An integrated study of threonine-pathway enzyme kinetics in *Escherichia coli*

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We have determined the kinetic parameters of the individual steps of the threonine pathway from aspartate in *Escherichia coli* under a single set of experimental conditions chosen to be physiologically relevant. Our aim was to summarize the kinetic behaviour of each enzyme in a single tractable equation that takes into account the effect of the products as competitive inhibitors of the substrates in the forward reaction and also, when appropriate (e.g. near-equilibrium reactions), as substrates of the reverse reactions. Co-operative feedback inhibition by threonine and lysine was also included as necessary. We derived the simplest rate equations that describe the salient features of the enzymes in the physiological range of metabolite concentrations in order to incorporate them ultimately into a complete model of the threonine pathway, able to predict quantitatively the behaviour of the pathway under natural or engineered conditions.

Key words: amino acid, enzymology, model fitting, parameter estimation, rate equation.

INTRODUCTION

Threonine is an essential amino acid for birds and mammals and so there is considerable interest in its economic industrial production for a variety of uses. The five-step metabolic pathway for its synthesis from aspartate in *Escherichia coli* (Figure 1) has long been known, so it would seem that this is a natural target for 'metabolic engineering', the improvement of industrial organisms using modern genetic tools [1]. The kinetics of all the five enzymes from *E. coli* have previously been studied extensively, the complete genome sequence of this organism is now known and there is an extensive range of genetic tools available. Nevertheless, a problem still exists in the rational design of modifications to the pathway, which is a key characteristic of the metabolic-engineering approach [2]: most of the information collected on the kinetics was directed at determining mechanisms of reaction and co-operative interaction, and has not been applied to producing a global model of the pathway under conditions *in vivo*. For example, kinetics have been studied at pH values well outside the physiological range, and some of the

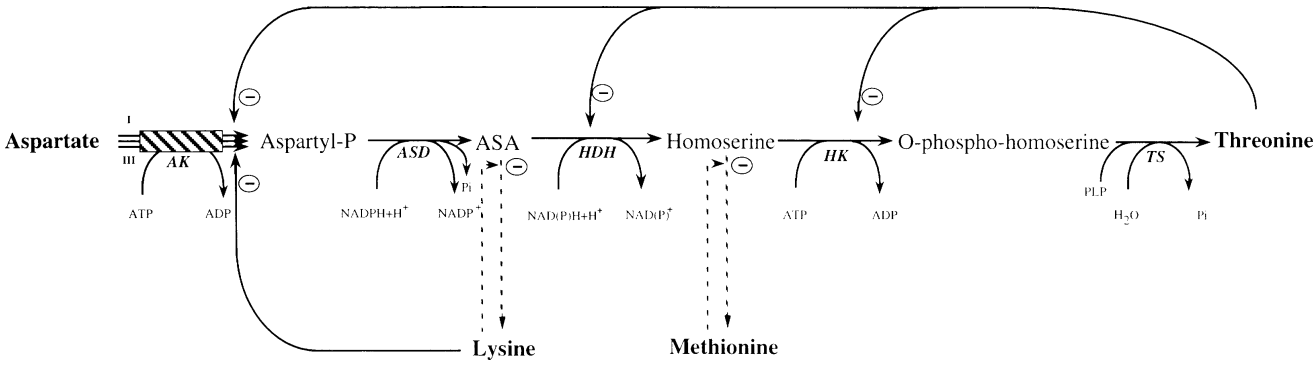

Figure 1 Threonine pathway from aspartate in *E. coli*

The different steps are catalysed by aspartate kinases I, II and III (AK I–III), aspartate semialdehyde dehydrogenase (ASD), homoserine dehydrogenase (HDH), homoserine kinase (HK) and threonine synthase (TS). ASA, D,L-aspartic β-semialdehyde; aspartyl-P, β-aspartyl phosphate; —, retro-inhibition.

Abbreviations used: AK, aspartate kinase; aspp, β-aspartyl phosphate; ASD, aspartate semialdehyde dehydrogenase; ASA, D,L-aspartic β-semialdehyde; HDH, homoserine dehydrogenase; HK, homoserine kinase; LDH, lactate dehydrogenase; PK, pyruvate kinase; TS, threonine synthase.

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reactions have been studied in the reverse of the biosynthetic direction. One of the two integrated studies of the pathway in any micro-organism is that of Szczesiul and Wampler [3], who reconstituted the pathway with enzymes partially purified from *E. coli* and other organisms to study the dynamics and regulation of the overall conversion of aspartate into threonine. However, at that time, even if the kinetic data had been available, the tools for building a quantitative dynamic model and simulating it by computer were still at an early stage of development. Subsequently, Shames et al. [4] used ^{31}P-NMR to study the effects of anti-metabolites on the intermediates of a reconstituted pathway derived from purified enzymes from *E. coli*, but they were not attempting to relate their results to the kinetics of the individual enzymes.

Yet without a functioning quantitative model, it is not possible to take the first step in the application of metabolic engineering, which is to make predictions about the effects of modifying the pathway. Since powerful tools for the simulation of metabolic pathways are now readily available [5–8], we thought it now appropriate to build a model for the threonine pathway, which required that we tackle the problem of obtaining a coherent set of kinetic data for the enzymes under close to physiological conditions, and including the effects on enzyme activity of not only the substrates, but also the intermediates and products of the pathway. Our overall aim was first to construct a model of the pathway that, based on kinetic descriptions of each of the enzymes, would be able to describe quantitatively the behaviour of a threonine-synthesis system *in vitro* derived from a cell-free extract of *E. coli* incubated under physiological temperature and pH. We chose to use a cell-free extract rather than a system reconstituted from purified enzymes to avoid the possibility that the kinetic properties of the enzymes might be altered during purification. The second advantage of a cell-free system is that the relative activities of the enzymes are automatically the same as in the cell. Ultimately, once we had confidence that the model behaved correctly under these circumstances, we should then be able to use it to predict the properties of the pathway within the cell, where it is less easy to manipulate the conditions and measure the intermediates and fluxes.

Each of the enzymes of the threonine pathway of *E. coli* has been subject to previous kinetic studies. Apart from the problem that the choices of temperature, pH and direction were not always appropriate, in many cases the studies had not considered the effects of product inhibition or reverse reaction, which are relevant in both *in vitro* reconstituted and *in vivo* systems, and often had not culminated in the generation of a single kinetic equation incorporating all the relevant terms and associated values of their parameters. Furthermore, most of the studies had been made on purified enzymes, but for the same reason that we intended to work on the pathway in a cell-free extract, we wished to measure the kinetic parameters on unpurified cell extracts.

In *E. coli*, there are three separate isoenzymes of aspartate kinase (AK) catalysing the first step. AK I, a bifunctional enzyme incorporating homoserine dehydrogenase (HDH) [9], has had its substrate kinetics (aspartate and ATP) studied at pH 8.0 and 27 °C by Patte et al. [9,10] and at the same pH and 30 °C by Angeles and Viola [11]. This was one of the few studies where a product-inhibition parameter (the K_i for ADP) was measured, and is significant in this case because the equilibrium constant of the reaction (3.5×10^{-4} at pH 8.0 and 15 °C [12]) favours the formation of aspartate and ATP. This isoenzyme is subject to co-operative, competitive inhibition by threonine [13]. AK II (with HDH II) is present at much lower levels than the other two isoenzymes and therefore does not need further consideration for our purposes. AK III is co-operatively and non-competitively inhibited by lysine [13–15]; K_m values for aspartate and ATP have been reported previously [16].

The second enzyme is aspartate semialdehyde dehydrogenase (ASD), for which the equilibrium constant is 3×10^6 at room temperature [17]. An extensive study by Karsten and Viola [18] reported the kinetic parameters for both the forward and reverse directions at pH 7.0 and 30 °C, along with some product-inhibition studies, but did not lead to a single overall equation for the kinetics under reversible conditions. Many other kinetic studies measured the reaction only in the reverse direction [19], and using arsenate instead of phosphate to make the reaction irreversible.

There are two isoenzymes of the third step, HDH I and II, associated with AK I and II respectively. Again we can disregard HDH II on account of its low relative activity. Like its associated AK I, HDH I is co-operatively inhibited by threonine, but in this case the inhibition type is non-competitive [13]; the inhibition is generally found to be partial. The extensive kinetic study by Wedler and Ley [20] was unfortunately at pH 9.0, although it was at 37 °C; Angeles and Viola [11] measured substrate kinetics only at pH 8.0. The equilibrium constant is 10^{11} M^{-1} [20], implying that the reverse reaction may need to be considered at physiological pH.

Homoserine kinase (HK) is the fourth step, and the most detailed study of the homoserine and ATP substrate kinetics was by Shames and Wedler [21] at pH 7.6 and 27 °C. They observed substrate inhibition by homoserine, but did not report any investigation of product effects. Threonine is a competitive inhibitor with respect to homoserine [22].

The final step is threonine synthase (TS); the conversion of homoserine phosphate into threonine is mechanistically complicated, but it is a single-substrate enzyme with no reported physiological effectors. Szczesiul and Wampler [3] measured the kinetics at pH 8.0 and 30 °C, and found slight co-operativity ($h = 1.4$).

Thus it is apparent that, in spite of the extensive kinetic studies of the pathway, they have been conducted at a variety of temperatures and pH values, and there is a lack of some essential parameters for a complete description. Furthermore, even those parameters that have been obtained have not been consolidated into a single kinetic equation for each enzyme. The aim of the study presented here was to complete the determination of the kinetic parameters of the enzymes under a single set of experimental conditions chosen to be physiologically relevant, and to arrive at a tractable, consolidated kinetic expression for each step. This would then provide both a foundation for our subsequent attempts to construct a complete model of the pathway, and a useful data set for others wishing to engineer the threonine pathway, as well as providing partial information for the pathways to lysine, methionine and isoleucine.

EXPERIMENTAL

Cells and buffers

An *E. coli* strain K12 thiaisoleucine-resistant derivative (Tir-8) [23], de-repressed for the threonine operon [3], was used in the study. Bacteria were grown in a minimal medium at 37 °C with 0.4 % (w/v) glucose as the carbon source. At the end of the exponential phase, the cells were harvested, washed and frozen at −80 °C in extraction buffer (see below).

The extraction buffer contained 20 mM KH_2PO_4, pH 7.2, 150 mM KCl, 2 mM Mg-titriplex, 1 mM dithiothreitol, 1 mM threonine and 1 mM lysine. The assay buffer contained 23 mM Tes buffer, pH 7.5, 114 mM KCl and 6 mM $MgCl_2$.

Preparation of the crude extract

The cells were resuspended in extraction buffer (2.5 ml/g of cells), broken by sonication (three 30 s intervals separated by 30 s rests) and then centrifuged at 15000 ***g*** for 15 min at 4 °C. The supernatant was applied to a Sephadex G-25 column to remove the small molecules (amino acids and cofactors). The protein content was assessed by the Biuret method [24] with BSA as a standard. A fresh extract was used for each experiment and was kept for no more than 6 h at 4 °C. Enzyme activities measured at the end of the experiment were comparable with those at the start.

Chemicals

Aspartate, homoserine and threonine were from Sigma. D,L-Aspartic β-semialdehyde (ASA) was prepared by the ozonolysis of D,L-allylglycine as described by Black and Wright [17]. This compound was characterized in HPLC by observing which peak was transformed into homoserine by HDH. ASA concentration was assessed by an enzymic assay using HDH and by measuring the NADPH consumption at 340 nm [25]. Like Szczesiul and Wampler [3], we observed no significant loss of substrate activity in 0.1 M HCl at −80 °C in 1 year.

O-Phospho-homoserine was prepared enzymically by a method similar to that described by Farrington et al. [26]. The reaction mixture contained 150 mM homoserine, 150 mM ATP and 3 units·ml^{-1} purified *E. coli* HK [4] in assay buffer. The mixture was incubated at 37 °C with gentle agitation and the reaction was followed by HPLC. The reaction was stopped after 35 h by adding perchloric acid at 4% (w/v) final concentration. The solution was neutralized with 2 M KOH/0.2 M Mops and then centrifuged for 15 min at 4 °C and 10000 ***g***. The supernatant was stored at −80 °C. The *O*-phospho-homoserine peak was identified in HPLC by following its transformation into threonine by TS. This compound was quantified by enzymic assay with purified TS [4] and measurement of either the formation of threonine by HPLC or P_i liberation by a colorimetric method (see the TS assay, below); both methods gave identical results. The average yield of the reaction was approx. 95%.

β-Aspartyl phosphate (aspp) was prepared enzymically as described by Shames et al. [4]. The reaction mixture contained 10 mM aspartate, 30 mM ATP, 20 mM phosphoenolpyruvate, 20 units·ml^{-1} pyruvate kinase (PK) and purified *E. coli* AK (4 units·ml^{-1}). The mixture was incubated at 37 °C with gentle agitation. The solution was first passed through a 0.2 μm-pore-size filter (Millipore) and then centrifuged through an Ultrafree 4 unit (Millipore) with a 5000 Da cut-off, for 90 min at 4 °C and 4000 ***g***, which retains the enzymes and stops the reaction. Because of its extreme lability the aspp had to be prepared immediately before use. This compound was characterized by following the appearance of its peak by HPLC during the synthesis and was quantified as described by Black and Wright [12]. With hydroxylamine the aspp is converted into aspartohydroxamate. In the presence of Fe(III) [4% (w/v) trichloroacetic acid/2% (w/v) $FeCl_3$/1 M HCl] a red colour is formed that can be measured at 540 nm and standardized with known concentrations of aspartohydroxamate [27].

HPLC measurements of amino acid concentrations

The amino acid concentrations were determined by pre-column derivation with OPA (*o*-phthaldehyde-thiol). They were analysed on a Hypersil ODS (250 mm × 4.6 mm) 5 μm column in reverse phase with a concentration gradient of potassium acetate buffer, according to Joseph and Marsden [28].

Enzyme assays

All the enzyme activities were assayed according to Szczesiul and Wampler [3] with some modifications for measuring all the activities under the same conditions (same buffer, pH, ionic strength and temperature, 37 °C).

AK (EC 2.7.2.4) and HK (EC 2.7.1.39) activities were measured in a coupled assay with PK and lactate dehydrogenase (LDH) as described by Wampler and Westhead [29] by following the NADH oxidation at 340 nm. The reaction mixture for AK contained, in 1 ml of assay buffer, 5 mM aspartate, 4 mM ATP, 1.5 mM phosphoenolpyruvate, 0.3 mM NADH, 2 units of PK, 2.25 units of LDH and 100 μg of protein crude extract. For HK the reaction mixture was exactly the same except that aspartate was replaced by 2 mM homoserine.

ASD (EC 1.2.1.11) activity was measured as described by Hegeman et al. [19]. This activity was measured in the forward direction by following NAPDH oxidation at 340 nm and in the reverse direction by following $NAPD^+$ reduction at 340 nm. The reaction mixture in 1 ml of assay buffer contained, for the forward direction, 0.3 mM NADPH, 0.5 mM aspp and 100 μg of protein crude extract. For the reverse direction the reaction mixture, in 1 ml of assay buffer, contained 0.8 mM $NADP^+$, 40 mM arsenate, 0.92 mM ASA and 100 μg of protein crude extract.

HDH (EC 1.1.1.3) activity was measured by following NAPDH oxidation at 340 nm and for the reverse direction by following $NADP^+$ reduction at 340 nm. In 1 ml of assay buffer for the forward direction the reaction mixture contained 0.3 mM NADPH, 1 mM ASA and 40 μg of protein crude extract. For the reverse direction in 1 ml of assay buffer the reaction mixture contained 0.8 mM $NADP^+$, 10 mM homoserine and 100 μg of protein crude extract.

TS (EC 4.2.3.1) activity was measured by following the release of inorganic phosphate from *O*-phospho-homoserine. In 3 ml of assay buffer the reaction mixture contained 0.1 mM pyridoxal phosphate and 600 μg of protein crude extract. The reaction was started by adding 2 mM *O*-phospho-homoserine. At time 0 and at 15 min, 400 μl of reaction mixture was removed and mixed with 40 μl of 40% (w/v) perchloric acid; the solution was neutralized with 2 M KOH/0.2 M Mops and then centrifuged for 15 min at 4 °C and 10000 ***g***. In these final solutions the inorganic phosphate was measured as described by Sumner [30]; the difference between the original and the final inorganic phosphate concentrations gave the rate of the reaction.

Equilibrium-constant determination

AK equilibrium-constant measurement

This measurement was performed as described by Black and Wright [12], modified to be effected in the same conditions as the other kinetic parameters. The reaction mixture contained, in 3 ml of assay buffer, 250 mM aspartate, 21.3 mM ATP and 4.8 units·ml^{-1} AK. After 60 min 2.86 mM ADP (final concentration) was added to obtain a new equilibrium. From the initial concentrations and the aspp concentration measurements at specific time points, it is possible to derive all the concentrations and thus the equilibrium constant:

$$K_{eq}(\mathrm{AK}) = \frac{[\mathrm{aspp}][\mathrm{ADP}]}{[\mathrm{Asp}][\mathrm{ATP}]} \quad (1)$$

However, to obtain compatible estimates of the equilibrium constant before and after the addition of ADP, it was necessary to take account of the rate of spontaneous hydrolysis of aspp [12,18], although no value for the rate constant was available for

our temperature and pH. This was done by simulating the AK reaction according to eqn (7) (see below) plus a first-order hydrolysis of aspp with SCAMP [6] and SIMFIT [31]. The aspp concentrations were then fitted using SIMFIT with the equilibrium constant, the aspp hydrolysis rate and the inhibition constant of AK by aspp as the variable parameters, and all other parameters for AK fixed at the values described in the Results section.

ASD equilibrium-constant measurement

The reaction mixture, in 1 ml of assay buffer, contained 0.92 mM ASA, 0.2 mM $NADP^+$, P_i (2, 4, 8, 12 or 20 mM) and partially (without HDH activity) purified ASD activity (0.01 unit·ml^{-1}). The equilibrium was measured by following the NAPDH concentration at 340 nm, allowing the calculation of all other concentrations from the known initial concentrations. At the equilibrium point the pH was also measured. The following equilibrium was then calculated.

$$K_{eq}(\text{ASD}) = \frac{[\text{ASA}][\text{NADP}^+][\text{P}_i]}{[\text{aspp}][\text{NADPH}][\text{H}^+]} \quad (2)$$

Kinetic analysis

For the two-substrate/two-product reactions (and in one case three products), a mechanistically exact equation will contain more parameters than can be easily determined or are useful, even assuming that the mechanism is adequately known. Consequently, for a reaction $A + B \leftrightarrow P + Q$, with P competitive with A and Q competitive with B, our starting point will be a simplified form of the usual two-substrate rate equation [32]:

$$v = \frac{V_f\left(\text{AB} - \frac{\text{PQ}}{K_{eq}}\right)}{\left[K_A\left(1 + \frac{\text{P}}{K_P}\right) + \text{A}\right]\left[K_B\left(1 + \frac{\text{Q}}{K_Q}\right) + \text{B}\right]} \quad (3)$$

where V_f is the maximal velocity (V_{max}) of the forward reaction. This form of equation gives a reasonable representation of the kinetics in a physiological range of substrate and product concentrations; more importantly, it takes into account the effects of products, both as inhibitors because of their binding at the active site in competition with the substrates and, when appropriate, as substrates of the reverse reaction.

The derivation assumes the existence of a Haldane relationship relating the parameters of the above equation to the limiting velocity (V_{max}) of the reverse reaction, V_r:

$$K_{eq} = \frac{V_f K_P K_Q}{V_r K_A K_B} \quad (4)$$

This can be seen to be compatible with eqn (3) for saturating values of P and Q at zero concentrations of A and B.

The experimental data were fitted to the theoretical equations by non-linear least squares using several different programs: SIMFIT [31], Kaleidagraph 3.0.2 (Abelbeck software) and the Excel V solver (Microsoft). For each enzyme, the empirical rate equations used for the determination of the different kinetic parameters (V_{max}, K_m and K_i) were developed and chosen as the experiments progressed; they are presented in the Results section. The kinetic parameters are summarized in Table 1.

Table 1 Kinetic parameters of the threonine-pathway enzymes

hsp, *O*-phospho-homoserine; α, the partial inhibition coefficient.

Enzyme	K_m (mM)		Inhibition	K_{eq}
AK I	Aspartate,	0.97 ± 0.48	K_{iThr}, 0.167 ± 0.003 mM	6.4×10^{-4}
	ATP,	0.98 ± 0.5	h_{Thr}, 4.09 ± 0.26	
	aspp,	0.017 ± 0.004	α, 2.47 ± 0.17	
	ADP,	0.25*		
AK III	Aspartate,	0.32 ± 0.08	K_{iLys}, 0.391 ± 0.08 mM	6.4×10^{-4}
	ATP,	0.22 ± 0.02	h_{Lys}, 2.8 ± 1.4	
	aspp,	0.017 ± 0.004		
	ADP,	0.25*		
ASD	aspp,	0.022 ± 0.001		2.84×10^5
	NADPH,	0.029 ± 0.002		
	ASA,	0.11 ± 0.008		
	$NADP^+$,	0.144 ± 0.02		
	P_i,	10.2 ± 1.4		
HDH	ASA,	0.24 ± 0.03	K_{iThr}, 0.097 mM	1×10^{11} M^{-1}†
	NADPH,	0.037 ± 0.006	h, 1.41	
	Homoserine,	3.39 ± 0.33	α, 3.93	
	$NADP^+$,	0.067 ± 0.006		
HK	Homoserine,	0.11	K_{iThr}, 1.09 mM	
	ATP,	0.072	K_{iLys}, 9.45 mM	
			$K_{ihomoserine}$, 4.7 mM	
			K_{iATP}, 4.35 mM	
TS	hsp,	0.31 ± 0.03		

* Values from [11].
† Value from [41].

RESULTS AND DISCUSSION

We have determined the kinetic parameters of the five enzymes of the threonine pathway under the same experimental conditions, as described in the Experimental section (assay buffer and 37 °C). The results are summarized in Table 1.

AK I

AK I is believed to follow a random mechanism [11], so a generic simplified reversible two-substrate equation, eqn (3), could be used. The activity of AK I was only measured in the forward direction. The K_m values for ATP and aspartate were determined by measuring the initial rate of the forward reaction for a set of initial concentrations of these two substrates. The K_m values were fitted to the simplified eqn (5):

$$v = \frac{V_f([\text{Asp}]\cdot[\text{ATP}])}{(K_{Asp} + [\text{Asp}])(K_{ATP} + [\text{ATP}])} \quad (5)$$

The values obtained, $K_{Asp} = 0.97 \pm 0.48$ mM and $K_{ATP} = 0.98 \pm 0.5$ mM, are close to the values of 0.71 mM and 0.5 mM reported for 30 °C and pH 8.0 by Angeles and Viola [11]. Patte et al. [10] reported 1 mM and 2 mM respectively, but with a different enzymic assay.

For the equilibrium constant, the fitting of the time course of the aspp concentrations (Figure 2), taking into account the hydrolysis of aspp, gives a value of 6.4×10^{-4} (see the Experimental section), with a rate constant for the hydrolysis of 0.049 min^{-1}. Our value is similar to the one reported by Black and Wright [12], 3.5×10^{-4} at 15 °C and pH 8. We also made this measurement at 15 °C and pH 7.5 and obtained a value of 3.9×10^{-4}.

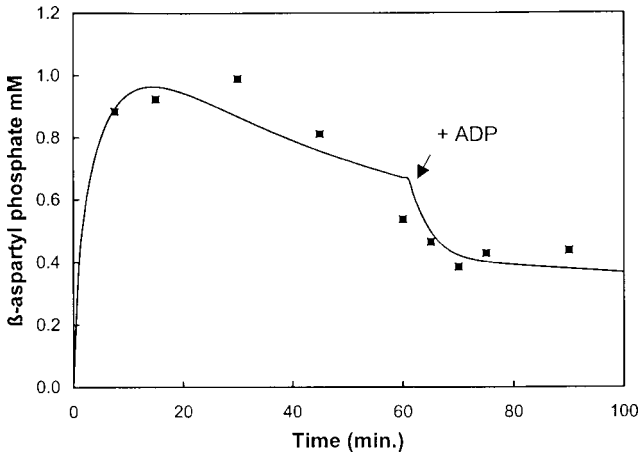

Figure 2 Determination of the AK equilibrium constant

The solid curve represents the time course fitted by SIMFIT for $K_{eq} = 6.4 \times 10^{-4}$, as described in the Experimental section. The fitting procedure takes into account the hydrolysis of aspp, with a fitted rate constant of 0.049 min^{-1}. The arrow shows addition of ADP (2.86 mM).

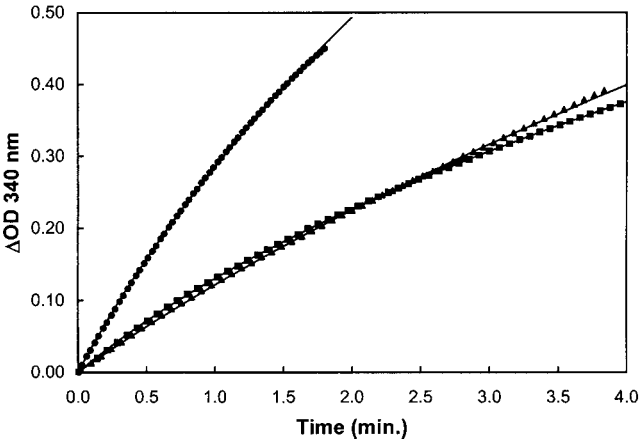

Figure 3 Determination of the product-inhibition constant of aspp on AK I

The symbols represent the experimental values of the absorbance at 340 nm for different substrate conditions (●, 5 mM aspartate, 4 mM ATP; ■, 1 mM aspartate, 4 mM ATP; ▲, 5 mM aspartate, 1 mM ATP) due to the oxidation of NADH in the coupled assay of AK in the presence of PK and LDH. 1 mol of oxidized NADH corresponds to 1 mol of synthesized aspp. The curve fitted by SIMFIT was adjusted according to eqn (3) by varying the values of K_{aspp} and V_f. Other parameters are fixed at the values in Table 1. OD, absorbance.

The reverse reaction could not be measured because of the ATP content of our aspp preparation, so for K_{aspp} we took the product-inhibition constant (K_{iaspp}), competitive with aspartate. This value was assessed by following the departure from linearity of the progress curve of aspp synthesis, which is followed stoichiometrically by NADH formation in the enzymic coupled test (Figure 3). The data were fitted using SIMFIT to integrate the AK I eqn (5), in which K_{Asp} was replaced by $K_{Asp}(1+[aspp]/K_{aspp})$ to take into account the competitive product inhibition of aspp (or eqn 7 for zero concentrations of threonine and ADP); in the fitting procedure, the other K_m values were fixed at the values determined previously. The value of $K_{aspp} = 0.017 \pm 0.004$ mM is the mean of three fitted experiments.

The K_m value for ADP, $K_{ADP} = 0.25$ mM, was taken from Angeles and Viola [11].

Threonine inhibition of AK I activity is allosteric and competitive with aspartate [27,33]. Because partial inhibition has been reported [34], the results were fitted to a partial sigmoidal competitive inhibition curve, by arbitary modification of Segel's [32] equation for partial competitive inhibition. The new apparent K_m for aspartate, $K_{Asp,\,app}$, with partial allosteric competitive inhibition is thus:

$$K_{Asp,app} = K_{Asp}\frac{1+\left(\frac{[Thr]}{K_{iThr}}\right)^{h_{Thr}}}{1+\left(\frac{[Thr]}{\alpha K_{iThr}}\right)^{h_{Thr}}} \quad (6)$$

where K_{iThr} is the threonine inhibition constant (0.167 ± 0.003 mM), h_{Thr} is the Hill coefficient (4.09 ± 0.26) and α is the partial inhibition coefficient (2.47 ± 0.17). This ascribes all the residual activity at high concentrations of lysine and threonine to uninhibited AK I (i.e. lysine inhibition of AK III is assumed to be complete). We do not exclude the possibility that a portion of the residual activity was due to AK II. However, whether the residual activity occurs because of incomplete inhibition or a small proportion of uninhibitable enzyme is immaterial for simulation provided that the kinetic equation fits the data. Accordingly, the complete equation used for AK I activity was:

$$v_{AKI} = V_{AKI}\left([Asp]\cdot[ATP] - \frac{[aspp]\cdot[ADP]}{K_{eq}}\right) \div \left\{\left[K_{Asp}\frac{1+\left(\frac{[Thr]}{K_{iThr}}\right)^{h_{Thr}}}{1+\left(\frac{[Thr]}{\alpha K_{iThr}}\right)^{h_{Thr}}} + [aspp]\frac{K_{Asp}}{K_{aspp}} + [Asp]\right] \times \left[K_{ATP}\left(1+\frac{[ADP]}{K_{ADP}}\right)+[ATP]\right]\right\} \quad (7)$$

AK III

AK III is completely inhibited by lysine in a non-competitive, co-operative manner [13–15]. The function used for this enzyme was analogous to that for AK I except for a non-competitive Hill term in lysine:

$$v_{AKIII} = V_{AKIII}\left([Asp]\cdot[ATP] - \frac{[aspp]\cdot[ADP]}{K_{eq}}\right) \div \left\{\left[1+\left(\frac{[Lys]}{K_{iLys}}\right)^{h_{Lys}}\right]\left[K_{Asp}\left(1+\frac{[aspp]}{K_{aspp}}\right)+[Asp]\right] \times \left[K_{ATP}\left(1+\frac{[ADP]}{K_{ADP}}\right)+[ATP]\right]\right\} \quad (8)$$

The equilibrium constant and the K_m values for aspp and ADP are assumed to be the same as for AK I above. The fitting of the kinetic curves when aspartate and ATP are varied gives $K_{Asp} = 0.32 \pm 0.08$ mM and $K_{ATP} = 0.22 \pm 0.02$ mM; these are lower values than those reported by Truffa-Bachi and Cohen [16] ($K_{Asp} = 4.7$ mM and $K_{ATP} = 4.8$ mM) but with a different assay. The values of K_{iLys} (0.39 ± 0.08 mM) and h_{Lys} (2.8 ± 1.4) are similar to those already published [13–15,35].

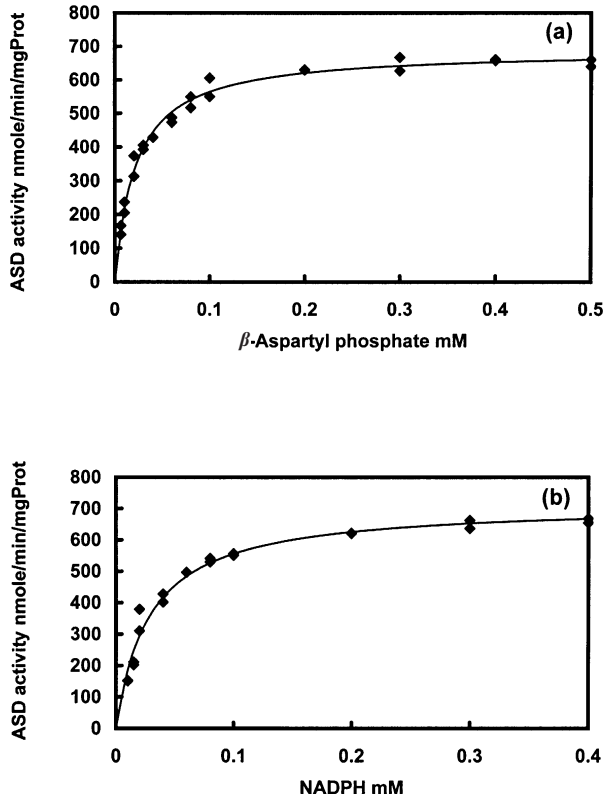

Figure 4 Kinetics of ASD

(**a**) ASD activity as a function of aspp. (**b**) ASD activity as a function of NADPH. All curves were fitted according to the Michaelis–Henri equation.

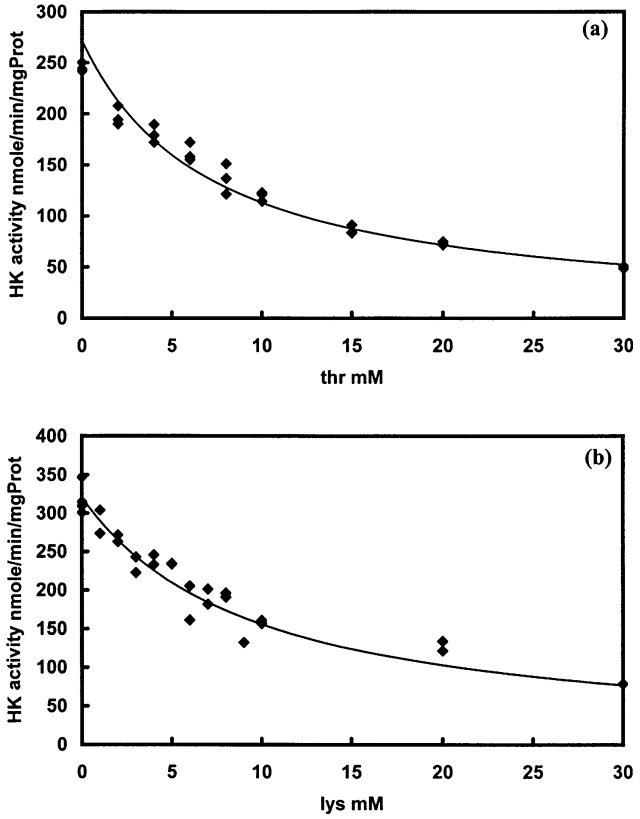

Figure 5 Inhibition of HK as a function of (a) threonine and (b) lysine

The experimental points were fitted according to eqn (11) with the values in Table 1.

ASD

The kinetics of this enzyme posed several difficulties. A fully accurate mechanistic equation for a reversible two-substrate/three-product reaction would be too complex and have too many parameters for the purposes of modelling. We therefore used a minimal equation possessing the main features:

$$v_{ASD} = V_{ASD}\left([aspp]\cdot[NADPH] - \frac{[ASA]\cdot[NADP^+]\cdot[P_i]}{K_{eq\,ASD}}\right) \div \left\{\left[K_{aspp}\left(1+\frac{[ASA]}{K_{ASA}}\right)\left(1+\frac{[P_i]}{K_{P_i}}\right)+[aspp]\right] \times \left[K_{NADPH}\left(1+\frac{[NADP^+]}{K_{NADP^+}}\right)+[NADPH]\right]\right\} \quad (9)$$

It is important to represent the reversibility of the reaction and the involvement of phosphate, because the experiments on cell-free threonine synthesis lead to accumulation of phosphate as the aspp is hydrolysed. Also, although the model requires the kinetics represented in the forward direction, it is simpler to assay the enzyme in the reverse direction, and also with arsenate replacing phosphate. Thus it is necessary to know the forward maximal rate and its relationship with the two reverse rates (with phosphate and arsenate) for routine evaluation of the amount of enzyme, as well as the equilibrium constant.

For the determination of the equilibrium constant we used a partially purified ASD preparation, which did not contain the other activities of the threonine pathway, to avoid any consumption of the substrates/products by the adjacent reactions. We started with 0.2 mM $NADP^+$ and 0.92 mM ASA and different concentrations of P_i (2, 4, 8, 12 and 20 mM). The NADPH concentration at equilibrium was measured and allowed the calculation of all the other concentrations. The calculation of the K_{eq} according to eqn (2) gives $(2.84 \pm 1.12) \times 10^5$ (mean $\pm$ S.D. of the five measurements at different P_i concentrations). This value is comparable with the value of 3×10^6 at 25 °C (instead of 37 °C) found by Black and Wright [17].

In the forward direction, we have measured the K_m values for aspp and NADPH (Figure 4). These values have never been determined in *E. coli*, as far as we know, but can be compared with the corresponding values found in *Saccharomyces cerevisiae* [25]. We obtained a lower value for K_{aspp} (0.022 ± 0.001 mM instead of 0.27 mM) and a similar value for K_{NADPH} (0.029 ± 0.002 mM instead of 0.03 mM). In the reverse direction the studies were done on the partially purified ASD preparation, to avoid any re-utilization of the NADPH formed by HDH. The presence of P_i instead of arsenate does not appear to have any influence on the K_m values: for instance we found a K_{NADP^+} value of 0.144 ± 0.018 mM in the presence of P_i, instead of 0.13 mM with arsenate. The other K_m values were 0.11 ± 0.01 mM for ASA and 10.2 ± 1.4 mM for P_i. These values are similar to the ones determined by Hegeman et al. [19] in *E. coli* K12 at pH 9 and 25 °C (0.2, 0.09 and 9 mM for the K_m values of ASA, $NADP^+$ and P_i respectively). Karsten and Viola [18] also studied this activity in the backwards direction and found a K_m of 7–14 μM for ASA and of 8 mM for P_i.

The measurements in the forward direction necessitate the preparation of aspp immediately before use and thus cannot be used routinely. For this reason we measured on the same free extract the ratio between ASD activity in the forward and backward (with arsenate) directions; the mean of three measurements gives the relationship:

$$\text{Activity}_{\text{aspp}\rightarrow\text{ASA}} = 1.35 \times \text{Activity}_{\text{ASA}\rightarrow\text{aspp}}$$

HDH

The kinetic parameters for HDH have been determined in both directions. Product-inhibition experiments by Angeles and Viola [11] confirm that any rate equation should display competition between NADPH and $NADP^+$ and between ASA and homoserine. Threonine inhibition is non-competitive according to Patte and Cohen [13] and Datta [36]. Wedler and Ley [20] also suggested that the inhibition was more non-competitive in their isotope-exchange experiments. Rees and Hay [34] expressed AK I/HDH I in animal cells, and observed that at 10 mM homoserine the maximal inhibition by threonine was 60 %, clearly implying, as for AK I, that the inhibition is only partial. In *E. coli* extracts, HDH II will be present as well in small amounts, but in effect is being combined in this model with the residual activity of HDH I in the presence of threonine ($\alpha = 3.93$). On fitting the results to a partial inhibition model, a slightly better fit, particularly at low threonine levels, was obtained with co-operative inhibition ($h = 1.41$). This leads to the equation:

$$v_{\text{HDH}} = V_{\text{HDH}} \left([\text{ASA}]\cdot[\text{NADPH}] - \frac{[\text{hs}]\cdot[\text{NADP}^+]}{K_{\text{eq}}} \right) \div \left\{ \left[\frac{1+\left(\frac{[\text{Thr}]}{K_{\text{iThr}}}\right)^h}{1+\left(\frac{[\text{Thr}]}{\alpha K_{\text{iThr}}}\right)^h} \right] \left[K_{\text{ASA}} + [\text{hs}]\frac{K_{\text{ASA}}}{K_{\text{hs}}} + [\text{ASA}] \right] \times \left[K_{\text{NADP}} \left(1+\frac{[\text{NADP}^+]}{K_{\text{NADP}^+}} \right) + [\text{NADPH}] \right] \right\} \quad (10)$$

where hs is homoserine. The K_m values are listed in Table 1 and are not very different from the values determined by Patte et al.: $K_{\text{homoserine}} = 6.5$ mM, $K_{\text{NADP}^+} = 0.11$ mM, $K_{\text{ASA}} = 0.14$ mM and $K_{\text{NADPH}} = 0.08$ mM [33]. Angeles and Viola [11] at pH 8.0 found $K_{\text{ASA}} = 0.068$ mM and $K_{\text{NADPH}} = 0.028$ mM.

The ratio between the assayed forward and reverse reactions was experimentally found to be approx. 100. This is in good agreement with eqn (10), and its Haldane relationship (eqn 4) which, with the values listed in Table 1, gives $V_f/V_r = 126$.

HK

The equilibrium constant of this enzyme is not known, but other kinase reactions with alcoholic groups (such as hexokinase) have K_{eq} values of approx. 1000. We failed to detect the reverse reaction, even with 36.7 mM *O*-phospho-homoserine and ADP, which is in accordance with Burr et al. [22]. Thus it is only possible to measure this activity in the forward direction. Burr et al. [22] also did not detect product inhibition by homoserine phosphate at concentrations up to 16 mM.

Shames and Wedler [21] suggested a 'preferred order' mechanism, with ATP binding before homoserine. They reported substrate inhibition by homoserine and assumed a second inhibitory binding site for homoserine, but residual activity of the enzyme with two substrate molecules attached. We have confirmed this result and also observed inhibition by excess ATP. In our HK equation, we take into account that (i) homoserine shows substrate inhibition at high concentrations (> 1 mM); (ii) ATP shows significant substrate inhibition above 3 mM; (iii) threonine is a competitive non-allosteric inhibitor with respect to homoserine (Figure 5a), and (iv) as we have shown ([37,38] and Figure 5b), there is a lysine inhibition, which can be regarded as simply non-competitive towards homoserine. Simultaneous variation of lysine and threonine shows that there is no significant interaction between the two (additive inhibition).

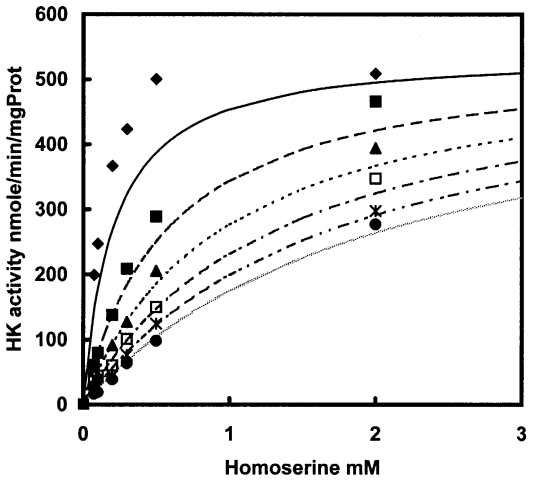

Figure 6 Kinetics of HK as a function of homoserine at different threonine concentrations

The experimental points were fitted according to eqn (11) with the values in Table 1. Threonine concentrations were: ◆, 0 mM; ■, 2 mM; ▲, 4 mM; □, 6 mM; ×, 8 mM; ●, 10 mM.

Fitting the experiments separately is not a particular problem, but in any equation that could give the range of effects observed above, the kinetic constants interact so that it is difficult to assess consistent and appropriate values for the global equation. To overcome this problem, two combined data sets were obtained, one containing homoserine, ATP and threonine variations, and the other where lysine and threonine were varied together (Lys, 0, 2, 4 and 6 mM; Thr, 0, 2, 4, 6, 8 and 10 mM). The results were fitted using a least-squares method with the Excel solver. The equation used was:

$$v_{\text{HK}} = V_{\text{HK}}\cdot[\text{hs}]\cdot[\text{ATP}] \div \left\{ \left[K_{\text{hs}} \left(1+\frac{[\text{ATP}]}{K_{\text{iATP}}} \right) \left(1+\frac{\text{Thr}}{K_{\text{iThr}}} \right) + [\text{hs}] \right] \times \left[K_{\text{ATP}} \left(1+\frac{[\text{hs}]}{K_{\text{ihs}}} \right) + [\text{ATP}] \right] \left(1+\frac{[\text{Lys}]}{K_{\text{iLys}}} \right) \right\} \quad (11)$$

The values we obtained (listed in Table 1) are lower than those of Shames and Wedler [21], but it has to be taken into account that eqn (11) involves an inhibition term by ATP. Figure 6 gives an example of the fitting by eqn (11) of some of the experimental points with various threonine and homoserine concentrations.

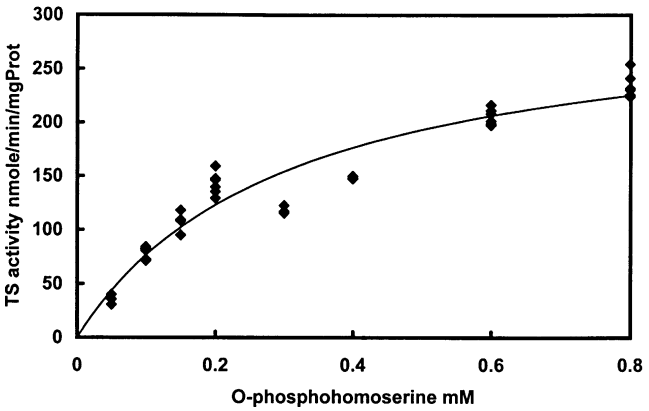

Figure 7 Kinetics of TS as a function of *O*-phospho-homoserine

The experimental points were fitted according to eqn (12) with the values in Table 1.

TS

This activity is only detectable in the forward direction; with 1.2 mM threonine and P_i no consumption of threonine could be detected after 1 h. The enzyme obeys simple Michaelis–Menten kinetics for one substrate [39], with no inhibition by threonine. The data of Figure 7 were fitted with the equation:

$$v_{TS} = \frac{V_{TS} \cdot [hsp]}{K_{hsp} + [hsp]} \quad (12)$$

where hsp is *O*-phospho-homoserine. The equation gives a K_m value for *O*-phospho-homoserine of 0.31 ± 0.03 mM. This value is comparable with the values of Szczesiul and Wampler [3] (0.23 mM) and of Laber et al. [40] (0.5 mM at pH 8).

Conclusions

In this study we have developed equations for each of the steps of the threonine-synthesis pathway, and have determined the parameters of the equations under a single set of conditions, chosen to be relevant to the conditions found when the pathway is operating in *E. coli* cytosol. There are two critical differences between the rate equations used for identification of the kinetic mechanism of an enzyme and those required for either simulation of metabolic pathways in particular or other methods for analysing the properties of functioning metabolic systems. (i) A rate equation used for simulation only needs to be accurate for metabolite concentrations within the physiological range, not for all possible combinations of concentrations from zero to saturating; it often realizes a compromise between different complex kinetic properties and the simplicity of a global equation, acceptable with the conditions under which the enzyme operates in a metabolic pathway. (ii) The rate equations used for simulation must represent the effects of products, both as inhibitors because of their binding at the active site in competition with the substrates and, when appropriate, as substrates of the reverse reaction.

Where direct comparisons can be made with previously published work, the parameters we have obtained are not greatly different, given the differences in assay conditions. The novel result from this paper is that, for the first time, the effects of the principal variables affecting the pathway enzymes have been compiled in a compact and mutually compatible form. Together, this set of equations will provide a basis for predicting the physiological response of the pathway to changes in conditions, whether natural or artificial, such as engineered changes in the amounts of one or more of the enzymes.

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