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An ATP-independent Phosphotransferase in Higher Plants. The Formation of O-Phospho-L-homoserine from O-Succinyl-L-homoserine and Phosphate

An ATP-independent phosphotransferase was newly found to be widely distributed in higher plants. This novel enzyme catalyzes the synthesis of O-phospho-L-homoserine from O-succinyl-L-homoserine and K-phosphate. About 1/15—1/18-fold activity was observed when O-acetyl-L-homoserine replaces O-succinyl-L-homoserine, but L-homoserine itself could not serve as a donor of the 3-amino-3-carboxypropyl-moiety.

Some other properties of the enzyme are also described.

Keywords——enzyme; ATP-independent phosphotransferase; homoserine kinase; amino acid; O-phospho-L-homoserine; homoserine; cystathionine; biosynthesis; *Spinacia oleracea*; *Nicotiana tabacum*

O-Phospho-L-homoserine (**6**) has recently been found to be an important intermediate in the biosynthesis of threonine (**7**) from L-homoserine (**1**) in higher plants [1–4] as it is in many bacteria and fungi.[5–7] L-Cystathionine (**4**) is also synthesized in plants from O-phospho-L-homoserine (**6**), as the activated form of L-homoserine (**1**), rather than from O-acetyl-L-homoserine (**2**) or O-succinyl-L-homoserine (**3**) as in bacterial systems. Although a number of alternative pathways (Figure 1) are available for the synthesis of cystathionine (**4**) and homocysteine (**5**) from L-homoserine in bacteria[8] and plants,[4,9,10] O-phospho-L-homoserine is a key branch-point intermediate in plants for the biosynthesis of threonine (**7**)-isoleucine and cystathionine (**4**)-methionine from L-homoserine by the aspartate pathway.

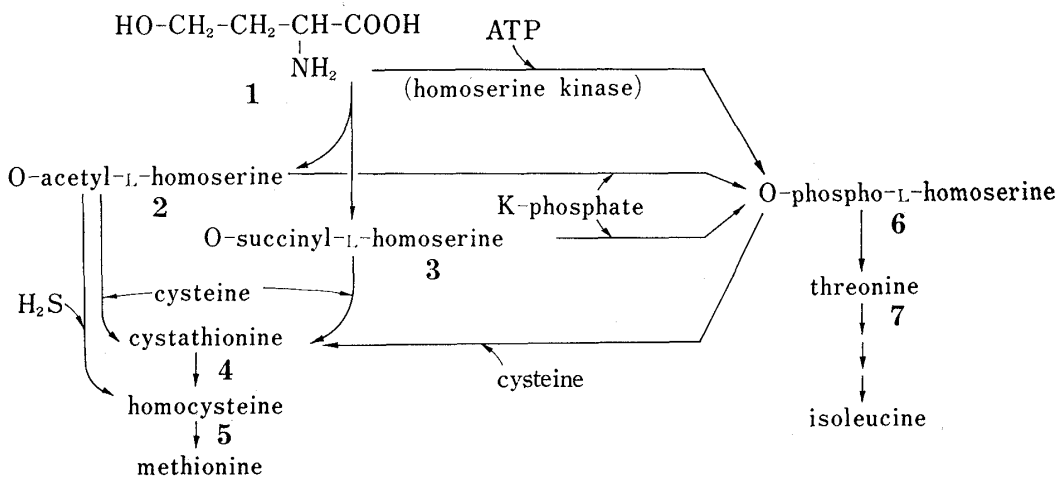

Fig. 1. The Pathway of Biosynthesis of Amino Acids derived from Homoserine in Plants

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Although the formation of O-phospho-L-homoserine (**6**) in bacteria, fungi[8] and plants[3,4,11] can occur by an ATP-dependent reaction catalyzed by homoserine kinase, we have now found a novel ATP-independent phosphotransferase capable of forming O-phospho-L-homoserine (**6**) from O-succinyl-L-homoserine and K-phosphate in the crude extracts of a wide phylogenetic range of higher plants as shown in Figure 2.

This communication presents preliminary evidence for the APT-independent L-homoserine O-phosphotransferase in higher plants and describes some properties of the new enzyme.

The enzyme preparations were obtained predominantly from the relatively young leaves of spinach (*Spinacia oleracea*), tobacco (*Nicotiana tabacum*) and other plants shown in Figure 2. Unless otherwise stated, enzyme fractions were prepared from the spinach leaves, purchased at local markets, essentially by methods described in previous papers[12,13] for β-substituted alanine synthases: plant material (30 g) was macerated in 0.1 M Tris-HCl buffer (120 ml), pH 7.6, containing 0.1% (v/v) 2-mercaptoethanol and 0.5% (w/v) Na-erythorbate at 0—4° in the presence of 15 g of polyvinylpolypyrrolidone (Sigma). Clear supernatant solutions, recovered after centrifugation at 25000 ***g*** for 30 min, were used to prepare a partially purified (*ca.* 5-fold) enzyme: a protein fraction precipitated between 35 and 50% $(NH_4)_2SO_4$ saturation was dissolved in 0.05 M Tris-HCl buffer (pH 7.6) and desalted by elution from a column of Sephadex G-25 (fine) equilibrated in the same buffer. The eluate was used directly as the source of enzyme.

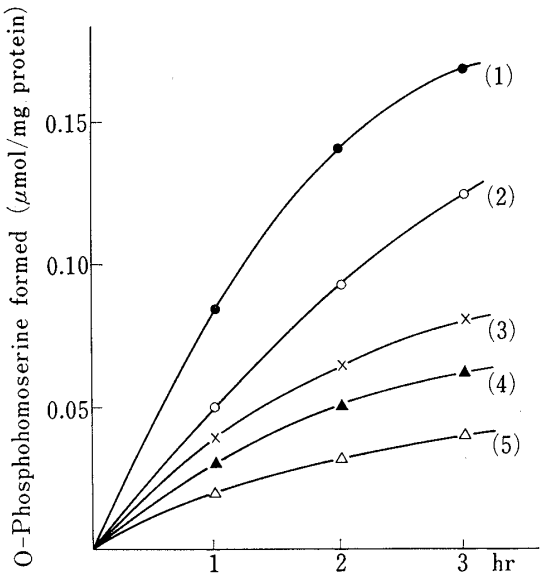

Fig. 2. Comparative Specific Activity for the O-Phosphohomoserine Formation by An ATP-independent Phosphotransferase in the Leaves of (1) *Spinacia oleracea*, (2) *Brassica juncea*, (3) *Datura alba*, *Datura metel* and *Lycium chinense*, (4) *Solanum tuberosum*, *Nicotiana tabacum* and *Citrullus vulgaris*, (5) *Papaver bracteatum*, *Atropa belladonna*, *Pisum sativum* and *Mentha ratundifolia*

Standard reaction mixtures used to demonstrate the formation of O-phosphohomoserine contained O-succinyl-L-homoserine (10 μmol), K-phosphate (30 μmol) and 0.2 ml of enzyme preparation (containing 2—3 mg of the soluble protein derived from 1 g fresh wt. of plant material) in a final volume of 0.6 ml. Reaction mixtures were normally incubated at 28° for appropriate periods. Reactions were terminated by the addition of 3 volumes of ethanol. Precipitated protein was removed by centrifugation and samples of the residual supernatant solution were examined chromatographically and chemically for the presence of O-phosphohomoserine. The presence of O-phosphohomoserine in final reaction mixtures was established by paper chromatography using the following solvent systems: 1, 2-propanol–formic acid–water (20:1:5, v/v); 2, 2-propanol–acetic acid–water (7:1:2, v/v); 3, 1-butanol–formic acid–water (3:1:1, v/v).

These solvents indicated clearly the presence of a product, reacting positively with 0.5% ninhydrin (reddish-violet), 0.1% bromocresol green (yellow) for acids and 5% ammonium molybdate-vanadyl chloride (blue) for phosphate[14] as chromogenic reagents. The *Rf* values

11) H. Aarnes, *Plant Sci. Lett.*, **13**, 103 (1978).
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for O-phosphohomoserine obtained in these solvents were 0.15, 0.08 and 0.32, respectively, whilst O-succinylhomoserine gave the following values; 0.50, 0.36 and 0.56, respectively. Under the same conditions, homoserine moved at *Rf*'s of 0.41, 0.29 and 0.51, respectively. Succinic acid and phosphoric acid also moved at *Rf*'s of 0.83 and 0.42 in solvent 1.

The formation of O-phosphohomoserine was not detected in incubation mixtures from which K-phosphate or O-succinyl-L-homoserine were omitted, nor when the enzyme preparation was pretreated at 100° for 15 min.

The further identity of the reaction product as O-phosphohomoserine was confirmed by stoichiometric convertion by 15% HCl into homoserine and phosphoric acid[15] by a modification of the methods of Watanabe, *et al.*[16] and G. Agren.[17]

The properties of the enzyme were examined to a preliminary manner. O-Succinyl-L-homoserine was the preferred activated form of L-homoserine for the ATP-independent O-phosphohomoserine synthase; the activity found with O-acetyl-L-homoserine as substrate was only about 6% of that with O-succinyl-L-homoserine and L-homoserine itself could not serve as a donor of the 3-amino-3-carboxypropyl-moiety.

The enzyme exhibited a pH optimum of 7.6 and half maximal activity was measured at pH 6.8 and 8.2, using 0.05 M Tris-HCl buffers.

Under the assay conditions employed the reaction rate was linear with time for at least 60 min, and with the amount of enzyme added in the range (0—9.2 mg) tested.

The synthase activity for O-phosphohomoserine was very dependent upon the concentration of phosphate used: a relatively low final concentration of K-phosphate (40—50 μmol) was required to give maximum rate of O-phosphohomoserine formation in the presence of fixed level (10 μmol) of O-succinyl-L-homoserine and higher concentration of phosphate lead to progressively more marked substrate inhibition. From the Lineweaver-Burk plot of these results, the K_m values were found to be 1.47×10^{-5} M for O-succinyl-L-homoserine and 6.45×10^{-6} M for phosphate.

The partially purified enzyme preparation had a specific activity of 1.417×10^{-3} μmol/mg protein/min and was reasonably stable: when stored at 0° for 24 hr, the residual enzyme activity for O-phosphohomoserine formation was about 75—78% of the activity initially assayed.

The addition of pyridoxal phosphate, as a possible coenzyme, to the reaction mixtures at 15—20 μg/ml enhanced the rate of O-phosphohomoserine synthesis about 62—65%. Complete inactivation of the enzyme was effected by NH_2OH(1.42×10^{-2} mM) in experiments where pyridoxal phosphate was omitted from the reaction mixtures. In the presence of phenylhydrazine (0.5 mM) or KCN (7.85 mM) the activity was inhibited by more than 50%.

Addition of $(NH_4)_2SO_4$ (0—42 mM) had no influence on the reaction rate, but NaCl and KCl (42—84 mM) inhibited the reaction by about 21—37%. $MgSO_4$ (42 mM) also produced significant inhibition (40%) due to complex formation between Mg ion and phosphate. When cysteine was present in the reaction mixtures, cystathionine was also produced.[18]

Enzyme preparations from a number of other plant species were also examined for their ability to catalyze the formation of O-phosphohomoserine from O-succinyl-L-homoserine and K-phosphate. In consequence the O-phosphohomoserine synthase activity was found to be widely distributed in higher plants as shown in Figure 2.

The specific activity of homoserine kinase present in the *Spinacia* enzyme preparation was approximately 7.5-fold greater than that detected with O-succinyl-L-homoserine and

15) Phosphoric acid was quantitatively determined by the method of D.F. Boltz and M.G. Mellon (*Anal. Chem.*, **19**, 873 (1947)).
16) Y. Watanabe and K. Shimura, *J. Biochem.* (Tokyo), **43**, 283 (1956).
17) G. Agren, *Acta Chem. Scand.*, **16**, 1607 (1962).
18) Cystathionine γ-synthase activity detected with O-succinylhomoserine or O-phosphohomoserine and cysteine was presented in *Spinacia* enzyme preparations examined.

K-phosphate. In contrast, the phosphorylation reaction catalyzed by the *Nicotiana* enzyme preparations was much more active with O-succinyl-L-homoserine and K-phosphate than that for the homoserine kinase.[19]

These observations suggest that this novel ATP-independent phosphotransferase may play a major physiological role by providing an alternative pathway for O-phosphohomoserine synthesis in such plants as *Nicotiana tabacum*.

A more detailed investigation of the enzyme from higher plants is in progress in our laboratory.

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19) Homoserine kinase activity in *Nicotiana* enzyme preparations was negligible under the same conditions.