THREONINE SYNTHETASE FROM HIGHER PLANTS: STIMULATION BY S-ADENOSYLMETHIONINE AND INHIBITION BY CYSTEINE

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SUMMARY

S-Adenosylmethionine greatly stimulates the formation of threonine from O-phosphohomoserine by an enzyme from sugar beet leaves. The stimulation due to S-adenosylmethionine is inhibited by cysteine. Cysteine and O-phosphohomoserine are incorporated into cystathionine by another enzyme. The results suggest that the conversion of O-phosphohomoserine to either threonine or cystathionine is regulated by the relative amounts of cysteine and S-adenosylmethionine present.

Threonine synthetase (E.C. 4.2.99.2) from many microorganisms and fungi (1,2,3,4,5) converts HSP[1] to threonine. Recently, this enzyme was demonstrated in higher plants (6) but the activity was difficult to detect. In Neurospora crassa, cystathionine synthetase is inhibited by SAM and stimulated by N^5-methyltetrahydrofolic acid (7). To determine if cystathionine synthetase in higher plants was similarly regulated, labelled HSP was synthesized with homoserine kinase, and cystathionine synthetase and threonine synthetase were assayed. The pronounced stimulation of threonine synthetase by SAM shows that threonine formation is affected by another product derived from aspartate. The inhibition of threonine synthetase by cysteine would increase the amount of HSP that is converted to cystathionine and methionine when the cysteine level is high and increase the amount of HSP that is converted to threonine when the cysteine level falls.

[1]Abbreviations: HSP, O-phosphohomoserine; SAM, S-adenosylmethionine; DTT, dithiothreitol; ACS, aqueous counting scintillant from Amersham-Searle.

MATERIALS AND METHODS

All amino acids were of the L-form, unless otherwise stated. Unlabelled HSP was prepared as described by Schnyder and Rottenberg (8) except that N-carbobenzoxy-L-homoserine p-nitrobenzyl ester was prepared as described by Pande et al. (9). [^{3}H]HSP was prepared by incubating in a total volume of 0.4 ml: 20 µCi [^{3}H]homoserine (260 µCi/µmole); KCl, 40 µmoles; Tris-HCl, pH 8.9, 50 µmoles; Mg-ATP, 10 µmoles; DTT, 2 µmoles; 0.04 ml glycerol and 0.16 ml of a radish leaf extract (see below) at 30° for 2 hours. After the reaction was stopped by boiling for 2 min, and the protein removed by centrifuging, the supernatant was put through a 0.9 x 3 cm column of Dowex[2] 50-H$^+$ (X8, 200-400 mesh). The HSP was recovered by rinsing the column with 15 ml of H_2O. The radish leaf extract was prepared by homogenizing 100 g of leaves with 100 ml 0.1 M KPO_4 buffer, pH 7.5, mM DTT, in a Waring blendor. The homogenate was strained through cheesecloth and centrifuged at 65,000 g for 30 min. The supernatant was made 75% saturated with $(NH_4)_2SO_4$, the precipitate collected, and resuspended in 0.05 M Tris-HCl, pH 7.5, 2 mM DTT. The proteins were purified of salts by passage through a Sephadex G-25 column in the same buffer. The protein fraction was frozen.

Assay for threonine synthetase. The sugar beet enzyme was prepared essentially as the radish leaf extract except that 50 ml of homogenizing media containing 10 g of purified polyvinylpyrrolidone, 0.1 M KPO_4 buffer (pH 7.0), 2 mM DTT, 1 mM mercaptobenzothiazole and 20% glycerol were used for 25 g of sugar beet leaves. The incubation mixture contained 50 µmoles of morpholinopropanesulfonic acid (pH 7.3), 2 µmoles DTT and 0.07 µCi [^{3}H]HSP (260 µCi/µmole or 0.64 µCi/µmole) and extract in 0.2 ml. After incubation for 30 min at 30° C, the reaction was stopped by the addition of 0.8 ml of 5% trichloroacetic acid. The protein was removed by centrifugation and the supernatant was applied to a 1 x 0.5 cm column of Dowex 50-H$^+$. The HSP was rinsed through the resin with 5 ml of H_2O and discarded. The homoserine formed by phosphatase action and threonine were eluted with 4 ml of 0.4 N HCl and 1.0 ml of H_2O. A 0.8 ml aliquot of HCl eluate was mixed with 0.2 ml of 2 N NH_4OH and 5 ml of ACS solution and counted with an efficiency of about 35%.

Threonine was distinguished from homoserine in the HCl eluate by degradation with periodate. The HCl eluate (4 ml) was evaporated to dryness in vacuo. The residue was dissolved in 0.3 ml of 0.1 M Na phosphate buffer (pH 7.0) and 0.2 ml of 0.25 M $NaIO_4$, and incubated 30 min at 30° C. After the excess periodate was removed by the addition of 0.5 ml of 0.25 M ethyleneglycol, the solution was placed on a column of Dowex 50-H$^+$ (1.5 x 0.5 cm) and was washed through with 4 ml of H_2O. Threonine degradation products were measured by scintillation counting as above. The absorbed homoserine was eluted with 1 ml of 2 N NH_4OH and the resin was rinsed with 4 ml of H_2O. A 1-ml aliquot was counted. Controls containing no enzyme were run with each series.

Proof of the identity of the threonine produced was obtained by paper chromatography of aliquots of the HCl eluate. Most of the counts co-chromatographed with authentic threonine by two-directional paper chromatography in phenol:H_2O (100:28, v:v) and in n-butanol:acetic acid:H_2O (12:3:5, v:v:v). The radioactive material that co-chromatographed with threonine agreed quantitatively with the radioactivity that was degraded by periodate.

Cystathionine synthetase. Cystathionine was synthesized in an incubation mixture similar to that used for threonine synthesis except that 0.2 µmoles of

[2]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

cysteine were included. The incubation conditions, cessation of reaction, and handling on resin were carried out as above. However, cystathionine is not eluted along with threonine and homoserine with HCl, but is eluted (after HCl elution) with 1 ml of 2 N NH_4OH and 4 ml of H_2O. Cystathionine formed was measured by liquid scintillation counting. The identity of cystathionine was established by chromatography and electrophoresis with authentic cystathionine.

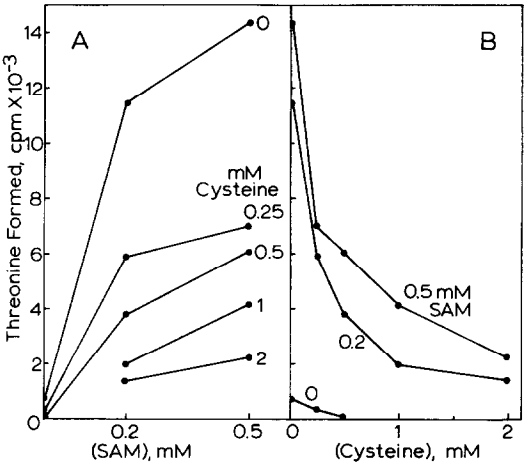

Figure 1. Threonine formed after sugar beet enzyme was incubated with the indicated concentrations of SAM and cysteine, and high specific activity HSP.

RESULTS

Figure 1A shows that SAM markedly (up to 20 fold) stimulated threonine synthetase while Figure 1B shows that the stimulation due to SAM was reduced by cysteine. To rule out the possibility that the SAM stimulation was an artifact caused by the extremely low concentration of HSP used in the assays shown in Figure 1, threonine synthetase was assayed with low specific activity HSP as shown in Figure 2. The Km for HSP and the relative V_{max} with various additions are shown in Table 1. These results show that the conclusions based on Figure 1 were valid at all concentrations of HSP used. In addition, the rate of cystathionine synthesis is intermediate between that of threonine synthesis in the presence of 0.5 mM SAM and that of threonine synthesis when both cysteine and SAM were present. The Km and V_{max} calculations demonstrated that the effect of

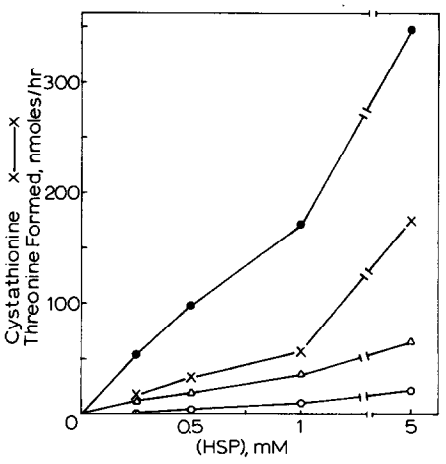

Figure 2. Activity of threonine synthetase and cystathionine synthetase as a function of HSP concentration. Cystathionine synthetase x—x; threonine synthetase: no additions o—o; in the presence of 0.5 mM SAM ●—●; in the presence of SAM and 1 mM cysteine Δ—Δ.

TABLE I. Km for HSP and Relative V_{max} for Threonine Synthetase and Cystathionine Synthetase

	Km (mM)	Rel. V_{max} (nmoles/hr)
Threonine synthetase		
No additions	2.7	38.2
Plus SAM (0.5 mM)	2.2	534.3
SAM plus cysteine (1 mM)	1.3	84.7
Cystathionine synthetase	6.6	446

SAM on threonine synthetase was to increase the rate of the reaction and not to increase the affinity of the enzyme for the substrate, HSP. Usually, the effect of an allosteric activator is to increase the affinity for the substrate (10),

but V_{max} effects do occur (11). The Km for cysteine in the cystathionine synthetase reaction was less than 0.1 mM.

At intermediate cysteine concentrations a biphasic curve was seen for the stimulation due to SAM (data not shown). The stimulation due to SAM was completely reversible, since when threonine synthetase was incubated with SAM for 30 min and then dialyzed overnight to remove SAM, the enzyme was inactive unless SAM was added. This indicates that SAM was not methylating threonine synthetase. The above results are consistent with SAM being an allosteric activator of threonine synthetase.

The stimulation due to SAM was quite specific. S-adenosylethionine stimulated about 40% as much as SAM. Spermine, spermidine, S-adenosylhomocysteine, and S-methylmethionine did not stimulate threonine formation. The inhibition by cysteine was also specific; homocysteine or D-cysteine were less than 20% as effective as L-cysteine.

Stimulation of threonine synthetase by SAM and inhibition of the SAM stimulation by cysteine was also observed with an enzyme from radish leaves (data not shown) indicating that the SAM stimulation may be a general characteristic of plant threonine synthetase. It was not necessary to add pyridoxal phosphate to either the sugar beet or radish leaf threonine synthetase.

The first unique step in methionine synthesis starting from homoserine (if cystathionine is an obligatory intermediate) (12) is the condensation of HSP and cysteine by cystathionine synthetase. Therefore, we thought that this enzyme might be inhibited by methionine or SAM, but no significant inhibition by either of these compounds was found (data not shown).

DISCUSSION

The question that is raised by the results shown is whether or not the SAM stimulation of threonine synthetase and the inhibition of this stimulation by cysteine are important parts of the regulation of the synthesis of the amino acids in the aspartate family. Figure 3 shows some of the pathways and the regulatory interactions involved in the aspartate family of amino acids in

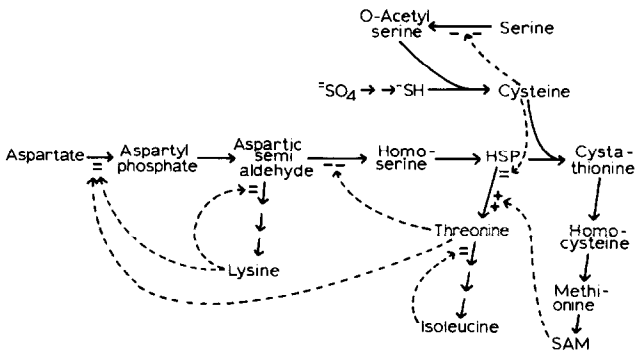

Figure 3. Metabolic pathways of the amino acids derived from aspartate. The dashed lines show regulatory interactions; plus signs indicate activation and minus signs inhibition of enzymes.

higher plants. The scheme, as shown, suggests that threonine plays a central role in the regulation of these amino acids and in the coordination of the formation of amino acids of the aspartate family with the pathway leading from sulfate to cysteine. The level of threonine would play a critical role in the flow of carbon into homoserine by regulation of the activity of homoserine dehydrogenase (13,14) and aspartokinase (15,16,17,18,19). Threonine synthetase, depending on the amount of SAM that was present, could determine the relative amount of HSP that is converted to cystathionine and methionine or threonine. If cysteine were absent no cystathionine could be formed, but if cysteine were present, the inhibition of threonine synthetase would make more HSP available for cystathionine synthesis. If adequate amounts of methionine and SAM were present, more of the HSP would be converted to threonine and isoleucine.

Although threonine is not nearly as effective as lysine in inhibiting the aspartokinase from some monocotyledons (15,18,19), threonine plus lysine inhibited this enzyme more than lysine alone. In cucumbers, both threonine and lysine were required to inhibit aspartokinase (17) while in five other plant species both threonine and lysine were inhibitory. So threonine probably plays a role in regulating the activity of aspartokinase.

Cheshire and Miflin (23) found that the aspartokinase from maize was inhi-

bited by lysine, but not by threonine. In addition, however, they found that lysine inhibited dihydrodipicolinic acid synthetase (the first enzyme in the pathway from aspartic semialdehyde to lysine) so that lysine could not accumulate, and shut off the synthesis of threonine, isoleucine and methionine. Cysteine inhibits serine acetylase (20) and isoleucine inhibits threonine deaminase (21,22).

It seems likely that the reason that threonine synthetase had not been detected in plants before 1975 (6) was that the activity is very low without SAM. Schnyder et al. incubated their pea seedling extract 25 hr at 37° C and added pyridoxal phosphate to demonstrate threonine synthesis.

Demonstrations of positive allosteric effects in higher plants are rare. Cotton and Gibson (24) found that tryptophan stimulated chorismate mutase from pea seedlings, but the stimulation was small (3 fold) as compared to the 20-fold stimulation of threonine synthetase by SAM.

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