson and Corkey, 1969). The sample was diluted with 0.1 M potassium phosphate buffer such that the total volume was 2 mL. Ten microliters of NADH (1 mg/mL) was added, and the fluorescence was recorded. Ten microliters of Glutamate dehydrogenase (GDH, 20 mg protein/mL at specific activity of 120 U/mg, Boehringer Mannheim, Mannheim) was added, and the decrease in fluorescence was determined after completion of reaction. Fluorescence of NADH was measured using an LS-50 spectrofluorometer (Perkin-Elmer, CT). The monochromator excitation wavelength was 360 nm, while the emission was monitored at 460 nm. The α-ketoglutarate concentration was determined by the measured depletion of NADH. Data recorded were averages of duplicate (for α-ketoglutarate) and triplicate (for all amino acids) experimental determinations. Standard deviations for all data were within 12%.

Modeling or Theoretical Aspects

A kinetic model previously developed (Malmberg and Hu, 1991) for describing cephamycin biosynthesis from α-aminoadipic acid, cysteine, and valine was extended to include the synthesis of α-aminoadipic acid from lysine.

The reaction mechanism of lysine 6-aminotransferase (LAT) in *S. clavuligerus* has not been studied. However, on the basis of analogy with other pyridoxyl phosphate requiring aminotransferases (Velick and Vavra, 1962) as well as LAT from *Achromobacter liquidum* (Soda et al., 1968), it can be assumed that the enzyme reaction follows a ping−pong bi bi mechanism. Although rigorous kinetic expressions for ping−pong-type enzymes are available, for whole pathway flux modeling it is adequate to use the simpler multiplicative form of saturation type or Michaelis−Menten type kinetics (Malmberg and Hu, 1991). LAT from *A. liquidum* has also been reported to be virtually irreversible (Yagi et al., 1991). The rate expression for LAT was assumed to obey the following relation

$$r_{LAT} = V_{max}\left(\frac{S_{Lys}}{K_{Lys} + S_{Lys}}\right)\left(\frac{S_{KG}}{K_{KG} + S_{KG}}\right) \quad (1)$$

where K_{Lys} and K_{KG} are the apparent Michaelis−Menten constants with respect to the substrate lysine and cofactor α-ketoglutarate respectively and V_{max} is the maximum activity. The effect of the second enzyme piperideine-6-carboxylate dehydrogenase (PCD) was not explicitly accounted for in the model. PCD activity that has been reported in the literature (de La Fuente et al., 1997) is 10-fold of the LAT activity reported here. Also the K_m value of PCD for its substrate piperideine-6-carboxylate is a relatively low value of 14 μM (de La Fuente et al., 1997). Keeping these data in mind, this reaction step was assumed to be non-rate-limiting.

The model consists of a set of first-order ordinary differential equations (Malmberg and Hu, 1991; Khetan et al., 1996), describing the rate of change in intermediate concentrations. The intracellular concentration of each intermediate is a balance of its synthesis, consumption by subsequent reactions, and the dilution due to biomass expansion.

$$\frac{dC_i}{dt} = \sum_{j=1}^{k} \alpha_{ij} r_j - \mu C_i \quad i = 1,...n \quad (2)$$

where C_i denotes the intracellular concentration of the reaction intermediate i, n is the number of intermediates in the system, r_j the activity of the enzymes catalyzing the reaction j, k the number of reactions in the system, α_{ij}, the stoichiometric coefficient, and μ, the specific growth rate. The specific growth rate, μ, is defined as the following:

$$\mu = \frac{1}{x}\frac{dx}{dt} \quad (3)$$

In comparison to the earlier model (Malmberg and Hu, 1991), the present one has an additional equation to account for the conversion of lysine to α-aminoadipic acid, with the assumption that the first reaction catalyzed by LAT is controlling of the two reactions:

$$\frac{d[AAA]}{dt} = r_{LAT} - r_{ACVS} - k_{AAA}[AAA] - \mu[AAA] \quad (4)$$

r_{LAT} and r_{ACVS} represent the rates of the reactions catalyzed by LAT and ACV synthetase, respectively. An additional term was added to account for the excretion of α-aminoadipic acid into the medium, as experimental results indicate that the excretion rate can be significant (Malmberg et al., 1995). It was assumed that the excretion rate was linearly dependent on the intracellular concentration. k_{AAA} is the transport rate constant. The expression for the rate law of ACV tripeptide formation, r_{ACVS}, was assumed to be a multiplicative type as enunciated earlier (Malmberg and Hu, 1991). In the present model, a term for excretion of ACV into the medium was incorporated. This modified equation is as follows

$$\frac{d[ACV]}{dt} = r_{ACVS} - r_{IPNS} - k_{ACV}[ACV] - \mu[ACV] \quad (5)$$

r_{ACVS} and r_{IPNS} represent the rates of the reactions catalyzed by ACV synthetase and isopenicillin N synthetase, respectively. k_{ACV} is the transport rate constant for ACV. k_{AAA} and k_{ACV} were estimated to be 2.8×10^{-4} and 0.3 min^{-1} respectively, by fitting the intracellular

Table 1. Known Michaelis Constants for the Enzymes in the Cephamycin C Biosynthetic Pathway

enzyme	substrate	K_m (μM)	reference
LAT	lysine	1200	this paper
	α-ketoglutarate	8600	
dehydrogenase	piperideine-6-carboxylic acid	14	(de La Fuente et al., 1997)
	NAD	115	
ACV synthetase	α-aminoadipic acid	560	(Jensen et al., 1988)
	cysteine	70	
	valine	1140	
cyclase	ACV tripeptide	320	(Jensen et al., 1986)
epimerase	isopenicillin N	300	(Usui and Yu, 1989)
	penicillin N	780	
DAOCS	penicillin N	29	(Dotzlaf and Yeh, 1989)
	α-ketoglutarate	18	
DACS	DAOC	59	(Baker et al., 1991)
	α-ketoglutarate	10	

data to the experimentally measured rates of excretion of α-AAA and ACV (data not shown). The other equations accounting for the balance on the intermediates downstream (IPN, Pen N, DAOC, and DAC) remain as described before (Malmberg and Hu, 1991).

As in the development of the previous model, a number of assumptions were made (Malmberg and Hu, 1991). The kinetic data determined in vitro are assumed applicable in vivo, and cofactors other than α-ketoglutarate are present in saturating concentrations. The steps downstream of deacetylcephalosporin C are also assumed to be non-rate-limiting.

Experimentally determined time profiles of intracellular concentrations of the precursors lysine, cysteine, and valine, α-ketoglutarate concentration, and the specific growth rate were used for the solution of the equations. In addition, enzyme activities determined in vitro and kinetic constants of enzymes as shown in Table 1 were also used. To solve the system of equations, we carried out numerical integration of these simultaneous nonlinear differential equations using the DDASSL integrator-based program DDASAC (Caracotsios and Stewart, 1985). The program enables the simultaneous derivation of the flux and the time profiles of the intermediate metabolites. The DDASSL integrator, which employs the use of predictor−corrector integration method based on Gear's approach, is capable of handling stiff systems of coupled ordinary differential and algebraic equations.

Results

To obtain the kinetic parameters of LAT, we measured the apparent K_m for lysine and α-ketoglutarate using cell free extract. The concentrations of lysine and α-ketoglutarate were varied independently, and the initial reaction rates were measured. The results are shown in Figure 2. The data were fit with multiplicative type reaction kinetics as shown in eq 1. The parameter values were estimated on MATLAB (The MathWorks, Inc., Natick, MA). The best-fit values were determined by local minimization of the squared error between the set of experimentally determined values and those obtained by calculation, using a given set of the three parameters in eq 1. The apparent K_m values obtained were 1.2 and 8.6 mM for lysine and α-ketoglutarate, respectively. Apparent K_m values of 3.2 mM for lysine and 3.6 mM for α-ketoglutarate have been reported using partially purified LAT (Romero et al., 1997).

The requirement of the enzyme for α-ketoglutarate as an amino group acceptor appeared to be specific, as has been reported previously for LAT from *Nocardia lactamdurans* (Kern et al., 1980). Replacing α-ketoglutarate with pyruvate at equal concentrations reduced the amino-

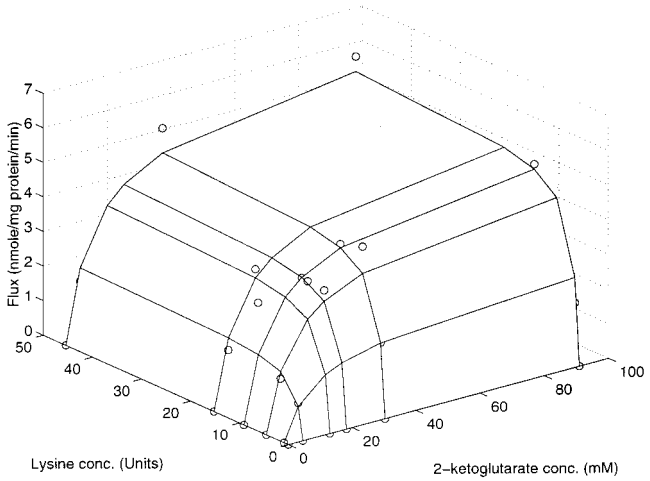

Figure 2. Characterization of the kinetics of the LAT enzyme. A relatively high K_m value of 8.6 mM was identified for the cofactor α-ketoglutarate.

transferase activity by more than 90%. In addition, the reverse reaction was not appreciable at up to 40 mM glutamate concentrations.

For simulation of the cephamycin biosynthesis, the time profiles of the enzyme activity were necessary. As previously shown, literature values of in vitro determined enzyme activities can be used to adequately describe the production kinetics of cephamycin C (Malmberg and Hu, 1991). For extension of the kinetic model, LAT activity during the fermentation was required. The LAT activity profile was determined using culture conditions reported earlier for measuring the activities of ACVS, IPNS, Epimerase, and DACS (Zhang et al., 1989). Figure 3b shows the LAT enzyme dynamics along with those of other enzymes.

The intracellular concentrations of the three precursors, lysine, cysteine, and valine, were measured under growth conditions as described earlier (Malmberg et al., 1995) (Figure 3c). The intracellular concentration of lysine stayed relatively constant at around 0.15 mM in the early growth phase but increased to about 0.5 mM toward the end of the growth. The valine concentration varied from 0.6 to 1.1 mM in the early growth stage and increased to 2.0 mM in the late growth phase, followed by a decrease in the stationary phase. The cysteine concentration was about 0.5 mM in most growth periods and dropped to approximately 0.25 mM in late growth and stationary phases. For the simulation of cephamycin biosynthesis, the time profiles of enzyme activities and precursor concentrations need to combined. The cultures used for intracellular amino acid measurement showed a different duration of lag phase as compared to cultures

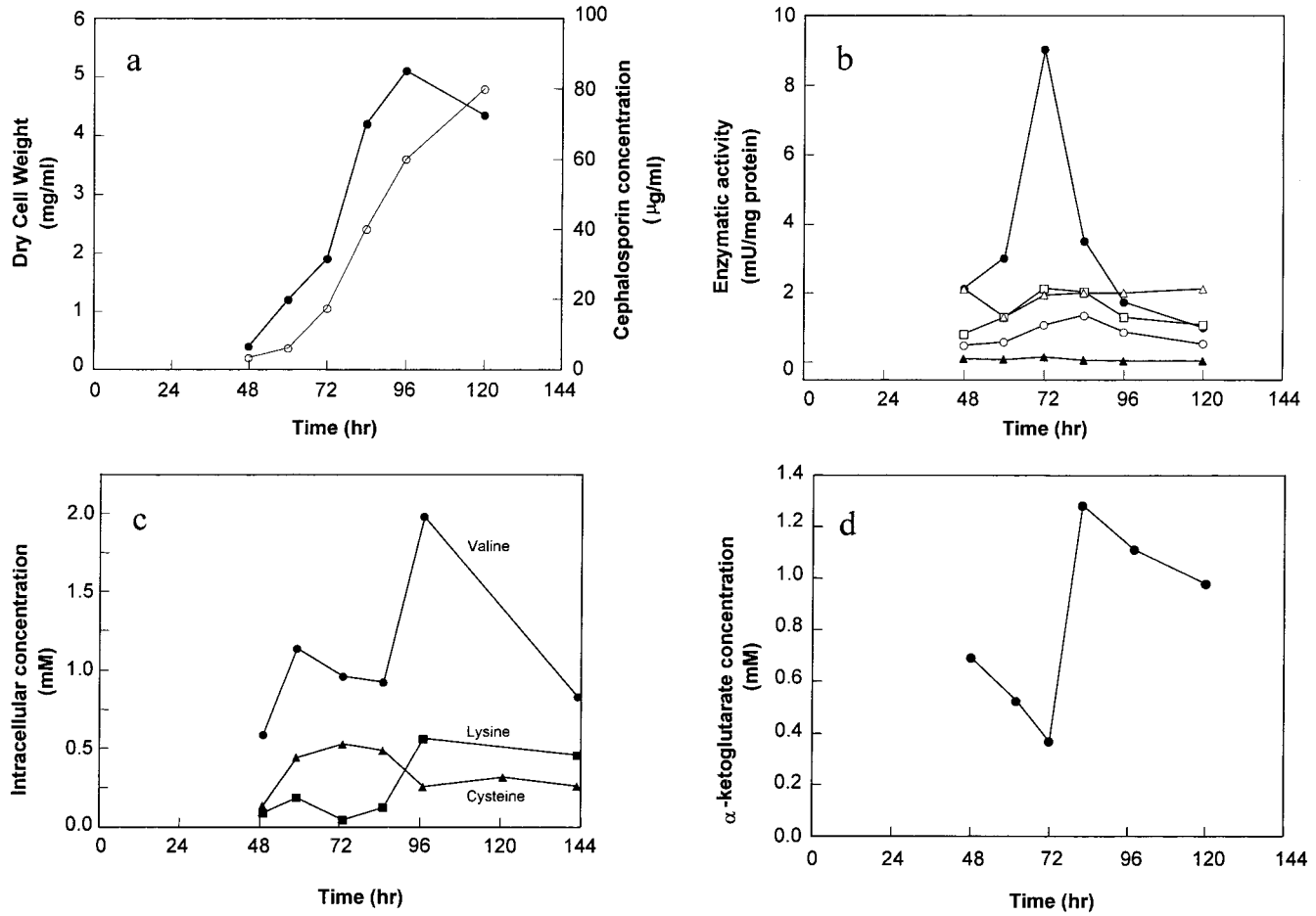

Figure 3. (a) Growth of *S. clavuligerus* (●) and the production of cephamycin C (○) in shake flask cultures. (b) Activities of biosynthetic enzymes in the cephamycin C biosynthetic pathway: LAT (●), ACV synthetase (▲), cyclase (□), epimerase (△), and expandase (○). LAT activity was determined as described in the materials and methods while the data for other enzymes was incorporated from Zhang et al., 1989. One unit of enzyme activity corresponds to 1 μmol of product formed per minute. (c) Intracellular pools of lysine (■), cysteine (▲), and valine (●), which are the primary precursors of the cephamycin C biosynthetic pathway. The time profile has been shifted 25 h to match growth profile as explained in the text. (d) Intracellular α-ketoglutarate levels measured using a fluorometric assay.

used for enzyme activity measurements. To align the amino acid intracellular data such that growth profiles matched in all experiments, we introduced a time shift of 25 h.

In the model reported previously, the concentrations of cofactors including α-ketoglutarate for expandase and hydroxylase, ATP for the tripeptide condensation reaction, and oxygen for the cyclase and oxygenases were all assumed to be at saturation levels. However, the measured K_m value of α-ketoglutarate for LAT was relatively high. This prompted us to speculate that the intracellular concentration of α-ketoglutarate may not be at saturation level. The intracellular α-ketoglutarate concentration was measured as described in Materials and Methods. In the early growth phase the concentration ranged from 0.3 to 0.7 mM. The concentration increased to 1.0−1.3 mM in the later stages of growth (Figure 3d).

These experimentally measured enzyme activities, precursors, and α-ketoglutarate concentrations were used to simulate the kinetics of cephamycin C biosynthesis. The simulated results were compared to the specific productivity obtained experimentally (Figure 4). The simulated time profile of the specific productivity followed the same trend as that calculated from experimental measurement. A 3-fold difference in numerical value was observed. It should be noted that the specific production rate was calculated from the cephamycin titer measured using cephalosporin C as a standard in bioassay. The nearly 3-fold difference in the bacteriocidal activity against ESS between cephamycin C and cephalosporin C (Aharonowitz and Demain, 1978) could have accounted for the difference in simulated and experimentally obtained specific rates.

To evaluate the effect of α-ketoglutarate on the flux through LAT, we simulated the intracellular α-AAA concentration using experimentally determined values and compared it to one assuming that α-ketoglutarate is at a saturation level. These simulations were compared to the intracellular concentration of α-AAA measured over time. The intracellular concentration of α-AAA varied between 1 and 4 mM. With the high K_m value for α-ketoglutarate of LAT taken into account, the simulated α-AAA levels were within an order of magnitude of that of experimental measurement over the entire time course. In contrast, with the assumption that α-ketoglutarate was at saturation level, a 2 orders of magnitude difference discrepancy was observed between the measured and simulated values of the α-AAA intracellular concentration. (Figure 5).

Discussion

For the metabolic engineering of the secondary metabolite pathways, it is crucial to understand the factors regulating the flux from primary metabolism to second-

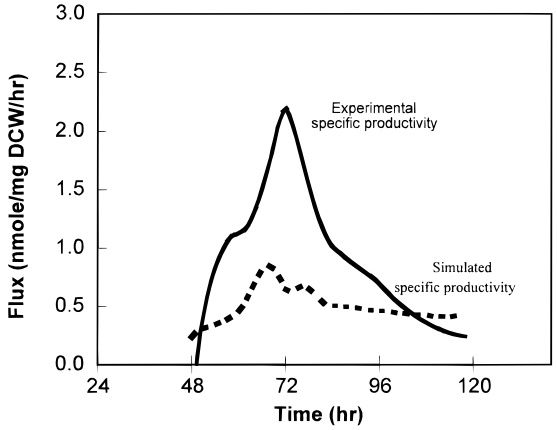

Figure 4. Comparison of the simulated and experimentally determined specific productivity of the antibiotic cephamycin C. The kinetic model incorporates the Lysine to α-aminoadipic acid pathway segment.

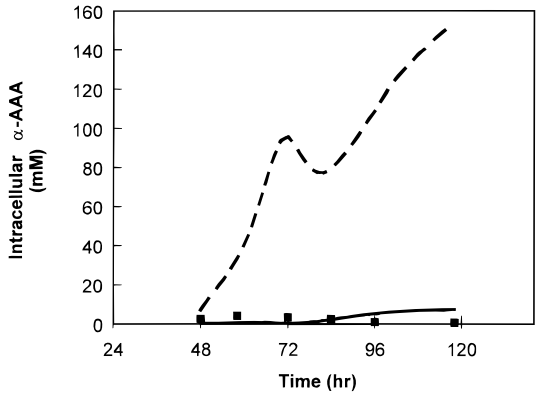

Figure 5. Simulated intracellular concentration profile of α-AAA compared to the experimentally measured values. The experimentally measured α-AAA (■) differs by 2 orders of magnitude from that simulated assuming saturated levels of α-ketoglutarate (---). Simulations using the experimentally determined K_m value of α-ketoglutarate for LAT and the intracellular profile of α-ketoglutarate (—) match within an order of magnitude.

ary metabolism. At the biochemical level, precursors and cofactors constitute the junction between primary and secondary metabolism. Their intracellular concentrations provide an effective regulation of the flux in secondary metabolism. In this study, the apparent K_m's of α-ketoglutarate and lysine for LAT were found to be in the millimolar range. The measured intracellular concentrations of both of these substrates were approximately an order of magnitude lower than their corresponding K_m values. In contrast to the 8.6 mM K_m value for LAT, the value for other enzymes in the pathway for which α-ketoglutarate is a cosubstrate, namely, Expandase, Hydroxylase, and OCDAC Hydroxylase, are only in 10s of micromolar (Dotzlaf and Yeh, 1989; Baker et al., 1991). Compared to K_m values of α-ketoglutarate of LAT which are not involved in secondary metabolism, 0.5 mM for LAT from *Achromobacter liquidum* (Soda et al., 1968), and 1.0 mM for LAT from *Pseudomonas aeroginosa* (Fothergill and Guest, 1977), that for LAT in *S. clavuligerus* is also much higher. It thus appears that the high K_m value of α-ketoglutarate for LAT is "by design." A high K_m value in LAT will help ensure that α-ketoglutarate is not channeled to secondary metabolism if the demand for it in primary metabolism is high.

The precursor lysine is required for protein synthesis. Thus, incorporation of lysine into the protein via aminoacyl-tRNA-synthetase can be viewed as a major reaction competing for lysine with LAT. Most tRNA-synthetases have K_m values for amino acids in the micromolar range (Freist et al., 1992). A consequence of such a high K_m value for lysine of LAT, the enzyme mediating the first committed step of the pathway, is that secondary metabolism is not favored at the expense of protein synthesis.

The modulation of cephamycin biosynthetic flux by precursors and cosubstrate can be reversed by metabolic engineering. Previously it has been shown that increasing LAT activity causes an increase in the rate of production of cephalosporins (Malmberg et al., 1993; Malmberg et al., 1995). Lysine added exogeneously has also been shown to have a similar stimulatory effect on cephamycin production (Mendelovitz and Aharonowitz, 1982). The use of α-ketoglutarate as carbon source gave a high value of cephalosporin to DCW ratio in shake flask cultures (Aharonowitz and Demain, 1978). Of course, lysine and α-ketoglutarate added exogenously may have many other physiological effects in addition to increasing the precursor/cosubstrate flux. Thus, it is not clear that the positive effect on antibiotic production observed was due to an increased supply of lysine and α-ketoglutarate as precursor and cosubstrate. However, simulations by doubling the concentration of intracellular α-ketoglutarate, lysine, or the activity of LAT all showed an approximate doubling of specific productivity of cephamycin C over control (data not shown). It is postulated that enhanced intracellular α-ketoglutarate levels should lead to greater productivity of cephalosporins. One possible way to increase the intracellular concentration of α-ketoglutarate is to supplement the medium with α-ketoglutarate. Another method would involve engineering the primary metabolism to enhance intracellular α-ketoglutarate levels. Alternatively, site-directed mutagenesis or gene shuffling (Patten et al., 1997) can be used to modify the kinetic constants of the LAT to achieve the same purpose.

We have shown previously that, among the biosynthetic enzymes, ACV synthetase is the most important rate-limiting enzyme (Malmberg and Hu, 1991), and among the three substrates for ACV synthetase, the supply of α-aminoadipic acid has the most profound effect on the biosynthetic rate. Subsequent cloning and amplification of gene encoding LAT demonstrated that increased LAT activity led to increased cephamycin C biosynthesis. These observations are consistent with the notion that precursor supply is at least partially responsible for regulating the flux into secondary metabolism. In this study, the K_m of lysine and α-ketoglutarate for LAT as well as their intracellular concentrations were measured. During the rapid growth state, the intracel-

lular concentrations for both compounds are merely about one-tenth of K_m. This interplay of high K_m and low concentration ensures that lysine and α-ketoglutarate are directed chiefly for primary metabolism. This observation of a precursor and a cosubstrate as control valve for cephamycin production further demonstrates the nature of multiple overlayering control of secondary metabolism. A comprehensive metabolic engineering strategy for enhanced secondary metabolite production will need to tackle all of these control points.

Notation

ACV	ACV tripeptide
C_i	concentration of intermediate I
DAC	Deacetylcephalosporin C
DAOC	Deacetoxycephalosporin C
IPN	Isopenicillin N
k_{AAA}	transport constant for α-AAA
k_{ACV}	transport constant for ACV
K_m	Michaelis constant
LAT	lysine-6-aminotransferase
PCD	piperideine-6-carboxylate dehydrogenase
PenN	Penicillin N
r_j	activity of enzyme catalyzing reaction j
t	time
V_{max}	maximum activity of enzyme
x	biomass concentration
α-AAA	α-aminoadipic acid
α_{ij}	stoichiometric coefficient
μ	specific growth rate

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References and Notes

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