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Biochemistry

Cotranslational insertion of selenocysteine into formate dehydrogenase from *Escherichia coli* directed by a UGA codon

(UGA suppression/localized mutagenesis)

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ABSTRACT **The structural gene (*fdhF*) for the 80-kDa selenopolypeptide of formate dehydrogenase (formate:benzyl viologen oxidoreductase, EC 1.2.—.—) from *Escherichia coli* contains an in-frame UGA codon at amino acid position 140 that is translated. Translation of gene fusions between N-terminal parts of *fdhF* with *lacZ* depends on the availability of selenium in the medium when the hybrid gene contains the UGA codon; it is independent of the presence of selenium when an *fdhF* portion upstream of the UGA position is fused to *lacZ*. Transcription does not require the presence of selenium in either case. By localized mutagenesis, the UGA codon was converted into serine (UCA) and cysteine (UGC and UGU) codons. Each mutation relieved the selenium dependency of *fdhF* mRNA translation. Selenium incorporation was completely abolished in the case of the UCA insertion and was reduced to about 10% when the UGA was replaced by a cysteine codon. Insertion of UCA yielded an inactive *fdhF* gene product, while insertion of UGC and UGU resulted in polypeptides with lowered activities as components in the system formerly known as formate hydrogenlyase. Altogether the results indicate that the UGA codon at position 140 directs the cotranslational insertion of selenocysteine into the *fdhF* polypeptide chain.**

Escherichia coli can synthesize two selenocysteine-containing enzymes—namely, formate dehydrogenase N (FDH_N; formate:ferricytochrome B_1 oxidoreductase, EC 1.2.2.1), which interacts with the nitrate reductase complex, and formate dehydrogenase H (FDH_H; formate:benzyl viologen oxidoreductase, EC 1.2.—.—), which is a component of the system formerly called formate hydrogenlyase. The size of the selenopolypeptide of FDH_N is 110 kDa, while that of FDH_H is 80 kDa (1, 2).

To elucidate the pathway and the mechanism by which selenocysteine enters a polypeptide chain, we have cloned and sequenced the structural gene (*fdhF*) of the 80-kDa selenopolypeptide of FDH_H (2). It was found that *fdhF* contains an in-frame UGA codon at position 140. Experiments with gene fusions between *fdhF* and *lacZ* indicated (*i*) that selenocysteine incorporation may be directed by the opal codon, and (*ii*) that incorporation occurs cotranslationally. Direct proof that selenocysteine insertion in the *fdhF* gene product is coded by this UGA codon is not yet available. However, corroborating evidence that an opal stop codon may be used generally for directing selenocysteine incorporation came from the analysis of a mammalian enzyme: the structural gene for glutathione peroxidase from mouse contains a UGA codon (3), and this corresponds to the highly conserved position of the selenocysteine residue in the proteins of bovine (4) and rat (5) origin.

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Here we report on the localized mutagenesis of the UGA codon at position 140 of the *fdhF* gene. Variants were constructed with a serine (UCA) or with cysteine (UGC or UGU) codons in this position. The results demonstrate that selenocysteine is incorporated during the translation process at the position of the UGA codon.

MATERIALS AND METHODS

Strains, Plasmids, Media, and Growth Conditions. The *E. coli* strains and the plasmids used in this study are listed in Table 1. Unless indicated otherwise, bacteria were grown anaerobically in the buffered (pH 6.5) rich medium described by Begg *et al.* (14). The selenium-deficient minimal medium contained 150 mM KH_2PO_4, 15 mM NH_4Cl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 μM $FeCl_3$, 10 μM Na_2MoO_4, and 5 μM $NiCl_2$. The pH was adjusted to 6.5 with NaOH. L-Cysteine (80 μg/ml) was added as the sulfur source. Glucose was used as a carbon source at 0.8% for anaerobic and 0.4% for aerobic growth. Anaerobic culture techniques using N_2 as the gas phase were adopted from the procedure of Balch and Wolfe (15).

Genetic Techniques. Basic genetic techniques were as described by Miller (16). *recA* mutations were transferred either by conjugation with JC10240 (7) or by P1kc-mediated transduction with JC10289 (pKY102) as the donor (8). Recombinant DNA methods were as compiled by Maniatis *et al.* (17).

Site-Directed Mutagenesis. Site-directed mutagenesis of the UGA codon in position 140 of the *fdhF* gene was either performed with the "gapped-duplex" procedure of Kramer *et al.* (9) or with the single priming method of Zoller and Smith (18). The gapped-duplex procedure was used to convert UGA into UGC. The 590-base-pair (bp) *Kpn* I–*Sph* I fragment of the *fdhF* gene from plasmid pFM20 (Fig. 1) was cloned into vector pUC19. The flanking *Hin*dIII–*Eco*RI sites of this construct were used for subsequent subcloning of the fragment into phage M13 vector mp9 yielding M13 FM100. The gapped-duplex DNA was formed between single-stranded M13 FM100 DNA, double-stranded linearized M13mp19 DNA, and the oligonucleotide 5′-GTACCAATAACGTTG-ACTGCTGCGCTCGTGTCTGC*CACGGCCCATCGG-3′ (where the asterisk represents the site of mutation). It was used to transfect strain BMH71-18mutS, and the resulting mixed lysate was taken to infect strain MK30-3 (*sup*^{0}) with a low multiplicity of infection. Ten plaques were selected and screened for the presence of the base change by sequencing with the chain-termination method (19). Four of them showed the desired mutation. The nucleotide sequence of the whole *Kpn* I–*Sph* I fragment was then determined by the chemical-cleavage method (20) to confirm that no other change was

Abbreviations: FDH_H and FDH_N, formate dehydrogenases H and N.

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Table 1. Bacterial strains and plasmids used

	Relevant genotype or phenotype	Ref.
E. coli strains		
MC4100	Δ(*argF-lac*)U169	6
FM420	MC4100, *recA56*	2
FM911	MC4100, Δ*fdhF recA56*	2
WL31153	MC4100, Δ*fdhA* Δ(*srl-recA*)*306*::Tn*10*	This work
JC10240	Hfr, *srl-300*::Tn*10 recA56*	7
JC10289	Δ(*srl-recA*)*306*::Tn*10*	8
BMH71-18mutS	Δ(*pro-lac*) *supE mutS215*::Tn*10*/F' *lacI*^{q} ZΔM15 *proA*$^+$*B*$^+$	9
MK30-3	Δ(*pro-lac*) *recA*/F' *lacI*^{q} ZΔM15 *proA*$^+$*B*$^+$	9
FM1226	Δ(*pro-lac*) *supE* Δ*fdhF*/F' *lacI*^{q} ZΔM15 *proA*$^+$*B*$^+$	This work
Plasmids		
pACYC184	Cm^{R}Tc^R	10
pFM20	Cm^R *fdhF*$^+$ (TGA)*	2
pFM201	Cm^R *fdhF* (TGC)*	This work
pFM202	Cm^R *fdhF* (TGT)*	This work
pFM203	Cm^R *fdhF* (TCA)*	This work
pMC1403	Ap^R *lac'ZYA*	11
pBN2	Ap^R φ(*fdhF'-'lacZ*)*hyb39*	2
pFM52	Ap^R φ(*fdhF'-'lacZ*)*hyb268*	2
pFM54	Ap^R φ(*fdhF'-'lacZ*)*hyb420* (TGA)*	2
pFM56	Ap^R φ(*fdhF'-'lacZ*)*hyb421* (TGC)*	This work
pFM57	Ap^R φ(*fdhF'-'lacZ*)*hyb422* (TGT)*	This work
pFM58	Ap^R φ(*fdhF'-'lacZ*)*hyb423* (TCA)*	This work
pKY102	Ap^R *recA*$^+$	8
Phage		
M13mp9	Iam IIam *lac'IPOZ'α*	12
M13mp19	*lac'IPOZ'α*	13
M13 FM99	M13mp9 + 1.3 kb *Nsi* I–*Bgl* II fragment from *fdhF*	This work
M13 FM100	M13mp9 + 0.59 kb *Kpn* I–*Sph* I fragment from *fdhF*	This work

Ap^R, Cm^R, Tc^R, ampicillin, chloramphenicol, and tetracycline resistant; Hfr, male donor strain of *E. coli*; ; Iam and IIam, amber mutations in M13 genes I and II, respectively.
*Letters in parentheses refer to codon 140 of *fdhF* in different plasmids, respectively.

present. The fragment was then cloned back into plasmid pFM20.

Conversion of UGA into UCA and UGU codons was achieved by following the procedure of Zoller and Smith (18).

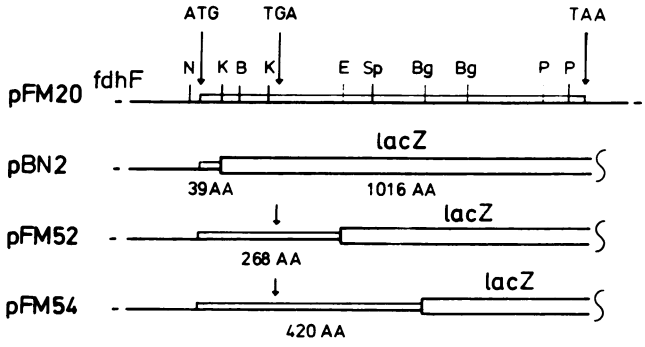

FIG. 1. Restriction map of *fdhF-lacZ* fusion plasmids. In-frame gene fusions on pBN2, pFM52, and pFM54 consist of different N-terminal parts of the *fdhF* gene fused to *lacZ* in these plasmids, respectively. For each fusion the number of amino acids (AA) of the *fdhF* moiety is indicated. Plasmid pFM20 contains the wild-type *fdhF* gene. The sites for restriction endonucleases are: B, *Bgl* I; Bg, *Bgl* II; E, *Eco*RV; K, *Kpn* I; N, *Nsi* I; P, *Pvu* I; Sp, *Sph* I.

The 1315-bp *Nsi* I–*Bgl* II fragment of the *fdhF* gene from plasmid pFM20 (Fig. 1) was cloned into M13mp9 to yield M13 FM99. Single-stranded DNA was prepared and hybridized to either one of the two mutagenic primers 5'-GGCCGTGT*G-AGACACGAGC-3' or 5'-GGCCGTGA*CAGACACGAGC-3'. Second-strand synthesis was carried out *in vitro* by using DNA polymerase (Klenow fragment) and phage T4 DNA ligase, and the DNA was transfected into strain FM1226. Presumptive "mutagenized" plaques were screened by hybridization with a radioactively labeled mutagenic primer; several positive plaques were picked and used to infect FM1226 again with a low multiplicity of infection. M13 clones were again identified by hybridization as described above. Replicative form DNA was prepared, and the 769-bp internal *Bgl* I–*Sph* I fragments were sequenced and cloned back into pFM20. Several clones were obtained showing the desired mutation; no other sequence change had occurred.

Blot-Hybridization Experiments. Strain FM911/pFM54 was grown anaerobically in glucose minimal medium supplemented with 10 μM Na_2SeO_3 or in the selenium-deficient minimal medium. After reaching an OD_{600} of 1, the cells of 20-ml cultures were harvested, and the RNA was prepared as described by Aiba *et al.* (21). Total RNA was electrophoretically separated in agarose gels containing formaldehyde by the method of Lehrach *et al.* (22). The separated RNA was transferred to GeneScreen (New England Nuclear) as described by the manufacturer. Hybridization with the radioactively labeled DNA probes described in Fig. 4 was performed as described by Maniatis *et al.* (17).

Other Techniques. Oligodeoxyribonucleotides were synthesized as described by Crea *et al.* (23). Incorporation of ^{75}Se into protein was followed as described by Cox *et al.* (1). β-Galactosidase activity was determined by the method of Miller (16). The immunoblotting experiments were performed by the procedure of Howe and Hershey (24) with modifications as described by Schmid and Böck (25).

RESULTS

Translation of UGA at Position 140 Requires Selenium. It has been shown (2) that the opal stop codon at position 140 of the *fdhF* gene is translated. If this UGA determines the insertion of selenocysteine, translation of the *fdhF* sequence downstream of the UGA should depend on the availability of selenium. To test this hypothesis, the *fdhF-lacZ* fusions previously described were used (2). In pBN2, pFM52, and pFM54, N-terminal portions of the *fdhF* gene corresponding to 39, 268, and 420 amino acids, respectively, are fused to *lacZ* (Fig. 1). These plasmids were transformed into strain FM420, and the transformants were tested for their β-galactosidase activity after growth in selenium-deficient and selenium-supplemented medium. The fusion carried by pBN2 expressed β-galactosidase irrespective of whether selenium was present or absent in the medium, whereas pFM52 and pFM54, which contain the UGA codon in the *fdhF* part of the fusion, expressed β-galactosidase only in the selenium-supplemented medium (Table 2). Wild-type *E. coli*

Table 2. Effect of selenium in the medium on the expression of *fdhF-lacZ* hybrid genes in strain FM420

	β-Galactosidase*	
Plasmid	Without Na_2SeO_3	With 10 μM Na_2SeO_3
pBN2	1800	1150
pFM52	30	1250
pFM54	60	1275

The medium contained 30 mM formate as inducer.
*Miller units (16).

cells containing an intact *lac* operon did not synthesize β-galactosidase under the anaerobic conditions required for expression of the *fdhF-lacZ* fusion protein unless a specific inducer of β-galactosidase such as isopropyl-β-D-thiogalactopyranoside was added to the medium (results not shown).

Quantitation of the effect of selenium on the read-through of the *fdhF-lacZ* fusion mRNA is depicted in Fig. 2. Under these conditions, synthesis of β-galactosidase activity was saturated by 0.1–0.2 μM selenite in the medium, a concentration range also sufficient to allow gas (H_2) production by the wild-type formate-hydrogenlyase complex.

In addition to assays for β-galactosidase activity, we also assessed the quantity of read-through protein by immunoblotting experiments with antibodies directed against β-galactosidase. The amount of anti-β-galactosidase cross-reacting material was drastically reduced when an *fdhF-lacZ* fusion strain containing the UGA codon was grown in selenium-deficient medium (Fig. 3).

Transcription of *fdhF* Is Independent of the Presence of Selenium. Indirect evidence that transcription of the *fdhF* promoter does not require selenium was provided by the observation that expression of β-galactosidase activity by the *fdhF-lacZ* fusion carried by plasmid pBN2 (lacking UGA) was selenium independent (Table 2). Direct demonstration of the selenium independence of *fdhF* transcription was provided by an RNA blot-hybridization experiment (Fig. 4). Electrophoretically separated total RNA from strain FM911 carrying plasmid pFM54 was hybridized with radioactively labeled DNA probes from an *fdhF-lacZ* segment upstream or downstream of the UGA codon (see Fig. 4). Both probes delivered one major hybridization signal with an RNA species of about 6 kb (compared to the migration position of 23S and 16S rRNA). The strong hybridization signal observed with RNA from cells starved for selenium indicates that transcription does not terminate at the UGA codon position in the absence of selenium.

Localized Mutagenesis of the UGA Codon. Localized mutagenesis experiments were performed to provide more direct proof that selenocysteine incorporation into the *fdhF* gene product is directed by a UGA codon. The UGA codon was converted into UGC (pFM201) and UGU (pFM202), which code for cysteine, and into UCA (pFM203), which directs serine incorporation. The resulting *fdhF* genes were tested for ability to direct selenocysteine incorporation. The UGA → UCA conversion abolished selenium incorporation completely, whereas a change in the wobble position (UGA → UGU or UGC) resulted in a quantitative reduction of selenium incorporation (Fig. 5*A*). All three replacements led to an

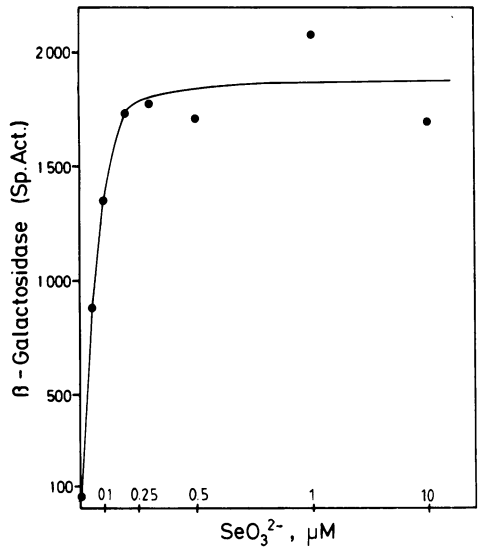

FIG. 2. Effect of selenite on the synthesis of β-galactosidase directed by the *fdhF-lacZ* fusion of plasmid pFM54. The host strain was FM420. Cells were grown anaerobically in selenium-deficient minimal medium in the presence or absence of 10 μM Na_2SeO_3 as outlined. Sp. Act., specific activity.

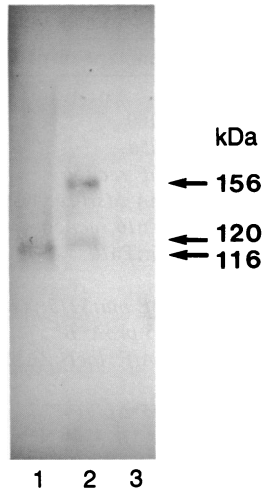

FIG. 3. Formation of anti-β-galactosidase cross-reacting material by strain FM911/pFM54. Cells were grown anaerobically in the presence (lane 2) or absence (lane 3) of 10 μM selenite, as indicated in the legend to Fig. 2. Lane 1 gives the migration position of β-galactosidase from Lac^+ wild-type cells induced by isopropyl β-D-thiogalactoside. An autoradiograph is shown of an immunoblot from a $NaDodSO_4$ electropherogram in which a $NaDodSO_4$ lysate of 0.5 OD_{420} unit of cells was separated. The material migrating at a position corresponding to an apparent size of 120 kDa is a breakdown product of the 156-kDa fusion protein (results not shown).

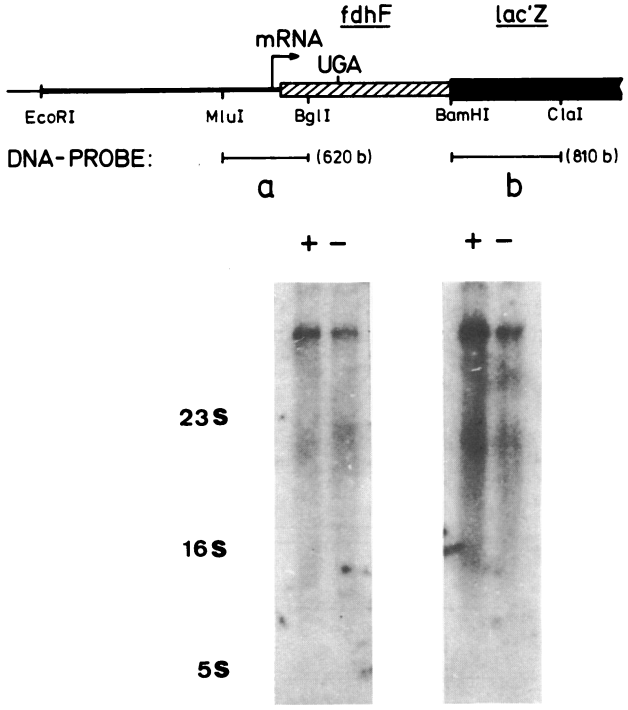

FIG. 4. Formation of *fdhF-lacZ* mRNA by strain FM911/pFM54 in the presence (lanes +) or absence (lanes −) of 10 μM selenite. For growth conditions, refer to the legend of Fig. 2. The DNA probes (a and b) used for hybridization are drawn above the autoradiographs. The migration positions of 23S, 16S, and 5S rRNAs are indicated; a size of ≈6 kb was determined for the hybridizing band. Each lane contains the total RNA from 3.75 OD_{420} units of cells. b, Bases.

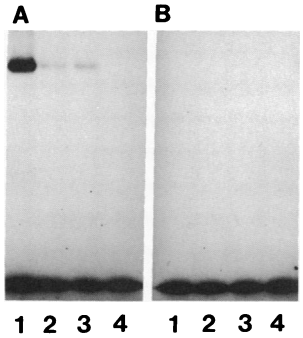

FIG. 5. Incorporation of ^{75}Se into the *fdhF* gene products directed by plasmids pFM20 (lanes 1), pFM201 (lanes 2), pFM202 (lanes 3), and pFM203 (lanes 4). (*A*) Incorporation in the genetic background of FM911. (*B*) Incorporation in the genetic background of WL31153. An autoradiograph of a $NaDodSO_4$ lysate electrophoretically separated on an 8% polyacrylamide gel in the presence of $NaDodSO_4$ is shown. The radioactive compounds at the front of the electropherogram are ^{75}Se-labeled tRNAs.

increase in the amount of *fdhF* polypeptide in Coomassie-stained $NaDodSO_4$ gels (not shown). Taking this increased formation of the *fdhF* gene product into account, the residual selenium incorporation directed by UGU or UGC amounts to approximately 10% of the UGA-containing gene.

In a similar experiment carried out with these plasmids in the genetic background of strain WL31153, no selenium label was detected in the wild-type and mutant *fdhF* gene products. Strain WL31153 was deficient in the capacity to incorporate selenium into either FDH_H or FDH_N but not into tRNA (Fig. 5; W.L., unpublished results). Its lesion mapped at the chromosomal position previously designated *fdhA* (26). Physiologically, this mutation mimics selenium starvation for formate dehydrogenase synthesis and provides an elegant means to circumvent the difficulties in preparing a completely selenium-deficient medium.

The *Kpn* I–*Sph* I restriction fragments (Fig. 1) of the mutagenized *fdhF* genes from plasmids pFM201, pFM202, and pFM203 were ligated into the fusion plasmid pFM54 to generate the *fdhF-lacZ* fusions pFM56, pFM57, and pFM58, in which the UGA codon at position 140 is replaced by UGC, UGU, and UCA codons, respectively. The plasmids were transferred into strain WL31153, and β-galactosidase activity was determined in anaerobically grown cells (Table 3). As expected, the short fusion of pBN2 was equally expressed in strain FM420, which possesses a functional system for selenium incorporation into formate dehydrogenase, and in strain WL31153, which lacks this ability. Expression of β-galactosidase directed by pFM54, on the other hand, depends on the presence of the active selenylation pathway. Conversion of UGA at position 140 into UGC, UGU, or UCA restored β-galactosidase formation in the genetic background of WL31153.

It was particularly interesting to see whether a replacement of selenocysteine by cysteine or serine is detrimental to formate dehydrogenase activity. For this purpose, plasmids pFM20, pFM201, pFM202, and pFM203 were transformed into strain WL31153, and the transformants were tested for gas formation. Cells harboring pFM20 (UGA codon, translation termination) and pFM203 (serine instead of selenocysteine) were completely devoid of formate hydrogenlyase activity. With cysteine substituted for selenocysteine (pFM201 and pFM202), cultures still exhibited detectable formate hydrogenlyase activity. These cultures also exhibited formate dehydrogenase activity (formate-dependent reduction of benzyl viologen under anaerobic conditions). The relative catalytic activities of the enzymes in which selenocysteine was replaced by cysteine can be assessed when the purified enzymes are available.

Table 3. β-Galactosidase activities in plasmid-containing strains grown anaerobically in medium supplemented with 10 μM selenite

Plasmid	Codon*	β-Galactosidase†	
		FM420	WL31153
pBN2	—	863	1020
pFM54	UGA	984	0
pFM56	UGC	1292	1510
pFM57	UGU	1248	1526
pFM58	UCA	1319	1632

*Codon in position 140 of the *fdhF* gene.
†Miller units (16).

DISCUSSION

The results described above demonstrate that a UGA codon in the structural gene *fdhF* of the selenopolypeptide of formate dehydrogenase directs the cotranslational insertion of selenocysteine. The arguments are as follows. (*i*) Whereas transcription of *fdhF* is independent of the presence of selenium in the medium, translation requires a functional and active selenium incorporation system. Lack of selenium blocks translation of the gene as determined by the analysis of β-galactosidase activity or antibody-reactive material. The formation of a putative selenocysteinyl-tRNA requires selenium, and when no such tRNA is available, the ribosomes translating the *fdhF* mRNA terminate at the UGA codon. Production of *fdhF-lacZ* mRNA from plasmid pFM54 is reduced slightly under conditions of selenium starvation (see Fig. 4); this reduction could be due to a decoupling of transcription and translation. Mutations (e.g., *fdhA*) have been described for *E. coli* and *Salmonella typhimurium* in which—because of a single mutation—both FDH_H and FDH_N activities disappear. This mutation is thought to abolish selenocysteine incorporation into the polypeptides. Furthermore, no cross-reacting material corresponding to a putative selenium-deficient polypeptide could be detected (27). (*ii*) Localized mutagenesis of the UGA codon affects selenocysteine incorporation; it is abolished after substitution by UCA (serine codon) or reduced when changed in the wobble position either to UGC or UGU (cysteine codons). In all of these mutant constructs, the absence of selenium incorporation no longer results in a blockage of translation. This indicates that it is the UGA codon that prevents downstream translation in the absence of selenium or in a mutant deficient in selenium incorporation. (*iii*) Finally, colinearity of a UGA codon in the gene sequence and a selenocysteine in the protein sequences of mammalian glutathione peroxidases provides a comparative argument (3).

An important question to be solved in the future is how the ribosome differentiates this internal UGA from the same codon used as a termination signal. Context effects of the mRNA sequences may play an important role. This is clearly suggested by the result that the UGC_{140}- and UGU_{140}-containing mutants still incorporate some selenium into the *fdhF* gene product. Although selenomethionine can be incorporated into polypeptides nonspecifically in place of methionine, selenocysteine residues are found only in specific locations of a few known proteins (28).

The finding of a nonsense codon directing the insertion of a specific amino acid is reminiscent of the fact that in bovine liver a UGA-suppressing tRNA species that is aminoacylated with *O*-phosphoserine has been detected (29, 30). In an *in vitro* translation system containing rabbit globin mRNA, this aminoacylated tRNA cotranslationally inserts *O*-phospho-

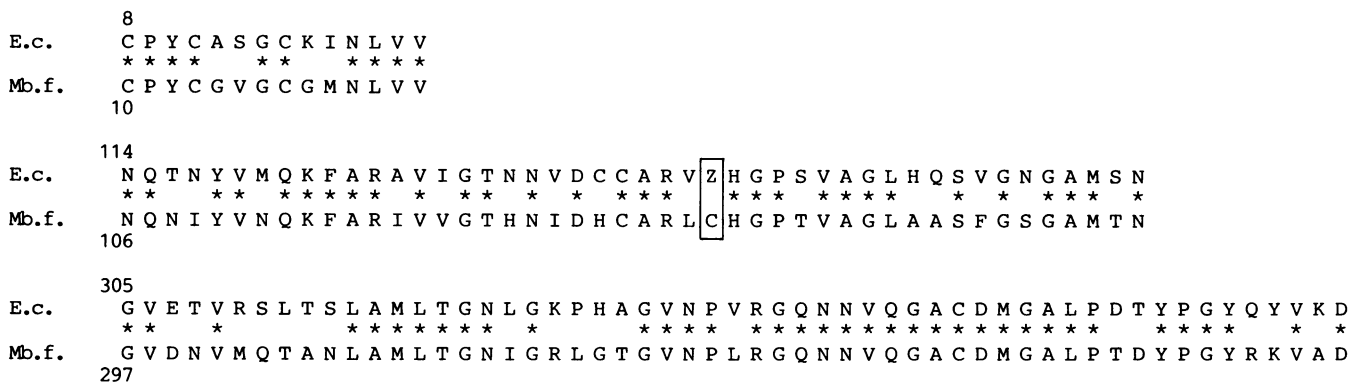

FIG. 6. Comparison of homologous regions of formate dehydrogenase from *E. coli* (lines E.c.) and *M. formicicum* (lines Mb.f.). Amino acids are abbreviated according to the standard code. The nonstandard letter "Z" (boxed) refers to selenocysteine. Occurrence of homologous amino acids in either protein is indicated by asterisks. Numbers refer to the positions of the first amino acids of the regions specified in the primary structure of the formate dehydrogenase from *E. coli* and *M. formicicum*, respectively.

serine into protein (31), although the precise location has not yet been determined.

Another point of interest is the effect of substitution of a sulfur amino acid (cysteine) for the selenocysteine residue in formate dehydrogenase on its catalytic activity. In addition to the mutant enzymes described here, such an enzyme also is found in nature. The nucleotide-derived amino acid sequence of the selenium-independent formate dehydrogenase of *Methanobacterium formicicum* (32) shows three striking homology regions to that of the *E. coli fdhF* gene product (Fig. 6). One of them is at the region encoding the amino acid sequence surrounding the selenocysteine of *E. coli* formate dehydrogenase. However, the *M. formicicum* enzyme contains a cysteine at the position corresponding to the selenocysteine in the *E. coli* enzyme. Incorporation of selenocysteine in the *M. formicicum* enzyme by site-directed mutagenesis could provide valuable additional experimental material for comparison of sulfur and selenium biochemistry.

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1. Cox, J. C., Edwards, E. S. & DeMoss, J. A. (1981) *J. Bacteriol.* **145,** 1317–1324.
2. Zinoni, F., Birkmann, A., Stadtman, T. C. & Böck, A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4650–4654.
3. Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W. & Harrison, P. R. (1986) *EMBO J.* **5,** 1221–1227.
4. Günzler, W. A., Steffens, G. J., Grossmann, A., Kim, S.-M., Ötting, F., Wendel, A. & Flohé, L. (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365,** 195–212.
5. Condell, R. A. & Tappel, A. L. (1982) *Biochim. Biophys. Acta* **709,** 304–309.
6. Casadaban, M. & Cohen, S. N. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4530–4533.
7. Csonka, L. & Clark, A. (1980) *J. Bacteriol.* **143,** 529–530.
8. Ihara, M., Yoshimitsu, O. & Yamamoto, K. (1985) *FEMS Microbiol. Lett.* **30,** 33–35.
9. Kramer, W., Drutsa, V., Jansen, H.-W., Kramer, B., Pflugfelder, M. & Fritz, H.-J. (1984) *Nucleic Acids Res.* **12,** 9441–9456.
10. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156.
11. Casadaban, M., Chou, J. & Cohen, S. N. (1980) *J. Bacteriol.* **143,** 971–980.
12. Messing, J. & Vieira, J. (1982) *Gene* **19,** 269–276.
13. Norrander, J., Kempe, T. & Messing, J. (1983) *Gene* **26,** 101–106.
14. Begg, Y. A., Whyte, J. N. & Haddock, B. A. (1977) *FEMS Microbiol. Lett.* **2,** 47–50.
15. Balch, W. E. & Wolfe, R. S. (1976) *Appl. Environ. Microbiol.* **32,** 781–791.
16. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
17. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
18. Zoller, M. J. & Smith, M. (1983) *Methods Enzymol.* **100,** 468–500.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
20. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–561.
21. Aiba, H., Adhya, S. & de Crombrugghe, B. (1981) *J. Biol. Chem.* **256,** 11905–111910.
22. Lehrach, H., Diamond, D., Wozney, J. M. & Boedtker, H. (1977) *Biochemistry* **16,** 4743–4751.
23. Crea, R., Kaszewski, A., Hirose, T. & Itakura, K. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5765–5769.
24. Howe, J. G. & Hershey, J. W. B. (1981) *J. Biol. Chem.* **256,** 12836–12839.
25. Schmid, G. & Böck, A. (1984) *Syst. Appl. Microbiol.* **5,** 1–10.
26. Mandrand-Berthelot, M.-A., Wee, M. Y. K. & Haddock, B. A. (1978) *FEMS Microbiol. Lett.* **4,** 37–40.
27. Graham, A., Jenkins, H. E., Smith, N. H., Mandrand-Berthelot, M.-A., Haddock, B. A. & Boxer, D. H. (1980) *FEMS Microbiol. Lett.* **7,** 145–151.
28. Sliwkowski, M. X. & Stadtman, T. C. (1985) *J. Biol. Chem.* **260,** 3140–3144.
29. Diamond, A., Dudock, B. & Hatfield, D. (1981) *Cell* **25,** 497–506.
30. Hatfield, D., Diamond, A. & Dudock, B. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6215–6219.
31. Mizutani, T. & Tachibana, Y. (1986) *FEBS Lett.* **207,** 162–166.
32. Shuber, A. P., Orr, E. C., Recny, M. A., Schendel, P. F., May, H. D., Schauer, N. L. & Ferry, J. G. (1986) *J. Biol. Chem.* **261,** 12942–12947.