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L-Selenohomocysteine: One-Step Synthesis from L-Selenomethionine and Kinetic Analysis as Substrate for Methionine Synthases

Zhaohui S. Zhou,[a] April E. Smith[b] and Rowena G. Matthews[a,*]

[a]*Biophysics Research Division and the Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109-1055, USA*

[b]*Department of Medicinal Chemistry, The University of Michigan, Ann Arbor, MI 48109-1055, USA*

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Abstract—A single-step convenient synthesis of L-selenohomocysteine (SeHcy) from L-selenomethionine (SeMet) using sodium in liquid ammonia is described. Methionine synthases convert SeHcy to SeMet at rates comparable to their rates of conversion of L-homocysteine (Hcy) to L-methionine (Met). This study suggests that SeHcy generated from SeMet metabolism can be efficiently recycled to SeMet in mammals.

Selenium is an essential trace element for humans, animals, and other living organisms.[1–3] Selenium deficiency in certain areas of China previously caused Keshan disease, which was associated with heart failure and cardiogenic shock.[4] Selenium supplementation has practically eliminated the incidence of this disease. In the past decade, changes in selenium metabolism have been associated with oxidant damage, cancer development, male infertility,[5] and other disease conditions.[1,2] A prevention trial study in the United States involving 1362 patients has shown that selenium supplementation of 200 μg per day significantly reduces both total mortality and total incidence of cancer of the lung, prostate, colon, and rectum.[6] Because of its beneficial effects, selenium has been added to many nutritional supplements. On the other hand, excess selenium uptake is toxic and causes injuries to plants, animal, and humans.[1–3] The toxicology of selenium is not well understood yet.

To provide general guidelines for the usage of and further research on selenium, the Institute of Medicine of the National Academy of Sciences recently issued an updated Dietary Reference Intake for selenium.[7] The report sets a recommended intake level for selenium at 55 μg per day, and for the first time, sets the upper intake level at 400 μg per day. It is worth noticing that these numbers refer to the total amount of selenium. Selenium exists in many forms in food, supplements, and human bodies.[3] For example, selenomethionine and Se-adenosylselenohomocysteine account for 85% of the total selenium in Selenomax from Nutrition 21 (Purchase, New York), the selenium-enriched yeast used in the cancer prevention trial mentioned earlier.[8] On the other hand, most health supplements contain inorganic forms of selenium. Detailed studies of the inter-conversions of different selenium compounds will not only provide a better understanding of the biological roles and toxicity of selenium, but also provide us with information to optimize the selenium supplementation to achieve maximal benefits and minimal side effects.[3]

To date, the biological functions of selenium have been associated with several natural selenoproteins, including glutathione peroxidase, iodothyronine deiodinase, and thioredoxin reductase.[1,2,9] Selenocysteine has been found to be the only essential seleno-amino acid in these proteins. Special proteins are used to synthesize selenocysteinyl-tRNA and deliver it to the ribosome for incorporation at UGA codons, which normally serve to terminate translation.[10,11] Nonetheless, at elevated concentrations, selenocysteine can be randomly incorporated into proteins,[10] replacing cysteine, which may contribute to selenium toxicity. In comparison, selenomethionine can be randomly incorporated into proteins in place of methionine without perturbation of the

*Corresponding author. Tel.: +1-734-764-9459; fax: +1-734-764-3323; e-mail: rmatthew@umich.edu

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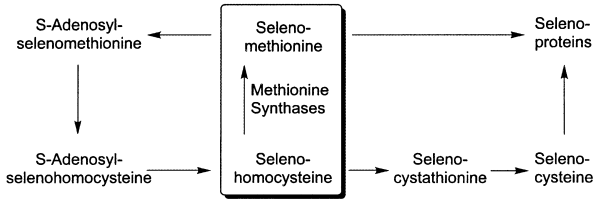
normal structures and functions.[12] Nevertheless, excess selenomethionine is toxic to human cells. The conversion of selenomethionine to selenocysteine via selenohomocysteine (as shown in Scheme 1) has been hypothesized to cause the toxicity in mammals.[13]

Each individual pathway in Scheme 1 has been characterized at the enzyme level, except the conversion from selenohomocysteine to selenomethionine (boxed in Scheme 1).[13,14] The biochemical pathways of selenium amino acids and their derivatives have been found to be very similar to those of their sulfur counterparts. In all cases, the enzyme for each individual step transforms the sulfur compounds and their selenium analogues at comparable rates.[13,14] For example, selenohomocysteine is converted to selenocystathionine by rat liver cystathionine beta-synthase at a rate 69% of that for conversion of homocysteine to cystathionine; and selenocystathionine is transformed to selenocysteine by rat liver cystathionine gamma-lyase at about 3 times the rate of cystathionine elimination.[14] Because selenohomocysteine is at a branch point of biochemical pathways to selenomethionine or selenocysteine, determination of whether selenohomocysteine can be converted to selenomethionine by methionine synthases and the reaction kinetics of that conversion are obviously crucial for having a complete picture of selenium metabolism.

In this paper, we report the one-step convenient synthesis of selenohomocysteine from selenomethionine using sodium in liquid ammonia shown in Scheme 2, and the kinetic characterization of the conversion of selenohomocysteine to selenomethionine by methionine synthases. This work provides key information relevant to the biochemical pathways of selenium metabolism.

Synthesis of Selenohomocysteine (SeHcy)

Selenohomocysteine has been synthesized chemically from α-amino-butyrate lactone via an α-amino-γ-benzylseleno-butyric acid intermediate,[15–17] and enzymatically from homoserine and sodium diselenide (Na_2Se_2) using *O*-acetylhomoserine sulfydrylase.[18] The former procedure involved multiple steps, and the later required a partially purified enzyme that is not readily available.



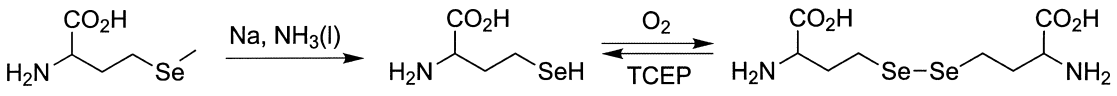
Scheme 1. Biochemical interconversions of selenium amino acids and their derivatives in mammals. The reaction catalyzed by methionine synthases is boxed.

Since L-selenomethionine is now commercially available, we have developed a convenient single step synthesis of L-selenohomocysteine from L-selenomethionine using metallic sodium in liquid ammonia as depicted in Scheme 2.[19] The protocol was modified from a literature procedure for the preparation of L-homocysteine from L-methionine.[20,21] L-Selenohomocysteine was oxidized in air to its diselenide form for storage, and was easily regenerated by in situ reduction with dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) in aqueous solution.[22]

The synthesis of selenohomocysteine reported here is more convenient than the literature procedures. The modest product yield (62%) was partially due to the slow oxidation of the selenol to the diselenide by oxygen in the air. After the synthesis had been completed, we found that our yields of product were low because it took a longer time than we expected to complete the oxidation, particularly in solutions at low pH. Addition of a catalytic amount of $FeCl_3$ has been reported to facilitate the oxidation process, which will conceivably improve the product yield.[17] The optical purity of amino acids normally does not change under the reaction conditions applied here, as evidenced by the complete retention of chirality on conversion of L-methionine to L-homocysteine under similar conditions.[21] The resulting selenium product was optically active,[21] $[\alpha]_D$ +31° (1.0 N HCl). Only L-homocysteine, but not the D-isomer, is a substrate for cobalamin-dependent methionine synthase (MetH). A fragment of MetH containing the substrate binding sites, MetH(2-649), catalyzed the quantitative conversion of the selenohomocysteine we synthesized and methyl cobalamin to cob(I)alamin and selenomethionine, suggesting that the selenohomocysteine obtained was in the L-configuration. In summary, a convenient single-step synthesis of optically pure L-selenohomocysteine from L-selenomethionine was achieved.

Conversion of SeHcy to SeMet by Methionine Synthases

There are two methionine synthases, the cobalamin-dependent methionine synthase (MetH, E.C. 2.1.1.13) and the cobalamin-independent methionine synthase (MetE, E.C. 2.1.1.14).[23] The overall reaction they normally catalyze is the same, namely, the transfer of a methyl group from 5-methyltetrahydrofolate to the thiol group of homocysteine to form tetrahydrofolate and methionine as depicted in Schemes 3 and 4. The MetH enzyme has a prosthetic group, cobalamin (B_{12}), which mediates the methyl transfer.[24] In humans and

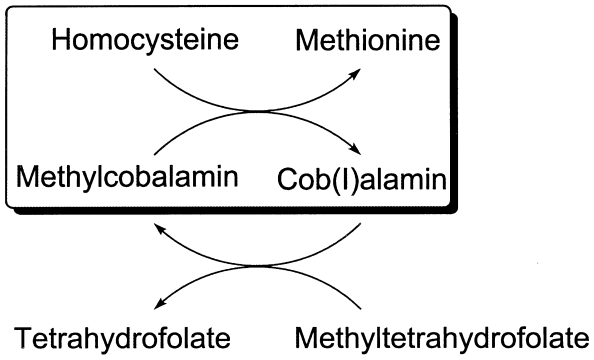


Scheme 2. Synthesis of L-selenohomocysteine and L,L-selenohomocystine from L-selenomethionine.

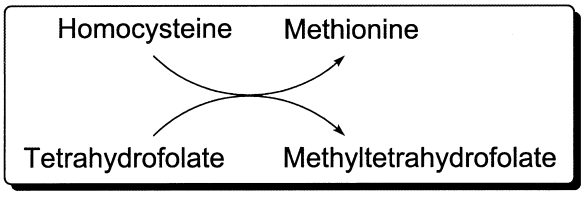
mammals, MetH is the sole form of methionine synthase. The mammalian and *Escherichia coli* MetH enzymes are highly similar, both in their amino acid sequences (which are 55% identical) and in their catalytic properties.[25] Since human MetH has not been purified to homogeneity, the *E. coli* enzyme was used to study the conversion of selenohomocysteine to selenomethionine.

A homocysteine-methylcobalamin assay for the *E. coli* MetH enzyme has been previously described.[26] In this assay (boxed in Scheme 3), the substrate binding region, MetH (2-649), was used to catalyze the transfer of the methyl group from exogenous methylcobalamin to the selenohomocysteine substrate, forming selenomethionine. Whereas conformational changes appear to limit the rate of the physiological reaction catalyzed by MetH, this assay directly assesses the reactivity of selenohomocysteine as a substrate. This assay was performed anaerobically, resulting in the formation of the cob(I)alamin product. The demethylation of methylcobalamin was monitored at 538 nm.[26] Selenohomocysteine was generated by the reduction of the diselenide with excess TCEP in aqueous solution containing 100 mM potassium phosphate at pH 7.2. An excess of TCEP did not interfere with the assay.

As previously described, the reaction of MetH (2-649) with exogenous methylcobalamin is first order in methylcobalamin.[26] The maximum second-order rate constant for selenohomocysteine in this assay was determined to be $(27\pm1)\times10^3$ M^{-1} s^{-1}, which is approximately four times that for homocysteine, $(6.5\pm0.5)\times10^3$ M^{-1} s^{-1}. The K_m for selenohomocysteine was determined to be 16 ± 5 μM as compared with the K_m for homocysteine of 69 ± 18 μM (Fig. 1).



Scheme 3. Reactions catalyzed by cobalamin-dependent methionine synthase (MetH). The half-reaction reported in this paper is boxed.



Scheme 4. Reaction catalyzed by cobalamin-independent methionine synthase (MetE).

The cobalamin-independent methionine synthase (MetE) catalyzes methyl transfer from methyltetrahydrofolate to homocysteine without formation of a detectable intermediate.[23,27] The enzyme catalyzes the terminal step of de novo synthesis of methionine in plants and other organisms that can not utilize cobalamin.[28] Both the MetE and MetH enzymes exist in *E. coli*. The activity assay of MetE is based on the conversion of the tetrahydrofolate product and formic acid to methenyltetrahydrofolate, which can be monitored at 350 nm, as previously reported.[29] The diselenide of selenohomocystine or the disulfide of oxidized DTT deactivates the MetE enzyme by releasing the catalytically essential zinc in MetE,[30] and excess TCEP interferes with the formation of methenyltetrahydrofolate. Thus, selenohomocysteine was generated by mixing excess solid selenohomocystine with an aqueous solution containing 100 mM TCEP in 500 mM bis[2-hydroxyethyl]-iminotris[hydroxymethyl]methane (Bis-Tris) at pH 6.2. The excess diselenide is insoluble at this

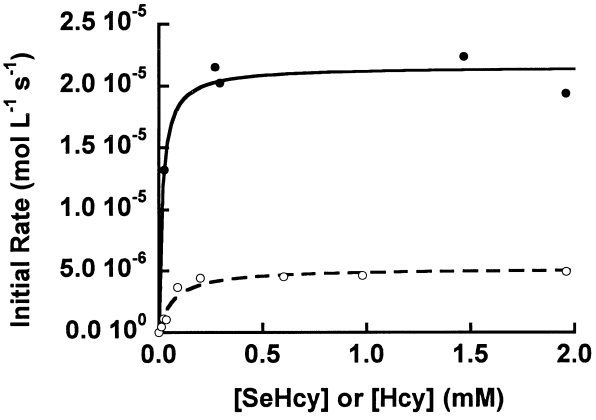


Figure 1. Dependence of initial reaction rates on substrate concentration for MetH-catalyzed methyl transfer from methylcobalamin. Data for selenohomocysteine and homocysteine are shown with filled and open circles, respectively, and were fit to the Michealis–Menten equation.

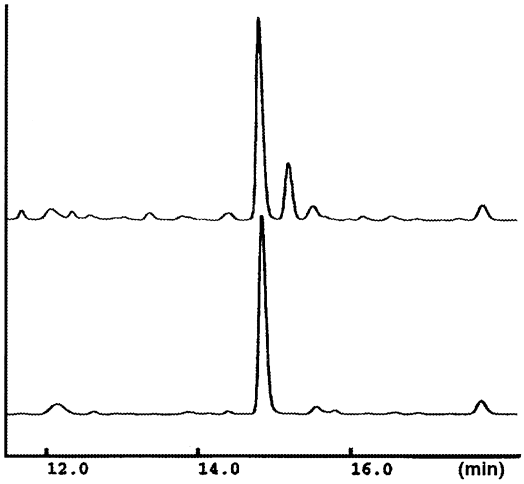


Figure 2. HPLC traces of authentic PTC-selenomethionine (bottom trace) and PTC-modified products of the MetH-catalyzed reaction (top trace). Amino acids were modified by treatment with phenylthiocyanate and separated by reverse phase HPLC.

pH and was removed by centrifugation. The specific activity for selenohomocysteine was 0.11±0.01 μmol min^{-1} mg^{-1} in comparison to 0.23±0.02 μmol min^{-1} mg^{-1} for the homocysteine substrate. The K_m values of homocysteine and selenohomocysteine could only be estimated to be below 125 μM, due to limitations of the assay.[29]

HPLC amino acid analysis confirms the formation of selenomethionine as the reaction product, as shown in Figure 2. After derivatization of the amino acids with phenylisothiocyanate (PTC) to form PTC-modified amino acids, reverse-phase HPLC analysis was carried out on the Applied Biosystems separation system at the Protein and Carbohydrate Structure Facility at the University of Michigan. PTC-modified selenomethionine (14.82 min) eluted after PTC-modified methionine (14.37 min).

Discussion and Conclusion

This study, for the first time, establishes that selenohomocysteine can readily be converted to selenomethionine by both purified cobalamin-dependent and cobalamin-independent methionine synthases at rates comparable to those for the conversion of homocysteine to methionine. A previous study with rat liver enzymes has shown that selenohomocysteine can be transformed to selenocysteine with similar efficiency to its sulfur counterpart.[14] The extent of conversion of selenohomocysteine to selenocysteine will be governed by the competition between methionine synthase and β-cystathionine synthase for selenohomocysteine.[31,32] β-cystathionine synthase is activated by S-adenosylmethionine,[33] so partitioning of selenohomocysteine will be critically dependent on the levels of S-adenosyl methionine and possibly also Se-adenosylselenomethionine in human cells.

Because selenium has similar, but nevertheless different, chemical and physical properties as compared to sulfur, selenohomocysteine can be used as an excellent probe to study the interactions between homocysteine and methionine synthases. Indeed, we have successfully applied X-ray absorption spectroscopic analysis to complexes of selenohomocysteine and methionine synthases (K. Peariso, Z. S. Zhou, A. E. Smith, R. G. Matthews and J. E. Penner-Hahn, submitted for publication). For example, because the methionine synthases do not contain selenium, the presence of a selenium in the coordination sphere of the active site zinc in the selenohomocysteine–enzyme complexes unambiguously proves the direct ligation of substrate to the zinc ion.

In conclusion, a convenient synthesis of selenohomocysteine has been developed, and methionine synthases have been shown to convert selenohomocysteine to selenomethionine at rates comparable to those for their sulfur analogues. This study will provide a better understanding of selenium metabolism, and the role of selenium in disease development and prevention.

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19. Experimental details for synthesis: To a solution of L-(+)-selenomethionine (0.98 g, 5 mmol; purchased from Acros Organics) in 40 mL liquid ammonia at −80 °C under stirring, small pieces of metallic sodium (~0.26 g, 11 mmol) were carefully added in 45 min until the solution remained blue for 15 min. The solution was stirred for another 50 min at −80 °C, solid ammonium chloride (1.27 g, 24 mmol) was then added to neutralize sodium amide. The reaction mixture was opened to air and slowly warmed up to room temperature overnight. The resulting yellow solid was mixed with 100 mL water. The mixture was adjusted to neutral pH and stirred vigorously open to air overnight. The pale-yellow precipitate collected by filtration was a mixture of selenomethionine and selenohomocystine as judged by NMR analysis. Selenohomocysteine is much more soluble than selenomethionine in aqueous solution

at pH 4–6, and selenohomocystine can be readily reduced by dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP). Thus, the diselenide was reduced to the selenol and separated from selenomethionine by filtration. The crude product was suspended in 35 mL of 250 mM aqueous citric acid buffer, pH 5.8, and dithiothreitol (0.5 g, 3 mmol) was then added. After stirring under nitrogen for 30 min at room temperature, the suspension was filtered, and the filtrate was stirred overnight in open air, allowing selenohomocysteine to be oxidized to selenohomocystine. The precipitate was collected by filtration, washed with water, and dried in vacuo to give a pale yellow solid as desired compound (0.39 g, 62% yield based on selenomethionine consumed). ^{1}H NMR (300 MHz, 1 N DCl in D_2O) δ 3.63 (2H, t, $J = 6.41$ Hz), 2.43 (4H, m), 1.72–1.92 (4H, m); ^{13}C NMR (300 MHz, 1 N DCl in D_2O with 2% (v/v) CD_3OD) δ 171.3, 52.5, 31.2, 22.8. DEPT 135: CH-CH_3, δ 52.5; CH_2, δ 31.2, 22.8. HRMS (MALDI, TFA/H_2O) calcd for $C_8H_{16}N_2O_4Se_2 + H$ 362.9527, found 362.9208. Mp (uncorrected): 235 °C (decomp, literature 214 °C,[18] 260 °C[16,17]). $[\alpha]_D^{21}$ +31° (1.0 N HCl).

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