Selenocysteine is co-translationally incorporated into prokaryotic and eukaryotic selenoproteins at in-frame UGA codons. However, the only component of the eukaryotic selenocysteine incorporation machinery identified to date is the selenocysteine-specific tRNA\(^{Sec}\). In prokaryotes, selenocysteine is synthesized from seryl-tRNA\(^{Sec}\) and the active selenium donor, selenophosphate. Selenophosphate is synthesized from selenide and ATP by the \(selD\) gene product, selenophosphate synthetase, and is required for selenocysteine synthesis and incorporation into bacterial selenoproteins. We have now cloned human \(selD\) and shown that transfection of the human \(selD\) cDNA into mammalian cells results in increased selenium labeling of a mammalian selenoprotein, type 1 iodothyronine deiodinase. Despite significant differences between the mechanisms of selenoprotein synthesis in prokaryotes and eukaryotes, human \(selD\) weakly complements a bacterial \(selD\) mutation, partially restoring selenium incorporation into bacterial selenoproteins. Human selenophosphate synthetase has only 32% homology with the bacterial protein, although a highly homologous region that has similarity to a consensus ATP/GTP binding domain has been identified. Point mutations within this region result in decreased incorporation of selenium into type 1 iodothyronine deiodinase in all but one case. Further analysis revealed that reduced selenium labeling was due to altered ATP binding properties of the mutant selenophosphate synthetases.

Selenocysteine is co-translationally incorporated into prokaryotic selenoproteins at UGA codons (1). It has been shown recently that translation of the selenocysteine UGA codon in the bacterial selenoenzyme, formate dehydrogenase, requires a specific stem-loop in the coding region immediately 3’ to the UGA codon (2). In addition, four genes, \(selA\), \(selB\), \(selC\), and \(selD\), are required for selenocysteine codon recognition and translation (3, 4, 5). The \(selC\) gene product is a selenocysteine-specific tRNA species that becomes charged with L-serine by seryl-tRNA synthetase (6). The product of the \(selA\) gene, selenocysteine synthase, converts seryl-tRNA\(^{Sec}\) to selenocysteyl-tRNA\(^{Sec}\) via an amminocrylyl intermediate (7, 8). The active selenium donor species in this reaction is selenophosphate, which is synthesized by the \(selD\) gene product, selenophosphate synthetase (9, 10). In the final step of bacterial selenoprotein synthesis, selenocysteyl-tRNA\(^{Sec}\) is bound by a specific translation factor, SELB, which also binds the stem-loop structure, and decodes the UGA specifying selenocysteine (11, 12). Selenophosphate is also required for conversion of 5-methylaminomethyl-2-thiouridine residues in the anticodon of certain bacterial tRNAs to 5-methylaminomethyl-2-selenouridine (5, 6, 13, 14, 15).

Several eukaryotic selenoproteins have also been identified, including glutathione peroxidases (16), selenoprotein P (17, 18), and the type I iodothyronine 5'-deiodinase (19), all of which contain selenocysteine encoded by UGA codons. However, the mechanisms of recognition and translation of selenocysteine codons differ between prokaryotes and eukaryotes. Although stable stem-loop structures are required for eukaryotic selenoproteins synthesis, these elements are situated within the 3'-untranslated region of the mRNAs (20). In addition, the sequences of prokaryotic and eukaryotic stem-loop structures are not conserved (2, 20). Mutagenesis studies have also indicated that the position of the stem-loop in bacterial formate dehydrogenase is essential to function (2), whereas in 5'-deiodinase, the position of the stem-loop is quite flexible (21). Finally, cloning of the rat and human selenoprotein P cDNAs revealed the presence of 10 in-frame UGA codons (17, 18) and two 3'-untranslated stem-loops (21). As yet, no prokaryotic selenoprotein containing multiple selenocysteine residues has been identified.

Apart from the tRNA\(^{Sec}\) (22, 23), none of the components of the eukaryotic selenocysteine incorporation machinery have been identified. This may be explained by poor conservation of the selenoprotein translational machinery. For example, eukaryotic selenoprotein mRNAs are unable to direct selenocysteine incorporation when expression is attempted in Escherichia coli (1). In addition, cloning of the tRNA\(^{Sec}\) from several organisms has revealed evolutionary differences in the sequences for this tRNA within both the eukaryotic (23) and the prokaryotic (24) kingdoms.

We have now cloned the human homologue of bacterial \(selD\) and shown that it is functional in mammalian cells and also in bacteria. Comparison of the human and bacterial selenophosphate synthetase peptide sequences reveals a high homology glycine-rich sequence that is similar to conserved sequences found in many ATP/GTP binding proteins and protein kinases (25, 26, 27, 28). Mutational analysis has revealed that conserved amino acids within this region are important for selenophosphate synthetase activity and for the ATP binding properties of the enzyme.

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†To whom correspondence should be addressed: Thyroid Div., Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115. Tel.: 617-732-6737; Fax: 617-731-4718.

1 M. J. Berry, unpublished observations.
EXPERIMENTAL PROCEDURES

Materials


t23Se was a gift from Dr. Dolph Hatfield (National Institutes of Health, Bethesda, MD) and was originally obtained from Dr. Kurt Zinn (University of Missouri Research Reactor, Columbia, MO). The Xenopus tRNA\textsuperscript{sec} gene in pGEM3 was kindly provided by Dr. Dolph Hatfield (22, 23). E. coli strain WL400, containing an inactivated selD gene, and pMN330, which contains the bacterial selD gene in pBR322 (5), were gifts from the laboratory of Dr. August Bock.

Isolation of a Human selD Clone

Partial Clone—A partial human selD cDNA clone was identified using a yeast two-hybrid system as described previously (29, 30, 31). The bait protein used to clone selD was the mammalian spliceosome-associated protein SAP62.

Full-length Clone—A human liver cDNA library (a gift of Dr. Brian Seed) in the plasmid CDM8 (33) was transformed into MC1061-P3, a strain of E. coli, and plated onto 150-nm agar plates. Clones were isolated using nylon filters (GeneScreen Plus, DuPont NEN) and denatured in 0.5 M NaCl, and cross-linked using a UV-Stratalinker 1800 (Stratagene, La Jolla, CA). Filters were prehybridized as described previously (34) and then hybridized overnight (34) with a random primed (Prime It, Stratagene, La Jolla, CA) probe of PsI-digested plasmid pCM1061-P3 (1,023-1,623) from the 1061-P3 strain of human selD. Membranes were washed to a final stringency of 0.1 × SSC (0.15 M NaCl and 15 mM sodium citrate), 0.1% SDS at 65°C. Four positive clones were identified and colony-purified.

Three of these, selD2 and selD4, were selected for further characterization. Dideoxynucleotide sequencing was carried out using a Sequenase kit (Version 2, U. S. Biochemical Corp.).

Transient Transfections

pSL21 was created by subcloning the entire E. coli selD cDNA from pMN330 (5) into the HindIII/NotI sites of CDM8. 10 μg of selD4, pSL21, or mutants of selD4 were transfected into HTA-1 cells (35) in combination with two other plasmids, pGZ-D10 (5 μg), encoding rat 5'-dectinase (36), and XKBNA (5 μg), encoding the Xenopus tRNA\textsuperscript{sec} gene in pGEM3 (23, 24). 3 days prior to transfection, cells were plated onto 60-mm dishes in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 1 day prior to transfection, the medium was changed to Dulbecco's modified Eagle's medium containing 1% fetal calf serum supplemented with 100 μM 3,5,3'-triiodothyronine, 10 μg/ml transferrin, 20 mg/ml insulin, and 50 μg hydrocortisone to decrease the endogenous serum and selenium concentrations. Plasmids were transfected by CaPO\textsubscript{4}, as described previously (37) and maintained in the supplemented low serum media. 1 day following transfection, cells were shocked with 10% Me2SO, and fresh medium containing 5 μM unlabeled selenium plus 6 μC of \textsuperscript{125}I was added. In vivo labeling was carried out for 18 h.

Cells were harvested, washed, and resuspended in 0.1 M potassium phosphate (pH 6.9), 1 mM EDTA containing 0.25 M sucrose. Aliquots of transfected cells were stored at −70°C until use. ATP-binding assays were performed using 8-azido-[\textsuperscript{32}P]ATP (specific activity 12.8 Ci/mmol, ICN Flow). The samples were processed as described above (37).

RESULTS

Isolation of a Human selD cDNA—Screening of a human liver cDNA library with a fragment of the partial selD clone resulted in the isolation of four positive clones with inserts varying from 1225 to 1672 nucleotides in length. All four clones showed significant sequence similarity to E. coli selD, with the fourth clone terminating at nuclotide 1374. No consensus polyadenylation signal at 1507–1512. A third clone extended 64 nucleotides in sense from the end of the first clone.

Bacterial Transformations

Since E. coli contain endogenous prokaryotic selD expression, a cloning vector was used to carry out the cloning experiments described above. Co-transfection of 3 μg of a constitutive human growth hormone-expressing plasmid, pTKGH, was used to monitor transfection efficiencies as described previously (37). Cells were harvested 2 days following transfection, washed, and resuspended in 0.1 M potassium phosphate (pH 6.9), 1 mM EDTA containing 0.25 M sucrose. Aliquots of transfected cells were stored at −70°C until use. ATP-binding assays were performed using 8-azido-[\textsuperscript{32}P]ATP (specific activity 12.8 Ci/mmol, ICN Flow), an analogue of ATP that can be cross-linked under UV irradiation. Reaction mixtures (30 μl) consisted of an aliquot of cell extracts corrected for the transfection efficiency. 100 mM Tricine (pH 7.2 with KOH) and 7.2 mM 8-azido-[\textsuperscript{32}P]ATP. Reactions were irradiated at 254 nm for 3 min on ice in the absence of visible light. The reaction was stopped by the addition of excess dithiothreitol. Labeled proteins were analyzed by 12% polyacrylamide SDS gel electrophoresis followed by autoradiography.

Time course experiments with 8-azido-[\textsuperscript{32}P]ATP were also carried out to examine potential differences between selD4 and the selD mutants. Reaction mixtures were assembled as described above and allowed to incubate on ice for varying lengths of time before UV cross-linking, then reactions were stopped by the addition of excess dithiothreitol. Labeled proteins were analyzed by polyacrylamide SDS gel electrophoresis and autoradiography.

RESULTS

Isolation of a Human selD cDNA—Screening of a human liver cDNA library with a fragment of the partial selD clone resulted in the isolation of four positive clones with inserts varying from 1225 to 1672 nucleotides in length. All four clones showed significant sequence similarity to E. coli selD; two clones, selD2 and selD4, were further investigated. Two alternative polyadenylation sites appear to be used in the human selD mRNA. The partial clone contains a consensus polyadenylation signal at nucleotides 1588–1593, followed by a poly(A) tail beginning at 1613. selD2 and selD4 are polyadenylated at an alternative site approximately 80 nucleotides 5' of the position in the partial clone. This site is also preceded by a polyadenylation signal at 1507–1512. A third clone extended 64 base pairs 3' of the partial clone and contained no poly(A) tail, while the fourth clone terminated at nucleotide 1374. No consensus polyadenylation sequence is present in this region.
the four clones isolated, selD2 extended the furthest 5', approximately 240 base pairs upstream of the ATG start codon. Included in this region were approximately 80 base pairs of sequence of >90% GC content, which was highly compressed and therefore difficult to sequence accurately.

The deduced amino acid sequence of human selD is 381 amino acids with a calculated molecular mass of approximately 45 kDa and shows overall 32% identity and 55% similarity with E. coli selD. Alignment of the human and E. coli peptide sequences (Fig. 1) revealed a conserved glycine rich region with vertebral lines. The glycine-rich, putative ATP binding consensus sequence is indicated by an asterisk; methionine residues in both sequences are indicated by *.

Expression of Human and Prokaryotic selD in Mammalian Cells—To examine the functional activity of selD in mammalian cells, human (selD4) and E. coli (pSL21) selD cDNAs were transfected into HTTA cells in combination with G21-D10, which expresses the mammalian selenoprotein 5'-deiodinase, and XtRNA, which encodes the Xenopus tRNA\(^{sec}\). Transfected G21-D10 alone is poorly labeled with \(^{75}\)Se (Fig. 2). However in the presence of co-transfected selD4, \(^{75}\)Se labeling of 5'-deiodinase is greatly enhanced, indicating that human selD cDNA is functional in mammalian cells (Fig. 2). In addition, labeling of 5'-deiodinase is increased by co-transfection of XrRNA, either in the absence or presence of selD. E. coli selD (pSL21) also leads to increased \(^{75}\)Se labeling of 5'-deiodinase compared with G21-D10 alone, indicating that the action of selD is probably very similar in bacteria and mammalian cells (Fig. 2).

Complementation of a Bacterial selD Mutation—E. coli strain BL21(DE3) contains endogenous selD, thus in vivo labeling with \(^{75}\)Se results in the incorporation of selenium into formate dehydrogenase and 5-methylaminomethyl-2-selenouridine residues in tRNAs (Fig. 3). Interestingly, when BL21(DE3) was transformed with pET-selD2, selenophosphate synthetase itself became labeled with \(^{75}\)Se. In contrast, in WL400-P3, which contains an interrupted and therefore inactive selD gene (5), neither the tRNAs nor formate dehydrogenase are labeled with \(^{75}\)Se for 3 h. Proteins were analyzed on 10% polyacrylamide SDS gels followed by autoradiography.
tations within the putative ATP-binding domain of human selD all but one of the transfection into HtTA-1 cells. Compared with wild-type selD, on selenium incorporation into 5'-deiodinase—

were created, and the effects of these amino acid substitutions (Fig. 3).

Gly271 also bind more ATP for a longer period of time than wild-type human selD. In particular, mutation of the glycine residue at position 274 to either asparagine, valine, or alanine resulted in an almost complete inability of these mutants to bind ATP. In addition, mutation of histidine 275 to aspartate also produced decreased ATP binding compared with wild-type selD (Fig. 5). In contrast, mutation of the same histidine to tyrosine resulted in increased ATP binding, indicating that the amino acid at position 275 is involved in ATP binding.

The amount of ATP binding by the His275 Tyr mutant decreases gradually over the same time period, suggesting less ATP hydrolysis by this mutant (Fig. 6). Gly269 Cys and Gly271 Arg also bind more ATP for a longer period of time than wild-type selD (Table I, and data not shown), suggesting that the glycine residues at these positions have roles in normal selenophosphate synthetase activity other than ATP binding.

Following ATP binding to selenophosphate synthetase, ATP is hydrolyzed to produce AMP and a pyrophosphate-enzyme intermediate (9, 10). In order to examine ATP hydrolysis by wild-type human and mutant selenophosphate synthetases, cell extracts were incubated with ATP, and the reaction products were analyzed by thin-layer chromatography. Data from this study indicated that the wild-type and mutant enzymes do hydrolyze ATP (data not shown). However, cell extracts alone also extensively degrade ATP, and thus rates of hydrolysis by the selenophosphate synthetases were difficult to measure accurately. Therefore, we have examined the time course of ATP binding. Wild-type selD4 initially exhibits efficient ATP binding, followed by a rapid loss of bound label (Fig. 6). In contrast, the amount of ATP binding by the His275 Tyr mutant decreases gradually over the same time period, suggesting less ATP hydrolysis by this mutant (Fig. 6). Gly269 Cys and Gly271 Arg also bind more ATP for a longer period of time than wild-type selD (Table I, and data not shown), suggesting that glycine residues at position 269 and 271 may be involved in increased ATP binding, indicating that the amino acid at position 275 is involved in ATP binding.

The amount of ATP binding by the His275 Tyr mutant decreases gradually over the same time period, suggesting less ATP hydrolysis by this mutant (Fig. 6). Gly269 Cys and Gly271 Arg also bind more ATP for a longer period of time than wild-type selD (Table I, and data not shown), suggesting that glycine residues at position 269 and 271 may be involved in increased ATP binding, indicating that the amino acid at position 275 is involved in ATP binding.

Expression of selD Mutants in Mammalian Cells—Point mutations within the putative ATP-binding domain of human selD were created, and the effects of these amino acid substitutions on selenium incorporation into 5'-deiodinase determined by transfection into HtTA-1 cells. Compared with wild-type selD, all but one of the selD mutants resulted in less efficient incorporation of 75Se into 5'-deiodinase (Fig. 4, a and b). Mutation of the histidine at position 275 to tyrosine resulted in increased selenium labeling of 5'-deiodinase, in contrast with the decreased 75Se incorporation observed when the same residue is mutated to aspartate (Fig. 4a). Mutational analysis of each of the conserved glycine residues indicates that all three are important for normal functioning of human selD, since selenium labeling of 5'-deiodinase is decreased in the presence of each of these mutants compared with wild-type human selD (Fig. 4b).

ATP Binding Properties of Mutant Selenophosphate Synthetases—Since the amino acid substitutions made were to the putative ATP-binding domain of human selD, we next tested the possibility that these mutants may have lost their ability to bind ATP, thus explaining the decreased selenium labeling of 5'-deiodinase. As shown in Fig. 5, several of the mutant selD proteins bind ATP less efficiently than the wild-type human selD. In particular, mutation of the glycine residue at position 274 to either asparagine, valine, or alanine resulted in an almost complete inability of these mutants to bind ATP. In addition, mutation of histidine 275 to aspartate also produced decreased ATP binding compared with wild-type selD (Fig. 5). In contrast, mutation of the same histidine to tyrosine resulted in increased ATP binding, indicating that the amino acid at position 275 is involved in ATP binding in wild-type selD. Mutation of the glycine residues at positions 269 and 271 to cysteine and arginine respectively, had no effect on ATP binding, although both of these mutations resulted in decreased 75Se incorporation observed when the same residue is mutated to aspartate (Fig. 4, a and b). Mutation of the same histidine to tyrosine resulted in increased ATP binding, indicating that the amino acid at position 275 is involved in ATP binding in wild-type selD. Mutation of the glycine residues at positions 269 and 271 to cysteine and arginine respectively, had no effect on ATP binding.
Fig. 6. ATP hydrolysis properties of selD. Extracts of HtTA-1 cells transfected with either wild-type selD (selD4; 10 μg/dish) or selD mutants (10 μg/dish) were incubated in the presence of 8-azido-[γ-32P]ATP for various time periods before being cross-linked under ultraviolet radiation for 3 min. The time course of ATP binding was analyzed on 12% polyacrylamide SDS gels followed by autoradiography.

| Mutant | 75Se of 5'-DeoDna | ATP binding | ATP hydrolysis |
|--------|-------------------|-------------|---------------|
| selD4  | ++                | +++         | +             |
| His275 → Tyr | ++        | +++         | +             |
| His275 → Asn | +        | −            | ND*          |
| Gly274 → Asp | +          | −            | ND           |
| Gly274 → Ala | +          | +            | ND           |
| Gly274 → Val | −          | −            | ND           |
| Gly299 → Cys | +          | +            | +            |
| Gly271 → Arg | +          | +            | +            |

* ND, not determined.

DISCUSSION

The role of prokaryotic selenophosphate synthetase in the production of the active selenium donor, selenophosphate, has been clearly demonstrated (3, 4, 5, 13, 14, 15). Mammalian selenoproteins have also been identified (16, 17, 18, 19, 20), although the mechanism whereby selenocysteine is synthetized and incorporated into these proteins is not yet clear. We have now cloned the human homologue of bacterial selD and shown that it is functional in both mammalian cells and bacteria. A partial clone of human selD was obtained using a yeast two-hybrid protein interaction system. The bait protein for this interaction was the mammalian spliceosome-associated protein SAP62, which is involved in mRNA splicing (42). Since appropriate control experiments were performed to minimize non-specific interactions with the bait protein, the interaction between selD and SAP62 may be specific, although the relevance of this and the implication that selD may be involved in RNA splicing is not known.

Transfection of E. coli and human selD into mammalian cells showed that both prokaryotic and eukaryotic selenophosphate synthetases are functional in higher eukaryotes. However, when human selD was transformed into bacteria containing an inactive selD gene, it only partially complemented the bacterial system and produced low levels of 75Se labeled RNAs and formate dehydrogenase. One possible explanation for this may be that the human selD gene product is improperly folded in bacteria. Alternatively, a factor(s) found in mammalian cells but not bacteria may be required in addition to the mammalian selD gene product to maintain normal selenophosphate synthetase activity. Nevertheless, these data do indicate that although human and bacterial selenophosphate synthetase sequences are poorly conserved (32% homology), and there is a great evolutionary distance separating the two proteins, the action of selD is probably very similar in bacteria and higher organisms. When the E. coli strain BL21(DE3) was transformed with human selD and grown in the presence of 75Se, in addition to the expected bacterial selenoproteins becoming labeled, selenophosphate synthetase itself was also labeled by 75Se (Fig. 3). This was unexpected, since the sequence of human selD does not indicate the presence of selenocysteine within the protein itself. However, when the selenium to sulfur ratio is higher than normal, selenium may be nonspecifically misincorporated into proteins in the form of selenomethionine (43, 44), or, if the methionine content of a protein is high, misincorporation of selenium into selenomethionine may also occur (45, 46). It was therefore interesting to note that human selenophosphate synthetase contains 18 methionine residues, and the bacterial protein contains 13, both of which are much higher than the average protein methionine content and may therefore explain the observed labeling of selD.

Mutational analysis of the putative ATP binding consensus sequence in the human selD cDNA indicated that all of the amino acids considered are important in normal selenophosphate synthetase activity. We have also shown that the residues within this region are important for normal ATP binding properties of selenophosphate synthetase, although direct measurement of ATP hydrolysis will require purification of wild-type and mutated enzymes. Recent work on bacterial selD involved mutational analysis of the sequence encoding the amino terminus of the protein, which also shared some similarity with the consensus ATP binding sequence (47, 48). It was shown that the cysteine residue at position 17 (Cys17) and the lysine residue at position 20 (Lys20) are both essential for the formation of selenophosphate from selenide and ATP (47, 48), while Cys17 was also shown to be required for ATP binding to selenophosphate synthetase. In contrast, mutation of Cys17 had no effect on selenophosphate activity. The significance of these results in relation to human selenophosphate synthetase activity is not clear since the only residue in this region that is conserved between the two sequences is Cys17, which was not essential for catalytic activity in E. coli selD. However it is possible that there are differences in the three-dimensional structure of the bacterial versus human selD gene products, which could explain the discrepancies between the two studies. Further work is dearly required to determine the importance of individual amino acids in both selD homologues for normal selenophosphate synthetase function, ATP binding, and ATP hydrolysis.

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