SelD Homolog from Drosophila Lacking Selenide-dependent Monoselenophosphate Synthetase Activity

Britt C. Persson1, August Böck1*, Herbert Jäckle2 and Gerd Vorbrüggen2

1Lehrstuhl für Mikrobiologie der Universität München Maria-Ward-Str. 1a D-80638 München, Germany
2Max-Planck-Institut für biophysikalische Chemie Abt. Molekulare Entwicklungsbiologie Postfach 2841, D-37018 Göttingen, Germany

The isolation and molecular characterization of an invertebrate gene that encodes a homolog of the human selenophosphate synthetase 1 is described. This Drosophila gene, termed selD-like, is located in the cytogenetic interval 50 D/E on the right arm of chromosome 2. It is expressed ubiquitously throughout embryogenesis and found to be highly enriched in the developing gut and in the nervous system of the embryo.

The SelD-like from Drosophila was purified after expression in Escherichia coli. The purified protein does not catalyze the selenide-dependent ATP hydrolysis reaction and its gene does not complement a selD lesion in E. coli. These results and the fact that selD-like possesses an arginine residue at the position of the essential Cys17 (E. coli nomenclature) indicate that the Drosophila gene exerts a function different from that of the classical selenophosphate synthetases. Two classes of SelD proteins can therefore be differentiated. The class I proteins contain a cysteine or selenocysteine residue in the active site and display selenide-dependent selenophosphate synthetase activity. Class II proteins, including Drosophila selD-like and human selenophosphate synthetase 1 are devoid of this activity and they possess other amino acids in position 17.

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Introduction

Monoselenophosphate is the key intermediate in selenium metabolism of both prokaryotes and eukaryotes. It serves as the selenium donor molecule in the conversion of seryl-tRNA Sec into selenocysteinyl-tRNA Sec and in the replacement of the sulfur in 2-thiouridine in tRNA by selenium yielding 2-selenouridine (for reviews see Baron & Böck, 1995; Stadtman, 1996). Monoselenophosphate (MSP) is the product of the reaction catalyzed by mono-selenophosphate synthetase in which the γ-phosphate group of ATP is transferred to HSe resulting in MSP production accompanied by the formation of inorganic phosphate and AMP (Leinfelder et al., 1990; Ehrenreich et al., 1992; Veres et al., 1992; Glass et al., 1993). A detailed kinetic analysis of the reaction mechanism of the enzyme from Escherichia coli revealed that a cysteine residue (Cys17) is essential for catalysis (Kim et al., 1992; Veres et al., 1994).

Intriguingly, two other forms of the enzyme related to this active-site-cysteine were discovered. Analysis of the total genome sequence of Haemophilus influenzae and Methanococcus jannaschii revealed that the selD gene that codes for selenophosphate synthetase in those organisms possesses a UGA codon at the position of the essential Cys17 (E. coli nomenclature) indicate that the Drosophila gene exerts a function different from that of the classical selenophosphate synthetases. Two classes of SelD proteins can therefore be differentiated. The class I proteins contain a cysteine or selenocysteine residue in the active site and display selenide-dependent selenophosphate synthetase activity. Class II proteins, including Drosophila selD-like and human selenophosphate synthetase 1 are devoid of this activity and they possess other amino acids in position 17.

Abbreviations used: MSP, monoselenophosphate; IPTG, isopropyl-β-d-thiogalactopyranoside; FDH, formate dehydrogenase; PEI, polyethyleneimine.
Drosophila is an excellent model organism to unravel the function of the gene products, we have initiated its genetic and biochemical analysis.

Results

Isolation, structure and expression of a selD-like gene of Drosophila

The clone carrying the apparent homolog of the gene encoding the human selenophosphate synthetase, SelD, was isolated from a Drosophila embryo cDNA library. Cytogenetic localization of the gene by in situ hybridization of the cDNA to polytene salivary gland chromosomes showed that selD-like is located in region 50D/E on the right arm of the second chromosome (Figure 1a).

In order to determine the structure of the selD-like transcription unit, the cDNA was used as a probe to isolate genomic DNA clones from a λ phage library. Restriction enzyme analysis of both the selD-like cDNA and its genomic counterpart, cross-hybridization experiments on Southern blots and DNA sequence comparison between cDNA and the corresponding genomic DNA allowed us to establish the structure of the gene (Figure 1b and d). The transcription region of selD-like expands over roughly 2 kb. The primary selD-like transcript contains a single intron in the 5′ untranslated region and is spliced into a 1.5 kb mature transcript.

Sequence analysis of the selD-like gene and comparison of the Drosophila SelD to other known SelD proteins

Sequence analysis of the selD-like cDNA (Figure 1d) revealed that the 1540 bp transcript contains 123 bp of non-translated 5′ sequence and a single polyadenylation signal followed by a poly(A) tail extending the 193 bp 3′ untranslated region. The putative translation start site (AGCAA-GATG) corresponds to the Drosophila consensus sequence C/AAAA/CATG (Cavener, 1987). The coding sequence extends over a 1194 bp open reading frame that codes for a protein of 398 amino acid residues. This putative protein can be aligned with other SelD proteins from all evolutionary domains (Figure 2). Interestingly, the amino acid residue in the catalytic site, corresponding to cysteine 17 in the E. coli sequence (Leinfelder et al., 1990), is arginine. With one exception, namely the human SelDI, all other known SelD proteins have cysteine or a selenocysteine in this position.

Expression in E. coli and purification of the Drosophila selD-like gene product (Dm-SelD)

In the plasmid isolated from the original 2AZAP clone (pDmD-SelD) the selD-like gene is already under the control of the lac promoter. However, no band corresponding to the product of selD-like, Dm-SelD, could be detected in Coomassie brilliant blue-stained gels when cells harboring this plasmid were induced with 0.5 mM IPTG. This was not unexpected, since the selD-like gene has no ribosome binding site of E. coli type and, hence, will probably not be translated. In αZAP there is a possibility for a fusion between lacZ and the cloned gene, in pDMD-SelD, however, the reading frame of lacZ is in the −1 frame compared to that of selD. To overcome this problem and to increase the production of Dm-SelD, two plasmids were constructed. In one of them, pBP46, the expression of the selD-like gene was set under the control of both the lac promoter and the lacZ Shine-Dalgarno motif. In the other, pT7-DmD, selD-like expression was controlled by the phage T7 φ10 promoter and a synthetic Shine-Dalgarno sequence. For details on the plasmid construction, see Experimental Procedures.

The strain BL21/pT7-DmD produced large amounts of Dm-SelD at 37°C. However, when S30 extracts were made from these cells, the Dm-SelD protein was found almost exclusively in an insoluble form. This problem was overcome by cultivation of strain BL21/pT7-DmD at 37°C to an A_{600} of 0.5, whereafter the culture was shifted to +14°C. After one hour at this temperature, production of Dm-SelD was induced by the addition of 0.1 mM IPTG and the culture was left at +14°C for approximately 30 hours. Generally about 50% of the protein produced at +14°C turned out to be in a soluble form (Figure 3). Dm-SelD was purified from cells grown in LB medium at +14°C. Cells were broken and an S100 extract was made. After ammonium sulfate-precipitation the fractions containing Dm-SelD were used for further purification by ion-exchange chromatography and gel filtration (see Experimental Procedures). After the gel-filtration chromatography the Dm-SelD protein was in an apparently homogeneous state (Figure 3). N-terminal sequencing proved its authenticity with the predicted product of selD-like and confirmed the translational start site deduced from the nucleotide sequence (see Figure 1), with the N-terminal methionine residue being cleaved off (data not shown).

In vitro activity of Dm-SelD

Selenophosphate synthetase catalyzes the reaction:

\[ \text{ATP + HSe}^- \rightarrow \text{selenophosphate + AMP + Pi} \]

The selenide-dependent hydrolysis of ATP to AMP was used as an assay for the selenophosphate synthetase activity of Dm-SelD (Ehrenreich et al., 1992). The experiments were performed under anaerobic conditions. Controls without selenide were included as well as controls with E. coli SelD instead of Dm-SelD. As shown in Figure 4, no selenophosphate was found with Dm-SelD, whereas a strong selenide-dependent activity was obtained with E. coli SelD.
One of the selenocysteine-containing proteins in *E. coli* is the formate dehydrogenase H (FDH-H). The *in vivo* activity of this enzyme can be measured by providing cells with formate as the natural substrate and benzyl viologen as an artificial electron acceptor (Mandrand-Berthelot *et al.*, 1978).

The plasmid pBP46 was used to transform the selenophosphate synthetase-deficient strain BP005.
(selD::cat). To examine whether the transformants contain FDH activity they were first grown on plates in an anaerobic vial filled with N₂ at 30°C or 37°C, and then shifted to +14°C, as it was shown that the Dm-SelD is produced in a soluble form only at 14°C. It was found that at none of these temperatures could the pBP46 or similar selD-like containing plasmids complement the FDH deficiency of the E. coli selD mutant. Thus, both in vitro and in vivo experiments consistently show that the SelD-like protein of Drosophila lacks selenide-dependent selenophosphate synthetase activity.

Expression of selD-like in the Drosophila embryo

The expression pattern of the selD-like gene during embryogenesis was examined by in situ hybridization with the digoxigenin-labeled selD-like cDNA probe to whole-mount preparations of embryos. Figure 5c to f indicates that selD-like transcripts are ubiquitously distributed, at low levels, throughout the developing embryo. From early gastrulation onwards (Figure 5c), selD-like transcripts became highly enriched in the endodermal anlagen of the midgut especially in the gastric caeca and in the developing nervous system (Figure 5d and e). In both locations they remain at high levels until the end of embryogenesis (Figure 5f).

Discussion

Genes coding for components required for selenocysteine biosynthesis and insertion into polypeptides have been sequenced in the last few years from a number of organisms. A comparison of the sequences revealed that SelD, the monoselenophosphate synthetase, was much more conserved during evolution than the primary structures of tRNAsec (Tormay et al., 1994), SelB (Kromayer et al., 1996) or selenocysteine synthase (unpublished results). This is surprising, since SelD was thought to exclusively catalyze the transfer of a phosphate moiety from ATP to the HSe⁻ anion, a reaction that appeared a priori not to be subject to such strong sequence constraints as those for the other sel gene products that undergo macromolecular interactions.

The SelD homologs characterized in the past few years and in this work can be divided into two major classes. Class I is characterized by the existence of a cysteine or selenocysteine residue in the active site and by catalyzing the selenide-dependent formation of MSP (Leinfelder et al., 1990; Guimaraes et al., 1996; Wilting et al., 1997). Replacement of the selenocysteine residue by cysteine decreased but did not abolish enzyme activity.
(Kim et al., 1997), whereas exchange of the Cys17 residue of the E. coli enzyme against serine destroyed activity (Kim et al., 1992).

Class II SelD variants have now been demonstrated for man (Low et al., 1995) and for Drosophila in the present work. This class, although sharing a high level of sequence similarity with class I enzymes, does not have the active-site cysteine or selenocysteine residue in position 17 of the E. coli enzyme. Our results show that the SelD-like protein from Drosophila does not catalyze the selenide-dependent selenophosphate formation in vitro and that its gene is unable to complete a selD deletion in an E. coli mutant in vivo. This finding correlates well with the fact that targeted replacement of Cys17 from E. coli SelD inactivates the enzyme completely (Kim et al., 1992) and that the human and Drosophila variants with threonine and arginine possess chemically diverse amino acids in the equivalent position. Finally, recent experiments by Kim et al. (1997) with the human class I enzyme showed that the replacement of selenocysteine by cysteine in position 17 (E. coli nomenclature) drastically reduced enzyme activity, indicating that this amino acid residue, and not some other cysteine residue is essential also for eukaryotic selenophosphate synthetase activities.

Our opinion, that class II SelD proteins are unable to catalyze the selenide-dependent selenophosphate synthesis, at first sight appears to be at variance with a report in the literature that the gene for human SelD1 complements an E. coli selD lesion (albeit weakly) and also stimulates $^{75}$Se incorporation into a selenoprotein in mammalian cells (Low et al., 1995). In view of the fact that organisms that possess class II enzymes also contain a class I protein, it may well be that the SelD class II proteins have some function in selenium metabolism different from catalyzing selenide-dependent selenophosphate formation. From the

![Figure 3. Purification of Dm-SelD. Lane 1, whole-cell extract before induction. Lane 2, whole-cell extract after 20 hours of induction with 0.1 mM IPTG at +14 C. Lane 3, S30 supernatant. Lane 4, S30 pellet. Lane 5, S100 supernatant. Lane 6, Proteins precipitated in the 35 to 50% saturated ammonium sulfate fraction. Lane 7, Dm-SelD after the Q-Sepharose anion-exchange chromatography. Lane 8, Dm-SelD after the Superdex 75 gel-filtration.](image)

![Figure 4. The selenide-dependent hydrolysis of $[^2\text{P}]\text{ATP}$ by Dm-SelD from Drosophila and SelD from E. coli. Samples were taken at the time-points indicated and separated by PEl-cellulose thin-layer chromatography. The selenite added is chemically reduced by DTT in the reaction assay.](image)

![Figure 5. Expression of selD-like as revealed by in situ hybridization to whole-mount embryos preparations of embryos at various stages of Drosophila embryogenesis. a, Embryo at cellular blastoderm stage; note the strong maternal contribution. b, Embryo during gastrulation shows ubiquitous expression. c, Embryo at maximum elongated germband (stage 11) shows weak overall expression and enriched levels of transcripts in the invaginating gut anlagen and in the mesoderm. d, At stage 13, transcripts are strongly expressed in the midgut and in the nervous system. e, At stage 14 transcripts continue to persist in the central nervous system and the midgut. Note the concentration of the transcript in the anlagen of the gastric caeca (arrowhead). f, In the differentiated embryo (stage 17) selD-like is expressed in the central nervous system, brain and midgut with strong enhancement in the gastric caeca (arrowhead). Stages are according to Campos-Ortega & Hartenstein (1985).](image)
strong conservation of the ATP-binding sequence motif (Figure 2) and from the fact that mutagenesis of residues within this motif affects the stimulation of $^{75}\text{Se}$ incorporation into protein in transfected mammalian cells (Low et al., 1995), it appears likely that this unidentified reaction requires ATP binding and/or hydrolysis. The sequence identity of 67% between human SelD1 and Drosophila SelD supports the notion that this function is homologous in invertebrates and vertebrates. Since Drosophila genes are easily accessible to mutagenesis we shall perform targeted inactivation of selD-like to elucidate its role in vivo.

**Experimental Procedures**

**Cloning and characterization of selD-like DNA**

The selD-like cDNA clone was obtained during a low stringency screening of a λ ZAP cDNA phage library prepared from 0 to 18 hours old embryos (Stratagene). The corresponding genomic DNA of the selD-like transcription unit (see Figure 1b) was isolated from a λ Fix genomic Drosophila DNA library (Canton S; Stratagene). Screening of DNA libraries, the structural analysis of the selD-like gene and its transcripts as well as cytogenetic analysis by in situ hybridization to polytene chromosomes obtained from third instar salivary glands were done as described (Hartmann & JaÈckle, 1995). Purification of Dm-SelD was performed as follows. Cells were harvested and resuspended in buffer A (50 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT), and broken by three passages through a French press cell at 16,000 psi (1 psi = 6.9 kPa). The lysate was centrifuged at 30,000 g for 30 minutes to remove cell debris, and the supernatant was further centrifuged at 100,000 g for two hours to obtain an S100 extract. The proteins precipitating between 35% and 50% ammonium sulfate saturation were dialyzed and used for further purification. The first chromatographic step was a Q-Sepharose anion-exchange chromatography. The proteins were eluted with an 0.1 M to 1 M KCl gradient in buffer A containing 100 mM KCl. After the gel filtration chromatography the Dm-SelD protein contained no detectable contaminating protein (Figure 3).

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**Selenide-dependent ATPase assay of SelD**

As test for the selenophosphate synthetase activity of SelD the selenide- dependent hydrolysis of ATP to AMP was used (Ehrenreich et al., 1992). These experiments were performed under strictly anaerobic conditions. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 20 mM ATP, 5 mM MgCl$_2$, 2 mM DTT, 100 μM selenide and 2000 cpn/ml [32P]ATP in a total volume of 100 μl. Controls without selenide were included as well as controls with E. coli SelD instead of Drosophila Dm-SelD. The enzyme reaction was started by the addition of 600 pmol of SelD. At the time-points given in Figure 4 samples (10 μl) were withdrawn and the reaction stopped by addition of an equal volume of 2 M formic acid. The samples were lyophilized and resuspended in 2 μl of water; 1 μl of each sample was chromatographed on polyethyleneimine-cellulose thin-layer plates with non-radioactive ATP, ADP and AMP as markers. The plates were dried and autoradiographed for four hours.

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