Threonine synthesis from aspartate in *Escherichia coli* cell-free extracts: pathway dynamics

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We have developed an experimental model of the whole threonine pathway that allows us to study the production of threonine from aspartate under different conditions. The model consisted of a desalted crude extract of *Escherichia coli* to which we added the substrates and necessary cofactors of the pathway: aspartate, ATP and NADPH. In this experimental model we measured not only the production of threonine, but also the time dependence of all the intermediate metabolites and of the initial substrates, aspartate, ATP and NADPH. A stoichiometric conversion of precursors into threonine was observed. We have derived conditions in which a quasi steady state can be transiently observed and used to simulate physiological conditions of functioning of the pathway in the cell. The dependence of threonine synthesis and of the aspartate and NADPH consumption on the initial aspartate and threonine concentrations exhibits greater sensitivity to the aspartate concentration than to the threonine concentration in these non-steady-state conditions. A response to threonine is only observed in a narrow concentration range from 0.23 to 2 mM.

Key words: amino acids, biosynthetic pathway, HPLC, *in vitro* model, pathway flux.

INTRODUCTION

The industrial production of amino acids using micro-organisms has expanded in recent years, in terms of both the amount and number of amino acids made this way. However, the production of threonine lags behind that of several of the others in spite of its importance as an essential amino acid for birds and mammals. Traditional approaches, such as mutation and selection, for increasing the productivity of bacterial biosynthetic pathways for amino acids gradually cease to deliver continuing improvements, and therefore researchers are turning more to 'metabolic engineering', the improvement of industrial organisms using modern genetic tools [1]. Implicit in the concept of metabolic engineering is that intended modifications to the pathway arise from a rational design process, which in turn requires some form of model of metabolism that can be used to predict the outcomes. Indeed, despite extensive knowledge of the kinetic features of the individual enzymes [2], it is difficult to anticipate the global behaviour of the pathway when some of these individual features are modified. For this reason, we have developed an experimental model of the threonine pathway (see Figure 1 in [2]) that allows us to study the production of threonine from aspartate under different conditions. In this experimental model we measure not only the production of threonine, but also the time dependence of all the intermediate metabolites and initial substrates, aspartate, ATP and NADPH. We have derived conditions in which a quasi steady state can be transiently observed and used to simulate physiological conditions of functioning of the pathway in the cell. This *in vitro* model has been used to determine the pathway response to changes in threonine concentration, which is difficult to infer from the enzyme kinetics as three of the steps are susceptible to inhibition by this end product. Variations in aspartate concentration have also been studied to determine the sensitivity of the pathway to any changes in the concentration of this starting material under different physiological conditions. The model has other potential applications that will be discussed further below.

EXPERIMENTAL

Chemicals

Aspartate, homoserine and threonine were from Sigma. D, L-Aspartic β-semialdehyde (ASA), *O*-phospho-homoserine and β-aspartyl phosphate (aspp) were synthesized by using the methods described by Black and Wright [3], Farrington et al. [4] and Shames et al. [5], respectively, with some modifications as described by Chassagnole et al. [2].

Cells

An *Escherichia coli* strain K12 thiaisoleucine-resistant derivative (Tir-8) [6], de-repressed for the threonine operon [7], was used in the study. Bacteria were grown in a minimal medium at 37 °C with 0.4 % glucose as the carbon source. At the end of the exponential phase, the cells were harvested and washed in the extraction buffer [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 for 15 min at 4 °C and 15000 ***g***. 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 [8] 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 remained comparable with those taken at the start.

Enzyme assays

All the enzyme activities were assayed according to Szczesiul and Wampler [7] with some modifications for measuring all the

Abbreviations used: AK, aspartate kinase; aspp, β-aspartyl phosphate; ASA, aspartic β-semialdehyde.

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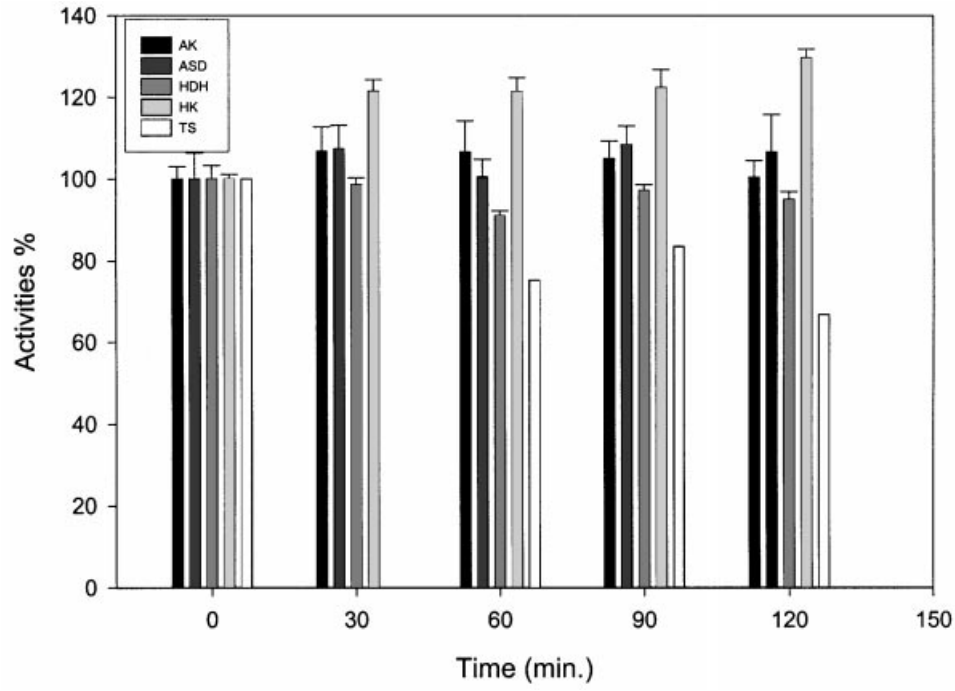

Figure 1 Activities (%) of the threonine pathway as a function of incubation time at 37 °C

At each time point, the activities of aspartate kinase (AK), aspartate semialdehyde dehydrogenase (ASD), homoserine dehydrogenase (HDH), homoserine kinase (HK) and threonine synthase (TS) are represented by bars, arranged from left to right respectively.

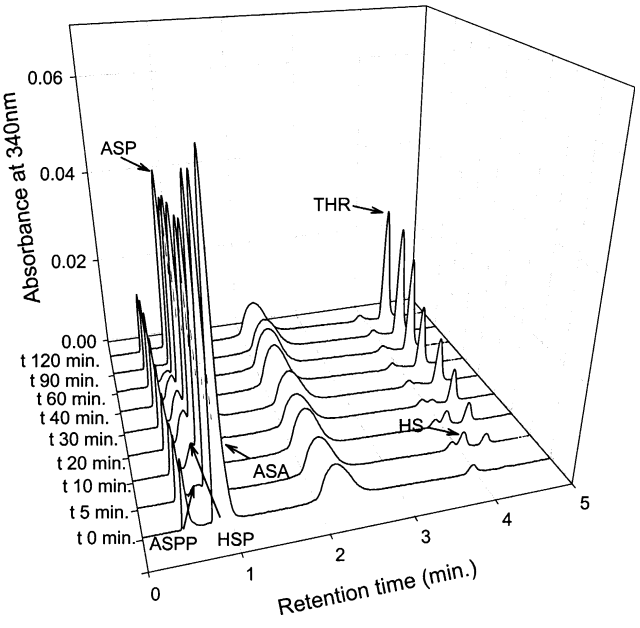

Figure 2 HPLC chromatograms corresponding to the experiments shown in Figure 4(a) (2 mM aspartate, 0 mM threonine)

The arrows indicate the different metabolite peaks. ASP, aspartate; HSP, *O*-phospho-homoserine; HS, homoserine; THR, threonine.

activities under the same conditions (same buffer, pH, ionic strength and temperature, 37 °C), as described by Chassagnole et al. [2].

ATP measurement

The ATP concentration was determined by bioluminescence with the luciferin/luciferase reaction using the ATP bioluminescence assay kit HS II from Boehringer-Mannheim.

HPLC measurements of amino acid concentrations

Amino acid concentrations were determined by pre-column derivation with OPA (*o*-phthaldehyde-thiol) based on Joseph and Marsden's method [9] with some modifications. They were analysed on a Micra NPS ODS-1 (33 mm × 4.6 mm) 1.5 μm column (Eichrom Technologies) in reverse phase with a concentration gradient of sodium acetate buffer. This gradient was formed from two buffers, 100 mM sodium acetate, pH 5.9 (adjusted with 1 M HCl; buffer A), and pure methanol (buffer B), with a flow rate of 0.5 ml/min. The time course of the gradient was as follows: initial, buffer A/buffer B (98:2, v/v); 1 min, A/B (85:15, v/v); 5 min, A/B (50:50, v/v); 10 min, A/B (30:70, v/v); 18 min, A/B (2:98, v/v). The retention times and the response factors of the commercially available amino acids (aspartate, homoserine and threonine) were evaluated by injecting known amounts of these amino acids. The characterization of the aspp, ASA and *O*-phospho-homoserine peaks is described in the accompanying paper [2].

Flux measurement

The reaction mixture in assay buffer (described in [2]) contained, at 37 °C, 2 mM NADPH, 5 or 10 mM ATP, 0.1 mM pyridoxal phosphate and a variable concentration of aspartate (from 0.5 to 5 mM). The reaction was started by addition of crude extract (0.2 mg of protein/ml); this concentration has been chosen after

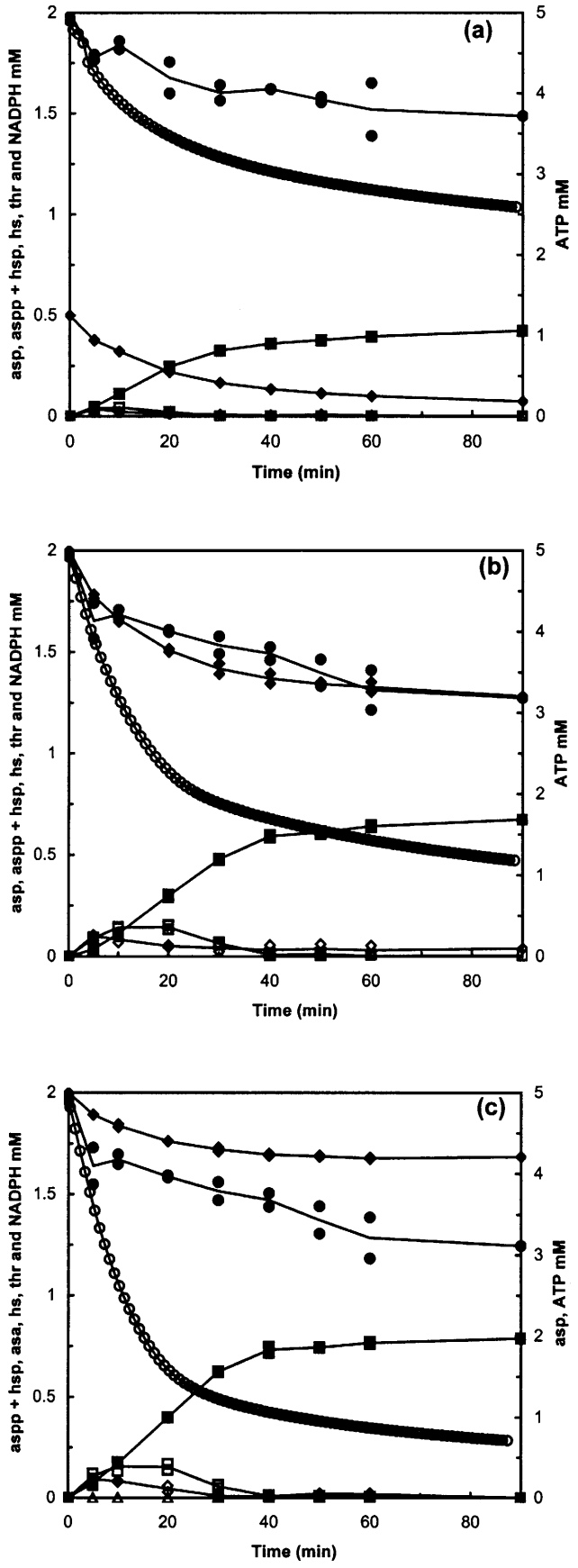

Figure 3 Time courses of the metabolites and co-metabolites of the threonine pathway

Initial concentrations: 2 mM NADPH, 5 mM ATP, no threonine and (**a**) 0.5 mM, (**b**) 2 mM or (**c**) 5 mM aspartate. Metabolites: ◆, aspartate (asp); □, aspp + *O*-phospho-homoserine (hsp); △, ASA (asa); ◇, homoserine (hs); ■, threonine (thr). Co-metabolites: ○, NADPH; ●, ATP.

preliminary experiments at different protein levels to approach the asymptote in the formation of a measurable quantity of threonine within a total reaction time of 2 h. In order to follow variations of NADPH, ATP and amino acid simultaneously, we made two equivalent reaction mixtures: the first one in a spectrophotometer cuvette to continuously follow the NADPH oxidation at 385 nm, and the second in a test tube to follow ATP and the amino acids. Aliquots were taken at set intervals. The reaction was stopped by adding perchloric acid at 4% (w/v), final concentration. The solution was neutralized with 2 M KOH/0.2 M Mops, centrifuged for 15 min at 10000 ***g*** and the supernatant was filtered with a 0.2 μm-pore-size filter (Millipore). Aspartate, threonine and intermediates of the threonine pathway were measured by HPLC; the supernatant could be kept for several days at -20 °C before analysis. On the other hand the ATP had to be measured immediately due to its instability.

RESULTS

Stability of the enzyme activities

Because we intended to follow the flux of threonine synthesis from aspartate over 2 h, it was necessary that all the activities were stable during this time in the reaction buffer at 37 °C. We followed all the enzyme activities for 2 h at 37 °C (Figure 1). The activities of aspartate kinase (AK; EC 2.7.2.4), aspartate semialdehyde dehydrogenase (EC 1.2.1.11) and homoserine dehydrogenase (EC 1.1.1.3) showed no significant changes (assessed by Student's t test of the slope of the regression line) over 120 min; the mean trends were changes of -0.4%, $+5.6\%$ and -4.6% respectively over this period. Homoserine kinase (EC 2.7.1.39), however, showed a highly significant ($P < 0.01$) increase of 22.5%. The largest apparent change was in the activity of threonine synthase (EC 4.2.3.1), with a decrease of 30%, although the change was not statistically significant because the measurements at the individual time points were not replicated.

Flux experiments

Endogenous consumption of NADPH and ATP

To measure correctly the changes in NADPH and ATP concentrations generated specifically by the threonine pathway, it was necessary to estimate any potential endogenous consumption by enzymes of the crude extract other than those of the threonine pathway and by other factors.

The endogenous consumption of NADPH was estimated by following NAPDH oxidation at 385 nm in four cuvettes containing the flux-measurement mixture without aspartate but with different NAPDH concentrations. We found that NADPH is slowly oxidized at a rate proportional to the NADPH concentration and to the crude-extract protein concentration (in mg/ml), according to the following equation:

$$V_{NADPH\_cons}\,(mM \cdot min^{-1}) = 0.0054 \cdot [protein] \cdot [NADPH]$$

The endogenous consumption of ATP was measured by following the ATP concentration in flux-measurement mixture without aspartate. The ATP is slowly degraded at a rate independent of

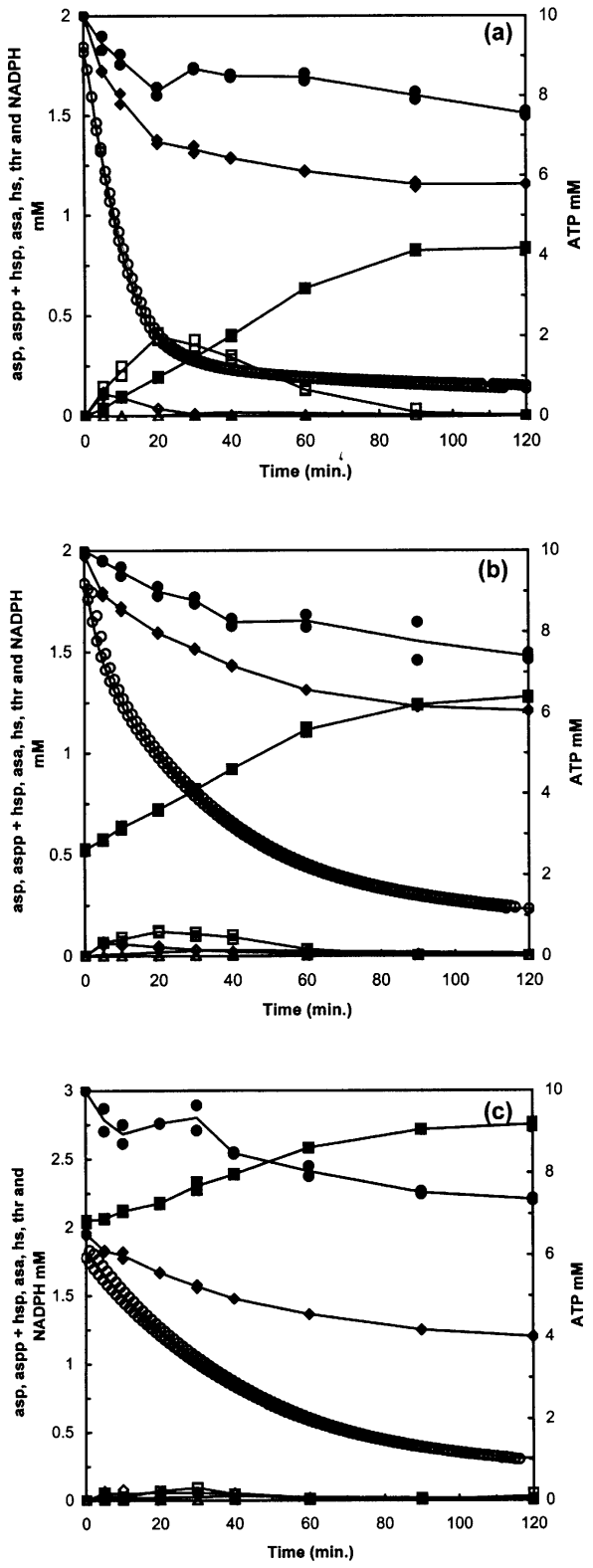

Figure 4 Time courses of the metabolites and co-metabolites of the threonine pathway

Initial concentrations: 2 mM NADPH, 10 mM ATP, 2 mM aspartate and (**a**) 0 mM, (**b**) 0.5 mM and (**c**) 2 mM threonine. Metabolites: ◆, aspartate (asp); □, aspp + *O*-phospho-homoserine (hsp); △, ASA (asa); ◇, homoserine (hs); ■, threonine (thr). Co-metabolites: ○, NADPH; ●, ATP.

Table 1 Stoichiometry in the inter-conversion of aspartate and threonine

The values correspond to Figure 4(a) with the following initial conditions: 2 mM aspartate, 2 mM NADH and 10 mM ATP.

Time (min)	Aspartate consumed (mM)	Threonine synthesized (mM)	NADPH consumed* (mM)	ATP consumed* (mM)
0	0	0	0	0
30	0.67	0.3	1.53	1.08
60	0.78	0.64	1.61	1.06
90	0.85	0.82	1.63	1.29
120	0.85	0.83	1.64	1.49

* Values corrected for endogenous consumption.

Table 2 Threonine synthesis at various initial concentrations of aspartate

The data are taken from Figure 3.

Aspartate (mM)	Threonine-synthesis rate (nmol/min per mg)	Threonine-synthesis plateau (mM)
0.5	68	0.42
2	92	0.67
5	115	0.79

its concentration, but proportional to the crude-extract protein concentration (in mg/ml), according to the following equation:

$$V_{ATP\_cons}\,(mM \cdot min^{-1}) = 0.064 \cdot [protein]$$

Time course of metabolite concentrations in the pathway to threonine

All metabolites, other than NADPH and ATP, were followed by HPLC. Figure 2 shows a typical series of HPLC chromatograms (corresponding to Figure 4a, see below), demonstrating the interconversion of aspartate and threonine. We observed a decrease in aspartate and the synthesis of threonine, and also the transients of the pathway intermediates. The ASA peak is usually difficult to see, due to its instability at neutral pH; in the experiments described in Figure 2 there is only a small peak of ASA between 20 and 90 min (hidden by the aspartate peak in this three-dimensional representation). A homoserine peak could be observed during the first 30 min. The aspp and *O*-phospho-homoserine peaks migrate to almost the same position, close to that of aspartate, which is at a much higher concentration. This renders the quantification of these individual peaks impossible and in Figures 3 and 4 the sum of aspp and *O*-phospho-homoserine concentration is plotted. However, it is seen qualitatively in Figure 2 that the aspp peak appears during the first 10 min, followed by the *O*-phospho-homoserine peak, which becomes the major peak.

In order to obtain a general description of the flux from aspartate to threonine we varied aspartate and threonine concentrations in a range where the flux is sensitive to these metabolites. This led us to two sets of experiments. In the first one (Figure 3), we measured the evolution of the metabolite concentrations and of threonine synthesis at three aspartate concentrations: 0.5, 2 and 5 mM. In the second set of experiments (Figure 4), threonine was varied at a fixed initial 2 mM aspartate concentration to see how threonine synthesis was inhibited by threonine itself.

In both sets of experiments it can be seen that threonine synthesis reached a plateau after a quasi-linear synthesis (between 5 and 30 min). The plateau phase suggests that the catabolism of threonine is negligible in our conditions, which is confirmed by

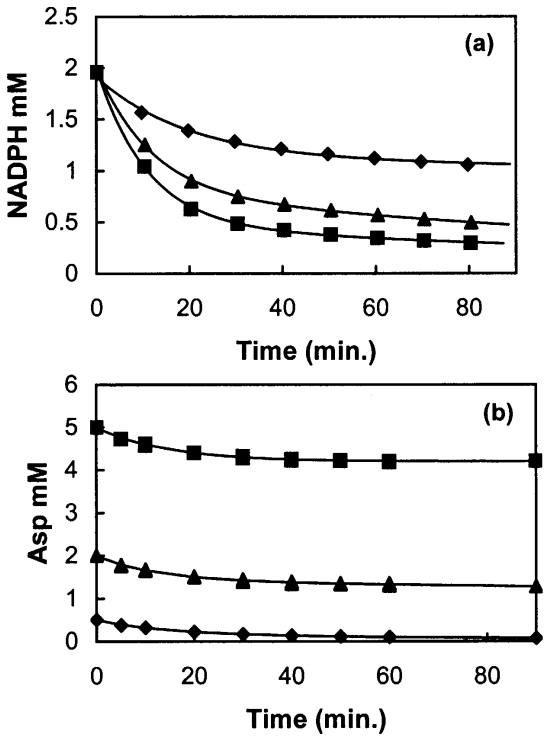

Figure 5 Comparison of the time courses of NADPH (a) and aspartate (b) for different initial concentrations of aspartate

◆, 0.5 mM aspartate; ▲, 2 mM aspartate; ■, 5 mM aspartate.

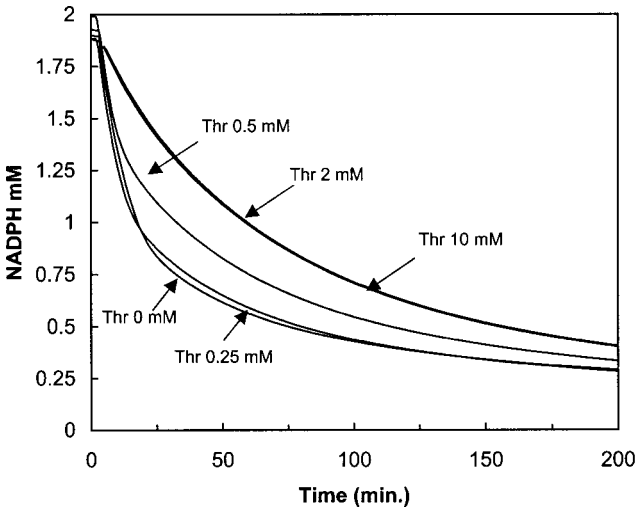

Figure 6 Time courses of NADPH consumption at different initial concentrations of threonine

Table 4 Threonine synthesis at variable threonine concentrations

The data are taken from Figure 4. △Threonine was calculated as (threonine-synthesis plateau)–(initial threonine).

Threonine (mM)	Threonine-synthesis rate (nmol/min per mg)	Threonine-synthesis plateau (mM)	△Threonine (mM)
0	51	0.83	0.83
0.5	48	1.28	0.78
2	47	2.77	0.77

the stoichiometric analysis below. A lag phase is expected in threonine synthesis due to the compulsory synthesis of the precursors. This lag phase is sometimes difficult to observe, perhaps because the first concentration measurement at 5 min is too late.

It is important to check that the stoichiometry is preserved during the inter-conversion of metabolites. Table 1 (corresponding to Figure 4a) gives a representative example of stoichiometric analysis of the curves in Figures 3 and 4; nearly all the consumed aspartate has been transformed into threonine using twice the concentration of NADPH and ATP. This means that threonine catabolism is negligible under our conditions, as mentioned above.

Variation of aspartate concentration

The concentration time courses as a function of aspartate concentration are shown in Figure 3. NADPH and ATP are shown corrected for their endogenous consumption. The plateau value in threonine synthesis depends upon the aspartate concentration as well as the rate of threonine synthesis itself (Table 2). The variation in the rate of threonine synthesis as a function

Table 3 NADPH and aspartate disappearance as a function of time at different aspartate concentrations

The data taken from Figure 5 were fitted with two exponential functions according to the equation $A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2) + C$ for aspartate decay and $A_1 \cdot \exp(-t/\tau_1) + A_3 \cdot \exp(-t/\tau_3) + C$ for NADPH decay, using the Excel Solver function. Other initial concentrations were 2 mM NADPH and 5 mM ATP.

	Aspartate time course			NADPH time course		
Initial [aspartate] ...	0.5 mM	2 mM	5 mM	0.5 mM	2 mM	5 mM
A_1 (mM)	0.31	0.54	0.76	0.75	1.21	1.54
A_2 or A_3 (mM)	0.18	0.3	0.058	0.47	0.8	0.47
C (mM)	0.0	1.16	4.16	0.7	0.0	0.0
τ_1 (min)	13.7	11.6	13.8	18	11.6	10.8
τ_2 or τ_3 (min)	100	100	100	323	170	185

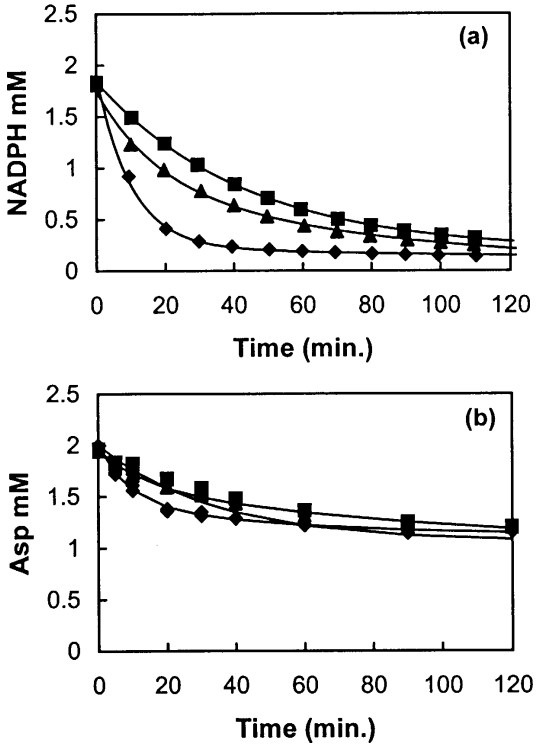

Figure 7 Time courses of NADPH (a) and aspartate (b) at different initial concentrations of threonine

◆, 0 mM threonine; ▲, 0.5 mM threonine; ■, 2 mM threonine.

of aspartate concentration (Table 2) can be fitted by a hyperbola with a maximal rate of 132 nmol/min per mg and a half-saturation in aspartate, aspartate$_{0.5}$, of 0.7 mM, close to the K_m of the AKs for aspartate [2]. This responsiveness to aspartate could indicate either a major role of the first enzyme in the control of the threonine pathway or a high sensitivity (elasticity) of AK to aspartate, or both [10–12]. The answer will be given in the following paper [13], where all these parameters will be calculated using a mathematical model.

The decreases in NADPH and aspartate concentrations shown in Figure 5 cannot be fitted by a single exponential function, indicating a complex dynamic process. Derivation of two exponential components from the curves indicates initial rapid processing of aspartate and NADPH (relaxation time in the order of 10 min) in every case (Table 3), followed by a slower process (with a constant relaxation time of 100 min when aspartate was varied, but times between 323 and 170 min when NADPH was varied). Both relaxation times for aspartate consumption are independent of the initial aspartate concentration. However, the amplitudes of the processes do depend on the aspartate concentration and could indicate different amounts of aspp being synthesized. The relaxation times τ_1 and τ_3 of the exponential functions fitting the NADPH decay are longer at 0.5 mM aspartate than at the other concentrations. This could indicate that the second and/or third steps are dependent on the initial aspartate concentration (through the aspp concentration for τ_1 and through the ASA concentration for τ_3).

Variation of threonine concentration

To determine the threonine concentration range to use, we performed a preliminary flux-measurement experiment where only the NADPH concentration was followed at different threonine concentrations (Figure 6). There were no observable differences between the curves at 0 and 0.25 mM threonine, nor between 2 and 10 mM. An intermediate NADPH curve was obtained at 0.5 mM threonine. Hence the progress curves of all the metabolites were determined at three threonine concentrations: 0, 0.5 and 2 mM (Figure 4). The final extent of threonine synthesis was nearly the same in all cases when the initial threonine concentration was taken into account (see Table 4). Unlike the case when aspartate was variable, the threonine-synthesis rate at steady state was constant (Table 4). Figure 7 shows the time courses of NADPH and aspartate at the three initial threonine concentrations. None of them can be fitted by a single exponential function, again stressing the complexity of the dynamics. Fitting the experimental points with two exponential functions again yields the same relaxation times (10 and 100 min) at 2 mM aspartate in the absence of threonine (Table 5). However, the first constant increases with threonine concentration, indicating the effect of threonine on AK activity and perhaps also on homoserine dehydrogenase activity.

DISCUSSION

We have designed an *in vitro* model of the threonine biosynthetic pathway to experimentally follow its global behaviour. For this, we used a crude extract from *E. coli* with the addition of aspartate. The main advantage of using a crude extract is being able to retain in our experiments the proportions of all the activities of the pathway as they are *in vivo* in *E. coli*. Although it is not possible to be certain that no loss of activity occurs

Table 5 NADPH and aspartate disappearance as a function of time at different threonine concentrations

The data, taken from Figure 7, are fitted with two exponential functions according to the equation $A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2) + C$ for aspartate decay and $A_1 \cdot \exp(-t/\tau_1) + A_3 \cdot \exp(-t/\tau_3) + C$ for NADPH decay, using the Excel Solver function. Other initial concentrations were 2 mM NADPH, 2 mM aspartate and 10 mM ATP.

	Aspartate time course			NADPH time course		
Initial [threonine] ...	0 mM	0.5 mM	2 mM	0 mM	0.5 mM	2 mM
A_1 (mM)	0.62	0.34	0.95	1.69	0.9	1.41
A_2 or A_3 (mM)	0.33	0.61	0	0.22	0.83	0.43
C (mM)	1.04	1.0	1.05	0	0	0
τ_1(min)	9.73	13	34	10	17.7	38
τ_2 or τ_3 (min)	100	99	–	308	84	167

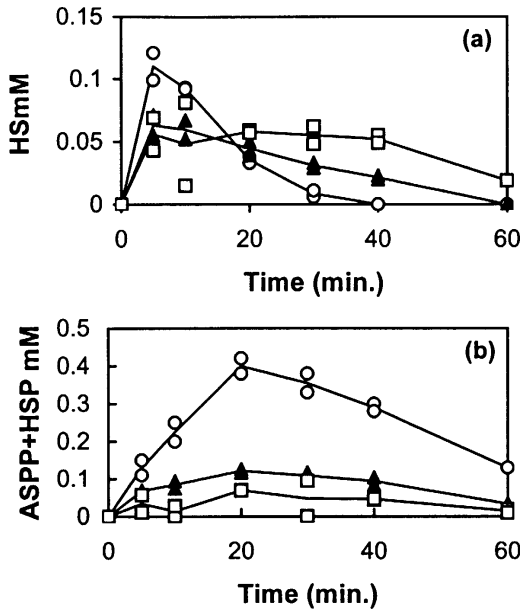

Figure 8 Time courses of the intermediates homoserine (HS; a) and aspp + *O*-phospho-homoserine (ASPP + HSP; b) at different initial concentrations of threonine

○, 0 mM threonine; ▲, 0.5 mM threonine; □, 2 mM threonine.

during the extraction procedure itself, this is an inescapable problem that would also be encountered in reconstitution of the pathway from purified enzymes in the proportions of the activities measured in a crude extract, as Szczesiul and Wampler [7] did, for instance. It appears in Figure 2 that the concentrations of all intermediate metabolites are much lower than those of aspartate and threonine. Furthermore the individual peaks of aspp and *O*-phospho-homoserine are difficult to separate in the analysis of the global flux because they both migrate very close to the large aspartate peak (Figure 2), so that we plotted the sum of both metabolites in Figures 2 and 3. We can see the transient appearance of the homoserine peak, which corresponds to a quasi steady-state synthesis of threonine. In their reconstituted threonine pathway from isolated enzymes, Szczesiul and Wampler [7] also found a transient accumulation of the intermediate metabolites, with a subsequent drop in threonine production attributed by the authors to inhibition of AK I. In our experiment the slowing of threonine synthesis could simply be due to the exhaustion of NADPH (or of aspartate when the initial aspartate concentration was 0.5 mM).

In our experiments, we have checked that the sum of all the metabolites is equal to the initial concentration of aspartate. We have also checked that the amounts of NADPH and ATP consumed are twice those of the threonine formed (taking into account the endogenous consumption of these cofactors). An example of such a stoichiometric analysis is shown in Table 1.

Threonine synthesis

Threonine synthesis reaches a plateau after a quasi-stationary phase. The plateau value (see Tables 2 and 4) does not correspond to the maximum that could theoretically be reached (usually 1 mM, corresponding to 2 mM NADPH). This could be due to some inhibition by the threonine synthesized itself, since the threonine plateau slightly decreases in the presence of 0.5 and 2 mM threonine (Table 4). However the plateau level increases with the aspartate concentration (Table 2); this could be due to the equilibrium of the first two reactions, particularly the first one, which is not in favour of threonine synthesis. In addition, side reactions consuming NADPH and/or some of the metabolites of the pathway themselves can decrease the yield of threonine synthesis from aspartate. A slight decrease in the total threonine synthesized is observed as a function of threonine concentration (see the last column of Table 4).

Analysis of the quasi steady state

The analysis of the quasi steady-state phase shows that the rate of threonine synthesis responds to the aspartate concentration in a hyperbolic manner, with the half-maximal synthesis rate in the region of the K_m of AK for aspartate (see Table 2). However, the rate of threonine synthesis appears to decrease only slightly with the initial threonine concentration (Table 4). This corresponds to a very slight decrease in the transient homoserine peak, which is also weakly delayed in time (Figure 8a). The time course of aspp + *O*-phospho-homoserine concentration shows a strong decrease as a function of threonine concentration (Figure 8b), which can be attributed to a decrease in aspp correlating with the concomitant decrease in NADPH consumption (Figure 7a) in accordance with AK inhibition by threonine. This means that the inhibition by threonine is more potent on AK than on the other steps, which are also inhibited by threonine, otherwise accumulation of some intermediate metabolites would be seen; this is particularly true for homoserine, which does not change very much as a function of threonine concentration, as already observed by Szczesiul and Wampler [7]. In contrast, these authors also observed an increase in threonine synthesis when the AK activity was increased and the homoserine dehydrogenase activity decreased (using threonine-insensitive AK and homoserine dehydrogenase of *Rhodopseudomonas spheroides*). This stresses the sensitivity of threonine synthesis to the AK step, whether it is modulated by changing the aspartate or threonine concentrations or by the AK concentration itself.

Analysis of the pre-steady state

Like Szczesiul and Wampler [7] we find a biphasic decrease in NADPH concentration and, in our case, also in aspartate concentration. We have analysed the time courses of both metabolites with the sum of two exponential components and found that the two relaxation times were dependent on threonine concentration but not on aspartate concentration in the case of the aspartate decay, whereas they were both dependent on aspartate and threonine concentration for the NADPH decay (Tables 3 and 5). One has to be cautious in the interpretation of the decomposition of these time courses into only two exponential components. Indeed, a five-step process could readily show, in principle, five exponential terms that would be dependent on the rate constants of the individual enzyme reactions. Furthermore, the rate function of each of the steps is complex and could readily give rise to more than one exponential component. However, it is futile to analyse our data with more than two exponential terms because any additional parameters would be ill-determined. Furthermore, the aspartate and NADPH time courses are not completely identical because aspartate is a substrate of the first reaction whereas NADPH is a cofactor of the second and third reactions; thus one can expect that analysis of both time courses will give information about the first three steps. Indeed, consideration of Tables 3 and 5 suggests three time constants. τ_1 is approx. 10 min, which corresponds to the same fast decrease

of aspartate and NADPH; τ_1 is independent of aspartate concentration and increases with threonine concentration. τ_2 is constant at approx. 100 min; it corresponds to the slower step in aspartate decay and is insensitive to aspartate and threonine, and thus could correspond to the second step catalysed by aspartate semialdehyde dehydrogenase. τ_3 corresponds to the slower decay of NADPH variation and varies between 84 and 323 min; it is sensitive to both aspartate and threonine; however, this time constant is rather difficult to estimate because the time course of NADPH was not recorded on an appropriate time scale for this slow exponential. The fact that both exponential time constants of aspartate variation are independent of aspartate concentration could indicate that AK reactions are more rapid than the following reactions.

Conclusions

The conditions used in this paper to study the behaviour of the whole threonine pathway are still far from physiological conditions in terms of enzyme and metabolite concentrations. However, we would like to stress that the relative concentrations of the enzymes are the same as they are *in vivo* because we used a crude extract. In the accompanying paper [13], the complete time course of all the metabolites (i.e. not only the quasi-stationary section) will be used for precise assessment of the individual kinetic parameters involved in a mathematical model of the pathway. This theoretical model will be used, in turn, to make predictions for conditions that cannot be easily performed or accessed experimentally, especially *in vivo* conditions with the measured *in vivo* concentrations of enzymes and metabolites. This will show the benefits of such an approach under non-steady-state conditions, not only for the experimental study of a biochemical pathway but also for model building.

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