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SELENIUM METABOLISM AND SELENIUM-DEPENDENT ENZYMES IN MICROORGANISMS[1]

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SELENIUM SUBSTITUTION FOR SULFUR

Selenium (Se) was recognized as a potent toxic element for many forms of life long before its role as an essential nutrient was discovered. Selenium toxicity has been assumed to be at least partially due to replacement of chemically similar sulfur (S) atoms in biomolecules with selenium, thus altering the functions of these biomolecules. For example, when levels of selenomethionine are elevated in response to high selenium concentrations, selenomethionine is incorporated into nascent proteins in place of methionine, a change that could alter the structure and reactivity of these proteins. These effects would be additive, and the greater the percentage of selenomethionine residues in a protein the greater the total effect. Incorporation of one or a few selenomethionine residues in a polypeptide molecule may be accommodated, whereas more may not.

As would be expected, bacteria can develop resistance to high levels of selenium. Selenium-resistant bacteria have been isolated from polluted areas containing high levels of selenium (1, 2). Bacteria and fungi that take up and accumulate high levels of selenium in organic form have been isolated. A number of these species also are able to convert inorganic forms of selenium to volatile compounds such as dimethylselenide. Such organisms are proposed to be useful in reducing selenium levels at polluted sites.

Increasing the selenomethionine content of proteins is possible in vivo. A strain of *Pseudomonas aeruginosa* has been isolated that requires methionine for growth (6). These bacteria were cultured in a medium containing selenomethionine in place of methionine, and the enzyme azurin was subsequently purified. The report claims that five of the six methionine residues in each azurin polypeptide were replaced by selenomethionine.

The labile sulfide of several bacterial proteins has been replaced with selenium in vitro (17, 19, 30, 32). The iron-sulfur clusters of redox-active proteins can be extruded and reformed as iron-selenium clusters in the enzymes. These derivatized proteins retain biologic activity, although they may be less stable than native forms. Alterations in redox activity and spectroscopic properties of these selenium proteins can be detected.

A synthetic method has been developed for the preparation of one of the metallothioneins, a class of low–molecular weight, cysteine-rich proteins that bind metal ions such as Cu, Cd, Zn, and Hg (29). In this case the copper metallothionein of *Neurospora crassa,* a 25–amino acid polypeptide that contains seven cysteinyl residues and binds six copper ions, was synthesized by the Merrifield method. The same procedure was used to prepare a polypeptide in which every cysteine (Cys) residue was replaced by selenocysteine (Secys). Studies of the metal binding and spectroscopic properties of these proteins are in progress.

Two of the 20 primary amino acids of proteins contain sulfur: methionine and cysteine. If selenium nonspecifically replaces sulfur during biosynthesis of these amino acids, one would expect to see increases in the selenium analogs of these amino acids when selenium is increased relative to sulfur. In other words, the fraction of the sulfur amino acids replaced by the selenium analogs would be proportional to the ratio of selenium versus sulfur available to the organism. In many instances this proportional representation has been shown to be the case for selenomethionine; the levels of this amino acid in proteins can vary according to the molar ratio of Se/S presented to the organism. A well-known example is the "selenized yeast" sold as selenium supplements in health food stores. For this product, baker's yeast is grown in a low-sulfur medium supplemented with selenite. The major form of selenium in such yeast is selenomethionine. Selenomethionine residues were shown to be randomly distributed in place of methionine throughout the polypeptide

chains of a thiolase isolated from *Clostridium kluyveri* (26). At present the highly specific incorporation of selenomethionine into a protein has not been reported.

In contrast, incorporation of selenocysteine residues into proteins appears to be highly specific over a considerable range of Se and S concentrations. Several proteins from different organisms are known that necessarily contain selenocysteine. The selenocysteine residues are found at specific sites in the polypeptides, and the proteins are not synthesized in the absence of selenium. A recently documented exception is a *Salmonella typhimurium* mutant defective in ability to synthesize selenium-dependent formate dehydrogenases and selenated tRNAs. In this abnormal strain, selenocysteine is synthesized and incorporated into numerous proteins of the cell, presumably replacing cysteine (12; T. C. Stadtman, unpublished information). This apparently unusual case may be the result of a metabolic imbalance brought about by the missing enzyme. Although the thiolase of *C. kluyveri* contains several cysteine residues in addition to being rich in methionine, no selenocysteine substitution for cysteine could be detected in numerous enzyme preparations that were examined. In this apparently normal situation, only selenomethionine substitution was allowed (26).

The reason cells have evolved to treat selenium differently with respect to cysteine and methionine is not completely clear. Selenium may be tolerated as the selenoether in selenomethionine as it is fairly stable in the reducing environment of the cell. Since the sulfur of methionine is not known to play a catalytically active role in enzymes, replacement by selenomethionine may affect a protein structurally but not catalytically. The thiol of cysteine, on the other hand, is a key reactant in many enzyme mechanisms, particularly in redox-type reactions. Substitution of a selenol with its lower pK_a and differing redox chemistry for cysteine would thus greatly alter the catalytic properties of the enzyme in question.

SELENIUM-DEPENDENT ENZYMES IN MICROORGANISMS

The selenium-dependent enzymes found to date in microorganisms are glycine reductase of *Clostridia,* several formate dehydrogenases, certain hydrogenases, a clostridial nicotinic acid hydroxylase, and xanthine dehydrogenase present in purine-fermenting *Clostridia* (28). These enzymes are all expressed in strict anaerobes such as *Clostridia* or methane-producing bacteria or under anaerobic conditions in facultative anaerobes such as *Escherichia coli* and *Salmonella typhimurium*. The selenium requirements for growth of a freshwater dinoflagellate (15), a bloom-forming planktonic algae from lakes (31), and a coastal marine diatom, *Thalassiosira pseudonana* (23), suggest a

much wider distribution of selenoenzymes among microorganisms than was earlier suspected.

Selenium is present in the chemical form of selenocysteine in glycine reductase, several formate dehydrogenases, and at least one hydrogenase (28). Nicotinic acid hydroxylase differs in that selenocysteine is not found in this enzyme; instead selenium occurs in a component that is readily dissociated from the protein. The chemical properties of derivatives of this component so far determined are not sufficient to identify its original structure (3).

Glycine Reductase Complex

Selenoprotein A, one of the protein components of clostridial glycine reductase, is a heat-stable, acidic glycoprotein of 12,000 molecular weight that contains one selenocysteine and two cysteine residues. All three of these residues react rapidly with oxygen. The oxidized protein, detected frequently in the form of a dimer, can be converted to the fully active reduced form by treatment with dithiothreitol or borohydride. The partial amino acid sequence of selenoprotein A (27) is as follows: blocked amino terminus-(Met,Ala,Ala,Leu,Asn,Gln)-Cys-Phe-Val-Secys-Thr-Ala-Ala-Gly-Ala-Met-Asp-Leu-Glu-Asn-Gln-Lys/. In vitro studies to determine the mechanism of insertion of selenocysteine in this polypeptide await the availability of the gene and its mRNA product that encodes selenoprotein A. Attempts to isolate the gene are in progress.

The two other larger molecular weight components of the glycine reductase complex are protein B, approximately 240,000, which contains an essential carbonyl group, and protein C, approximately 200,000, a sulfhydryl protein. The reductase, reconstituted from the three purified proteins, catalyzes the reductive deamination of glycine to acetate and ammonia with the concomitant esterification of one equivalent of orthophosphate, which reacts with ADP to form one ATP. In the in vitro system, a dithiol such as dithiothreitol can serve as the electron donor substrate. This in turn reduces the selenoprotein. The phosphate ester intermediate generated in the reaction is believed to be a derivative of one of the proteins of the enzyme complex.

Selenium-Dependent Formate Dehydrogenases

The first indication that selenium is involved in formate metabolism came from the report of Pinsent in 1954 (22) that selenium and molybdenum supplements were required by *E. coli* for expression of formate dehydrogenase activity. Later workers demonstrated association of radioactive selenium with these enzymes (25), and Enoch & Lester in 1975 succeeded in purifying the formate dehydrogenase that links to nitrate reductase (4). This large molecular weight enzyme complex (approximately 600,000) of $\alpha_4\beta_4\gamma_{(2}$

or 4) structure contains 4 gram atoms of selenium present in the four 110,000 α-subunits. Other components are 4 gram atoms of Mo, 56 nonheme Fe, 52 acid-labile sulfide, and 4 B-type heme groups. This enzyme is expressed when *E. coli* is grown anaerobically with nitrate as electron acceptor. Under anaerobic conditions in the absence of nitrate, another formate dehydrogenase that is linked to a hydrogenase (formate hydrogen lyase) is expressed (9). This enzyme, molecular weight approximately 120,000, contains an 80,000-selenopolypeptide subunit, an additional smaller subunit, FeS centers, and molybdenum (Table 1). The 110,000 selenopolypeptide of the nitrate reductase–linked enzyme and the 80,000 selenopolypeptide of the hydrogenase-linked enzyme are products of two different genes (21). Detailed genetic and biochemical studies dealing with the mechanism of biosynthesis of the selenocysteine residue in the latter enzyme are described below. Although the nitrate reductase–linked enzyme has been characterized in some detail in terms of protein structure, it has not been subjected to genetic analysis. In contrast, there is considerable information at the molecular biology level concerning the hydrogenase-linked enzyme, but the protein has not yet been prepared in a homogeneous, catalytically active form. Formate dehydrogenases comparable to these *E. coli* enzymes also occur in *Salmonella* species.

The methane-producing organism *Methanococcus vannielii,* which grows on formate as the sole carbon and energy source, also produces two types of formate dehydrogenases (11). One of these, which enables the organism to survive under selenium-deficient conditions, is a 105,000–molecular weight protein that contains 1 Mo and 10 FeS groups but no selenium (Table 1). When adequate selenium is provided (0.5–1 μM) in the medium, a large formate dehydrogenase complex consisting of multiples of a selenocysteine-

Table 1 Formate dehydrogenases with similar subunit composition[a]

Methanobacterium formicicum enzyme; α_1, β_1; 177,000[b]
α subunit: 85,000 (76,000[c]), contains Cys instead of Secys
β subunit: 53,000 (44,000[c]), presumed location of FeS centers
Escherichia coli enzyme (H_2ase-linked); approximately 120,000[d]
α subunit: 80,000, contains selenocysteine
β subunit: 40,000–45,000, presumed location of FeS centers
Methanococcus vannielii enzyme; α_1, β_1; 105,000; non-Se form
α subunit[e]: 60,000 (may be analogous to *M. formicicum* α subunit)
β subunit[e]: 33,000, possible location FeS centers

[a] Molybdopterin cofactor location in these enzymes is unknown.
[b] From Ref. 24.
[c] Calculated from amino acid sequence data (Ref. 24).
[d] T. C. Stadtman, A. Böck, et al, unpublished information.
[e] Subunit composition determined by S. Yamazaki, unpublished information.

containing polypeptide (approximately 100,000) and the 105,000 molybdo-FeS polypeptide is present in the cells. A markedly increased rate of growth that is correlated with synthesis of the selenium-containing formate dehydrogenase implies that the large enzyme complex is a more effective catalyst than the Mo-FeS protein alone.

A formate dehydrogenase that contains FeS centers and a molybdopterin but no selenium (24) is present in high concentration (up to 2% of the protein of the cell) in another formate-utilizing methane organism, *Methanobacterium formicicum* (Table 1). This organism apparently does not produce a selenium-containing formate dehydrogenase. As deduced from gene-sequence data (24) the 76,000–molecular weight subunit of the *M. formicicum* enzyme contains a cysteine residue in the position corresponding to the selenocysteine residue in the 80,000–molecular weight selenoprotein subunit of the hydrogenase-linked *E. coli* enzyme. Considering that the latter enzyme is much less active catalytically when the selenocysteine residue is replaced by cysteine (see below), the high level of the nonselenium formate dehydrogenase in *M. formicicum* may be necessary to compensate for its relatively lower activity as a catalyst.

A selenium-dependent formate dehydrogenase of *Clostridium thermoaceticum* (33) is a 340,000–molecular weight protein of $\alpha_2\beta_2$ structure that contains two gram atoms of selenium, two tungsten, and 36 or more FeS centers. In this enzyme molybdenum is replaced, apparently normally, by tungsten. Unlike several of the other selenium-dependent formate dehydrogenases, it uses $NADP^+$ as electron acceptor.

A common property of all of the formate dehydrogenases mentioned above is their marked sensitivity to molecular oxygen.

Selenium-Dependent Hydrogenases

A hydrogenase isolated from *M. vannielii* was the first enzyme of this type to be shown to contain selenium (34). The selenium is present as selenocysteine residues in the protein. Although other selenium-containing hydrogenases are now known, the selenium moiety in these proteins has not been isolated and identified as selenocysteine. However, the TGA codon has been reported in the open reading frame of the gene encoding the hydrogenase of *Desulfovibrio baculatus* (16). The *M. vannielii* hydrogenase (34, 35) is a 340,000–molecular weight enzyme of $\alpha_2\beta_4\gamma_2$ structure that contains 3.8 gram atoms of selenium as selenocysteine residues in the four β-subunits (42,000). Two 56,000 α-subunits (actually dimers of 27,000) and two 35,000 γ-subunits also are present in the complex. Two gram atoms of nickel, 18–20 FeS groups, and two equivalents of FAD are also bound per mole of enzyme. Similar subunit and cofactor compositions have been reported for several other hydrogenases. For example, a hydrogenase purified from *Methanococcus voltae*

with subunits of 45,000, 37,000, and 27,000 was reported to contain selenium, nickel, iron, and FAD (20). The hydrogenase purified from *M. thermoautotrophicum* ΔH is composed of three subunits of similar sizes: α, 47,000, β, 31,000, and γ, 26,000 (5). Nickle, iron-sulfur centers, and FAD are present in the enzyme. An 8-hydroxy-5-deazaflavin cofactor that is abundant in methane bacteria is the natural electron acceptor for all of these hydrogenases. In *M. vannielii,* the reduced deazaflavin is then used as a substrate by an $NADP^+$-dependent reductase for the generation of NADPH (10, 36). Stereochemical studies on the course of the coupled oxido-reduction reactions showed that both the hydrogenase and the $NADP^+$ reductase recognize the same face of the 8-hydroxy-5-deazaflavin ring system (35, 36).

Although the hydrogenases from anaerobic and facultative bacteria generally are oxygen sensitive, the inactivation due to oxygen exposure usually is reversible. For example, when the hydrogenase from *M. vannielii* is isolated under aerobic conditions, the resulting inactive form of the enzyme can be converted subsequently to the catalytically active form by incubation at an appropriate ionic strength with dithiothreitol and molecular hydrogen (34). This is an important advantage when large amounts of pure enzyme are needed for spectroscopic studies on the precise catalytic roles of nickel and iron sulfur centers in these hydrogenases.

Preparations of the *M. thermoautotrophicum* ΔH and the *M. voltae* hydrogenases still retain the ability to reduce methyl viologen after inhibition of deazaflavin-reducing capacity by heat treatment or by titration with antibodies, which indicates that the dye and the natural cofactor react at spacially distinct sites on the enzymes (5, 20). By analogy with other iron-sulfur proteins, the viologen dye probably interacts at an iron-sulfur center on the enzymes. Although there is evidence from electron paramagnetic resonance studies that the nickel in these enzymes is redox active, the precise role of this metal in these nickel-containing hydrogenases has not been determined. Studies to elucidate the roles of the various potential redox centers of these enzymes are in progress in several laboratories.

MECHANISM OF SELENOCYSTEINE INCORPORATION INTO PROTEINS

Until recently, no definitive evidence distinguished between a cotranslational and a posttranslational mechanism of the specific insertion of selenocysteine into proteins. Now, evidence shows that although the selenocysteine residue is incorporated into the growing polypeptide chain, free selenocysteine itself is not esterified directly to a tRNA but rather is synthesized on the tRNA from a serine residue that undergoes O activation and then a selenium-replacement reaction. Much of the work toward solving this puzzle has been carried out by

A. Böck and co-workers in Munich (references below) using the hydrogenase-linked formate dehydrogenase of *E. coli*. The selenopolypeptide of this enzyme, as pointed out above, is an 80,000-dalton protein that contains a single selenocysteine residue essential for activity of the enzyme.

The DNA sequence of the gene encoding the selenopolypeptide surprisingly revealed the presence of a TGA codon in the open reading frame corresponding to a UGA opal termination codon in the mRNA (38). Although the position of the selenocysteine residue in the protein has not yet been determined, a number of lines of evidence show that it indeed is located at position 140, as deduced from the DNA sequence, and that its incorporation occurs at the ribosome level (37). When *N*-terminal portions of the gene were fused to the β-galactosidase gene, expression of β-galactosidase activity depended on the presence of selenium in the medium when the fusion point was located 3' to the TGA codon but was independent of selenium when the fusion point was located upstream of the TGA. Conversion of TGA to cysteine codons, TGC or TGU, or to TCA, serine codon, also allowed β-galactosidase synthesis in the absence of selenium. In a T7 expression system, short polypeptides corresponding in length to the distance between the *N*-terminus and the UGA codon at position 140 were formed in the absence of selenium, but complete read-through occurred when UGA was replaced with UGC, UGU, or UCA.

The effect of substitution of selenocysteine with cysteine in the 80,000-dalton subunit of the formate dehydrogenase on catalytic activity of the enzyme has been estimated in partially purified preparations using antibody titrations as reference (W. Leinfelder, A. Böck, M. J. Axley, & T. C. Stadtman, unpublished information). These preliminary studies indicate that the selenocysteine-containing enzyme is four to five times more active than the sulfur-substituted species. When the selenocysteine is substituted by serine, the enzyme in inactive.

Several *E. coli* mutants have been isolated that are unable to insert selenocysteine into formate dehydrogenases (13). These were isolated by monitoring their inability to utilize formate under anaerobic conditions. In these strains, excretion of formate caused a detectable acidification of the culture medium. The various mutants were grouped into four classes according to genetic complementation, and the mutant genetic loci were designated *selA, selB, selC,* and *selD*. Whereas none of the mutants inserts selenocysteine into the formate dehydrogenases, only the *selD* lesion additionally abolishes normal selenium incorporation into tRNAs. A mutant of *Salmonella typhimurium* isolated by Kramer & Ames and referred to as *selA1* (12) is identical phenotypically with the *E. coli selD* mutant. The genes corresponding to the four selenium metabolism mutants of *E. coli* have been cloned and isolated (13, 14; A. Böck, personal communication). Polypeptides are en-

coded by the genes for *selA*, *selB*, and *selD*, with respective molecular weights of 50,000, 70,000, and 37,000. At present the functions of these gene products are unknown. The gene for *selD*, when inserted into the *Salmonella selA1* mutant, complements the mutation, which indicates that the lesion in this case is also the inability to synthesize a 37,000–molecular weight protein (A. Böck et al, personal communication).

The *selC* gene has also been cloned and isolated. Sequencing revealed that the gene codes for an unusual tRNA (14). The anticodon of this tRNA, UCA, recognizes the UGA codon, and the tRNA is charged with L-serine. In addition to the anticodon complementary to UGA, the tRNA differs markedly from other *E. coli* tRNAs in size and nucleotide sequence. The recent demonstration that this tRNA occurs in the cell as the selenocysteyl-esterified form establishes its role as the direct precursor of selenocysteine in the formate dehydrogenase selenopolypeptide (W. Leinfelder, A. Böck, & T. C. Stadtman et al, unpublished information).

Although the reactions involving the conversion of L-serine esterified to the tRNA to the selenocysteyl derivative have not been identified in *E. coli*, the process may be analogous to that in eukaryotes. As shown by Hatfield & co-workers (7, 8), an L-serine accepting tRNA complementary to UGA (anticodon UCA) is converted to an O-phosphoseryl-tRNA by reaction with a specific kinase (18). This conversion then sets the stage for a nucleophilic attack by a selenol group on the serine beta carbon, which displaces the phosphate group and results in the formation of selenocysteyl-tRNA. That this process actually occurs has been demonstrated recently by isolating all three of the esterified forms of the $tRNA^{Ser}_{UCA}$ from a rat mammary tumor cell line and identification of each of the three appropriately labeled amino acids after deacylation (B.-J. Lee, T. C. Stadtman, & D. Hatfield, unpublished information). The nature of the selenol donor remains to be identified in both the eukaryotic and prokaryotic systems.

The uncharacterized proteins encoded by *selA*, *selB*, and *selD* are also known to be involved in selenocysteine incorporation into polypeptide, and one or more of these could be responsible for catalysis of some of the steps leading to synthesis of selenocysteyl-tRNA in *E. coli*.

The UGA codon generally signals translational termination. As selenocysteine residues are not inserted at most UGA codons, mRNAs that encode selenocysteine-containing polypeptides must contain further contextual information to override translational termination and allow selenocysteine insertion. Where selenocysteine is inserted, the UGA codon should not be considered a termination codon, as it codes for incorporation of an amino acid into polypeptide. The tRNA involved in selenocysteine incorporation should not be considered a termination-suppressor tRNA, as it functions only in the context of specific sequences.

This new information raises many intriguing questions. It might be proper to consider selenocysteine the 21st natural amino acid, as it is a necessary component of several known proteins and is cotranslationally inserted by a distinct tRNA. As this amino acid is found in a variety of organisms, there must be an evolutionary requirement for it that is not satisfied by cysteine. One can also wonder whether the primordial use for the UGA codon was as termination codon or selenocysteine codon, or whether the two uses evolved independently.

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