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The protozoa dinoflagellate *Oxyrrhis marina* contains selenoproteins and the relevant translation apparatus☆

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Abstract

In the phylogenetic tree, selenoproteins and the corresponding translation machinery are found in *Archaea*, *Eubacteria*, and animals, but not in fungi and higher plants. As very little is known about *Protozoa*, we searched for the presence of selenoproteins in the primitive dinoflagellate *Oxyrrhis marina*, belonging to the *Protoctista* kingdom. Four selenoproteins could be obtained from *O. marina* cells cultured in the presence of ^{75}Se. Using *O. marina* or bovine liver cytosolic extracts, we could serylate and selenylate in vitro total *O. marina* tRNAs. Moreover, the existence of a tRNA^{Sec} could be deduced from in vivo experiments. Lastly, an anti-serum against the specialized mammalian translation elongation factor mSelB reacted with a protein of 48-kDa molecular mass. Altogether, our data showed that *O. marina* contains selenoproteins and suggests that the corresponding translation machinery is related to that found in animals.

Keywords: Selenocysteine; Selenoprotein; tRNA; Selenium; Dinoflagellate; *Protozoa*; *Oxyrrhis marina*

Selenocysteine (Sec) is the 21st amino acid [1]. This was suspected three decades ago by the work of Hatfield and Portugal [2] who found that one species of tRNA^{Ser} in the liver and brain from chicken, bovine, and rabbits can recognize the UGA codon. Meanwhile, the cDNA of the murine selenoprotein glutathione peroxidase (GPx) was sequenced and it was discovered that the Sec codon is UGA [3]. Selenocysteine biosynthesis starts from serine, as demonstrated in the case of GPx that the carbon backbone of Sec originates from ^{14}C-Ser [4]. Subsequently [^{75}Se]Sec could be prepared in vitro using this natural tRNA^{Ser} species or the corresponding T7 transcript, bovine liver cytosol as the enzyme source [5], and [^{75}Se]H_2Se as the Se donor [6]. Whereas two tRNA^{Ser} and one tRNA^{Sec} species are recognized and aminoacylated by one kind of seryl-tRNA synthetase (SerRS) dimer [7,8], only the Ser-tRNA^{Sec} is recognized by the selenocysteine synthase (SecS) that converts it to Sec-tRNA^{Sec}. In the tRNA^{Sec}, the identity elements for selenylation by the SecS are the 9-bp long amino acyl-stem and the 6-bp long D-stem [9,10]. The biologically active selenium donor is the selenophosphate (SeP) that is produced by the enzyme selenophosphate synthetase (SePS) from H_2Se and ATP [11]. The Sec-tRNA^{Sec} is brought to the A site of the ribosomes by the specialized translation elongation factor called mSelB/eEFSec in mammals [1,12–15].

The mechanism employed to discriminate between the in-frame UGA Sec and the UGA stop codon is still unclear, but the SECIS (Sec insertion sequence) RNA in the 3′ untranslated region of selenoprotein mRNAs may play a key role in this respect [16]. The SECIS RNA is recognized by the SECIS binding protein SBP2 [17]. In order to show that SECIS is essential for expression of selenoproteins, most studies have dealt with natural

☆ *Abbreviations:* Sec, selenocysteine; SecS, Sec synthase; SerRS, Seryl-tRNA synthetase; SECIS, Sec insertion sequence; GPx, glutathione peroxidase.

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selenoprotein mRNAs. Rarely, however, the conversion of non-selenoproteins to selenoproteins could be achieved from an mRNA in which the UGA Sec codon and the SECIS element were engineered. We reported earlier a model showing, in addition to the SECIS element, a conserved region upstream of the in-frame UGA Sec codon in most Se-protein mRNAs [18,19]. The selenocysteine incorporation machinery has been found in *Archaea*, *Eubacteria*, and animals, but not in fungi and higher plants. It is considered that animals and plants originate from protozoa [20]. Few data are available about Sec in *Protozoa*, except for the presence of selenoproteins in *Chlamydomonas reinhardtii*, a species containing chloroplasts, although authors describe that *C. reinhardtii* belongs to the Plant kingdom [21,22].

In this report, we provide evidence for the existence of selenoproteins and components of the selenocysteine incorporation machinery in the Dinoflagellata *Oxyrrhis marina*, a Protozoan commonly found in tide pools along the Coast.

Materials and methods

Oxyrrhis marina cultures and preparation of tRNA and proteins. *Oxyrris marina* was cultured in artificial seawater Jamarin U (Jamarin Lab., Osaka, Japan) essentially, as described previously [23]. Quail yolk was used as food [24]. A 20-l culture yielded 7 g *O. marina* cells. Total tRNA was prepared by the standard method with phenol–SDS, as for bovine liver tRNA [25]. The cytosol used as the enzyme source was the supernatant obtained after a 100,000*g* centrifugation of cell extracts.

Preparation and analyses of O. marina [^{75}Se]Sec-tRNA and [^{75}Se]selenoproteins. One microliter (10 μCi) [^{75}Se]SeO_2 was added to 1 ml of culture medium and incubated for 2 days at 15 °C. Cells were collected by centrifugation and washed three times with physiological saline to remove [^{75}Se]SeO_2 in the medium. For analysis of selenoproteins, the precipitate was suspended in SDS–dye solution for electrophoresis, heated for 1 min at 95 °C, and then analyzed by SDS–PAGE on 8% or 15% gel. To detect the [^{75}Se]Sec charged in vivo, the aminoacylated tRNAs were extracted in acetate buffer at pH 4.6 by the standard SDS–phenol method. The aqueous phase was recovered and the aminoacylated tRNAs were precipitated by addition of 2 volumes of ethanol. A mild alkaline hydrolysis of the precipitate content released the tRNAs and [^{75}Se]Sec that was analyzed by the TLC method described below.

In vitro charging of [^{75}Se]Sec. [^{75}Se]Sec was charged in vitro in a reaction mixture (50 μl) containing tRNA (20 μg), 5 mM ATP, 0.4 mM Ser, 0.1 μM [^{75}Se]H_2Se (0.02 μCi), SerRS, and Sec-synthesizing enzymes (partially purified cytosolic SecS and SePS), according to a previous report [5]. After a 2-h incubation at 30 °C, the [^{75}Se]Sec-tRNA was collected by ethanol precipitation and the precipitate was treated for 30 min with 5 μl of mild alkaline solution. The amino acid released from the aminoacyl-tRNA^{Sec} was spotted on silica gel G. Authentic selenocysteine (a gift from Prof K. Soda, Kyoto University), was also spotted and co-chromatographed. The amino acids were chromatographed in *n*-butanol:acetic acid:water (4:1:1) for the first dimension, in phenol:water (3:1) for the second one. After development, the position of Sec was revealed by ninhydrin staining. [^{75}Se]Sec was detected on TLC plates with a Fuji BAS 2500 image analyzer.

Western blotting. Proteins were separated on 8% or 10% SDS–PAGE, transferred onto PVDF membranes, and analyzed by Western blotting with rabbit anti-sera against bovine cellular GPx [26,27] and against mSelB [13].

tRNA and enzymes from bovine liver. Total tRNA was prepared according to [25]. The T7 tRNA^{Sec} transcript, SerRS, and SecS were prepared according to [5,10]. The [^{14}C] serylation activity was measured as in [7].

Results

We first wished to determine whether *O. marina* contains tRNA^{Sec} and the enzymes responsible for the serine to selenocysteine conversion. The [^{75}Se]Sec released from the Sec-tRNA^{Sec} was analyzed by two-dimensional TLC. The position of [^{75}Se]Sec was confirmed from the position of the spot of the authentic Sec colored with ninhydrin as shown by circles and arrows. Fig. 1A

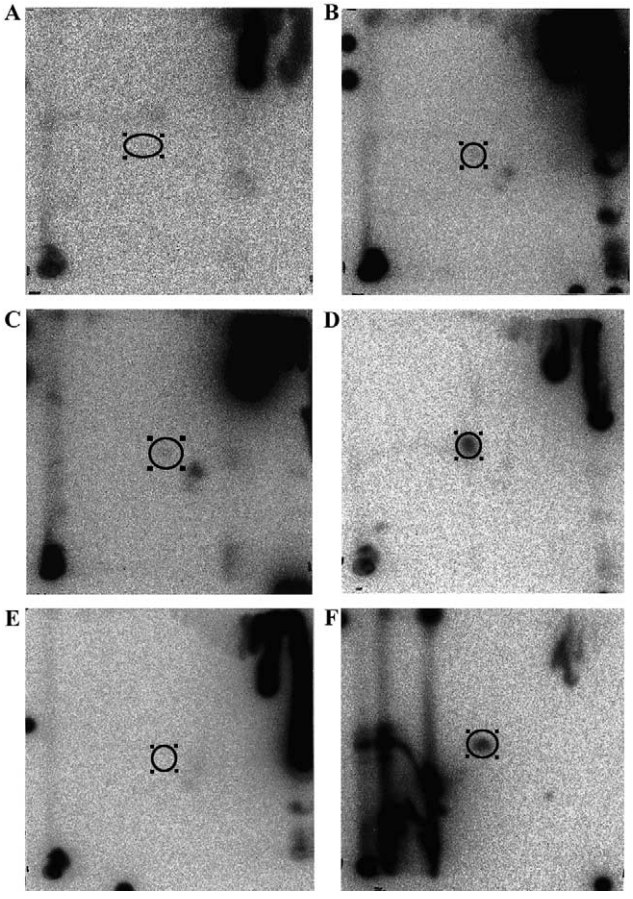

Fig. 1. TLC patterns for analysis of [^{75}Se]Sec released from Sec-tRNA^{Sec}. (A–E) are the results with Sec-tRNA produced in vitro. The first (horizontal) and the second (vertical) dimensions are *n*-butanol:acetic acid:water and phenol:water, respectively. Combinations of tRNA and enzymes are bovine liver and *O. marina* for (A), *O. marina* and bovine liver for (B), *O. marina* and *O. marina* for (C), and bovine liver and bovine liver for (D), respectively; (E) a control experiment without tRNA; and (F) the result of [^{75}Se]Sec-tRNA obtained in vivo. The positions of authentic Sec are indicated by circles at the center of the pictures.

Table 1
Serylation and selenylation levels of total tRNA from *O. marina* and bovine liver

tRNA	[^{14}C]Ser[a], dpm/A_{260} unit	[^{75}Se]Sec, pmol/A_{260} unit/2 h	
		Bovine enzyme	*O. marina* cytosol[b]
Bovine liver	1231	27.2 (Fig. 1D)	~0.2 (Fig. 1A)
O. marina	1034	1.3 (Fig. 1B)	~1.3 (Fig. 1C)

[a] Enzyme used is purified SerRS from bovine liver and the values show the saturated level of Ser on tRNA.
[b] This value (indicated by ~) is tentative and estimated from Figs. 1A and C.

shows that the *O. marina* cytosol is able to selenylate the bovine tRNA^{Sec} but the intensity is weak. For comparison, the selenylation by the cognate bovine enzymes is shown by a clear spot in Fig. 1D. Conversely, selenylation of the tRNA prepared from *O. marina* extracts was assayed with bovine enzymes (Fig. 1B) or *O. marina* cytosolic extracts (Fig. 1C), both showing clear spots of the same level. Fig. 1E shows a control experiment without tRNA. The spot with the highest intensity was obtained with the bovine tRNA and enzymes (Fig. 1D). These results suggest that the bovine and *O. marina* tRNA^{Sec} possess near common identity elements for the SerRS and SecS. Quantification of the data indicated that the yield of Sec-tRNA^{Sec} in *O. marina* with bovine enzyme is about 1/20 of that in bovine liver and the level in *O. marina* with *O. marina* enzyme is higher than that in bovine liver under the conditions described in Table 1. This is very likely due to the species specific interactions between tRNA^{Sec} and SecS. Table 1 also shows that crude tRNA preparations of bovine liver and *O. marina* can charge [^{14}C]Ser to the same extent. Next, we asked whether the selenylation observed in vitro reflects the existence of [^{75}Se]Sec-tRNA^{Sec} in vivo. To this end, *O. marina* cells were cultured in the presence of [^{75}Se]selenite. The bulk of aminoacylated tRNAs was isolated under acidic conditions and the amino acids were released by a mild alkaline treatment. Fig. 1F shows the presence of a [^{75}Se] spot migrating at the same position as the authentic selenocysteine revealed by ninhydrin. From this experiment, we conclude that the [^{75}Se]Sec present in *O. marina* is aminoacylated to the tRNA^{Sec}.

We also looked for the presence of ^{75}Se-labeled selenoproteins in *O. marina* cultured in the presence of [^{75}Se]SeO_2. Cell lysates were analyzed by SDS–PAGE. Fig. 2A shows a protein of 52-kDa molecular mass on 8% gel. The faster bands at the bottom of the gel were better resolved on a 15% gel as 25-, 21-, and 18-kDa molecular mass proteins as shown in Fig. 2B. Thus, we found in *O. marina* four major and other minor selenoproteins. They were analyzed by Western blotting with an antibody against the mammalian glutathione peroxidase (GPx). The result is shown in Fig. 2C where a main band appeared at 21 kDa. This is coincident with one of the [^{75}Se]selenoproteins shown in Fig. 2B.

Having shown that *O. marina* contains selenoproteins and selenocysteine, we wished to determine whether this organism contains a specialized translation elongation factor homologous to the mammalian mSelB/eEFSec [13]. *O. marina* extracts were analyzed by Western blotting with an anti-mSelB antibody. Anti-mSelB reacted with the authentic mSelB of 67-kDa molecular mass in Fig. 3A. In the pattern (Fig. 3B) with the *O. marina* extracts, we clearly found a band of 48-kDa molecular mass, smaller than mSelB. This 48-kDa protein is slightly smaller than the general elongation factor eEF1α that has 52 kDa of molecular mass. This is rather surprising because we showed in this work that the tRNA^{Sec} and selenylation enzymes are interconvertible between mammals and *O. marina*, but the molecular mass of the specialized elongation factor is clearly different. Thus, it may be that some components of the Sec machinery are similar between Protozoa and Mammals, but that others differ. Finally, we concluded that one of the protozoa, *O. marina*, possesses the Sec system including tRNA^{Sec} and selenoproteins.

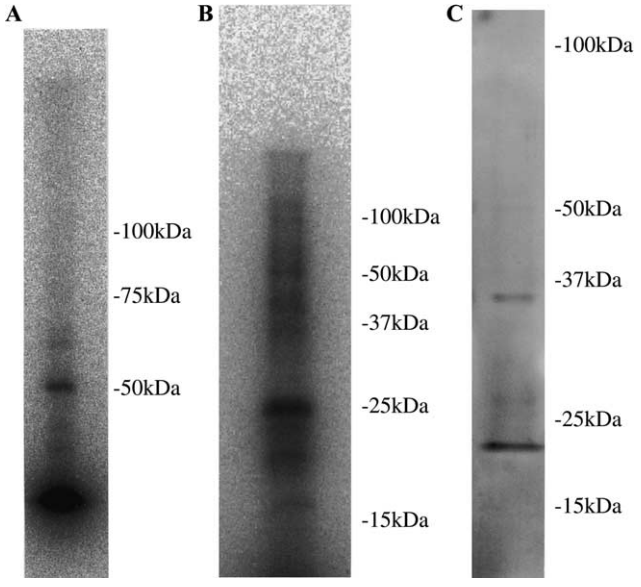

Fig. 2. Fractionation of *O. marina* selenoproteins labeled in vivo with [^{75}Se]. (A) Autoradiography of in vivo [^{75}Se]Se-proteins on 8% gel. (B) Autoradiography of Se-proteins on 15% gel. (C) Western blot with rabbit anti-serum against bovine cellular GPx.

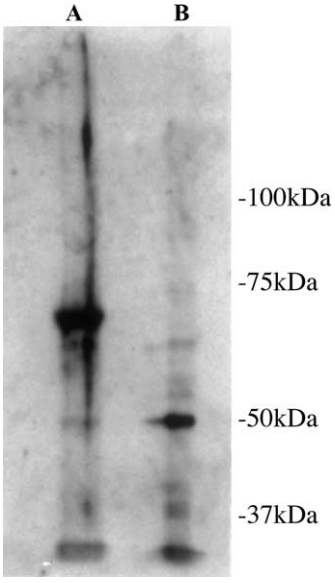

Fig. 3. Western blot with rabbit anti-serum against mSelB: (A) with the authentic mSelB; (B) with the *O. marina* cytosol.

Discussion

The Sec incorporation machinery containing the $tRNA^{Sec}$ and selenylating enzymes is distributed in *Archaea*, *Eubacteria*, and animals, but yeast and higher plants do not possess it. In the evolutionary tree, Protozoa reside at the origin of plants (not having Sec) and mammals (having Sec) [20]. It was therefore interesting to establish whether Protozoa are able to synthesize and incorporate Sec. *Euplotes*, one of the ciliated Protozoa, has a $tRNA^{Cys}$ with the anti-codon complementary to UGA [28] which is one of stop codons in the universal codon table. During the course of preparation of this manuscript, two papers were published that described the existence of Sec in *C. reinhardtii* [21,22]. Fu et al. [21] reported that the cDNA of the GPx homolog contains an internal TGA codon and the sequence analysis by MALDI-TOFMS showed the presence of a Sec residue at the predicted site. Novoselov et al. showed that *C. reinhardtii* contains four selenoproteins of 52, 22, 18, and 7 kDa and that a tRNA fraction containing Se recognizes UGA [22]. They speculate that *C. reinhardtii* belongs to Plant kingdom, because *C. reinhardtii* has chloroplast. However, we considered that *C. reinhardtii* does not belong to the *Plantae* kingdom but rather to the *Protoctista*. Thus, in consideration of the study of Sec tRNA and selenoproteins in *C. reinhardtii*, we provided evidence of the presence of Sec system in another Protozoan, *O. marina*, leading to the deduction that Protozoa universally have $tRNA^{Sec}$, the Sec system, and selenoproteins, specific to Protozoa.

In Table 1 and Fig. 1, we showed the cross-reactivity of tRNA and selenylation enzymes between bovine and *O. marina*. The level of [^{75}Se]Sec-$tRNA^{Sec}$ produced in vitro with tRNA of *O. marina* was lower than that with the bovine liver. It is not clear whether this is due to a lower level of $tRNA^{Sec}$ or poor recognition of the *O. marina* tRNA by the bovine liver enzymes. It could also arise from structural differences between the $tRNA^{Sec}$ of *O. marina* and bovine liver. It is possible that purified selenylation enzymes from *O. marina* give rise to the higher level of Sec-tRNA from *O. marina*, like in Fig. 1D. The data in Table 1 show that the serylation level is similar, suggesting that the identity elements on $tRNA^{Ser}$ for SerRS are similar. Both tRNAs have the same structure whose identity elements for SerRS are the discrimination base G74 and the long extra arm [29]. In animals, the $tRNA^{Sec}$ identity elements for selenylation are the 9-bp long aminoacyl stem and the 6-bp D stem [9,10,30,31]. It is possible that these identity elements are slightly altered in the $tRNA^{Sec}$ of *O. marina*.

We showed the existence in *O. marina* of a protein reactive to an antibody against the mammalian specialized translation elongation factor mSelB. The protein we have detected has a molecular mass of 48 kDa, slightly lower than the general elongation factor eEF1α and clearly different from mSelB of molecular mass 67 kDa [13]. It is interesting that mSelB in *O. marina* has a lower molecular mass of 48 kDa. It is considered that, in mammals, several proteins may function to discriminate the Sec UGA from the stop UGA codon [12–15]. In *O. marina*, the protein species for Sec translation may be smaller and different from those in animals. It was established that the $tRNA^{Sec}$ from *C. reinhardtii* recognizes the UGA Sec codon, but its sequence has not been determined yet [22]. In the case of *O. marina*, the sequence of the $tRNA^{Sec}$ and its codon specificity are still to be investigated.

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