PAPS-reductase of *Escherichia coli*

Correlating the N-terminal amino acid sequence with the DNA of gene *cys* H

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The DNA of the gene complementing a PAPS-reductase-deficient strain of *Escherichia coli* was sequenced. The N-terminal amino acid sequence of the purified PAPS-reductase confirmed that *cys* H is the structural gene for this enzyme. The open reading frame extends 732 bases and encodes for a peptide of $M_r = 27927$. The gene product is functionally active when supplemented with thioredoxin and immunologically related with the wild type enzyme.

3'-Phospho-adenylylsulfate reductase; Phenotypic complementation; *cys* H DNA; PT7 expression; Gene product; (*Escherichia coli*)

1. INTRODUCTION

In most bacteria, fungi and plants cysteine is formed from sulfide and *O*-acetylserine. The requirement for sulfide can be met by the assimilatory reduction of inorganic sulfate. This process consists of a sequence of 5 enzymatic steps. It involves the action of ATP-sulfurylase, APS-kinase, PAPS-reductase, sulfite-reductase and *O*-acetylserine sulfhydrylase [1]. The PAPS-reductase catalyzes the first reductive step leading to the formation of sulfite from the activated sulfate (3'-phospho-adenylylsulfate) with thioredoxin as reductant [2,3].

Auxotrophic mutants of *E. coli* or *S. typhimurium*, mainly obtained by deletion or conjugation experiments, were used for assigning gene loci and enzymes (i.e. *cys* D: ATP-sulfurylase, *cys* C: APS-kinase, *cys* H: PAPS-reductase, and *cys* IJ: sulfite reductase [4]).

The structural genes of the sulfate-reducing enzymes are located in a cluster at 59–60 min of the chromosomal map. So far, information about the organization and structure of the operons still is fragmentary or, as in the most recent publications, relies entirely on the expression of cloned restriction fragments and complementation of the corresponding auxotrophic phenotypes [5,6]. Overexpression, as indicated by increased activity of the enzyme expected to be coded by the cloned fragment, was found for *cys* DN expressing ATP-sulfurylase and of *cys* C expressing APS-kinase activity [5]. By complementing *cys* HIJ mutants with a large fragment (18.5 kb) increased sulfite reductase activity was observed but a functional product coded by *cys* H, i.e.: the PAPS-reductase, was not identified [6,7].

The major aim of the present work is to correlate the DNA structure of *cys* H with the enzyme protein it is encoding for. Hence, the gene was isolated, identified and sequenced. Correspondingly, the recombinant PAPS-reductase was purified as to establish its amino acid sequence and, finally, the functional gene product was compared by biochemical and immunological methods with the enzyme protein as obtained from a cysH$^+$ wild type of *E. coli*. The data presented in this note are focusing on the correlation between DNA and amino acid sequence of the gene *cys* H – a complete report will be published separately.

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Abbreviations: APS, adenylylsulfate; PAPS, 3'-phospho-adenylylsulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession number no. Y07525

2. EXPERIMENTAL

E. coli strain JM 96 [8] (CGSC No.: 5746) was complemented with *Sau*3A restricted wild type DNA (3–6 kb average length) ligated into pBR322. Of the resultant complementing plasmids, pCH10 containing a 3.5 kb *Eco*RI/*Sal*I insert was subcloned into the sequencing vectors pBluescript SK$^+$ and KS$^+$. Deletion mutants were constructed according to Henikoff [9] and sequenced by the chain terminator method [10]. The cloned gene was expressed with the Tabor and Richardson [11] pT7 expression system subcloning the 3.5 kb *Eco*RI/*Sal*I fragment of pCH10 in pBluescript KS$^+$ plasmids which were propagated in strain K38 containing the plasmid pGP1-2.

Thioredoxin-dependent PAPS-reductase was assayed as described in [3]. It was purified from the complemented cysH$^+$ mutant JM96

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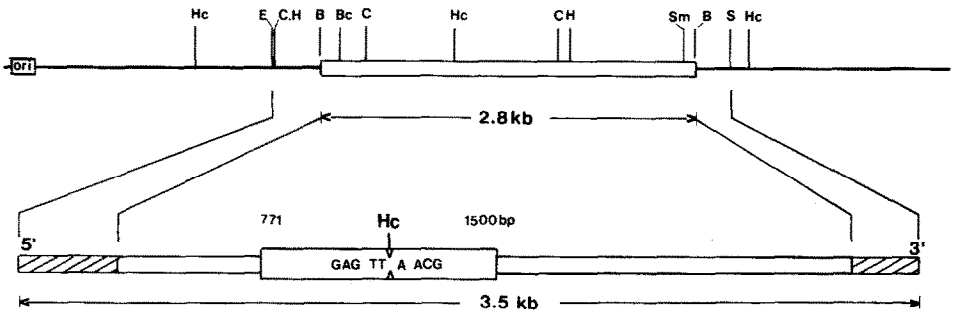

Fig.1. Plasmid pCH10. Structure of the plasmid pBR322 containing the 2.8 kb *Sau*3A insert (boxed); the *Eco*RI/*Sal*I fragment (3.5 kb) was sequenced. The *cys* H gene is contained in the large box (bp 771 to bp 1500). Restriction sites: B, *Bam*HI; Bc, *Bcl*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*dII (*Hin*cII); Sm, *Sma*I.

grown on M9 minimal medium containing 0.2% glucose. A nearly homogeneous enzyme protein was obtained within three chromatographic steps: employing (a) Phenyl Sepharose CL 6B, (b) Blue Sepharose, and (c) Red Sepharose (unpublished). For amino acid sequencing the protein was purified to homogeneity by PAGE using a combination of buffer and SDS gels [12,13]. The N-terminal amino acids were determined by automated Edman degradation with on-line detection of the amino acids [14].

Polyclonal antibodies against electrophoretically pure protein were raised in rabbits following the immunization regimen described in Catty [15]. Validation of the immunoreaction was by cross-dot blotting (unpublished) and of specificity by Western immunoblotting according to [16].

Protocols for transformation were as described in [17], and isolation of genomic DNA or plasmids, ligation and restriction followed the procedures as outlined in [18] or as specified by the manufacturer of the enzymes. Substrates (PAPS and thioredoxin) were prepared as described in [19,20].

3. RESULTS

Complementation of the *E. coli* S-auxotrophic mutant JM96 (*cys* H56) with *Sau*3A restricted wild type DNA ligated into pBR322 yielded 75 cysH$^+$ phenotypes per 10^5 transformants. The high rate of transformation was obtained through P1 transduction of *hsd* R$^-$ from a Tn10 mutated donor (LCK8) to JM96 as recipient (details omitted). Of these complemented cysH$^+$ phenotypes 12 were analyzed for PAPS-reductase activity – in two of the complementing clones the activity of the enzyme was increased by a factor of ≥ 100 (pCH07 and pCH10) as compared to the wild type strain K12. The enzyme isolated from both clones retained its requirement for thioredoxin as cofactor (table 1). The plasmids did not complement the mutant strains *cys* D, *cys* I or *cys* J (i.e.: JM221, JM246 and AT713, data omitted).

Table 1

PAPS-reductase activity in mutant JM96 complemented with plasmids pCH

Strain	Thioredoxin	
	Omitted	Added
Control (wild type K12)	0.018	0.170*
pCH7	0.021	1.87
pCH10	0.02	2.05

* nmol ^{35}S sulfite formed from PAPS per mg protein per min [3]

The plasmid pCH10 was used for further investigations of the DNA and the gene product. The length of the inserted DNA was 2.8 kb. It was ligated into the *Bam*HI site of the plasmid though sequencing of the DNA revealed that the ligation site was not retained (details of the sequence will be published separately). For subcloning the plasmid pCH10 was restricted with *Eco*RI and *Sal*I yielding a 3.5 kb restriction fragment including small parts of the right and left arm of the vector DNA. The restriction pattern was as outlined in fig.1. *Hin*dII (*Hinc*) was the only restriction enzyme to cut within the gene *cys* H.

A total of 40 deletion mutants was sequenced in order to establish the DNA sequence of *cys* H, 95% of the gene was sequenced in both directions. The structural

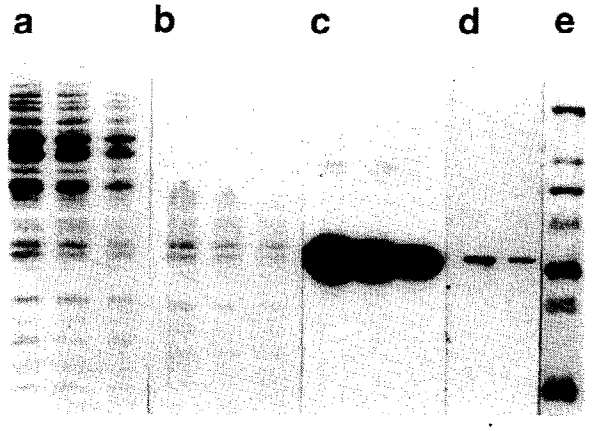

Fig.2. Expression of the plasmid encoded PAPS-reductase. The *Eco*RI/*Sal*I$^-$ fragment of pCH10 was subcloned in pBluescript KS$^+$ which was used to transform the strain K38 harbouring pGP1-2. After thermal induction and rifampicin treatment the cell homogenates were analyzed by SDS-gradient PAGE for ^{35}S-methionine labelled proteins. a, K38 transformed with pBluescript KS$^+$; b, proteins as expressed after induction; c, autoradiography of lanes in b; d, recombinant PAPS-reductase 5 and 1 μg; e, weight standards: 66, 45, 36, 29, 24, 20, 14 kDa.

gene for PAPS-reductase was 732 bp in length coding for a peptide of M_r 27 927 (calculated from its deduced amino acid sequence). Expression of the cloned DNA using the Tabor and Richardson pT7-pGP1-2 expression system [11] gave rise to a single protein of M_r ≈ 28 000 (fig.2). No products were expressed from the 3′-5′ anti-sense strand of the cloned fragment.

In the cysH$^+$ complemented mutant of JM96 a peptide of similar weight (i.e. 28 000) was formed in the late phase of growth. Western immunoblotting confirmed the identity of this peptide with the protein purified as PAPS-reductase and used as the antigen. In cell extracts from the wild type K12 this protein was barely detectable by immunoblotting, yet reactivity towards the enriched enzyme purified from the same source confirmed its identity as PAPS-reductase (fig.3). The protein expressed in the mutant strain JM96 grown on sulfate was purified to homogeneity and sequenced by Edman degradation. An alignment of 15 residues of the N-terminal amino acids with the amino acids deduced from the DNA sequence showed a complete homology except for methionine (fig.4) which is lacking from the mature protein. As excision of the methionine is a common phenomenon in protein processing, the N-terminal amino acids were a precise translation of the cloned DNA's first 15 coding triplets. Moreover, it confirms the correct positioning of the reading frame.

4. DISCUSSION

The structural gene for PAPS-reductase from *Escherichia coli* was detected by complementation of the *cys* H56 mutant JM96 with a 2.8 kb *Sau*3A restriction fragment of genomic DNA from *E. coli* K12. Using pBR322 as vector the fragment was ligated into the *Bam*HI cloning site. The resultant plasmids were used for transformation of the *cys* H56 mutant. As the mutant JM96 was made restriction deficient (*hsd* R2) by P1 transduction the transformation rate was considerably higher than reported by Li et al. [6] employing the same vector yet in the unmodified mutant as host.

The cloned DNA in pCH10 consisted of 2866 bp with an open reading frame of 732 bp. pCH10 did not complement the mutants *cys* I, *cys* J, or *cys* C. The open reading frame coded for a peptide with a mass of 27 927. In vivo expression of this gene with the pT7-pGP1-2 expression system [10] gave a single product with a size of ~28–29 000. Li et al. [6] used the maxicell expression of a 18.3 kb *Pst*I insert comprising *cys* HIJ which yielded peptides of M_r 27 000, 60 000, and 57 000. The authors only ascertained sulfite reductase as being expressed from this insert but suggested that the M_r 27 000 gene product was the PAPS-reductase. A similar observation concerning the *cys* H gene product was most recently published by Ostrowski et al. [7].

Two (out of 12) *cys* H complementing plasmids (pCH07 and pCH10) conferred high levels of PAPS-reductase activity. The recombinant enzyme protein retained its requirement for thioredoxin as cosubstrate. The level of plasmid-encoded activity in the complemented mutant exceeded the activity of the strain K12 by a factor of ~100. (That this increase in activity was truly caused by overproduction of the enzyme rather than by modulation of specific activity was confirmed by Western immunoblotting, see below.)

The PAPS-reductase was purified to homogeneity from the complemented strain of JM96 containing pCH10. Practically pure enzyme (98%) was obtained by three chromatographic steps employing hydrophobic and pseudo-affinity matrices. The molecular mass de-

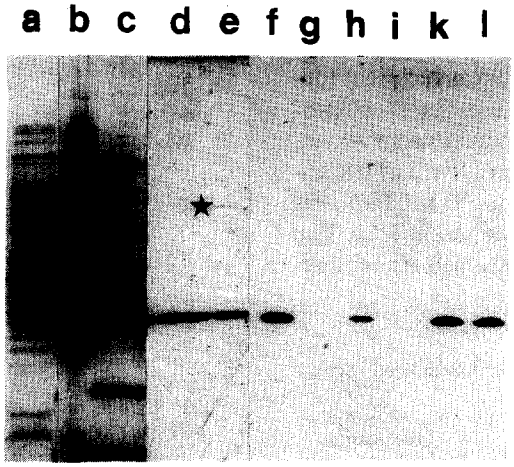

Fig.3. Western immunoblotting of recombinant and wild-type PAPS-reductase. a, cell homogenate of JM96 harbouring pCH10; b, enriched PAPS-reductase from *E. coli* K12; c, molecular weight marker proteins: M_r 66, 45, 36, 29, 24, 20, 14 kDa; d, immunoblot of lane a, lane e corresponds to lane b, asterisk indicates the position of the dimer; f, JM96 harbouring pCH10 grown on sulfate (M9 medium); g, mutant JM96 grown on rich medium (LB); h, JM96 harbouring pCH10 grown on LB; i, wild-type K12 grown on LB; k and l, JM96 harbouring pCH10 grown on sulfate + 1.5 mM (k) and + 5 mM cysteine (l).

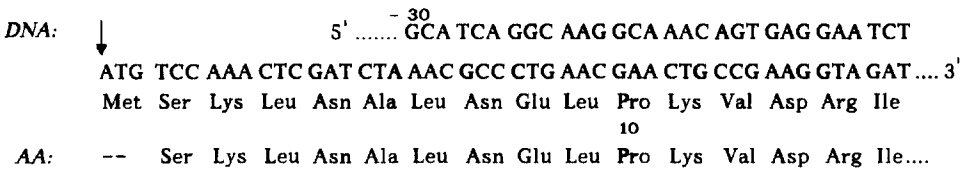

Fig.4. DNA sequence in pCH10 representing the initiation site of translation for *cys* H (start codon as indicated by arrow) was identified by comparison of the deduced amino acid sequence with the amino terminal sequence of the purified PAPS-reductase from *E. coli.*

termined by SDS-PAGE was 28–29 000. This weight agrees well with previous data using the in vivo expression system. In addition, it supports an earlier observation by Tsang [2] stating an M_r of 58 000 for the active dimer of the K12 PAPS-reductase.

The cross-reactivity of polyclonal immunoglobulins raised against the gene product of pCH10 with the PAPS-reductase isolated from a wild type K12 was interpreted as evidence for identity of the antigens. As the immunoglobulins were monospecific the protein was identified in complete cell extracts of the mutant strain separated by SDS-PAGE. In mutants grown on rich medium and in the wild type the antigenic protein was barely detectable, but when the mutant harbouring pCH10 was transferred from rich to minimal medium (details not shown) the amount of immunodetectable PAPS-reductase increased drastically within 60 min.

The most conclusive evidence of identity was obtained when the homogeneous PAPS-reductase was sequenced by Edman degradation and the amino acid sequence aligned with the DNA sequence. Of the 15 amino acid residues sequenced from the N-terminus all gave a perfect match with the coding triplets of the pCH10 DNA sequence. Moreover, as the reading frame thus identified coded for a peptide of M_r 27 927 we believe to have isolated the gene *cys* H, i.e.: the structural gene coding for PAPS-reductase.

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