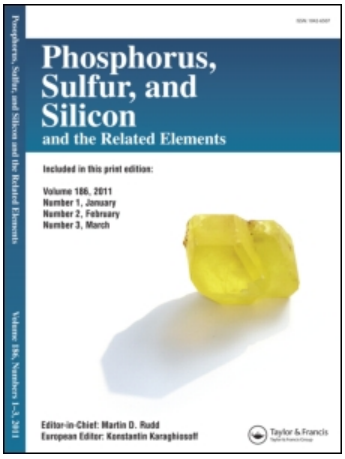

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Specific Occurence of Selenium in Certain Enzymes and Amino Acid Transfer Ribonucleic Acids

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SPECIFIC OCCURENCE OF SELENIUM IN CERTAIN ENZYMES AND AMINO ACID TRANSFER RIBONUCLEIC ACIDS

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Abstract Some enzymes known to contain selenium are enumerated. In four of them which catalyze coupled oxidation-reduction reactions the selenium occurs exclusively in the form of selenocysteine residues. Their structure and function are described in detail. Two other bacterial enzymes which contain selenium in the form of a labile, readily dissociable component are also described.
Results on the isolation, identification and structure determination of selenium-containing amino acid transfer ribonucleic acids are presented. These seleno-t RNA's are shown to contain either 5-methylaminomethyl-2-selenouridine or other 2-selenouridine derivatives. The role of selenium in a glutamate isoaccepter species is discussed.

Introduction

The recognition of selenium as an essential micronutrient for animals and many bacteria dates from the 1950's but identification of this element as a highly specific component of several enzymes and amino acid transfer ribonucleic acids (tRNAs) is much more recent. The amount of selenium required for optimal synthesis of several known selenium-containing macromolecules is usually in the 0.1 to 1 μM range for bacteria and cultured mammalian cells whereas sulfur levels in the media normally are at least three orders of magnitude higher. For adult humans a daily intake of 1 to 3 microequivalents of selenium is estimated to be sufficient. In the case of bacteria, it has been shown experimentally that the biosynthesis of essential seleno-enzymes and seleno-tRNAs is unaffected

by wide variations in sulfur levels. In several instances relatively constant levels of a particular selenoenzyme or seleno-tRNA are observed when organisms are cultivated in the presence of o.1 to 1 μM selenite while the sulfur is varied from o.5 to 1o mM.

In view of the many chemical properties common to sulfur and selenium, the question of the possible biological advantages of the use of selenium instead of sulfur frequently is posed. The fact that selenols, in contrast to thiols, are largely ionized at neutral pH indicates that selenols in enzymes will be anionic and thus good nucleophiles. Also, the lower redox potential, e.g. of selenocysteine as compared to cysteine, could serve as an important advantage for an enzyme catalyst operative at the lower end of the potential scale. The occurrence of selenium in several enzymes from strictly anaerobic bacteria and the apparent lack of selenium in the corresponding enzyme catalysts present in strictly aerobic species, tends to support this idea. To date six enzymes have been identified that contain selenium essential for their catalytic activities. Five of these enzymes are of bacterial origin; clostridial glycine reductase [1], formate dehydrogenases from Escherichia coli [2] and certain anaerobic bacteria [3-6], a hydrogenase from Methanococcus vannielii [7] nicotinic acid hydroxylase from Clostridium barkeri [8,9] and xanthine dehydrogenase [1o] from certain purine fermenting anaerobic bacteria. The only selenoenzyme known to occur in animals is glutathione peroxidase [11,12]. However, at least two other selenium-containing proteins of unidentified catalytic function have been detected and partially purified from mammalian sources. One of these, a 75,ooo dalton selenoprotein, is found in liver, kidney and serum [13,14]. An-

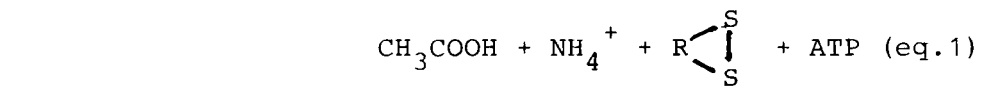
other selenoprotein, about 15,ooo daltons, is present in spermatozoa [15,16] and is believed to be a factor involved in selenium-responsive fertility of domestic animals. Two additional bacterial enzymes, thiolase and β-hydroxybutyryl-CoA dehydrogenase, that were isolated by Hartmanis and Sliwkowski [17] from Clostridium kluyveri, contain conspicuous amounts of selenium when the organism is cultured in standard media. However, in these proteins, the selenium occurs as selenomethionine residues that are distributed randomly throughout the polypeptide structures in place of methionine [18,19]. Although thiolase also contains several cysteine residues, there was no detectable selenocysteine in the protein. This is of particular interest because it shows that in the case of cysteine no nonspecific substitution of the selenium analog occurred whereas random replacement of methionine residues by selenomethionine was appreciable. Whether there is any special biological significance of the occurrence of selenomethionine in the enzymes is unknown at present.

Enzymes that contain selenocysteine

Four of the selenoproteins mentioned above, e.g. glycine reductase, formate dehydrogenase, hydrogenase and glutathione peroxidase, catalyze coupled axidation-reduction reactions and can be considered as typical redox catalysts. These have all been shown to contain selenium exclusively in the form of selenocysteine residues, and in each case the selenocysteine appears to serve as a catalytically active redox center. The selenoprotein A component of the clostridial glycine reductase complex was the first protein in which the selenium was identified as selenocysteine [2o]. This small (12,ooo dalton) selenoprotein is acidic, heat stable and contains glycosyl

groups. In addition to the one selenocysteine residue there are also two cysteine residues within the polypeptide chain [21]. All three residues are reduced by the dithiol that is employed as electron donor for the in vitro reaction. Both the selenol and the two thiol groups are rapidly oxidized upon exposure to air. Reduction of the selenoprotein at pH 8 with borohydride generates a chromophore with an electronic absorption maximum at 238 nm that is equivalent to the contribution expected of one ionized selenol group. This chromophore is destroyed when oxygen is added and the original spectrum of the protein is regained. Reaction of the reduced ^{75}Se-labeled protein with a variety of alkylating agents and isolation of the corresponding alkylated ^{75}Se-labeled amino acid after acid hydrolysis or enzymic digestion was used to identify the selenium-containing moiety as selenocysteine. The alkyl derivatives isolated from the protein proved to be indistinguishable by a variety of chromatographic and electrophoretic procedures from the corresponding alkyl derivatives of authentic selenocysteine. A number of other derivatization procedures commonly used for identification of small amounts of naturally occurring compounds were rendered impractical because of the marked lability of selenocysteine. The reaction in which the selenoprotein participates as a catalyst is shown in equation 1. In this overall reaction, glycine is reductively deaminated to acetate and ammonia concomitant with the esterification of orthophosphate and the synthesis of ATP. The enzyme complex that catalyzes this reaction consists of the selenoprotein, a 200,000 dalton carbonyl group protein and a third 250,000 dalton protein [22]. When these three purified proteins are added to a reaction mixture, the

active enzyme complex is reconstituted, and the reaction is catalyzed.



$$(NH_2)CH_2COOH + R(SH)_2 + HPO_4^{2-} + ADP \longrightarrow CH_3COOH + NH_4^{+} + R\begin{matrix}S\\|\\S\end{matrix} + ATP \quad \text{(eq.1)}$$

The identity of the enzyme bound phosphate ester intermediate and the precise role of the selenoprotein component in the reaction mechanism are particularly interesting aspects of this problem.

The two other bacterial selenoenzymes that are known to contain selenocysteine are certain formate dehydrogenases [23,24] and a hydrogenase [7] from a methane-producing organism, M. vannielii. The selenium-containing formate dehydrogenases are enzyme complexes made up of subunits that contain iron-sulfur centers and molybdenum in the form of a molybdopterin cofactor together with selenoprotein subunits. A formate dehydrogenase that is synthesized by E. coli when the organism is grown anaerobically with nitrate as electron acceptor also contains cytochrome b subunits [2]. In this enzyme that couples to nitrate reductase, the selenium occurs in a 11o,ooo dalton selenoprotein subunit. A different selenoprotein subunit (8o,ooo daltons) is present in the formate dehydrogenase that is elaborated when E. coli is cultured anaerobically in the absence of nitrate [25]. Under these conditions, reducing equivalents generated from formate by the dehydrogenase are transferred to a hydrogenase and molecular hydrogen is evolved. The selenium-containing formate dehydrogenase of M. vannielli consists of a selenoprotein subunit of about 11o,ooo daltons (selenium present as selenocysteine) and another type of sub-

unit (1o5,ooo daltons) that contains one molybdopterin cofactor and ten iron-sulfur centers [6]. Certain clostridial formate dehydrogenases contain selenium as selenocysteine residues and, in some cases, tungsten replaces molybdenum as the metal constituent of the molybdopterin cofactor [24].

The only hydrogenase presently known to contain selenium was isolated from M. vannielli by Yamazaki [7]. This enzyme (34o,ooo daltons) contains four gram atoms of selenium in the form of selenocysteine residues that are located in four 42,ooo dalton subunits. Two other types of subunits, 35,ooo and 27,ooo, are present in the enzyme. Other redox centers of this enzyme are two gram atoms of nickel, 18-2o gram atoms of iron as iron-sulfur centers, and two equivalents of flavine adenine dinucleotide [26].

In M. vannielii the formate dehydrogenase and the hydrogenase are relatively non-specific as regards electron acceptor and can reduce flavins and several dyes in addition to the naturally occurring 8-hydroxy-5-deazaflavin cofactor which is abundant in the microorganism. However, the deazaflavin, an obligatory two electron acceptor, serves as a specific substrate for a third enzyme, an 8-hydroxy-5-deazaflavin dependent $NADP^+$ reductase [27,28]. This reductase utilizes the reduced deazaflavin as substrate for generation of NADPH from $NADP^+$. Since M. vannielii is limited to the use of formate or molecular hydrogen as its major oxidizable substrate for growth and selenium occurs in the two prominent enzymes that metabolize these substances, a nutritional requirement for selenium is readily demonstrable [29].

Glutathione peroxidase which was isolated in pure form, crystallized, and extensively characterized [3o-32] prior

to its identification as a selenoprotein [11,12], catalyzes the reduction of organic peroxides and H_2O_2 using reduced glutathione (GSH) as electron donor (equations 2 and 3).

$$2\ GSH + ROOH \longrightarrow GSSG + ROH + H_2O \quad (eq.2)$$

$$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O \quad (eq.3)$$

This selenoprotein (76,000 to 84,000 daltons), consisting of four identical subunits of 19,000 to 21,000 daltons, is widely distributed in mammalian and avian tissues but has not been reported to occur in microorganisms. Each subunit of the enzyme contains one selenocysteine residue and four cysteine residues. The selenocysteine residue is in the reduced or selenol form after reaction of the enzyme with its reduced substrate, GSH. The precise oxidation state of the selenium after reaction with a peroxide substrate has not been determined.

The complete amino acid sequence of bovine glutathione peroxidase was reported recently from the laboratory of Flohe [33]. A portion of this sequence near the amino terminus of the polypeptide chain which contains the selenocysteine residue is shown in Figure 1. There is good agreement in this portion of the molecule with the partial sequence of the rat enzyme reported by Tappel's laboratory [34]. It is interesting that x-ray crystallographic analysis of the bovine enzyme [35] detected only one of the four cysteine residues present in each subunit (Figure 1).

GLUTATHIONE PEROXIDASE

Enzyme is made up of 4 identical subunits. Subunit M.W. is 21,9oo by amino acid sequence analysis. About 198 residues per subunit.

Composition of enzyme from bovine erythrocytes (Flohe et al.)

```
  3o                                              4o
-Ser-Leu-Arg-Gly-Lys-Val-Leu-Leu-Ile-Glu-Asn-Val-Ala-Ser-

       45                    5o
Leu-SeCys-Gly-Thr-Thr-Val-Arg-
```

The 4 cysteine residues in the subunit are present at:

Residue 74
Residue 91 (identified as His by X-ray)
Residue 111 (identified as Met by X-ray)
Residue 152 (identified as Gln by X-ray)

Figure 1

A comparison of the structures of the four known selenocysteine containing enzymes is shown in Table 1. In each of these enzymes the selenol group apparently serves as a redox center. In at least two of the enzymes, the selenoprotein component of glycine reductase and glutathione peroxidase, cysteine residues in addition to selenocysteine are present in the same polypeptide chain. Thus, it is apparent that selenocysteine occurs as a highly specific component of these enzymes but the mechanism of its incorporation remains to be established.

TABLE 1 Selenium-dependent redox enzymes that contain selenocysteine

1. Glycine Reductase Complex (M_r ~ 45o,ooo)
   Selenoprotein component — ~12,ooo M_r glycoprotein
   Contains 1 selenocysteine and 2 cysteine residues
   Two other protein components —— 2oo,ooo and 25o,ooo M_r

2. Formate Dehydrogenases (M_r 3oo,ooo - 6oo,ooo)
   Methanococcus vannielii enzyme:
   Selenoprotein component — ~11o,ooo M_r
   Se present as selenocysteine
   Molybdopterin - FeS protein component —— 1o5,ooo M_r
   Contains 1 Mo and 1o FeS / 1o5,ooo
   Two types of subunits: 6o,ooo and 33,ooo M_r

3. Hydrogenase (M_r 34o,ooo) $\alpha_2\beta_4\gamma_2$ subunit composition
   β-subunit —— 42,ooo M_r —— contains selenocysteine
   α-subunit —— 57,ooo M_r is dimer of 27,ooo
   γ-subunit —— 35,ooo M_r
   Native protein contains per mol
   4 Se, 2 Ni, 18-2o Fe and 2 FAD

4. Glutathione peroxidase (M_r ~ 8o,ooo: 4 gm atoms Se/mol)
   4 selenoprotein subunits —— 2o,ooo M_r
   1 selenocysteine and 4 cysteine residues/subunit

Selenoenzymes that contain an unidentified selenium moiety

In contrast to the enzymes that contain selenium in the form of selenocysteine residues there are two bacterial selenoenzymes in which selenium occurs as a readily dissociable component. These are nicotinic acid hydroxylase [9] and xanthine dehydrogenase [10]. The first step in the overall fermentation of nicotinic acid to ammonia, propionate, acetate and carbon dioxide by C. barkeri is the hydroxylation of the substrate forming 6-oxonicotinic acid [36,37]. The enzyme (about 300,000 daltons) that catalyzes the anaerobic hydroxylation reaction contains flavine adenine dinucleotide, molybdenum as molybdopterin cofactor, numerous iron sulfide centers and selenium in the form of a labile, readily dissociable component [9,38]. The hydroxylase is extremely sensitive to alkylating agents and is immediately inactivated upon addition of an alkylating agent to the reaction mixture. When enzyme labeled with ^{75}Se is inactivated by alkylation, all of the ^{75}Se is found in a compound that is easily separated from the protein and has the properties of a dialkyl selenide. Thus, reaction of [^{75}Se]enzyme with acidic, neutral or basic alkylating agents converts the ^{75}Se to the predicted dialkyl [^{75}Se]selenide. A typical example is shown in equation 4.

$$[^{75}Se]\text{enzyme} + 2\ ICH_2COOH \longrightarrow {}^{75}Se(CH_2COOH)_2 + 2\ I^- \quad \text{(eq. 4)}$$

No selenocysteine or selenomethionine has been detected in the nicotinic acid hydroxylase. When the native active enzyme is treated with guanidine hydrochloride or

urea under strictly anaerobic conditions, both the selenium and the intact molybdopterin cofactor are dissociated quantitatively from the protein. Based on observations that the selenium and the molybdopterin cofactor then comigrate during molecular sieve chromatography, it has been suggested by Dilworth [39] that the selenium might be present in the cofactor as an external ligand to the molybdenum. For example, in the nicotinic acid hydroxylase cofactor selenium might occur in place of the "cyanide labile sulfur atom" present in the molybdopterin cofactor of xanthine oxidase [40]. In any event it is clear that the selenium in nicotinic acid hydroxylase is readily alkylable and in the presence of excess alkylating agent is eliminated as a dialkyl selenide.

Selenium-containing amino acid transfer ribonucleic acids (tRNAs)

Recent studies in our laboratory have dealt extensively with another type of naturally occurring macromolecule that contains selenium. Amino acid transfer ribonucleic acids (tRNAs) are a class of nucleic acids that deliver activated amino acids (in ester linkage to an adenosine residue at the 3'-terminus of the polynucleotide chain) to the site of protein synthesis. Instead, of only 20 different species of these molecules, one for each of the amino acids commonly found in proteins, there are actually more than 50. This means that for some of the amino acids there may be 3 or 4 isoacceptor tRNA species. These differ mainly in the presence of one or more modified bases which apparently confer additional specificity as regards regulatory roles. For example, a modified base occurring in the first position of the anticodon region can regulate base pairing interactions

necessary for codon interaction. Additionally, in many bacterial tRNAs the 8th nucleoside from the 5'-end of the molecule is a sulfur modified uridine, 4-thiouridine. The specific function of this thionucleoside is unknown.

Selenium occurs as a specific component of a few tRNA species in E. coli and some anaerobic bacteria that we have studied [41-44]. The amount of selenium in the tRNA population is largely independent of the S to Se ratio in the culture media in which the bacteria are grown and thus its incorporation is highly specific. The most abundant source of seleno-tRNAs (15-20% of the total population are modified) that we have found is the methane-producing organism that contains the two prominent selenoenzymes discussed above [44]. Whether this is more than mere coincidence is not known.

The marked affinity of selenium compounds for mercury was exploited in many experiments to achieve enrichment of the selenium-containing tRNA species present in the total tRNA populations [43]. For this purpose organomercury affinity chromatographic procedures were employed. Purification of individual seleno-tRNA species was accomplished by reversed phase high pressure liquid chromatography. Enzymic digestion of the total ^{75}Se-labeled tRNA population from E. coli and HPLC analysis of the resulting nucleoside mixture showed the presence of a single radioactive nucleoside [45]. From the electronic absorption properties of this isolated nucleoside together with its elution position just after a known sulfur modified nucleoside (5-methylaminomethyl-2-thiouridine) present in E. coli, it was surmised that the selenonucleoside might be the corresponding 2-selenouridine. Accordingly, authentic 5-methylaminomethyl-2-selenouridine was synthesized by Dr. Lin Tsai [45] using a

procedure analogous to that employed previously for the preparation of the sulfur nucleoside [46]. A starting material for preparation of the selenouridine was the light sensitive and unstable selenourea. The authentic 5-methylaminomethyl-2-selenouridine and the ^{75}Se-labeled nucleoside isolated from the tRNAs were indistinguishable chromatographically and spectrally and gave rise to identical acid decomposition products [45]. Thus, it is quite certain that the natural compound is indeed 5-methylaminomethyl-2-selenouridine. Two anaerobic bacteria, C. sticklandii [45] and M. vannielli [44], contain the same selenonucleoside in their tRNAs. Additional, chromatographically distinct, 2-selenouridines also occur in the tRNAs of these microorganisms [44,45].

A pure glutamate specific seleno-tRNA (seleno-$tRNA^{glu}$) that was isolated from C. sticklandii [43] was digested to the nucleoside level and analyzed by HPLC [45,47]. A single radioactive nucleoside was detected in the mixture and this was shown to be identical with 5-methylaminomethyl-2-selenouridine. The amount of this seleno-nucleoside present is equivalent to one mol per mol of the $tRNA^{Glu}$. Partial sequence analysis of the pure seleno-$tRNA^{Glu}$ carried out by Dr. Wei-Mei Ching [48] showed that the 2-selenonucleoside is located in the first position of the anticodon region. The analogous sulfur modified nucleoside, 5-methylaminomethyl-2-thio-uridine, occupies the same position in a glutamate tRNA species from E. coli. Extensive homology between the structures of these two glutamate specific tRNAs was demonstrated. The biological effects of sulfur and selenium substitutions for oxygen in the 2-position of the uridine in the anticodon of the tRNAs would be expected to progressively weaken hydrogen

bonding to the cognate base in the glutamate codon and thus achieve some type of regulatory effect.

The seleno-tRNA^{Glu} from C. sticklandii contains one equivalent per mol of the common sulfur modified nucleoside, 4-thiouridine, in addition to the 2-selenouridine in the anticodon [45]. Although a purified enzyme system from E. coli can modify a specific uridine residue of tRNA at the 4-position with selenium instead of sulfur if cysteine is replaced with an equivalent concentration of selenocysteine as donor [49,50], we have never detected 4-selenouridine in any of the numerous bacterial tRNA populations examined. Presumably the in vivo concentration of selenocysteine required for this substitution to be achieved would be toxic and thus is never reached. In contrast, the 2-selenouridine derivatives found in the tRNAs are synthesized when the available selenium is orders of magnitude lower in concentration than sulfur.

Initial observations that there seemed to be a correlation between the spontaneous loss of selenium from pure seleno-tRNA^{Glu} and a decrease in the ability of the tRNA to be acylated with its cognate amino acid [43], led to the discovery that the selenonucleoside in the anticodon portion of the molecule is essential for the enzymic esterification of the 3'-adenosine moiety of the tRNA with glutamate. Deliberate removal of selenium by treatment with base or with cyanogen bromide completely destroys the biological activity of the tRNA as a glutamate acceptor [47]. When the intact seleno-tRNA^{Glu} from C. sticklandii is esterified with glutamate and added to an in vitro protein synthesis system derived from wheat germ, it serves as an effective glutamate donor and thus exhibits the expected biological activity [47].

In addition to questions concerning the more precise roles or regulatory functions of the selenium modified tRNAs, present studies have as their aim the elucidation of the mechanism whereby selenium is specifically introduced into a precursor tRNA molecule. As yet, the chemical nature of the immediate selenium donor is unknown, although in the case of the anaerobic bacteria, selenide formed by reduction of added selenite is available. In E. coli where sulfur modified and selenium modified isoaccepting tRNA species coexist [41], the mechanism by which the normal ratio of these species is maintained is of particular interest.

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