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FEEDBACK INHIBITION OF HOMOSERINE KINASE FROM RADISH LEAVES

HOWARD J. BAUM,* JAMES T. MADISON and JOHN F. THOMPSON

U. S. Plant, Soil and Nutrition Laboratory, USDA–ARS, Tower Road, Ithaca, NY 14853, U.S.A.

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Key Word Index—*Raphanus sativus*; Cruciferae; radish; biosynthesis; regulation; inhibition; methionine; threonine; aspartate family; homoserine kinase.

Abstract—Homoserine kinase is a potential control point in the biosynthetic pathway for threonine, isoleucine and methionine. The radish leaf enzyme was tested for the effects of end products on its activity. L-isoleucine, L-threonine, but not their D-isomers, and L-*S*-adenosylmethionine were effective inhibitors. At 0.1 *mM*, L-*S*-adenosylmethionine and L-isoleucine showed a marked synergistic inhibition. The combination of isoleucine, threonine, methionine and *S*-adenosylmethionine, at levels found in the radish leaf, inhibited homoserine kinase *ca* 25%. This fact, plus the stereospecificity of the inhibition suggested that the inhibition is involved in physiological control of threonine, isoleucine and methionine biosynthesis. Synergistic inhibition by compounds differing in charge and shape indicated that there is one major homoserine kinase with two allosteric sites.

INTRODUCTION

Aspartic acid is a precursor of lysine, threonine, isoleucine and methionine. Because of the multiple products of the aspartate pathway there has been considerable interest in its regulation [1]. There is evidence for control by feedback inhibition of aspartokinase and homoserine dehydrogenase [1], but there is less persuasive evidence for the control of other enzymes in the pathway. Homoserine kinase as the last enzyme before bifurcation of the biosynthetic pathway towards methionine or towards threonine and isoleucine might be regulated.

Aarnes [2, 3] found no significant inhibition of homoserine kinase from barley seedlings by the amino acids he tested. On the other hand, Thoen *et al.* [4] found inhibition by amino acids using the enzyme from peas. However, peas may not be a representative species because they have an unusually large free homoserine pool [5].

The purpose of the present study was to determine if homoserine kinase from a dicotyledonous plant (other than peas) is under possible physiological regulation by the end products of the methionine and isoleucine biosynthetic pathways. Several amino acids, separately and in combination, were tested as inhibitors. Amino acid analogs were also used in an attempt to examine the size, shape and charge characteristics of the binding sites.

RESULTS

The product of enzymatic activity was identified as *O*-phosphohomoserine as follows. The product derived from homoserine was not retained on a sulfonic acid ion exchange resin (behavior like that of homoserine phosphate [5]). The action of phosphatase on the product yielded a compound that bound to the resin and cochromatographed on paper with homoserine. There was no significant phosphatase activity in the homoserine kinase preparation because when homoserine phosphate was incubated 15 min with the enzyme preparation there was no reduction in the amount of homoserine phosphate.

Radish leaf homoserine kinase had a pH maximum between pH 8.3 and 9.1. The enzymatic activity was the same in two buffers and without added buffer. Homoserine kinase was stimulated by KCl with a maximum at 0.2 M (Fig. 1) and required ATP and Mg^{2+}. Product formation was linear through 60 min with 0.01 ml of radish leaf extract (data not shown). The reaction conditions were chosen to be at the optimum pH and near the optimum KCl concentration. Product formation was proportional to incubation times and enzyme concentration.

All the amino acids and amino acid analogs tested gave an inhibition curve with the same general shape. The inhibition of homoserine kinase was stereospecific; both L-isoleucine and L-threonine (Table 1) were much more inhibitory than D-isoleucine and D-threonine. L-Isoleucine, L-threonine and L-*S*-adenosylethionine (SAE) were the most inhibitory of the non-polar, polar hydroxy

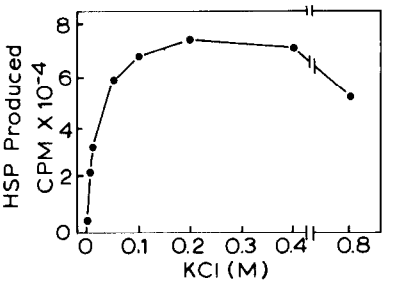

Fig. 1. Effect of KCl concentration on homoserine kinase activity. Homoserine phosphate (HSP).

*Present address: Department of Biochemistry, Brandeis University, Waltham, MA 02254, U.S.A.

Table 1. Inhibition of homoserine kinase by single amino acids and amino acid analogs

Amino acid group	Amino acid	Concn of amino acid (mM) 0.1	1.0	5.0	7.5	10.0	15.0
		% Inhibition					
Non-polar	L-Ile	4.4	40.6	76.8	—	87.9	92.1
	L-Val	2.9	22.4	67.6	—	79.1	86.1
	L-Leu	2.9	18.7	42.5	—	63.6	75.8
	L-Norval	6.8	18.7	43.9	—	63.1	74.1
	L-Norleu	0.0	6.6	26.7	—	40.3	56.3
	D-Ile	0.0	6.0	11.3	—	—	16.3
Polar-hydroxy	L-Thr	0.9	26.2	54.2	—	71.0	80.6
	L-Ser	0.8	4.6	25.4	—	39.9	49.9
	D-Thr	0.0	1.0	—	—	—	4.1
Sulfur compounds	L-Met	2.6	13.9	37.3	—	49.5	67.0
	L-Eth	0.0	1.9	19.1	—	24.3	39.7
	L-SAM	0.9	33.0	69.9	—	80.3	85.6
	L-SAE	10.9	51.0	89.0	93.6	—	—
	L-SAHC	0.0	14.1	58.5	70.8	—	—
	L-SMM	0.0	5.6	23.0	37.9	—	—

Abbreviations: Norval, norvaline; Norleu, norleucine; Eth, ethionine; SAM, *S*-adenosylmethionine; SAE, *S*-adenosylethionine; SAHC, *S*-adenosylhomocysteine; SMM, *S*-methylmethionine: others by normal convention.

and sulfur amino acids, respectively (Table 1). Both L-isoleucine and SAE inhibited homoserine kinase more than 90% at the higher concentrations. L-Valine and *S*-adenosylmethionine (SAM) also showed significant inhibition (e.g. > 20% at 1.0 mM) while the rest of the compounds tested were less inhibitory.

Combinations of amino acids were more inhibitory at low concentrations than single amino acids (Table 2). Both the SAM–threonine–isoleucine and SAM–isoleucine combinations inhibited *ca* 50% when each amino acid was present at 0.5 mM concentration. The SAM–threonine and isoleucine–threonine combinations were less inhibitory. The inhibitions were synergistic at 0.1 mM but less than additive at 1.0 mM concentrations of each amino acid (Table 3). At 0.1 mM, the SAM–isoleucine and SAM–threonine–isoleucine combinations were the most synergistic of the combinations tested.

Table 4 shows the inhibitions found when combinations of amino acids were used at the approximate concentrations found in radish leaves. [Since there was

Table 2. Inhibition of homoserine kinase by combinations of amino acids

Combination*	Concn of each amino acid (mM) 0.1	0.2	0.5	1.0
	% Inhibition			
SAM–Thr–Ile	19.2	29.8	50.9	65.5
SAM–Ile	20.1	30.2	48.8	63.6
SAM–Thr	4.7	16.2	31.3	46.5
Ile–Thr	6.8	13.3	28.2	43.1

*See Table 1 for abbreviations.

Table 3. Expected and measured inhibition of homoserine kinase by amino acid combinations

Combination*	0.1 mM Calc.	Found	Diff.	1.0 mM Calc.	Found	Diff.
	% Inhibition					
SAM–Thr–Ile	6.2	19.2	13.0	99.8	65.5	−34.3
SAM–Ile	5.3	20.1	14.8	73.6	63.6	−10.0
SAM–Thr	1.8	4.7	2.9	59.2	46.5	−12.7
Ile–Thr	5.3	6.8	1.5	66.8	43.1	−23.7

*See Table 1 for abbreviations.

Expected additive inhibitions were calculated from Table 1 while actual inhibitions were from Table 2.

Table 4. Inhibition of homoserine kinase by the concentrations of amino acids found in radish leaves

Amino acids present*	No. of tests	% Inhibition (mean ± s.e.)
Ile, Thr, Met, SAM, SMM	7	27.0 ± 2.0
Thr, Met, SAM, SMM (−Ile)	6	8.8 ± 3.2
Ile, Thr, Met, SMM (−SAM)	2	29.5 ± 1.1
Ile, Thr, Met, SAM (−SMM)	5	24.0 ± 2.7
Ile, Thr, SAM, SMM (−Met)	2	26.1 ± 4.6
Ile, Met, SAM, SMM (−Thr)	2	27.6 ± 1.4
Met, SAM, SMM (−Ile, −Thr)	4	7.8 ± 4.2

*See Table 1 for abbreviations.

considerable variation in the concentration of *S*-methylmethionine (SMM) found in radish leaves, a somewhat higher concentration was used than the average that was measured.] The data in Tables 4 and 5 suggest that the inhibition of homoserine kinase by amino acids is physiologically significant and that isoleucine is the most important effector. When isoleucine was present, the combinations of amino acids (at concentrations similar to those found in radish leaves) inhibited homoserine kinase *ca* 25–30%. Without isoleucine the combinations of amino acids were much less inhibitory (*ca* 8%). The fact that the L-forms of isoleucine and threonine were much more inhibitory than the D-forms suggests that the inhibitions observed may be of physiological importance.

Table 5. Concentrations of amino acids found in radish leaves and used in the inhibition experiments shown in Table 4

Amino acid*	Found in radish leaves	Concns used in expts shown in Table 4
	mM	
Ile	0.2	0.2
Thr	0.49	0.5
Met	0.05	0.04
SAM	0.017	0.02
SMM	0.11	0.32

*See Table 1 for abbreviations.

DISCUSSION

The end products of the aspartate family biosynthetic pathway are better inhibitors of homoserine kinase than other naturally occurring amino acids of similar structure; SAM and isoleucine, end products of this pathway, show considerably more inhibition than methionine and threonine, which are intermediates. Since SAM promotes isoleucine and threonine production by stimulating threonine synthetase [6], SAM appears to be important in regulation. Isoleucine inhibits its own production by inhibiting threonine deaminase [7] and homoserine kinase.

Threonine is a minor inhibitor of homoserine kinase but it plays an important regulatory role in the inhibition of homoserine dehydrogenase [8] and aspartokinase [9], two of the enzymes involved in the synthesis of homoserine from aspartate. L-Valine may also cause slight physiological inhibition even though it is not part of the aspartate family, presumably because of its similarity to isoleucine. The cellular level of L-valine in turnip leaves is *ca* 0.6 mM [10]. L-Valine shows *ca* 10% inhibition at 0.6 mM which might be significant. None of the other protein amino acids showed physiologically significant inhibition. SAE was more inhibitory than any other amino acid or amino acid analog. SAE is not normally present in plants and therefore has no regulatory role. Why SAE should be a better inhibitor than SAM is not clear.

Aarnes [3], working with homoserine kinase from barley seedlings, found no inhibition by any amino acid at concentrations up to 10 mM. Since barley is a monocotyledon, its regulatory mechanisms could be different from those in dicotyledons such as radishes. Thoen *et al.* [4], working with pea seedlings, found significant inhibition of homoserine kinase by SAM, isoleucine and valine. They found that only isoleucine and valine were physiologically inhibitory while SAM at 50 μM concentrations was not inhibitory. Radishes, on the other hand, showed physiological inhibition with isoleucine, threonine and SAM. However, Giovanelli *et al.* [5] found that the homoserine metabolism in peas is unusual in that peas have a large pool of free homoserine and is the only higher plant known to synthesize O-acetylhomoserine. Thoen *et al.* [4] found that pea homoserine kinase had a low affinity for homoserine, which could account for the large pool of free homoserine. They concluded that in peas homoserine fulfils other roles besides being an intermediate in the biosynthesis of amino acids.

Threonine appears to have no effect when combined with isoleucine. The SAM–threonine–isoleucine and SAM–isoleucine combinations inhibit to about the same extent. Also isoleucine–threonine and isoleucine give about the same amount of inhibition. This is consistent with the observation that the isoleucine–threonine combinations were the least additive and the SAM–isoleucine combinations were the most synergistic. The work of Datko *et al.* [11] offers one explanation for these phenomena. They suggested that there are two separable homoserine kinases in plants, one involved in threonine biosynthesis, the other involved in methionine biosynthesis. Isoleucine and threonine are in the same branch of the pathway and could inhibit the same isozyme. This hypothesis fails to explain why isoleucine or SAE, when alone, can inhibit greater than 90%. Walter *et al.* [8] found that homoserine dehydrogenase, with two separable isozymes, cannot be completely inhibited by any single amino acid.

A more likely explanation for the data is the existence of one enzyme with two allosteric sites (one for isoleucine and one for SAM), since it seems unlikely that SAM with its adenosyl-ribose group can share the same allosteric site with isoleucine. This explanation can also account for the synergistic effect at lower concentrations and the less than additive effect at higher concentrations of inhibitors.

The amino acid analog data can be explained by the assumption that the neutral amino acids (i.e. all except SAM, SAE, SAHC and SMM) act at the isoleucine allosteric site. Valine, threonine and serine would not fit the isoleucine allosteric site as well as isoleucine because their side chains are smaller. Threonine and valine would have similar inhibitions because of their similar size and shape. Leucine and norvaline have carbon chains of the same length as isoleucine, but differ in their side chains, so they are less effective than isoleucine. Methionine, norleucine, ethionine and *S*-methylmethionine are less inhibitory than isoleucine because they have a longer main chain (and lack the branch at the β-carbon). (In addition, *S*-methylmethionine could bind at the SAM allosteric site because of its positive charge.) The β-carbon branch appears to be more important than the length of the chain, but both are important for maximum inhibition. A study of the size and shape of the putative SAM allosteric site must wait for studies with more analogs.

From these results, it appears that regulation of the formation of methionine, threonine and isoleucine may depend on the synergistic action of several end products to inhibit homoserine kinase as well as inhibition of aspartokinase and homoserine dehydrogenase [1].

EXPERIMENTAL

Materials. White Icicle (short top) radish seeds were purchased from Agway Inc. (Syracuse).* L-[U-^{14}C]Homoserine and aqueous counting scintillant (ACS) were obtained from Amersham. All amino acids and analogs were from Sigma Chemical Co. Bacterial alkaline phosphatase was from Worthington Biochemicals. Homoserine phosphate was synthesized by the procedures in ref. [12], except that *N*-carbobenzoxy-L-homoserine *p*-nitrobenzyl ester was prepared as described in ref. [13].

Homoserine kinase preparation. The radish leaf extract was prepared by homogenizing 15 g of leaves with 25 ml 0.1 M KPO_4 buffer (pH 7.0), 2 mM dithiothreitol (DTT), 1 mM mercaptobenzothiazole, 1 mM ethylenediamine tetraacetic acid in an Omni Mixer (Servall) at 0°. The homogenate was filtered through two layers of cheesecloth and centrifuged at 26 000 *g* for 30 min. Ammonium sulfate was added to the supernatant to 70% saturation and the soln centrifuged at 20 000 *g* for 20 min. The ppt. was suspended in buffer A (50 mM Tris–Cl, pH 8.0, 1 mM DTT) and passed through a Sephadex G-25 column (12 × 3 cm) equilibrated with buffer A to remove salts. Glycerol was added (10%) and the protein fraction was frozen (−80°).

Homoserine kinase assay. The incubation mixture contained 0.1 μCi L-[U-^{14}C]homoserine (40 mCi/mmol), 0.1 M KCl, 0.1 M Tris–Cl (pH 8.90), 5 mM ATP, 5 mM Mg^{2+} (acetate), 5 mM DTT, 10% glycerol and 0.05 ml of enzyme extract in a total vol. of 0.2 ml. The mixture was allowed to incubate for 60 min at 30° and the reaction was stopped at 100° for 1 min. The reaction mixture was applied to a 1.5 × 0.5 cm Dowex 50-H^+ (× 8200–400 mesh) column. The homoserine phosphate was rinsed through the column with 4.8 ml H_2O while the unreacted homoserine remained on the resin. ACS (5 ml) was added to the homoserine phosphate soln and counted by liquid scintillation. All values presented are the average of at least 2 determinations. There was less than a 5% difference between duplicates.

Procedures for identification of homoserine phosphate. To identify the enzymatic product, the incubation procedures were scaled up 20-fold. After incubation, the mixture (4 ml) was heated at 100° for 3 min and diluted with 4 ml H_2O. The suspension was centrifuged and the supernatant was applied to a 4 × 0.5 cm column of Dowex 50-H^+ and washed with H_2O. The effluent and wash were neutralized with 1.5 N NH_4OH. ATP was removed from this soln. by adsorption on 5 ml of acid-washed activated charcoal. The charcoal was removed by centrifugation, the supernatant evapd to dryness and the residue dissolved in 1.5 ml H_2O.

One 50 μl aliquot was mixed with 450 μl of Tris–HCl (pH 9.6) and 10 μl (4.5 units) of *E. coli* alkaline phosphatase was added. In a control sample, the phosphatase solution was replaced with water. After the 2 samples were incubated 30 min at 37°, they were heated to boiling, diluted with 1 ml H_2O and centrifuged. Supernatant (0.9 ml) aliquots were passed through a 3 × 0.4 cm column of Dowex 50-H^+ and washed with 4 ml H_2O. The effluent and wash were mixed with 15 ml of ACS and radioactivity was measured by liquid scintillation. After elution with 2 N NH_4OH, the eluates were also counted.

A second pair of 50 μl aliquots of charcoal supernatant were treated as above except that Dowex 50-H^+ effluent and wash were dried. The dried effluents were mixed with homoserine phosphate and homoserine and chromatographed on paper with phenol–H_2O (25 : 7). After drying the paper, the amino acids were located by spraying with a soln of ninhydrin (0.2%) in EtOH. The spots were cut out and suspended in 5 ml 50% EtOH. After the addition of 15 ml ACS, the radioactivity was measured by liquid scintillation.

Determination of the effect of pH *on enzyme activity.* The Tris–Cl buffer in the normal incubation mixture was replaced by a 0.1 M Tris–Cl buffer in the range from pH 7.0 to 9.0 and a 0.075 M CAPS (cyclohexylaminopropane sulfonic acid)–0.025 M Tris–Cl buffer from pH 8.5 to 11.5. In one of the tubes, water replaced the buffer. The incubation mixture was then allowed to react with homoserine kinase according to the procedure above. The pH was measured on a duplicate set of incubation mixtures.

Determination of amino acids in radish leaves. About 10 g of radish leaves were ground in a mortar and pestle with cold 80% EtOH and centrifuged. The pellet was extracted 3 × 80% EtOH at room temp. The extracts were combined and divided in half. One half was passed through a column (0.9 × 6 cm) of Dowex 50-H^+ (× 8, 200–400 mesh) in 80% EtOH. The column was rinsed with 80% EtOH; the amino acids were eluted with 15 ml 2 N NH_4OH followed by 15 ml H_2O. The eluates were dried in a stream of air, transferred to test-tubes, redried and treated with 1 ml 2 M 2-mercaptoethanol for 1 hr at 100° to reduce methionine sulfoxide to methionine. After this reduction, samples must be analysed within a day or two because we have found that a compound that chromatographed with methionine was formed when glutamate was stored with mercaptoethanol in 0.1 M HCl at −15°. Amino acids were determined on an amino acid analyser (Beckman 119CL).

The other half of the ethanol extract was passed through a 0.9 × 6 cm column of Dowex 50-NH_4^+. The column was rinsed with 10 ml 80% EtOH and then 10 ml H_2O. The basic amino acids were eluted with 10 ml each of 0.01, 0.02, 0.05, 0.2, 0.4, 0.5, 0.6 and 0.7 M NH_4HCO_3. The absorbancies were determined at 260 nm. The *S*-adenosylmethionine content was calculated from the A_{260} determination of the 0.2, 0.4 and 0.5 M solutions. The 0.02–0.4 M solns were combined, dried in an air stream and the *S*-methylmethionine content was determined on an amino acid analyser.

*Mention of 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.

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