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COMPARATIVE ENZYMOLOGY OF CYSTATHIONINE AND SELENOCYSTATHIONINE SYNTHESIS OF SELENIUM-ACCUMULATOR AND NON-ACCUMULATOR PLANTS

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Key Word Index—*Astragalus* spp.; *Neptunia amplexicaulis*; Leguminosae; cystathionine; selenocystathionine; *O*-phosphorylhomoserine; cysteine; selenocysteine; selenium.

Abstract—Crude extracts of *Astragalus racemosus*, a selenium-accumulator plant, supported the incorporation of [^{3}H]cysteine into cystathionine in the presence of *O*-phosphorylhomoserine (PHS) (K_m cysteine = 0.14 mM). Unlabelled selenocysteine inhibited [^{3}H]cysteine incorporation; at (cysteine plus selenocysteine) concentrations less than 0.3 mM the kinetics most closely approximated competitive inhibition (K_i selenocysteine = 0.07 mM). Extracts of *A. racemosus* also supported the incorporation of [^{14}C]PHS into cystathionine in the presence of cysteine; an analogous reaction proceeded at similar rates when selenocysteine replaced cysteine (K_m selenocysteine = 0.07 mM). The product was presumed to be selenocystathionine. The total [^{14}C]PHS incorporation in the presence of both cysteine and selenocysteine did not exceed that of either substrate alone. It was concluded that cysteine and selenocysteine act as alternative substrates of the cystathionine γ-synthase (CγS) of *A. racemosus*. Crude extracts of the selenium accumulator plants *A. bisulcatus* and *Neptunia amplexicaulis* and the non-accumulators *A. sinicus* and *A. hamosus* exhibited similar properties thus implying that differences in the metabolism of selenium between accumulator and non-accumulator plants cannot be attributed to differences in the CγS activities of these species.

INTRODUCTION

Seleniferous soils support the growth of plants which accumulate high concentrations of selenium. These plants, known as selenium accumulators, accumulate most of their selenium as selenium isologues of various sulphur-containing amino acids. Some selenium accumulator plants grown on seleniferous soils contain high concentrations of selenocystathionine, the selenium isologue of cystathionine, an intermediate of the trans-sulphuration pathway of methionine synthesis. Selenocystathionine is reportedly the predominant form of selenium in the Australian accumulator species *Neptunia amplexicaulis* [1–3]. Large quantities of selenocystathionine have also been detected in the accumulator species *Astragalus pectinatus* while trace amounts have been detected in other *Astragalus* accumulator and non-accumulator species (see ref. [4] for review).

This paper reports a study of the properties of the cystathionine γ-synthase (CγS) activity in crude extracts of leaf tissue of several closely related selenium accumulator and non-accumulator species with respect to their activity towards cysteine and selenocysteine. The species examined include the selenium accumulators, *N. amplexicaulis* and two species of *Astragalus*, (*A. racemosus* and *A. bisulcatus*) and two non-accumulators (*A. sinicus* and *A. hamosus*). The synthesis of cystathionine and selenocystathionine by the CγSs from these sources is compared with those reported previously for crude spinach extracts [5].

RESULTS

Cystathionine synthesis by crude extracts of N. amplexicaulis *and* Astragalus *species*

Crude extracts of the five species examined catalysed the incorporation of [^{3}H]cysteine into cystathionine in the presence of *O*-phosphorylhomoserine (PHS). The identity of the product as cystathionine was established by oxidizing Dowex treated material with performic acid and, after a further Dowex treatment, subjecting the ^{3}H-labelled product to PE in buffer A; the ^{3}H-label co-migrated as a single compound with authentic cystathionine sulphone. Cystathionine was not synthesized in the absence of crude extract or PHS nor in the presence of 0.1 mM DL-propargylglycine, a potent inhibitor of CγS of *Lemna paucicostata* [6–8] and spinach [5]. Similarly, the extracts of all five species catalysed the incorporation of [^{14}C]PHS into cystathionine in the presence of cysteine. After Dowex treatment (but without performic acid oxidation) the ^{14}C-labelled product ran as a single component and co-migrated with authetic cystathionine on PC in solvent A. The product did not form in the absence of cysteine or extract nor in the presence of propargylglycine. These data are consistent with CγS activity in each of the species examined.

The CγS activity of the five species was examined with respect to cysteine concentration. The kinetics of the enzymes from the selenium-accumulator species *A. racemosus* are shown in a Hanes–Woolf plot in Fig. 1.

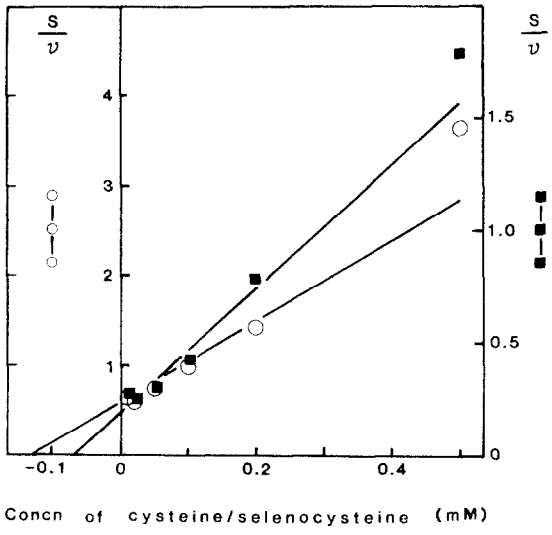

Fig. 1. Hanes–Woolf plot of the effect of concentration of cysteine (○) and selenocysteine (■) on the rate of incorporation of [^{14}C]PHS (10 mM) into cystathionine/selenocystathionine catalysed by crude extracts of *A. racemosus*. All other conditions were as described for method B (cysteine as substrate) and method D (selenocysteine as substrate). Separate extracts were used to investigate the effect of cysteine and selenocysteine concentration.

The rate of cystathionine synthesis increased with cysteine concentration up to 0.2–0.5 mM. Similar results were obtained with respect to cysteine concentration for the extracts from the other four species (results not shown); the K_m values for cysteine for all five species are summarized in Table 1. Concentrations greater than 0.5 mM cysteine decreased enzyme activity (data not shown); for *A. racemosus* the activity at 1 mM cysteine was *ca* 70% of the rate at 0.5 mM. The rate of cystathionine synthesis determined with extracts of *A. racemosus* by [^{3}H]cysteine incorporation (method A) increased with PHS concentration up to 20 mM. The K_m value for PHS (Table 1) was determined from Hanes–Woolf plots (results not shown). Concentrations greater than 20 mM PHS were slightly inhibitory (17% inhibition at 50 mM).

Incorporation of [^{14}C]*PHS in the presence of selenocysteine*

When crude extracts of *A. racemosus* were incubated with selenocysteine in place of cysteine [^{14}C]PHS was incorporated into a product which exhibited the same characteristics as cystathionine when treated on Dowex 50-H$^+$ and eluted with aqueous ammonia. The ^{14}C-labelled material ran as a single compound and co-migrated with authentic cystathionine/selenocystathionine when subjected to PC in solvent A. This product was not formed in the presence of 0.1 mM propargylglycine. Logically, the product formed under these conditions is selenocystathionine, a conclusion which is supported by experiments with the spinach enzyme which catalysed the incorporation of [^{75}Se]selenocysteine. Unfortunately, [^{75}Se]selenocysteine was not commercially available at the time the investigations with the *Astragalus* enzymes were conducted.

The rate of [^{14}C]PHS incorporation catalysed by extracts of *A. racemosus* increased with the concentration of selenocysteine up to 0.5 mM. Higher concentrations were inhibitory. This is reflected in the Hanes–Woolf plot (Fig. 1) from which the K_m value for this substrate was derived (Table 1). Due to limited supplies of [^{14}C]PHS direct determinations of K_m for selenocysteine were not made for the other four species. However, the rate of

Table 1. Some properties of CγS from leaf tissue of various selenium-accumulator and non-accumulator plants

	K_m PHS*† (mM)	K_m cyst† (mM)	K_m Secyst‡ (mM)	K_i Secyst§ (mM)	V Secyth‖	Ratio $\frac{V(\text{cyth})}{V(\text{Secyth})}$¶
Selenium accumulators:						
A. racemosus	2.85	0.14	0.07	0.07	0.35	0.82
A. bisulcatus	n.d.	0.12	n.d.	0.06	0.39	0.87
N. amplexicaulis	n.d.	0.15	n.d.	0.05	0.21	0.74
Non-accumulators:						
A. sinicus	n.d.	0.17	n.d.	0.06	0.26	0.66
A. hamosus	n.d.	0.24	n.d.	0.11	0.42	0.77
Spinach**	0.75	0.24	0.07	0.04	1.07	1.00

*Abbreviations: n.d., not determined; PHS, phosphorylhomoserine; cyst, cysteine; Secyst, selenocysteine; cyth, cystathionine; Secyth, selenocystathionine; V, V_{max}.

†Determined by [^{3}H]cysteine incorporation (method A).

‡Determined by [^{14}C]PHS incorporation with selenocysteine as substrate (method D).

§Determined from selenocysteine inhibition of [^{3}H]cysteine incorporation for combined (cysteine plus selenocysteine) concentrations less than 0.3 mM (see the text).

‖Determined by incorporation of 20 mM [^{14}C]PHS in the presence of 0.5 mM selenocysteine. Activity expressed as nmol/min/mg protein.

¶Ratio of rates of [^{14}C]PHS incorporation with 0.5 mM cysteine and 0.5 mM selenocysteine as substrates.

**Data from ref. [5].

Table 2. Effect of cysteine and selenocysteine on the CγS activity of crude extracts of *A. racemosus* as determined by [^{3}H]cysteine incorporation and [^{14}C]PHS incorporation

Labelled substrate	Unlabelled substrates and additions	CγS activity (nmol/min/mg protein)
[^{3}H]Cysteine (0.5 mM)	PHS (10 mM)	0.20
	PHS (10 mM) plus selenocysteine (0.5 mM)	0.02
[^{14}C]PHS (10 mM)	Cysteine (0.5 mM)	0.28
	Selenocysteine (0.5 mM)	0.35
	Cysteine (0.5 mM) plus selenocysteine (0.5 mM)	0.26

[^{3}H]Cysteine incorporation was measured as described for method A except for the modifications shown. [^{14}C]PHS incorporation was determined as for method B except that cysteine was replaced or supplemented with selenocysteine as shown. Under the latter condition some [^{14}C]PHS would be incorporated into selenocystathionine in addition to cystathionine.

[^{14}C]PHS incorporation was measured at 0.5 mM cysteine and 0.5 mM selenocysteine, concentrations which support maximum rates in spinach [5] and *A. racemosus*. The ratio of the activity with cysteine relative to the activity with selenocysteine varied between 0.66 and 1.00 (Table 1). No consistent differences in this ratio were detected between extracts from selenium accumulator and non-accumulator species.

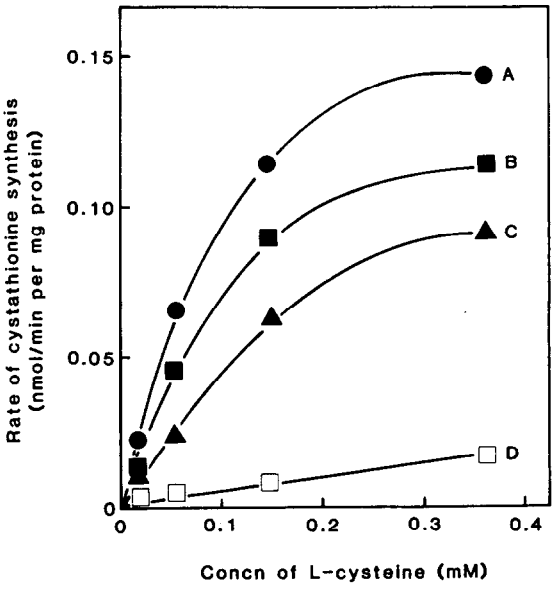

Fig. 2. Effect of selenocysteine on the incorporation of [^{3}H]cysteine into cystathionine catalysed by crude extracts of *A. racemosus* in the presence of 10 mM PHS. [^{3}H]Cysteine incorporation was measured by method A without any additions (A) and in the presence of 0.05 mM (B), 0.125 mM (C) and 0.50 mM (D) selenocysteine respectively.

Competition between cysteine and selenocysteine

Experiments were undertaken to determine whether the incorporation of cysteine and selenocysteine catalysed by crude extracts of *A. racemosus* involved common or independent mechanisms. The incorporation of [^{3}H]cysteine was studied in the presence and absence of unlabelled selenocysteine while concomitantly monitoring the incorporation of [^{14}C]PHS in the presence of unlabelled cysteine and/or selenocysteine (Table 2). Selenocysteine (0.5 mM) inhibited the incorporation of 0.5 mM [^{3}H]cysteine by *ca* 90%. The rate of [^{14}C]PHS incorporation in the presence of selenocysteine was some 25% greater than the rate with cysteine but when both substrates were supplied together the total rate of incorporation was less than the rate determined for either substrate alone. These data are consistent with the hypothesis that cysteine and selenocysteine compete for the same enzyme and that a selenocysteine-specific selenocystathionine γ-synthase is not involved. The substantial inhibition of [^{3}H]cysteine incorporation by equimolar concentrations of selenocysteine (90% inhibition at 0.5 mM, Table 2) is consistent with the higher affinity for selenocysteine than cysteine reported in Fig. 1 and Table 1.

The effect of concentration of unlabelled selenocysteine on [^{3}H]cysteine incorporation catalysed by extracts of *A. racemosus* is shown in Fig. 2. A Hanes–Woolf plot of this data is shown in Fig. 3. The data are not consistent with non-competitive competition between [^{3}H]cysteine and selenocysteine. Rather, the kinetic analysis resembles that obtained with crude spinach extract [5]. If all the experimental points are used to derive the line of best fit then the data are consistent with a mixed competitive/non-competitive inhibition. However, the data in Fig. 1 show that concentrations greater than *ca* 0.3 mM of either substrate, supplied alone, are inhibitory. If the data from incubations containing a total (cysteine plus selenocysteine) concentration greater than 0.3 mM are disregarded, then, for those few values falling within this limit (see Fig. 2), the kinetics closely approximate competitive inhibition of [^{3}H]cysteine incorporation by sel-

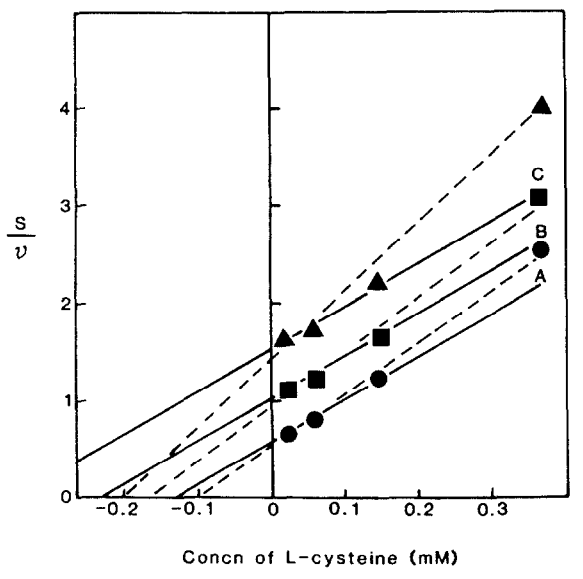

Fig. 3. Hanes–Woolf plot of the kinetics of selenocysteine inhibition of [^{3}H]cysteine incorporation into cystathionine catalysed by crude extracts of *A. racemosus*. The dotted line depicts the lines of best fit determined by the least squares method [15] for all available primary data shown in Fig. 2. The continuous lines are compiled only from those estimations for which the combined (cysteine plus selenocysteine) concentrations do not exceed the presumed inhibitory level of 0.3 mM. Without selenocysteine (A), 0.05 mM selenocysteine (B) and 0.10 mM selenocysteine (C).

enocysteine (Fig. 3). Assuming that selenocysteine is competitive towards cysteine, then the K_i value for selenocysteine, determined from the data at 0.05 mM selenocysteine (the only concentration for which there are sufficient points with a total amino acid concentration less than 0.3 mM) is 0.07 mM, a value identical to the K_m value for selenocysteine determined by [^{14}C]PHS incorporation (Table 1). Analogous experiments to those shown in Figs 2 and 3 for extracts from *A. racemosus* were performed with extracts from the other four species and similar results obtained (results not shown). Procedures analogous to those described above for *A. racemosus* were used to determine K_i values for selenocysteine. These values are shown in Table 1.

DISCUSSION

The results demonstrate the presence of CγS activity in crude extracts of the two non-accumulators and three selenium accumulators examined. The properties of the enzymes from these sources were not studied in detail but the K_m values for cysteine (Table 1) are similar to those reported for CγS in crude extracts of spinach [5], *Lemna paucicostata* [8] and sugar beet [9]. The K_m value for PHS for *A. racemosus* is some four-fold greater than for spinach but campares favourably with the values reported for *L. paucicostata* [8].

In the presence of selenocysteine, crude extracts of all the species examined catalysed the incorporation of [^{14}C]PHS into a product which logically, and on the basis of previous studies of [^{75}Se]selenocysteine and [^{14}C]PHS incorporation in spinach [5], was presumed to be selenocystathionine. The incorporation of [^{14}C]PHS by extracts of *A. racemosus* into a product which was indistinguishable from cystathionine/selenocystathionine and which did not form in the presence of 0.1 mM propargylglycine, is consistent with this proposal. The competition data involving cysteine/selenocysteine (Fig. 2, Table 2) imply that cysteine and selenocysteine compete for the same enzyme site and suggest that a selenocysteine-specific selenocystathionine γ-synthase is not involved in any of the species examined. Both the competition data (Table 2) and the kinetic data (Fig. 1) imply that the enzyme from *A. racemosus*, like the spinach enzyme [5], has a higher affinity for selenocysteine than cysteine. The lower K_i values for selenocysteine relative to the K_m values for cysteine (Table 1) suggest that the CγSs of the other species also exhibit higher affinity for selenocysteine. In this regard CγS shows some similarity to ATP sulphurylase and cysteine synthase in that both enzymes support reactions with the selenium isologues of the relevant sulphur-containing substrate and also exhibit higher affinity towards the selenium isologue [10–12]. However, CγS appears to be unique in that it exhibits V_{max} values with the selenium isologue similar to or, in some instances, greater than the V_{max} for the sulphur-containing substrate.

The capacity of CγS from selenium accumulators and non-accumulators to support a reaction involving the selenium isologue of the substrate and product, together with reports that cysteine synthase and β-cystathionase from both groups of plants [12, 13] also support reactions involving selenium isologues, implies that the transsulphuration pathway for methionine synthesis is open to selenium at least to the point of selenohomocysteine synthesis. The results described in this paper, however, do not provide any information which might explain the quantitative and qualitative differences in the selenium-containing compounds typically observed in selenium accumulator and non-accumulator plants in their natural environments. No consistent differences were observed in the properties of CγS between the two groups of plants with respect to the optimum rates of enzyme activity, the affinity for cysteine and selenocysteine and the V_{max} (cystathionine)/V_{max} (selenocystathionine) ratio. The foregoing discussion implies that if inorganic selenium is metabolised by the transsulphuration pathway *in vivo*, other factors must be responsible for the accumulation of selenocystathionine and other selenium isologues of the transsulphuration pathway in various accumulator and non-accumulator plants.

EXPERIMENTAL

Plant material. Seeds of *Astragalus sinicus*, *A. racemosus*, *A. bisulcatus*, *A. hamosus* and *Neptunia amplexicaulis* were raised as described in refs [11, 12]. Acetone powders were prepared from freshly harvested leaf material of each species and were stored at −10°.

Chemicals. All reagents and their sources are described in ref. [5].

Preparation of enzyme extracts. Crude extracts of *Astragalus* species and *Neptunia amplexicaulis* were prepared essentially as described for spinach [5, 14]. However, after the second Sephadex G-25 treatment to remove unbound pyridoxal-phosphate the protein fractions were too dilute to employ in assays of CγS activity. These fractions were usually pooled and the protein pptd

with $(NH_4)_2SO_4$ (0–80% saturation). After centrifugation (20 000 *g*, 30 min) pptd protein was dissolved in a small vol. of 10 mM Tris–HCl buffer (pH 8) and dialysed overnight against the same buffer. All operations were performed at 4°. Presumably some pyridoxal-phosphate, previously bound to protein, was lost during the $(NH_4)_2SO_4$ concn procedure since it was necessary to add 50 μM pyridoxal phosphate to enzyme incubations to obtain maximum CγS activity.

Assay of CγS. Three methods were used and were as described in ref. [5]. Method A involves [^{3}H]cysteine incorporation, method B involves [U-^{14}C]PHS incorporation with cysteine as substrate and method D involves [U-^{14}C]PHS incorporation with selenocysteine as substrate. In some experiments with method B or D assays the incubations were terminated by addition of 12.5 μl of a cold solution of trichloroacetic acid (32% (w/v)) containing L-cystathionine (1 mg/ml). This procedure was introduced in view of the low rates of CγS activity in extracts of *Astragalus* species and *Neptunia amlexicaulis*. Insoluble material was removed by centrifugation (3000 *g*, 10 min) and radioactive (seleno)cystathionine was isolated by subjecting an aliquot (55 μl) of the supernatant to PC in solvent A. Selenocystathionine/cystathionine (R_f 0.25) was well resolved from [^{14}C]PHS (R_f 0.57) and any [^{14}C]homoserine (R_f 0.49), produced non-enzymically, by this solvent. Maximum rates of CγS activity were observed at PHS concns of 20 mM (see text). However, PHS was routinely supplied at 10 mM to conserve the limited stocks of this compound. The rate of CγS activity in the presence of 10 mM PHS was *ca* 85% of that at 20 mM.

Other methods. All other methods were as described in ref. [5].

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