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Some properties of murine selenocysteine synthase

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Selenocysteine (Scy) was synthesized on natural opal suppressor $tRNA^{Ser}$ by conversion from seryl-tRNA. We studied the mechanisms of the synthesis of mammalian Scy-tRNA using hydro[^{75}Se]selenide ($H^{75}Se^-$). We found Scy synthase activity in the 105000 *g* supernatant of a murine liver extract. The supernatant was chromatographed on DEAE-cellulose, and the activity was eluted at 0.12 M-KCl. The reaction mixture for synthesis of Scy-tRNA contained suppressor tRNA, serine, ATP, seryl-tRNA synthetase (SerRS), HSe^- and the enzyme to synthesize Scy-tRNA. These are all essential for the synthesis of Scy-tRNA. Scy in the tRNA product was confirmed by five t.l.c. systems. The conversion from seryl-tRNA to Scy-tRNA was also confirmed with the use of [^{14}C]- and [^{3}H]-serine. The apparent K_m values for the substrates serine, tRNA, ATP and HSe^- were 30 μM, 140 nM, 2 mM and 40 nM respectively. The active eluates from DEAE-cellulose contained no tRNA kinase. This result showed that Scy-tRNA was not synthesized through phosphoseryl-tRNA. ATP was necessary when Scy-tRNA was synthesized from seryl-tRNA and HSe^-. Therefore ATP is used for not only the synthesis of seryl-tRNA but also for the synthesis of Scy-tRNA from seryl-tRNA. The active fraction from DEAE-cellulose was chromatographed on Sephacryl S-300, but the activity disappeared. However, the activity was recovered by mixing the eluates corresponding to proteins of 500 kDa and 20 kDa. In order to examine the binding of HSe^- to proteins, a mixture of the active fraction, $H^{75}Se^-$ and ATP was analysed by chromatography on Sephacryl S-300. The ^{75}Se radioactivity was found at the position of a 20 kDa protein in the presence of ATP. Thus the 20 kDa protein plays a role in binding HSe^- in the presence of ATP. The 500 kDa protein must have a role in the synthesis of Scy-tRNA. There are two natural suppressor serine tRNAs, $tRNA_{NCA}$ and $tRNA_{CmCA}$, in cell cytosol. The present paper shows that the suppressor tRNA fraction, eluted later on benzoylated DEAE-(BD-)cellulose, is a better substrate with which to synthesize Scy-tRNA. Thus we consider that murine Scy-tRNA is synthesized from a suppressor seryl-tRNA on the 500 kDa protein with the activated HSe^-, which is synthesized with ATP on the 20 kDa protein. This mammalian mechanism used to synthesize Scy is similar to that seen in *Escherichia coli*.

INTRODUCTION

Selenium is an essential trace element for humans, its deficiency causing Keshan disease, a condition endemic in China [1]. The syndrome was relieved by administration of selenious acid. Selenium was found as selenocysteine (Scy) in glutathione peroxidase (GSHPx) [2] and type-I iodothyronine deiodinase (ID-I) [3]. GSHPx plays an important role in the deoxidation of hydrogen peroxides, organic hydroperoxides and lipid peroxides. ID-I has the role of converting thyroxine into tri-iodothyronine, a more active thyroid hormone that is a key component of metabolic and developmental effects. Scy corresponds to the opal nonsense codon UGA on the mRNA of GSHPx [4,5] and ID-I [6]. The natural tRNA corresponding to UGA is called opal suppressor tRNA and accepts serine [7]. This tRNA is present in cytosol as Scy-tRNA *in vivo*. This Scy moiety was incorporated into selenoproteins by a co-translational mechanism [8,9]. In order to understand the mechanism of incorporation of Scy into GSHPx, an experiment with a perfused rat liver showed that the carbon source of the Scy in GSHPx came from serine by specific labelling of Scy with [^{14}C]serine [10]. Additionally, natural suppressor tRNA corresponding to UGA was found about two decades ago [7]. By using this suppressor tRNA, the readthrough protein of β-globin, having the UGA termination codon, was synthesized [11]. This opal suppressor seryl-tRNA was phosphorylated by tRNA kinase to become phosphoseryl[Ser(P)]-tRNA [12–14]. It has been presumed that Scy-tRNA in mammals was biosynthesized on seryl-tRNA by conversion of the serine moiety through an intermediate, Ser(P)-tRNA [9,15–17]. However, the mechanism in mammals is ambiguous, because the activities synthesizing Scy-tRNA, and the substrates employed, have not been clarified. In *Escherichia coli* the products of the *selA* and *selD* genes (SELA and SELD respectively) function in the biosynthesis of Scy-tRNA [18]. SELA produced Scy-tRNA from seryl-tRNA and SELD produced an active Se compound from ATP, Mg and hydrogen selenide (H_2Se). Se in the active Se compound was transferred to the seryl-tRNA–SELA complex in order to synthesize Scy-tRNA [19]. In a previous paper we showed the presence of the Scy synthase in murine liver extract [20]. Here we illustrate some properties of murine Scy synthase using $H^{75}Se^-$ as an Se donor.

MATERIALS AND METHODS

Materials

Natural opal suppressor tRNA was prepared from bovine liver and chromatographed on BD-cellulose [21]. The last fraction eluted from BD-cellulose was rich in suppressor tRNA and separated from major serine tRNA. This fraction was used for assay of the Scy-tRNA synthesis reaction. The serine-acceptor activity of this fraction was 20 pmol/A_{260} unit. Assays of the seryl-tRNA synthetase (SerRS) of molecular mass 65.5 kDa and tRNA kinase (450 kDa) were performed as described in [14] and [22] respectively. [^{75}Se]Selenite was a product of NEN. [γ-^{32}P]ATP

Abbreviations used: Scy, selenocysteine; Ser(P), phosphoser(ine/yl); GSHPx, glutathione peroxidase; ID-I, type-I iodothyronine deiodinase; SerRS, seryl-tRNA synthetase; FDH, formate dehydrogenase; H_2Se, hydrogen selenide; HSe^-, hydroselenide anion; BD-, benzoylated DEAE-; PLP, pyridoxal 5′-phosphate.

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was a product of Amersham International. [^{3}H]Serine, [^{14}C]serine and [^{3}H]ATP were products of ICN.

Methods

The enzyme of Scy-tRNA synthesis was prepared from the liver of an ICR-strain mouse as follows. The liver was minced with quartz sand in 5 vol. of 0.25 M-sucrose/10 mM-Tris/HCl/10 mM-mercaptoethanol at pH 7.5. This was centrifuged at 8000 *g* for 10 min, and the supernatant was centrifuged at 105000 *g* for 60 min. The supernatant was chromatographed on a DEAE-cellulose column. Chromatography on DEAE-cellulose and Sephacryl S-300 was performed, and the activity of SerRS in each eluate was measured, as described in [22]. The activity of Scy synthase was measured by the method described below. The active fraction of Scy synthase was dialysed against 50% (v/v) glycerol/10 mM-Tris/HCl/10 mM-$MgCl_2$/10 mM-mercaptoethanol at pH 7.5, and the dialysis residue was stored at −80 °C.

$H^{75}Se^-$ was prepared from [^{75}Se]selenite by the enzymic method of Ganther [20,23], as follows. [^{75}Se]Selenite (1 μl; 1.3 mM; 280 GBq/mmol; 370 MBq/ml) was mixed with 10 μl of 40 mM-GSH, 1 μl of 10 mM-NADPH, 5 μl of 0.2 M-mercaptoethanol, 5 μl of water and 2 μl of 1 M-Hepes/NaOH, pH 7.5, and 0.25 μl of yeast GSH reductase (Boehringer). This mixture was incubated overnight, and HSe^- was purified from the above H_2Se mixture by chromatography on Sephacryl S-200 in 10 mM-mercaptoethanol/10 mM-acetate at pH 4.6. The pattern of separation was shown in [20]. The ^{75}Se radioactivity in the eluates from the Sephacryl S-200 column was measured by a Gamma-Autowell counter (Aloka ARC 301). Subsequently eluates were analysed by t.l.c. on silica-gel G in butan-1-ol/acetic acid/water (4:1:1, by vol.). The fractions containing HSe^- were pooled and used to synthesize Scy-tRNA. Authentic unlabelled HSe^- was chemically prepared by the method given in [24]. The concentration of unlabelled Se was measured by fluorimetry using diaminonaphthalene [25].

The Scy synthase reaction was carried out as follows: suppressor tRNA (0.5 μmol) in 20 μl of 0.2 M-Hepes (pH 7.4)/20 mM-$MgCl_2$/20 mM-KCl/20 mM-mercaptoethanol was mixed with 1 μl of 40 mM-serine, 5 μl of 50 mM-ATP and 1 μl of SerRS. This was incubated for 20 min at 30 °C. Afterwards, 10 μl (60 kBq) of 1.2 μM-HSe^- and 10 μl of enzyme preparation for Scy-tRNA synthesis were added to the mixture and were further incubated for 2 h at 30 °C. Under these conditions the production of Scy was linear with the incubation time. For the estimation of apparent-K_m values for the above substrates (tRNA, serine, ATP and HSe^-), the concentrations in the reaction mixture were changed. After the incubation, the tRNA was collected by precipitation with the addition of ethanol. Part of this tRNA was chromatographed on Sephacryl S-200 in 0.15 M-NaCl/10 mM-acetate/10 mM-mercaptoethanol, pH 4.6. The precipitate was washed with ethanol and then hydrolysed in 5 μl of aq. 1 M-NH_3 in the presence of 1 M-mercaptoethanol for 2 h at 30 °C. This addition of mercaptoethanol was done to protect the Scy from oxidation. After the hydrolysis of aminoacyl-tRNA, the tRNA was precipitated by the addition of ethanol, and the supernatant was immediately analysed by t.l.c. on silica-gel-G plates. Authentic unlabelled Scy, which was prepared by reduction of selenocystine (generously given by Professor K. Soda of the University of Kyoto) with 50 mM-mercaptoethanol, was co-chromatographed. We confirmed that most of the Scy was not oxidized during spotting of the samples on, and developing, the TLC plates. Unlabelled Scy on the t.l.c. plates was stained with ninhydrin.

The solvent systems used for t.l.c. were as follows.

System 1. The first dimension was butan-1-ol/acetic acid/water (4:1:1, by vol.) and the second dimension was phenol/water (3:1, w/w) [2]. Occasionally the activity in enzyme fractions was measured by one-dimensional t.l.c. analysis (solvent: butan-1-ol/acetic acid/water); other t.l.c. systems for the confirmation of Scy (Systems 2–5) were also as described in [2].

System 2. The first dimension was chloroform/methanol/17% NH_3 (2:2:1, by vol.), and the second dimension was phenol/water (3:1, w/w).

System 3. This was a one-dimensional system on silica-gel-G plates and employed propan-1-ol/28% NH_3 (7:3, v/v).

Systems 4 and 5. These were two-dimensional thin-layer systems on cellulose MN 300 plates. In system 4 the first dimension was butan-1-ol/acetone/diethylamine/water (10:10:2:5, by vol.) and the second dimension was propan-2-ol/formic acid/water (4:2:1, by vol.); in system 5 the first dimension was butan-1-ol/acetone/diethylamine/water (10:10:2:5, by vol.) and the second dimension was phenol/water (3:1, w/w; the gas phase was equilibrated with 3% NH_3). The radioactivity of ^{75}Se on t.l.c. plates was measured by standard autoradiographic techniques or was occasionally analysed with a Fuji Bio-Image Analyzer BAS 2000.

For quantitative measurement of ^{75}Se radioactivity, the silica on t.l.c. plates was scraped off and the radioactivity in the silica was determined on a Gamma-Autowell counter. When the synthesis of [^{14}C]Scy-tRNA or [^{3}H]Scy-tRNA was studied, [^{14}C]serine or [^{3}H]serine was used instead of unlabelled serine; unlabelled HSe^- was used instead of $H^{75}Se^-$. Seryl-tRNA was prepared by chromatography of the reaction mixture of tRNA, serine, ATP and SerRS on Sephacryl S-200 in 0.15 M-NaCl/10 mM-acetate, and the synthesis of Scy-tRNA from seryl-tRNA was carried out at pH 6.5 to avoid the hydrolysis of aminoacyl-tRNA.

Dot-blot hybridization to measure the amount of opal suppressor tRNA was done by the method of Kumazawa *et al.* [26]. A DNA probe for suppressor tRNA was constructed at the position from the TψC arm to the aminoacyl arm, and the sequence was 5′-CGCCCGAAAGGTGGAA-3′. This sequence was specific to the tRNAs [27] and did not correspond to serine major $tRNA_{GCU}$ or $tRNA_{IGA}$. There were two opal suppressor tRNAs, $tRNA_{NCA}$ [28] and $tRNA_{CmCA}$ [11], and this probe hybridized to both tRNAs. Hybridization of this ^{32}P-labelled probe to the tRNA preparation was done by a standard hybridization method [27].

RESULTS

The supernatant 105000 *g* of the centrifuged murine liver extract contained Scy-tRNA-synthetic activity [20]. The supernatant was chromatographed on DEAE-cellulose, as shown in the upper portion of Fig. 1. Scy synthase activity in eluates is shown by the autoradiographic traces in Fig. 1(*a*). The activity was found in tubes 21–23 and the specific activity was 0.8 mol/h per mg. Other fractions, excepting tubes 21–23, showed no activity, as shown in [20]. Fractions 21–23 were collected and used later as the active DEAE-cellulose fraction. The peak of SerRS activity was eluted in tubes 22–26. The tRNA kinase was eluted at a higher concentration of KCl than was SerRS [14]. These elution positions are indicated in Fig. 1. Fig. 2(*a*) shows the elution pattern of the 105000 *g* supernatant on Sephacryl S-300. The autoradiographic results in Fig. 2 show that Scy-tRNA-synthetic activity was found in fraction 2 and was eluted at a K_d value of 0.15. This K_d value indicated that the main Scy-tRNA-synthetic activity had a molecular mass of 500 kDa. The activity in eluates from DEAE-cellulose appeared as a distinct band of [^{75}Se]Scy on autoradiography, but the density of the Scy band

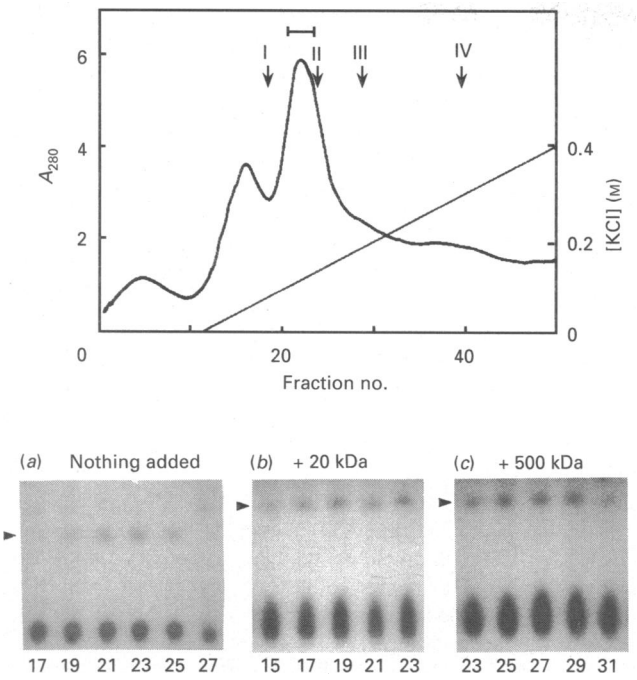


Fig. 1. Chromatograph of the 105000 *g* supernatant of murine liver extracts on DEAE-cellulose

The upper section is a chromatographic profile. Arrows I, II, III and IV are the elution positions of the 500 kDa protein, SerRS, the 20 kDa protein and tRNA kinase respectively. The lower section shows autoradiographs of [^{75}Se]Scy liberated from Scy-tRNA produced with fractions from the upper section as enzyme sources. The fraction number used is indicated under the autoradiographs. In (*a*) the activity was measured in the fractions as collected. In (*b*) and (*c*) the 20 kDa protein (tube 38 in Fig. 7) and 500 kDa protein (tube 24 in Fig. 7) was added to each eluate. Fractions 21–23 were collected and used later as the 'active DEAE-cellulose fraction'. The arrowheads (▶) indicate the position of Scy.

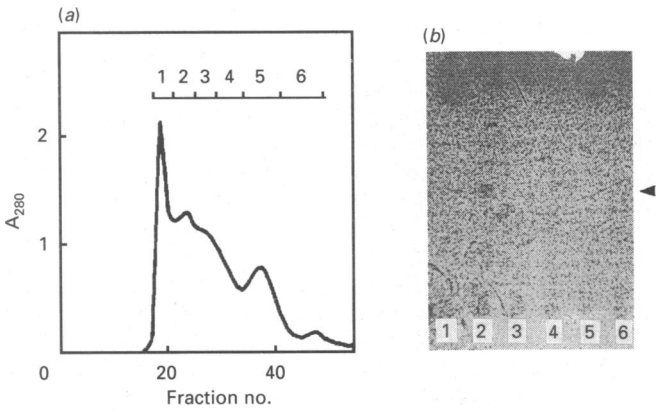


Fig. 2. Chromatographic profile of the supernatant on Sephacryl S-300

(*a*) Chromatographic profile; (*b*) autoradiograph of [^{75}Se]Scy produced with fractions 1–6 shown in (*a*). The arrowhead indicates the position of Scy.

produced with the eluates from Sephacryl S-300 was weak, as shown in Fig. 2(*b*). This result suggests that some factors promoting Scy synthesis may be removed from the main Scy synthase activity of 500 kDa on Sephacryl S-300. However, the eluates from DEAE-cellulose contained all the factors needed for Scy-tRNA synthesis. In the experiments described below we

Table 1. Chromatography of [^{75}Se]Scy products on thin-layer plates

The solvents used in each of the t.l.c. systems are described in the Materials and methods section.

T.l.c. system	R_F First dimension	R_F Second dimension
1	0.43	0.40
2	0.77	0.46
3	0.49	–
4	0.44	0.68
5	0.49	0.70

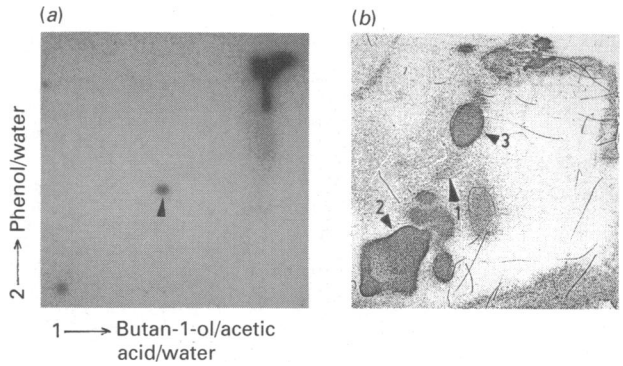


Fig. 3. Autoradiographs of the hydrolysates of aminoacyl-tRNA on silica-gel-G plates

The enzyme source is the active DEAE-cellulose fraction of Fig. 1. (*a*) Result with [^{75}Se]Scy. The arrowhead indicates the position of Scy. (*b*) The result with ^{14}C. The arrowhead 1 indicates the position of Scy. The arrowheads 2 and 3 indicate the positions of serine and pyruvate respectively.

studied the synthesis of Scy-tRNA with this active DEAE-cellulose fraction.

For further confirmation of Scy synthesis, we analysed the product (Scy) by five t.l.c. systems (see the Materials and methods section.) In all cases we found the spot of ^{75}Se radioactivity at the position of authentic unlabelled Scy which was co-chromatographed and detected by its ninhydrin colour reaction. The R_F values obtained with these five systems are summarized in Table 1. These values show that [^{75}Se]Scy is a product formed by weak alkaline treatment of aminoacyl-tRNA, and this shows that [^{75}Se]Scy is bound to tRNA.

In order to confirm the conversion of seryl-tRNA to Scy-tRNA *in vitro*, we studied the conversion of [^{3}H]seryl-tRNA and [^{14}C]seryl-tRNA to [^{3}H]Scy and [^{14}C]Scy. The Scy produced from seryl-tRNA, ATP, unlabelled HSe^- and the enzyme was analysed by t.l.c. The autoradiographic results are shown in Fig. 3. Fig. 3(*a*) shows the results with $H^{75}Se^-$. Fig. 3(*b*) shows the results with [^{14}C]Ser. Arrowheads 1, 2 and 3 in Fig. 3(*b*) indicate the positions of Scy, serine and pyruvate respectively. The ^{14}C radioactivity was found at the position of Scy. Serine represents the raw starting material and pyruvate may be an intermediate. The radioactivity of [^{14}C]Scy was 0.5% of the total ^{14}C radioactivity applied on the t.l.c. plate. The t.l.c. pattern of [^{3}H]Scy produced from [^{3}H]serine was similar to that of [^{14}C]Scy.

We investigated which substrates were essential for Scy synthesis. The reaction mixture contained suppressor tRNA, ATP, serine, HSe^- and the enzyme fraction. The results for substrate-dose-dependency are shown in Fig. 4 by ^{75}Se autoradiography.

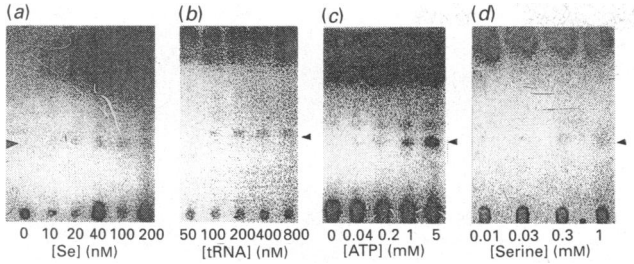


Fig. 4. Autoradiographs of [^{75}Se]Scy showing the dose-dependency of some substrates

The arrowheads indicate the position of Scy. (*a*) Shows [^{75}Se]HSe^--dependency on the reaction of Scy-tRNA-synthetic reaction. (*b*) tRNA-dependency in the [^{75}Se]Scy-tRNA-synthetic reaction. (*c*) and (*d*) show the concentration-dependency of ATP and serine in [^{75}Se]Scy synthesis respectively.

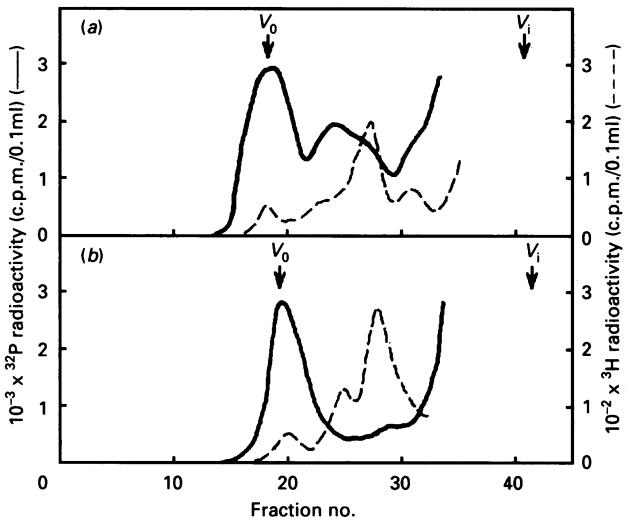


Fig. 5. Chromatographic profile of phosphoseryl-tRNA on Sephacryl S-200

(*a*) Result obtained with authentic tRNA kinase. The ^{32}P radioactivity in fractions 22–26 is Ser(P)-tRNA. The ^{3}H peak in fractions 26–28 is [^{3}H]seryl-tRNA. (*b*) Result obtained with the fraction containing Scy synthase (the active DEAE-cellulose fraction). The ^{32}P peak was not found in (*b*) at the position of Ser(P)-tRNA.

These substrates were all found to be essential, since Scy was not produced in the absence of any one of the substrates, as shown in Fig. 4. The apparent K_m value for HSe^- was 40 nM, as shown in Fig. 4(*a*). The concentration of total Se in our bodies is 2.5 μM, and more Se is present in the bound state on proteins. Therefore the amount of free Se might be near the concentration of the K_m value (40 nM), and the K_m value reasonably approximates to the natural concentration of HSe^-. Thus Scy synthase has a high affinity for its substrate HSe^-. The K_m value for suppressor tRNA is also 140 nM, and this value also is near the concentration of suppressor tRNA. Meanwhile, the K_m value of ATP was 2 mM, a value near that of ATP in cytosol. The K_m value for serine, 30 μM, is the value of SerRS [22]. Thus the K_m values for HSe^- and tRNA in the Scy-synthesis reaction showed a high affinity of HSe^- and suppressor tRNA for Scy synthase and were near the concentrations of those substrates in cytosol.

Mammalian Ser(P)-tRNA was presumed to be an intermediate between seryl-tRNA and Scy-tRNA. Thus it is a key point to clarify whether or not tRNA kinase is necessary to synthesize Scy-tRNA. Suppressor seryl-tRNA was incubated with the active DEAE-cellulose fraction or tRNA kinase in a reaction mixture containing [γ-^{32}P]ATP. The product was precipitated with ethanol and chromatographed on Sephacryl S-200. Ser(P)-tRNA and seryl-tRNA were eluted at tubes 23–26 and tubes 26–28 respectively. Fig. 5(*a*) illustrates the result using authentic tRNA kinase. Fig. 5(*b*) shows the result using the active DEAE-cellulose fraction in Fig. 1 instead of tRNA kinase. Fig. 5(*b*) shows that the active DEAE-cellulose fraction in Fig. 1 did not contain any tRNA kinase. Thus the DEAE-cellulose fraction in Fig. 1 had the ability to synthesize Scy-tRNA in the absence of tRNA kinase. Scy-tRNA was not synthesized via Ser(P)-tRNA, but was directly synthesized from seryl-tRNA by the active DEAE-cellulose fraction. The active fraction was separated from tRNA kinase because Scy synthase was eluted from DEAE-cellulose at a lower concentration than was SerRS. The kinase was eluted at a higher concentration than was SerRS. The active DEAE-cellulose fraction contained a small amount of SerRS, but did not contain any tRNA kinase.

In Fig. 4 we showed that ATP is essential for the synthesis of Scy-tRNA. ATP has a role in the synthesis of Scy-tRNA from seryl-tRNA as well as in the synthesis of seryl-tRNA from tRNA and serine. Fig. 6(*b*) shows the synthesis of Scy-tRNA from seryl-tRNA in the presence of ATP. However, in the absence of ATP we could not detect any synthesis of Scy-tRNA from seryl-tRNA (Fig. 6*a*).

Thus ATP was essential not only for the synthesis of Scy-tRNA from seryl-tRNA, but also for the synthesis of seryl-tRNA.

As a second step in the purification of Scy synthase, the active DEAE-cellulose fraction was chromatographed on Sephacryl S-300. The elution pattern is shown in Fig. 7. We looked for Scy synthase activity in each fraction, but none contained any. We thought it possible that the two components necessary for activity had become separated from each other. We searched for those activities by a complementation test involving the addition of two separate fractions. Among many combinations of two fractions we found activity when fractions 24 and 38 were mixed, as shown in Fig. 7*b* (the patterns of ^{75}Se on t.l.c. plates were the result of analysis of a Fuji Bio-Image Analyzer). We have omitted the results for combinations showing no activity. The molecular mass of the component in fraction 24 was estimated to be 500 kDa from the K_d value (0.15 on Sephacryl S-300). This fraction coincides with an active fraction in Fig. 2 and represents the main enzyme. The molecular mass of the component in fraction 38 was estimated to be 20 kDa from the K_d value (0.7 on Sephacryl S-300). Thus it was confirmed that the two components, of 500 kDa and 20 kDa, were necessary for the production of Scy-tRNA.

We studied the elution profiles of the 500 kDa protein and 20 kDa protein on DEAE-cellulose in Fig. 1. By addition of the 20 kDa protein to each fraction, a new peak (fractions 17–21) appeared in the earlier elution position of the original peak, as shown in Fig. 1(*b*). This indicated that the 500 kDa protein was eluted at a lower concentration of KCl from DEAE-cellulose. The 500 kDa protein had a molecular mass near to that of tRNA kinase (450 kDa), but that elution position (0.08 M-KCl) of the 500 kDa protein from DEAE-cellulose was clearly different from that of tRNA kinase (0.3 M-KCl). By addition of the 500 kDa protein, another peak appeared at the later elution position (fractions 27–29) of the original peak, as shown in Fig. 1(*c*). (In Figs. 1*b* and 1*c* we have omitted the results of inactive fractions before and after the main active fractions.) This indicated that the 20 kDa protein was eluted at a higher concentration of KCl. From these results, it was found that the original activity peak on DEAE-cellulose was the result of the overlapping of 500 kDa and 20 kDa proteins. From the results in Fig. 7 and Fig. 2, the

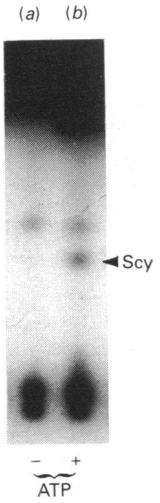


Fig. 6. Synthesis of [^{75}Se]Scy-tRNA from seryl-tRNA in the presence or absence of ATP

−, Absence of ATP (no Scy band was found); +, presence of ATP (a Scy band was found). The arrowhead indicates the position of Scy.

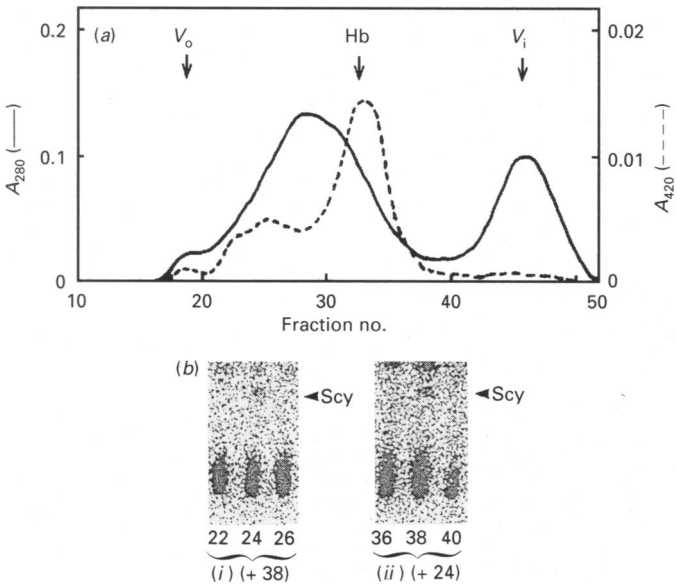


Fig. 7. Chromatographic profile of the active DEAE-cellulose fraction on Sephacryl S-300

(*a*) Chromatographic profile. (*b*) Autoradiograph of [^{75}Se]Scy: (*i*) result of mixing fractions 22–26 with fraction 38; (*ii*) result of mixing fractions 36–40 with fraction 24. The arrowheads indicate the position of Scy.

main enzyme component of the murine Scy synthase activity is judged to be the 500 kDa protein, and the 20 kDa protein must have a supporting role in the Scy synthase activity of the 500 kDa protein.

Fig. 7 shows the pattern measured at 420 nm. The 20 kDa protein did not have any absorbance at this wavelength, but the 500 kDa protein did. This result also supports the notion that the 500 kDa protein is the main enzyme producing Scy, because Scy synthase in *Escherichia coli* is a pyridoxal 5′-phosphate (PLP)-dependent enzyme [18] that absorbs at 420 nm.

In order to clarify the function of the 20 kDa protein, we studied the affinity of H^{75}Se$^-$ to the 20 kDa protein. A mixture of the active DEAE-cellulose fraction and HSe$^-$ in the absence or

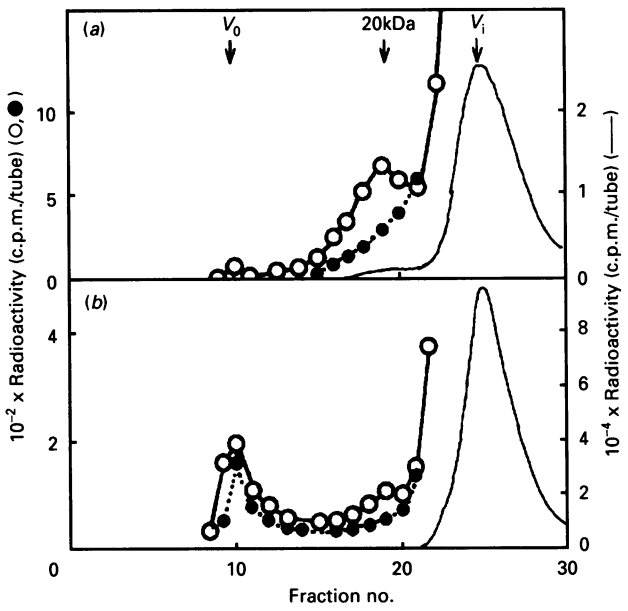


Fig. 8. Chromatographic profile of a complex of HSe$^-$, ATP and the active DEAE-cellulose fraction on Sephacryl S-300

(*a*) Mixture of [^{75}Se]HSe$^-$ (10 Ci/mmol), unlabelled ATP and the active DEAE-cellulose fraction (○); ●, absence of ATP. (*b*) is the result of a mixture of unlabelled HSe$^-$, [^{3}H]ATP (0.8 Ci/mmol) and the enzyme (○); ●, absence of HSe$^-$. ——, Radioactivity of unbound ^{75}Se and ^{3}H eluted at V_i (right ordinate scale).

presence of ATP was analysed by chromatography on Sephacryl S-300. Patterns of ^{75}Se elution are shown in Fig. 8(*a*). When ATP was present, the peak of ^{75}Se radioactivity coincided with the peak of the 20 kDa protein (○ in Fig. 8*a*) and was not found in the position corresponding to the 500 kDa protein. The peak of H^{75}Se$^-$ was not found at the position of the 20 kDa protein in the absence of ATP (● in Fig. 8*a*). Next we analysed a mixture of the DEAE-cellulose fraction, unlabelled HSe$^-$ and [^{3}H]ATP by chromatography on Sephacryl S-300 (Fig. 8*b*). One peak of [^{3}H]ATP was found at the position of the 20 kDa protein in the presence of HSe$^-$ (○), but was not found in the absence of HSe$^-$ (●). Binding of HSe$^-$ and ATP to the 20 kDa protein was also found when fraction 38 (Fig. 7) was used. The amounts of H^{75}Se$^-$ and [^{3}H]ATP bound to 12 μg of protein were 0.5 pmol and 0.4 pmol respectively. Thus the 20 kDa protein has the ability to bind equivalent amounts of HSe$^-$ and ATP. HSe$^-$ and ATP may be converted to activated HSe$^-$ on the 20 kDa protein and transferred to Scy synthase (500 kDa protein).

We determined the apparent-K_m value of suppressor tRNA in the Scy-tRNA synthesis reaction, as shown in Fig. 4(*b*). We used the last peak on BD-cellulose chromatography as a source, and the amount of suppressor tRNA was estimated from the serine-accepting activity of the tRNA. After the establishment of the '*in vitro*' Scy-tRNA-synthesis system we looked for the suppressor tRNA in the pattern of elution from BD-cellulose (Fig. 9*a*) by the dot-blot hybridization method [27] with a DNA probe which corresponded to the common position between two suppressor tRNAs ($tRNA_{NCA}$ and $tRNA_{CmCA}$) and was different from the major species, $tRNA_{GCU}$ and $tRNA_{IGA}$. The result is shown in Fig. 9(*b*). The probe most potently hybridized with BD-8 (i.e. fraction 8 from the BD-cellulose column) and BD-9 and weakly hybridized with BD-11. Thus BD-8 and BD-9 contained more suppressor tRNA. In cytosol, $tRNA_{NCA}$ was more evident than $tRNA_{CmCA}$ and was eluted earlier from the column than was $tRNA_{CmCA}$ [28]. Therefore BD-8 and BD-9 may contain $tRNA_{NCA}$ and the fractions eluted later, namely BD-10 and 11, may contain

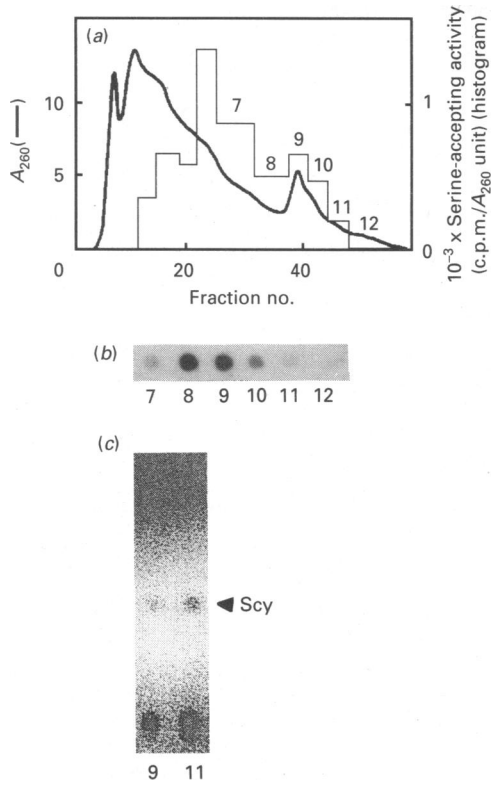


Fig. 9. Characterization of bovine tRNA fractions from BD-cellulose chromatography

(*a*) Elution pattern of tRNA on BD-cellulose. Suppressor tRNA was eluted at the later peak near fraction 40. The peak also coincided with the peak of serine-accepting activity. (*b*) Result of hybridization with a DNA probe for suppressor tRNA. (*c*) Result of autoradiography of [^{75}Se]Scy. The arrowhead indicates the position of Scy. The numbers in (*b*) and (*c*) correspond to the numbers against the histogram bars in (*a*) and also those used with a BD- prefix in the text.

$tRNA_{CmCA}$. Surprisingly, Fig. 9(*c*) shows that ^{75}Se in the Scy band corresponds less to the lane with BD-9 than to the lane with BD-11, although both lanes contained the same amount of tRNA (0.8 A_{260} unit). Therefore the fractions eluted later were a better substrate with which to synthesize Scy. We also show, in Table 2, the results for serine-accepting activity and [^{32}P]phosphorylation by tRNA kinase. These results accord with the results of hybridization shown in Fig. 9(*b*).

DISCUSSION

We studied the mechanism of mammalian Scy-tRNA synthesis, which is shown in Scheme 1. Natural opal suppressor tRNA was activated by SerRS of molecular mass 65.5 kDa to seryl-tRNA. This seryl-tRNA was converted into Scy-tRNA by Scy synthase of molecular mass 500 kDa. The Se donor for this reaction was HSe^-–ATP supplied by a 20 kDa protein. Thus 2 mol of ATP are necessary to produce 1 mol of Scy-tRNA. Another product from suppressor seryl-tRNA is Ser(P)-tRNA, which is synthesized by a tRNA kinase of molecular mass 450 kDa. It was thought that Ser(P)-tRNA might be an intermediate for the synthesis of Scy, since Scy-tRNA was synthesized in the presence of ATP [15]. However, we showed in the present study that tRNA kinase had no role in the synthesis of Scy. The tRNA kinase may have a role in regulating Scy-tRNA synthesis by phosphorylation of suppressor seryl-tRNA.

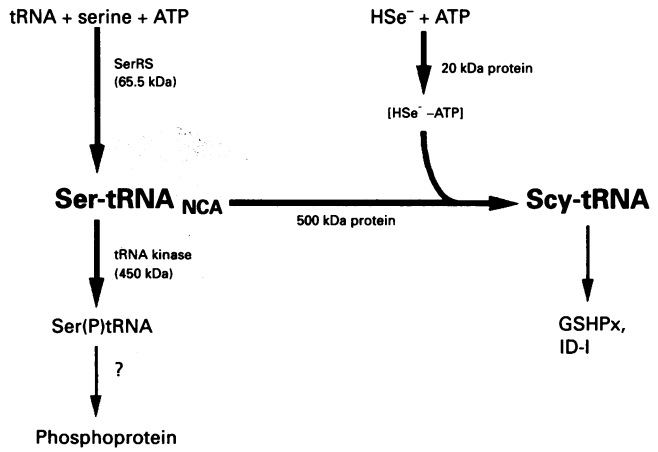
Careful consideration of certain papers [9,28,29] by Hatfield and co-workers shows that the suppressor tRNA eluted later may be a better tRNA for Scy synthesis, since a Figure presented in [9] indicates that $tRNA_{CmCA}$ is a major [^{75}Se]Scy-tRNA, although the authors claimed that both tRNAs ($tRNA_{NCA}$ and $tRNA_{CmCA}$) had equal amounts of Scy. The amount of $tRNA_{CmCA}$ in some cell lines was increased by supplementation of Se in the medium [29]. Our results, and the findings by Hatfield and co-workers, confirmed that $tRNA_{CmCA}$, which was eluted later, was a prominent tRNA for Scy synthesis. It was postulated that $tRNA_{NCA}$ and $tRNA_{CmCA}$ were synthesized by RNA editing (modification of mRNA precursors after transcription) from only one tRNA precursor which was produced from one tRNA gene [30,31]. Meanwhile, Kato *et al.* [32] reported the sequence of $tRNA_{NCA}$, which is identical with that of the above tRNA gene, but had three types of 5′-terminus (monophosphate, diphosphate and triphosphate). Therefore it is possible that the differences in the Scy-synthetic activity of suppressor tRNA is dependent upon differences in phosphorylation of the 5′-terminus of the tRNA. Hence we presume that one type among the three types of $tRNA_{NCA}$ described by Kato *et al.* [32] is a tRNA suitable for Scy synthesis.

We showed that SerRS recognized $tRNA_{GCU}$, $tRNA_{IGA}$ and suppressor tRNAs with similar K_m values [22,33]; therefore the common sequences among these serine tRNAs must be recognized by SerRS [22]. Recently, the identity of serine tRNA in *E. coli* was reported [34] and the tertiary structure of *E. coli* SerRS was established [35]. The different sequences among those tRNAs must be recognized by tRNA kinase (450 kDa) and Scy synthase (500 kDa). There is no tRNA kinase in *E. coli* [36]. In order to incorporate Se into proteins, a long extra arm in

Table 2. Summary of some activities of some fractions obtained by chromatography on BD-cellulose

Sample (fraction no. against histogram bars in Fig. 9*a*)	[^{14}C]Serine-accepting activity (c.p.m./unit*)	[^{32}P]DNA hybridized on tRNA (c.p.m./0.01 unit*)	[^{75}Se]Scy produced on tRNA ('density'†)	Ser(^{32}P) liberated from tRNA (c.p.m./unit*)
7	560	480	0	ND‡
8	510	1950	0	ND
9	640	1900	27	140
10	475	970	58	ND
11	190	320	75	35
12	11	50	5	ND

* A_{260} unit of tRNA.
† This is the relative autoradiographic density of [^{75}Se]Scy produced on 0.8 A_{260} unit of tRNA.
‡ ND, not determined.



Scheme 1. Mechanism of mammalian selenocysteinyl-tRNA synthesis

suppressor tRNA is essential [37]. Scy synthase and tRNA kinase have high molecular masses and may be able to recognize the difference between major tRNA and suppressor tRNA. Nevertheless, SerRS recognizes serine $tRNA_{GCU}$, $tRNA_{IGA}$, $tRNA_{CmCA}$ and $tRNA_{NCA}$. The tRNA kinase recognizes seryl-$tRNA_{NCA}$ and seryl-$tRNA_{CmCA}$. Scy synthase strongly recognizes seryl-$tRNA_{CmCA}$. Thus $tRNA_{CmCA}$ was recognized by SerRS, tRNA kinase and Scy synthase. Major $tRNA_{GCU}$ and $tRNA_{IGA}$ were only recognized by SerRS and were not recognized by tRNA kinase or Scy synthase. These systems are complex, but very interesting for the fine recognition mechanisms they show between serine tRNAs and those enzymes involving the reaction of aminoacyl-tRNAs.

In *E. coli* there are four genes (*selA*, *-B*, *-C*, and *-D*) relating to the synthesis of formate dehydrogenase. One of these genes, *selC*, codes for a Scy tRNA [38]. The product of *selB* is a translation factor (such as EF-Tu) specific to Scy-tRNA [39]. This translation factor resembles the anti-releasing factor in mammals [40]. The product of *selA* is Scy synthase. The molecular mechanisms of Scy synthase in *E. coli* were studied with [^{14}C]Ser-tRNA. In the present study, to clarify the mechanisms in mammals, we studied Scy synthesis with [^{75}Se]HSe^-. The product of *selD* plays a role in bringing HSe^- to the seryl-tRNA–SELA complex with ATP [18]. The 500 kDa protein described here is similar to SELA of molecular mass 600 kDa. SELA is composed of some subunits of 50 kDa and has pyridoxal 5′-phosphate (PLP) as a cofactor of the enzyme [18]. It is not clear whether or not the 500 kDa murine protein in the present study is composed of subunits. In preliminary experiments the active DEAE-cellulose fraction was inactivated by modification with hydroxylamine, suggesting that murine Scy synthase is a PLP-dependent enzyme. SELD, of molecular mass 37 kDa, has a role in activating HSe^- with ATP, and the mammalian 20 kDa protein described here is similar to this SELD. These results suggest that the mechanisms of Scy-tRNA synthesis in mammals are the same as those in *E. coli*. Properties of the *E. coli* and mammalian systems are compared in Table 3. The systems supplying Scy-tRNA to ribosomes in *E. coli* and mammals may also be similar.

However, the structure of mammalian $tRNA^{Scy}$ clearly differs from that of *E. coli* $tRNA^{Scy}$, as does every other amino-acid-specific tRNA in *E. coli* and mammals. From the standpoint of context effects (the situation whereby recognition of the codon on mRNA is influenced by the surrounding sequence) near the Scy UGA codon, mRNAs of formate dehydrogenase [41] and glycine reductase [42] adopted a specific secondary structure downstream of the UGA codon. However, we could not find any similar structure downstream of the UGA codon on the mRNAs of GSHPx [4] or ID-1 [7] by computer analysis. The expression of human GSHPx in *E. coli* was unsuccessful (Y. Sukenaga, personal communication) and this suggests that the context effect near the Scy UGA codon is different between bacterial selenoprotein mRNA and mammalian selenoprotein mRNA. Recently it has been shown that recognition of UGA as a Scy codon in ID-I requires sequences in the 3′-untranslated region [43]. Accumulation of information regarding mRNAs of certain other selenoproteins, such as plasma selenoprotein [44] and phospholipid hydroperoxide GSHPx [45], will help to understand the selenocysteine-insertion sequence motif in the long 3′-untranslated region of mRNA of selenoproteins [43].

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Table 3. Comparison (mammalian versus *E. coli*) of some factors involved in Scy incorporation into selenoproteins

Factor	Mammalian	*E. coli*
Codon	UGA	UGA
Precursor of Scy	Serine	Serine
tRNA for Scy	Opal sup $tRNA_{NCA}$ (90 nucleotides) 5′ mono-, di- and tri-phosphates A bulge on TψC arm	SELC ($tRNA_{UCA}$) (95 nucleotides) 8 bp of aminoacyl arm Long extra arm
SerRS	65.5 kDa (α2 type)	48.4 kDa (α2 type)
Scy synthase	500 kDa protein (labile with NH_2OH)	SELA (650 kDa) (PLP enzyme)
Se donor	HSe^-	HSe^-
HSe^- activator	20 kDa protein with ATP	SELD (38 kDa) with ATP
Translation factor (EF-1α- or Tu-like)	50 kDa protein*	SELB (68 kDa)
Scy incorporation	Co-translational	Co-translational
Context mechanism	The long 3′ untranslated region	A stem-loop at 3′ site of Scy codon
Final products	GSHPx(s), ID-I, selenoprotein-P	Formate dehydrogenase

*Molecular mass of the protein preventing the degradation of Scy-tRNA in the microsomal extracts (T. Mizutani, unpublished work).

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