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# An impaired peroxisomal targeting sequence leading to an unusual bicompartmental distribution of cytosolic epoxide hydrolase

Michael Arand, Michael Knehr, Helmut Thomas*, Hans-Dieter Zeller** and Franz Oesch

*Institute of Toxicology, University of Mainz, Obere Zahlbacherstr. 67, D-6500 Mainz, Germany*

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To gain an understanding of the mechanism by which the subcellular distribution of cytosolic epoxide hydrolase (cEH) is directed, we have analyzed the carboxy terminal region of rat liver cEH by means of cDNA cloning to define the structure of its possible peroxisomal targeting sequence (PTS). Purified cEH was subjected to peptide analysis following endoproteinase Glu-C digestion and HPLC-separation of the fragments. The obtained sequence information was used to perform PCR experiments resulting in the isolation of a 680 bp cDNA clone encoding the carboxy terminus of cEH. The deduced amino acid sequence displays a terminal tripeptide Ser-Lys-Ile which is highly homologous to the PTS (Ser-Lys-Leu) found in other peroxisomal enzymes. This slight difference appears to be sufficient to convert the signal sequence into an impaired and therefore ambivalent PTS, directing the enzyme partly to the peroxisomes and allowing part to reside in the cytosol.

cEH; Peptide analysis; PCR; cDNA sequence; Amino acid sequence; PTS

## 1. INTRODUCTION

Cytosolic epoxide hydrolase is an enzyme involved in the detoxication of a variety of potentially carcinogenic epoxides including *trans*-stilbene oxide [1–3]. It displays a low level of expression in the livers of untreated rats. Efficient induction of cEH may be achieved by hypolipidemic agents such as clofibrate and tiadenol [3–5]. These compounds also trigger the proliferation of peroxisomes in the liver and kidney of rodents [6–8]. Based upon this correlation, a peroxisomal location of cEH was postulated and has recently been further investigated by purification of the enzyme from hepatic peroxisomal fractions of mice [9,10]. Only minor, if any, differences between the proteins purified from either cytosol or peroxisomes were observed in these studies.

Peroxisomal proteins are synthesized on free ribosomes in the cytoplasm and are subsequently transported to the organelle resulting in the integration into the peroxisomal membrane or in the uptake into the peroxisomal lumen [11]. Recently, a peroxisomal targeting signal (PTS), i.e. the carboxy terminal tripeptide Ser-Lys-Leu and its conservative derivatives, has been identified as a consensus sequence in many of the peroxisomally localized proteins, and experimental evidence for its function in sorting out the peroxisomal proteins has been provided [12–14]. We now report the identification of a PTS-like tripeptide at the carboxy terminus of the cEH protein sequence as deduced from the cDNA sequence. In contrast to a previous report [14] which found this sequence to be inactive in targeting proteins to peroxisomes, we conclude that in the case of cEH it provides an ambivalent signal directing only a part of the protein to the peroxisomes while leaving a remainder residing in the cytosol.

*Abbreviations:* cEH, cytosolic epoxide hydrolase; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; TFA, trifluoro acetic acid; uv, ultra-violet; PTS, peroxisomal targeting sequence; dITp, 2′-deoxyinosine-5′-triphosphate.

**Present address:* Ciba-Geigy AG, R-1066.4.32, CH-4002 Basel, Switzerland.

***Present address:* Boehringer Mannheim GmbH, Dept. BN-Q, Nonnenwald 2, D-8122, Penzberg, Germany.

*Correspondence address:* M. Arand, Institute of Toxicology, University of Mainz, Obere Zahlbacherstr. 67, D-6500 Mainz, Germany. Fax: (49) (6131) 230506.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Tiadenol was obtained from Aldrich Chemie, Steinheim, Germany. AMV reverse transcriptase, endoproteinase Glu-C (protease V8), RNAsin and Klenow fragment were from Boehringer Mannheim, Mannheim, Germany. T4-polynucleotide kinase, a T7-sequencing kit and an mRNA purification kit were purchased from Pharmacia LKB, Freiburg, Germany. *Taq* DNA polymerase (AmpliTaq) was from Perkin Elmer, Ueberlingen, Germany.

### 2.2. *Peptide sequence analysis*

Cytosolic epoxide hydrolase was purified from the livers of 7 male tiadenol-induced rats according to a modified procedure (manuscript in preparation) based on the method of Hammock and coworkers [15]. The enzyme protein (900 μg) was directly digested with endoproteinase Glu-C (5% w/w) in potassium phosphate buffer (50 mM, pH 7.8) for 18 h. The incubation mixture was brought to pH 3 by addition of TFA, centrifuged for 1 min at 12 000 rpm, and the supernatant was taken for the HPLC-separation of the resulting peptides.

Chromatography was performed on a Vydac C18 RP column (4.6 × 250 mm) using a discontinuous gradient from 1% to 70% acetonitril in 0.1% aqueous TFA for 3 h at a flow rate of 1 ml per min. Eight separations were carried out under identical conditions with a protein load of approximately 100 μg each. The individual peaks of the separate runs were then pooled and re-chromatographed using the same column and solvent system under optimized conditions. The resulting pure fractions were lyophilized and subjected to gas-phase sequencing on an Applied Biosystems sequencer.

2.3. *Specific amplification of cEH-cDNA*

Different oligonucleotides containing dITP at ambiguous positions were synthesized on a Millipore DNA synthesizer using the information of the obtained peptide sequences. A cDNA pool was prepared by reverse transcription of mRNA from the liver of a tiadenol-treated rat. About 50 ng of this cDNA were subjected per experiment to subsequent PCR analysis. The incubation mixtures (50 μl) were set up according to the recommendations of the manufacturer containing one or more of the self-constructed *sense* cEH oligonucleotides as 5′-end primer (a total of 20 pmol) and a 27-mer oligo-dT as 3′-end primer (20 pmol). Samples were taken through 30 cycles of amplification (94°C for 1 min, 55°C for 1.5 min, 72°C for 2.5 min, each), preceded by 5 min of denaturation at 95°C before enzyme addition and followed by 5 min of terminal elongation at 72°C. Analyses of the PCR products were performed on 1% agarose gels containing ethidium bromide for uv detection of DNA.

2.4. *Isolation and characterization of a cEH-cDNA fragment*

One specific cDNA fragment (680 bp) resulting from the PCR amplification using the primer $O_{Ia}$ was isolated by extraction following preparative agarose gel electrophoresis. The DNA was prepared for blunt-end ligation by treatment with Klenow fragment and T4-polynucleotide kinase. The success of this procedure was checked by self-ligation resulting in the formation of concatemers. The fragment was ligated into a *Sma*I-cut and subsequently dephosphorylated pUC19 plasmid, and competent *E. coli* C600 were transformed with the resulting construct using standard procedures [16]. The recombinant plasmid was isolated and subjected to DNA sequencing according to Sanger [17] using a commercial kit.

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I:      Tyr-Tyr-Ile-Gln-Gln-Phe-Lys-Lys-Ser-Gly-Phe-Trp-Trp

  OIa:  TAC TAC ATI CAG CAG TTC AAG AAG

  OIb:  TAC TAT ATI CAG CAG TTT AAG AAG

  OIc:  TAT TAI ATI CAI CAI TTI AAI AAG

II:     Val-Asn-Gln-Ile-Leu-Ile-Lys-Trp-Leu-Lys-Trp

  OIIa: GTG AAT CAG ATI CTI ATI AAG TGG CTI AAG TGG

  OIIb: GTG AAC CAG ATC CTI ATI AAG TGG CTG AAG TGG

  OIIc: GTI AAI CAI ATI CTI ATI AAI TGG CTI AAG TGG

III:    Asn-Trp-Ile-Pro-Phe-Leu-Lys-Arg-Gly-?-Ile-Glu

IV:     Leu-Ala-Leu-Pro-Ser-Ile-Ala-Gly-Val-Leu

V:      Met-Lys-Gly-Lys-Ile-Thr-Phe

VI:     Arg-Ser-Ile-Pro-Val-Phe-Asn

VII:    Met-Gly-Gly-Ile-Leu

VIII:   Lys-Asn-Met

IX:     Thr-Gly
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Fig. 1. Peptide sequences of the endoproteinase Glu-C fragments of cEH. Below the sequences of peptides I and II, the derived oligonucleotides used as PCR primers are shown.

## 3. RESULTS

The peptide analysis of cytosolic epoxide hydrolase resulted in the identification of 9 amino acid sequences (Fig. 1). Peptide sequences I and II were selected for the construction of oligonucleotides (Fig. 1) in order to

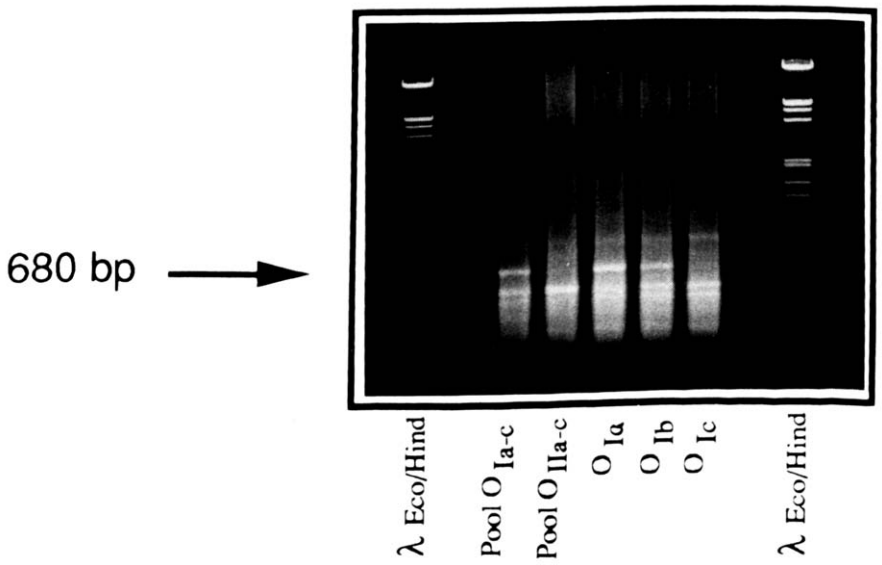


Fig. 2. Amplification of cEH-cDNA. PCR reactions were performed and analysed as described in section 2.3. using the indicated oligonucleotide(s) for specific priming. Aliquots (5 μl) of the incubation mixtures were subjected to electrophoresis on a 1% agarose gel containing 0.4 μg/ml of ethidium bromide for the direct visualization of DNA. *Eco*RI/*Hin*dIII-double-digested λ DNA was run along with the samples as a molecular weight marker (100 ng in the left lane, 200 ng in the right lane). The arrow marks the position of the amplified 680 bp cEH-cDNA.

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TAC TAC ATC CAG CAG TTC AAG AAG TCT GGC TTC AGA GGC CCT CTA AAC TGG TAT CGA AAC      60
Tyr Tyr Ile Gln Gln Phe Lys Lys Ser Gly Phe Arg Gly Pro Leu Asn Trp Tyr Arg Asn      20

ACA GAA AGA AAC TGG AAG TGG AGC TGT AAG GCG TTG GGA AGG AAG ATC TTG GTC CCT GCC     120
Thr Glu Arg Asn Trp Lys Trp Ser Cys Lys Ala Leu Gly Arg Lys Ile Leu Val Pro Ala      40

CTG ATG GTC ACA GCT GAG AAG GAC ATT GTA CTC CGT CCT GAA ATG TCC AAG AAC ATG GAA     180
Leu Met Val Thr Ala Glu Lys Asp Ile Val Leu Arg Pro Glu Met Ser Lys Asn Met Glu      60

AAC TGG ATC CCT TTC CTG AAA AGG GGA CAC ATC GAA GAC TGT GGT CAC TGG ACA CAG ATA     240
Asn Trp Ile Pro Phe Leu Lys Arg Gly His Ile Glu Asp Cys Gly His Trp Thr Gln Ile      80

GAG AAA CCG GCA GAG GTG AAC CAG ATT CTC ATC AAG TGG CTG AAG ACT GAA ATC CAG AAC     300
Glu Lys Pro Ala Glu Val Asn Gln Ile Leu Ile Lys Trp Leu Lys Thr Glu Ile Gln Asn     100

CCA TCG GTG ACC TCC AAG ATT TAG CCAGTGGCGTGTCCTCTGCTGGGACACATTTTCATTTCTGACGTGGC     371
Pro Ser Val Thr Ser Lys Ile ***                                                     107

CTTATCCACAGCCAGCAGCATCGTTCTTTTGCCAGCAGTGATTTTCTTTAAATGAAAATGATCAGATGTGATGTAATTT     450
TAGATCAGGAAGAAAGTGGTGTGTCTGATTCTTTTGAGGATGACTGTATCAGTAAAGGAGAGATCACACCCCAATAGGG     529
AGGCATGGGGCAGCCCAGTTTGTACCTTTGTAGCCAAACCCAAGCCTGCTCTTTCTGAAGCAGCTGATCAGAGAGTAGG     608
GACCTTCATTCAATAAAGCTAAGGCCTTGGTGCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA      677
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Fig. 3. Nucleotide sequence and deduced amino acid sequence of the 3'-end of cEH-cDNA. The underlined amino acid sequences have already been identified by the preceding peptide analysis. The carboxy terminal peroxisomal targeting sequence is typed in bold face.

specifically amplify cEH-cDNA fragments by PCR in separate experiments. A 27-mer oligo-dT oligonucleotide served as the second primer in each of the different settings to allow for the amplification of the 3'-end of the cDNA in question. This strategy resulted in a significant loss of specificity of the amplification (increased formation of non-specific signals) as compared to experiments using 2 specific primers. However, it would have been impossible to obtain cDNA fragments encoding the carboxy terminus of cEH using 2 specific peptide-derived primers, and we thus had to use the unspecific oligo-dT primer in order to cover the region of interest.

Three different oligonucleotides were constructed for each of the 2 peptide sequences that differed among each other with respect to the base composition at ambiguous sites. In a first experiment, oligonucleotides corresponding to the same amino acid sequence were pooled for the use in PCR amplification. Multiple fragments were obtained with both settings obviously due to the application of the general oligo-dT primer. However, the reaction employing the oligonucleotide pool corresponding to peptide I resulted in the generation of one prominent species of an estimated size of 680 bp. In a second experiment, the 3 different oligonucleotides encoding for peptide I ($O_{Ia}$–$O_{Ic}$) were tested in separate set-ups for their potential to prime the amplification of the observed 680 bp cDNA fragment. Under the conditions given in section 2.3, one of them ($O_{Ia}$) proved to be very efficient in supporting the synthesis of the fragment in question, while the others gave a weaker ($O_{Ib}$) or no ($O_{Ic}$) detectable amount of the specific product. The results of the 2 sets of PCR are given in Fig. 2.

The 680 bp cDNA fragment was subcloned into pUC19 for cDNA sequencing. The sequence analysis revealed a 677 cDNA that was flanked by the self-constructed oligonucleotide sequence at the 5'-end and an oligo-dA tail at the 3'-end (Fig. 3). An open reading frame of 315 bp was detected in frame with the peptide-encoding primer sequence. The deduced amino acid sequence contained another 3 of the peptides that had already been identified by peptide sequencing, including peptide II, thus proving the identity of the isolate as being a cEH-cDNA. It also revealed a sequence error in peptide II at the last carboxy terminal amino acid residue resulting in a fatal three-base mismatch at the 3'-end of the primers $O_{IIa}$–$O_{IIc}$ constructed for PCR with the cDNA template. Therefore, no efficient amplification could be achieved using the latter oligonucleotides.

At the carboxy terminus of the deduced amino acid

sequence a tripeptide Ser-Lys-Ile could be identified that is almost identical to the common peroxisomal targeting signal Ser-Lys-Leu. As already pointed out, Gould and co-workers have reported that this specific tripeptide was unable to direct protein import into peroxisomes under their experimental conditions [14].

The nucleic acid sequence and the deduced amino acid sequence reported here have been submitted to the EMBL Data Library and have been assigned the accession number X60328 RAR CEH.

## 4. DISCUSSION

The observation of the concomitant induction of cEH with peroxisomal $\beta$-oxidation by hypolipidemic compounds [4,5] raided the question of the proper subcellular localization of this enzyme. Recently, Chang and Gill have presented a detailed study on the comparison of what they called cytosolic and peroxisomal epoxide hydrolases [10]. Both isolates displayed virtually identical physicochemical properties and similar biochemical qualities. The authors concluded that both species are almost identical and further speculated that they may just be different in possessing or missing a peroxisomal targeting sequence.

The nature of signal sequences triggering the import of proteins into peroxisomes has been extensively studied by the group of Gould [12–14]. They have identified the carboxy terminal tripeptide Ser-Lys-Leu as a sufficient peroxisomal targeting sequence. While several conservative mutations of this sequence have been shown to be also efficient in functioning as a PTS in those studies, the tripeptide Ser-Lys-Ile that we have found at the carboxy terminus of cEH has been reported to be inactive [14].

The findings of Gould and co-workers concerning the subcellular localization of proteins carrying the different variants of the PTS are based on immunofluorescence data. Since the cytosol surrounds the peroxisomes in the cell, a bicompartmental localization of a protein under investigation, i.e. its presence in both the cytosol and the peroxisomes, might be difficult to distinguish from an exclusively cytosolic localization under these experimental conditions. A less efficient targeting signal may thus lead to an apparently exclusive cytosolic location. The result of the present study strongly indicates that the tripeptide Ser-Lys-Ile is such a weak PTS since it is present at the carboxy terminus of a protein that is found in the cytosol as well as in peroxisomes of rodent liver cells.

In conclusion, the identification of an impaired peroxisomal targeting sequence at the carboxy terminus of cEH is the clue to the explanation of previous findings strongly indicating that it is the same enzyme rather than 2 very similar species that is located in 2 different compartments of the liver cells.

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