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Mitochondrial 3-2*trans*-Enoyl-CoA Isomerase

Purification, Cloning, Expression, and Mitochondrial Import of the Key Enzyme of Unsaturated Fatty acid β-Oxidation

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Summary: 3-2*trans*-Enoyl-CoA isomerase (EC 5.3.3.8) is a key enzyme in mitochondrial β-oxidation of unsaturated fatty acids in bacteria, plant and animal cells. The enzyme was isolated from rat liver mitochondria and purified to homogeneity by two chromatographic steps. Partial polypeptide sequences of the 29 kDa protein were derived from cyanogen bromide, tryptic, Lys-C, and protease V8 fragments by Edman degradation. Peptide-derived synthetic oligonucleotides were used for the isolation of a 990 bp long isomerase-specific cDNA from rat liver cDNA libraries. 867 bp encode the 289 amino-acid residues of the preisomerase with a molecular mass of 32254 Da. The 1.3-kb mRNA is most strongly expressed in skeletal muscle followed by liver, heart, kidney, and weakly expressed in spleen and brain.

In vitro transcription and translation yielded a 32 kDa polypeptide which was immunoprecipitated by anti rat isomerase antibodies. In the presence of mitochondria the 32 kDa precursor isomerase was processed during mitochondrial import to the 29 kDa mature form of the 3-2*trans*-enoyl-CoA isomerase with 264 amino-acid residues (M_r 29706). A N-terminal signal sequence of 25 amino-acid residues directs the import into the mitochondrial matrix and is cleaved in two successive steps passing through an intermediate form of M_r 30475.

The two cysteine residues in positions 142 and 148 of the preisomerase are present as free thiol groups as shown by derivatization of the mature, native protein with the fluorescent label *N*-(iodoacetaminoethyl)-1-naphthylamine-5-sulfonic acid.

The mitochondrial 3-2*trans* enoyl-CoA isomerase shows significant homology and conserved amino-acid exchanges with the mitochondrial enoyl-CoA hydratase, the N-terminal domain of the bifunctional peroxisomal enoyl-CoA-hydratase:3-hydroxyacyl-CoA dehydrogenase and to extended domains of the α-subunit of the procaryotic β-oxidation complex sharing enoyl-CoA isomerase, D(−)3-hydroxyacyl-CoA epimerase, enoyl-CoA hydratase and L(+)3-hydroxyacyl-CoA dehydrogenase activity, encoded by the fad B operon of *E. coli*.

Enzymes:
Acetyl-CoA acetyltransferase, acyl-CoA:acetyl-CoA *C*-acyltransferase (EC 2.3.1.16);
Enoyl-CoA hydratase, (3*S*)-3-hydroxyacyl-CoA hydro-lyase (EC 4.2.1.17);
3-2*trans*-Enoyl-CoA isomerase, recommended name: dodecenoyl-CoA Δ-isomerase, dodecenoyl-Δ^3-*cis*-Δ^2-*trans*-isomerase (EC 5.3.3.8);
3-Hydroxyacyl-CoA dehydrogenase, (*S*)-3-hydroxyacyl-CoA:NAD® oxidoreductase (EC 1.1.1.35);
3-Hydroxybutyryl-CoA epimerase, 3-hydroxybutanoyl-CoA epimerase (EC 5.1.2.3).

Abbreviations:
BSA, bovine serum albumin; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylendiaminetetraacetate; ELISA, enzyme-linked immuno sorbent assay; FPLC, fast performance liquid chromatography; Hepes,4-(2-hydroxyethyl)-1-piperazinethanesulfonic aicd; I-AEDANS, 6-[2-(iodoacetaminoethyl)]-8-amino-1-naphthalinesulfonic acid; MIP, mitochondrial intermediate peptidase; MPP, mitochondrial processing peptidase; NEM, *N*-ethylmaleinimide; PAGE, polyacrylamide gel electrophoresis; pCMB; 4-chloromercuribenzoate; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; SSC, standard saline citrate (0.15M NaCl, 0.015M Na citrate, pH 7.0).

3-2trans-Enoyl-CoA-Isomerase aus Mitochondrien. Reinigung, Klonierung, Expression und mitochondrialer Import des Schlüsselenzyms für die β-Oxidation ungesättigter Fettsäuren

Zusammenfassung: 3-2*trans*-enoyl-CoA-Isomerase (EC 5.3.3.8) ist ein Schlüsselenzym in der mitochondrialen β-Oxidation von ungesättigten Fettsäuren in Bakterien, Pflanzen und tierischen Zellen. Das Enzym wurde aus Rattenleber-Mitochondrien isoliert und zur Homogenität durch zwei chromatographische Schritte gereinigt. Partialsequenzen des 29-kDa-Proteins wurden aus Bromcyan-, tryptischen, Lys C- und Proteinase-V8-Fragmenten durch Edman-Abbau gewonnen. Aus diesen Peptiden abgeleitete synthetische Oligonucleotide wurden für die Isolierung einer 990 bp langen Isomerase-spezifischen cDNA aus Rattenleber-cDNA-Banken verwendet. 867 bp kodieren die 289 Aminosäurereste-haltige Präisomerase mit einer molekularen Masse von 32254 Da. Die 1.3 kb lange mRNA wird am stärksten exprimiert im Skelettmuskel, gefolgt von Leber, Herz, Niere und nur schwach in Milz und Gehirn.

In-vitro-Transkription und Translation führten zu einem 32-kDa-Polypeptid, das durch Ratten-Isomerase-Antikörper immunpräzipitiert wurde. Das 32-kDa-Vorstufenpolypeptid wird beim mitochondrialen Import zu der 29 kDa reifen Form mit 264 Aminosäureresten (M_r 29706) prozessiert. Die N-terminale Signalsequenz von 25 Resten wird in zwei Stufen durch eine 30475 Da große intermediäre Form gespalten.

Die beiden Cysteinreste in Position 142 und 148 der Präisomerase liegen als freie Thiolgruppen vor, wie die Derivatisierung des reifen, nativen Proteins mit dem Fluoreszenzmarker *N*-(Iodacetamidoethyl)-1-naphthylamin-5-sulfonsäure zeigt. Die mitochondriale 3-2*trans*-Enoyl-CoA-Isomerase zeigt signifikante Homologie und konservierte Aminosäurenaustausche mit der mitochondrialen Enoyl-CoA-Hydratase, der N-terminalen Domäne der peroxisomalen Enoyl-CoA-Hydratase:3-hydroxyacyl-CoA-Dehydrogenase und ausgedehnten Domänen der α-Untereinheit des prokaryotischen β-Oxidationskomplexes, der die Enoyl-CoA-Isomerase, D(−)3-Hydroxyacyl-CoA-Epimerase, Enoyl-CoA-Hydratase und L(+)3-Hydroxyacyl-CoA-Dehydrogenase-Aktivität enthält und im fad B-Operon in *E. coli* kodiert ist.

Key terms: Mitochondrial β-oxidation, 3-2*trans*-enoyl-CoA isomerase, partial polypeptide sequences, expression, in vitro transcription-translation.

Saturated and unsaturated (monoenoic and polyenoic) fatty acids are present in the esterlipids (triacylglycerols, phospholipids, and cholesterol-esters) in comparable amounts. They are metabolized at the same rate and form an essential source of energy generated during their complete combustion in the mitochondria of muscle, heart and liver, but not of brain. A comprehensive view of the β-oxidation pathway of all naturally occurring fatty acids was only possible after the discovery of the mitochondrial 3-2*trans*-enoyl-CoA isomerase (EC 5.3.3.8) in mammalian tissue[1].

This enzyme shifts the 3*cis* double bonds of the intermediates of e.g. oleic and linoleic acid oxidation with positional and geometrical isomerization to the 2*trans* position, yielding the common intermediate of the β-oxidation spiral. 3*trans* isomers are also isomerized to their respective 2*trans* derivatives[2]. Isolated 2*cis*-enoyl-CoA substrates, such as 2*cis*-octenoyl-CoA, on the other hand, are hydrated to the D(−)3-hydroxyacyl-CoA intermediate, which is epimerized by an epimerase (EC 5.1.2.3) to the appropriate substrate of the mitochondrial β-oxidation enzyme L(+)3-hydroxyacyl-CoA dehydrogenase[1,3]. The epimerase and isomerase activities are also encoded on the fad B operon and expressed in the α-subunit of the procaryotic β-oxidation complex[4–7]. In addition a mitochondrial NADPH-dependant reductase (EC 1.3.1.34) has been postulated to reduce and isomerize 2*trans*,4-dienoyl-CoA intermediates occurring during the degradation of polyunsaturated fatty acyl-CoA derivatives with *cis* double bonds on even-numbered C-atoms, to the respective 3*trans*-enoyl-CoA derivatives[8]. The reaction products are again substrates of the key enzyme 3-2*trans*-enoyl-CoA isomerase. We have purified the 3-2*trans*-enoyl-CoA isomerase of rat and bovine liver to homogeneity[9,10] and also the D(−)3-hydroxyacyl-CoA epimerase (unpublished results). The native isomerase is a 58 kDa homodimer, consisting of two identical 29-kDa subunits.

Here we describe the molecular cloning of the 3-2-*trans*-enoyl-CoA isomerase, indispensable for the complete oxidation of unsaturated fatty acids in mitochondria. This enzyme was purified from rat liver mitochondria to homogeneity by a simplified procedure. Peptides isolated after enzymatic or chemical cleavage of the purified protein were analysed by Edman degradation. Screening of cDNA libraries with an

amino-acid sequence-derived synthetic oligonucleotide led to the isolation of isomerase-specific cDNA clones, one of which harboured the complete coding region. The preform of the 3-2*trans*-enoyl-CoA isomerase is a 289 amino-acid residue polypeptide of M_r 32254.

The expression of the enzyme was studied in liver, heart, muscle, brain, kidney and spleen by Northern blot hybridization.

The majority of mitochondrial proteins is synthesized with an N-terminal targeting sequence in the cytosol. During the import into the organelle, this leader sequence is proteolytically cleaved either in one step by the mitochondrial processing peptidase (MPP) alone or in two successive steps by MPP and the mitochondrial intermediate peptidase (MIP)[11,12]. Processing experiments *in vitro* revealed that the isomerase is synthesized as a preprotein, which is processed during its mitochondrial import to the 29-kDa mature form via an 30-kDa intermediate form probably by successive loss of the N-terminal heptadecapeptide and octapeptide.

Results

*Isolation and immunological characterization of 3-2*trans*-enoyl-CoA isomerase*

The isomerase was purified from the mitochondrial fraction (10000 × *g* sediment) of a rat liver homogenate in three steps:

a) mitochondria were lysed and submitted to the approved heat step[1], followed by
b) chromatography on Whatman CM52 cation exchange cellulose and
c) by isolation of the purified enzyme as a single peak by FPLC on a Mono Q cation exchange column, applying salt gradient elution. The table shows the purification steps and Fig. 1A presents their PAGE analysis.

Monospecific antibodies were obtained from rabbit antiserum against FPLC-purified 3-2*trans*-enoyl-CoA isomerase by affinity chromatography on a covalently coupled isomerase Sepharose 4B-CL column.

Table. Purification steps of 3-2*trans*-enoyl-CoA isomerase.

	Protein [mg]	Activity [U]	Spec. act. [U/mg]
500 × *g* supernatant	13600	–	–
Sonified mitochondria	2100	544	0.3
Heat denaturation	82	344	4.2
CM cellulose, pH 6.0	13	165	12.7
FPLC Mono Q	1.2	74	62.0

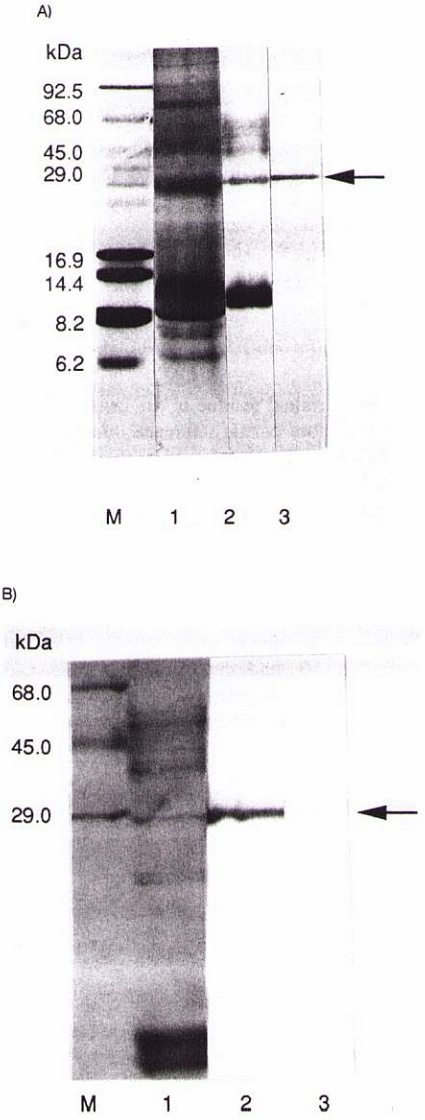

Fig. 1. A) Silver-stained SDS polyacrylamide gel (15%) of aliquots of protein of the three purification steps of 3-2*trans*-enoyl-CoA isomerase. M: marker proteins; lane 1: mitochondrial lysate after heat step; lane 2: pooled isomerase containing fractions after CM Cellulose cation exchange chromatography; lane 3: the homogenous enzyme after FPLC Mono Q anion exchange chromatography.
B) Western blot analysis of enriched 3-2*trans*-enoyl-CoA isomerase. M: Coomassie blue-stained marker proteins; lane 1: Coomassie blue-stained enriched isomerase fraction; lane 2,3: Western blot analysis of enriched isomerase fraction with anti-isomerase antibodies and non immune serum, respectively. The antibody exclusively detects the 29-kDa band.

The antibody solution reached a titer of 1:10000 in the ELISA[13]. The antibodies detected exclusively the 29-kDa isomerase band in Western blot analysis[14], Fig. 1B.

Aliquots of enzymatically active isomerase solution were preincubated with the antibodies against the 29-kDa band and the activity measured. Fig. 2 illustrates the immunotitration of the isomerase reaction. The antibody inhibits the enzyme activity completely up to a dilution of 1:80, whereas the nonimmune serum did not interfere with the isomerase activity.

Protein structural analysis of 3-2trans-enoyl-CoA isomerase

The amino-terminal residue of the isomerase is protected. Therefore partial sequence information was obtained from peptides released by chemical and proteolytic degradation of the purified enzyme.

The protein was degraded by cyanogen bromide into 10 fragments. They where separated by high resolution PAGE[15] and transferred to glass fibre membranes by electroblotting for gas phase microsequencing[16], Fig. 3A. The isomerase was also tryptically cleaved, the fragments separated by 17.5% SDS PAGE, Fig. 3B, and the Coomassie blue-stained bands eluted for microsequencing following the procedure recently elaborated in this laboratory[17]. With the same approach fragments of the Lys-C and V8 protease hydrolysis were isolated and sequenced. The following amino-acid sequences were obtained (underlined sequences were used for the synthesis of derived oligo-deoxyribonucleotide probes):

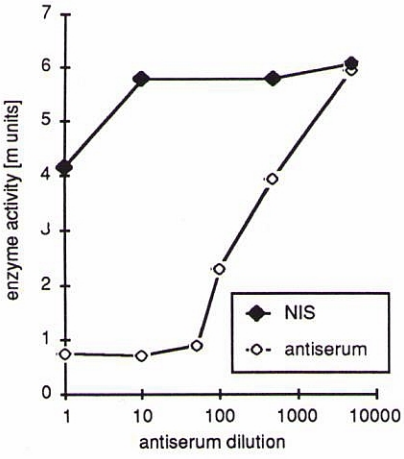

Fig. 2. Immunotitration of 3-2*trans*-enoyl-CoA isomerase reaction.

Inhibition of isomerase activity by titration with specific anti-isomerase antibodies. Non immune serum (NIS) serves as a control.

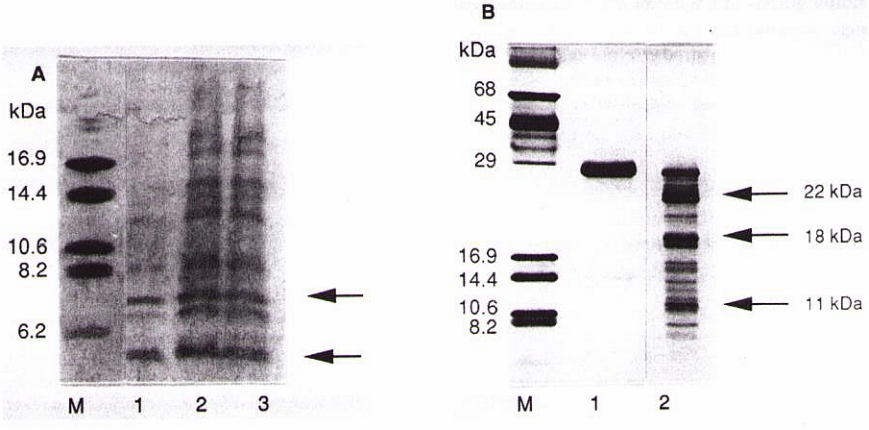

Fig. 3. A) Coomassie blue-stained electroblot of cyanogen fragments of purified 3-2*trans*-enoyl-CoA isomerase (lanes 1–3). Fragments were separated by high resolution PAGE (16.5% acrylamide). Peptides marked by arrows were directly sequenced. M: marker proteins.

B) SDS PAGE (17.5% acrylamide); lane 1: purified isomerase; lane 2: purified isomerase after tryptic digestion. M: marker proteins. Peptides marked by arrows were sequenced after buffer elution.

BrCN fragments:

① 8 kDa [Met]-Ala-Asp-Asn-Ser-Lys-Tyr-Thr-Ile-Gly-Leu-Asn

② 6 kDa [Met]-Lys-Phe-Lys-Asn-Pro-Pro-Val-Asn-Ser-Leu

Fragments from tryptic cleavage:

③ 22 kDa Phe-Ser-Asn-Lys-Arg-Val-Leu-Val-Glu-Lys-Glu-Gly-Glu-Ala-Gly-Ile-Ala-Val-Met-Lys-Phe-Lys-Asn-Pro

④ 18 kDa Phe-Ser-Asn-Lys-Arg-Val-Leu-Val-Glu-Lys

⑤ 11 kDa Glu-Lys-Glu-Gly-Glu-Ala-Gly-Ile-Ala-Val-Met-Lys

The 11-kDa fragment is part of the 22- and 18-kDa fragments.

Lys-C-cleavage:

⑥ 12 kDa Ser-Ile-Arg-Gly-Val-Ile-Leu-Thr-Ser-Glu-Arg-Pro

V8 protease cleavage:

⑦ Asp-Gln-Val-His-Ser-Lys-Ala

*Molecular cloning of rat liver 3-2*trans*-enoyl-CoA-isomerase*

On the basis of the amino-acid sequence derived from the overlapping 6 kDa BrCN fragment and the 22 kDa tryptic fragment (see underlined amino acids above) the following degenerated oligonucleotide was synthesized:

5' GTI ATG AA$\frac{A}{G}$ TT$\frac{C}{T}$ AA$\frac{A}{G}$ AA$\frac{C}{T}$ CCI CCI GTI AA 3'

This oligonucleotide, ^{32}P-labelled with T4-polynucleotide kinase, was used for screening of about 5×10^5 plaques of an oligo(dT)-primed rat liver cDNA library in λgt11. Four isomerase-specific cDNA clones were isolated and their inserts released by *Eco* RI digestion. They yielded two fragments due to an *Eco* RI site within the cDNA sequence. The randomly primed ^{32}P-labelled N-terminal 120 bp *Eco* RI fragment of the most 5′-extended clone was used for screening an oligo(dT) randomly primed rat liver cDNA library in λZAPII. Eight isomerase-specific cDNA clones, one bearing the whole coding region including the putative start methionine, were isolated. Restriction of the λZAPII clone with *Sac* I-*Xho* I released the intact 987 bp insert, which was cloned into the *Sac* I-*Sal* I site of pGEM3Z for *in vitro* transcription-translation experiments. The two *Eco* RI fragments were isolated and subcloned in pGEM3Z for direct double strand sequencing, using T7 and SP6 promoter-specific primers and synthetic oligonucleotides of the established isomerase nucleotide sequences. Fig. 4 shows the nucleotide sequence with the deduced amino-acid sequence of the 3-2*trans*-enoyl-CoA-isomerase of rat liver mitochondria. The cDNA encodes a protein of 289 amino acids with a molecular mass of 32254 Da. All amino-acid sequences obtained from Edman degradation of BrCN, trypsin, Lys-C and Protease V8 cleavage products described above are present in the nucleotide-derived amino-acid sequence.

*Expression of the 3-2*trans*-enoyl-CoA-isomerase*

Total RNA of rat liver, brain, heart, kidney, skeletal muscle and spleen were separated on a formaldehyde agarose gel for Northern blot hybridization analysis. The randomly primed ^{32}P-labelled 808 bp isomerase cDNA *Eco* RI fragment was used as probe. Only one transcript of appr. 1300 bp was present in all isomerase-positive organs. For quantitation, the RNA was hybridized with ^{32}P-labelled β-tubulin cDNA. Densitometry of the autoradiograms allowed an approximation of the relative expression of the 3-2*trans*-enoyl-CoA-isomerase in the different organs. The 1.3 kb mRNA is most strongly expressed in muscle, followed by liver, heart, kidney, and very weakly in spleen and brain, Fig. 5.

*Sulfhydryl groups of 3-2*trans*-enoyl-CoA-isomerase*

The 3-2*trans*-enoyl-CoA-isomerase contains two cysteine residues in position 142 and 148 of the preisomerase polypeptide chain, which might either form a disulfide bond or are present as free thiol groups. Aliquots of native, FPLC-purified enzyme were allowed to react with I-AEDANS before and after denaturation and reduction with DTT[18]. Pancreatic ribonuclease was treated in the same way as a control. PAGE of the products yielded fluorescent bands of the I-AEDANS-treated native and DTT-reduced isomerase. Native ribonuclease was not derivatized, whereas the reduced enzyme shows intensive fluorescence due to the eigth thiol groups liberated after denaturation and DTT treatment (Fig. 6). This indicates that the cysteine residues of the isomerase are present as free thiol groups and not as intramolecular disulfide bond.

*Mitochondrial import of the 3-2*trans*-enoyl-CoA-isomerase*

Isomerase-specific mRNA was synthesized by an *in vitro* transcription reaction, using the complete isomerase cDNA cloned in the *Sac*I-*Sal*I site of pGEM3Z as template. The capped isomerase-specific mRNA was translated with wheat germ extract and reticulocyte lysate in the presence of ^{35}S-methionine. After immunoprecipitation with the antiisomerase antibody, gel electrophoresis and fluorography, the translation product appeared as a 32 kDa band. Whole rat liver mitochondria were added to the *in vitro* translation mixtures under conditions for an effective import. The isomerase preform generated in reticulocyte lysate is processed to the mature 29-kDa form during mitochondrial import, whereas the wheat germ translation product is not processed. Subsequently added reticulocyte lysate did not lead to an import reaction (Fig. 7).

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-17  ..C CCA CCG ATA TCC AAA ATG GCG CTG GCT GCT GCG CGT CGC GTT CTG CTG CAG GCC GGA  42
                            Met Ala Leu Ala Ala Ala Arg Arg Val Leu Leu Gln Ala Gly

                                                                    S③,④
 43  TCC CGC CTC GGC CGC AGG GGG GCC GTG GAC GGC GCG CGT CGC TTC TCT AAC AAG CGG GTG 102
     Ser Arg Leu Gly Arg Arg Gly Ala Val Asp Gly Ala Arg Arg Phe Ser Asn Lys Arg Val

           S⑤ E④                                          S② E⑤              E③
103  TTG GTG GAG AAG GAG GGC GAG GCA GGA ATC GCA GTG ATG AAG TTC AAG AAC CCT CCA GTG 162
     Leu Val Glu Lys Glu Gly Glu Ala Gly Ile Ala Val Met Lys Phe Lys Asn Pro Pro Val

163  AAT TCC CTC AGC TTG GAG TTT CTG ACA GAG TTC GTC ATC AGC CTG GAG AAG CTG GAA AAT 222
     Asn Ser Leu Ser Leu Glu Phe Leu Thr Glu Phe Val Ile Ser Leu Glu Lys Leu Glu Asn

           S⑥                                                 E⑥
223  GAC AAG AGC ATC CGA GGT GTC ATC CTC ACT TCG GAG CGC CCG GGT ATC TTC TCG GCT GGC 282
     Asp Lys Ser Ile Arg Gly Val Ile Leu Thr Ser Glu Arg Pro Gly Ile Phe Ser Ala Gly

283  CTG GAC TTG ATG GAG ATG TAT GGC CGG AAC CCA GCC CAC TAT GCT GAG TAC TGG AAG GCT 342
     Leu Asp Leu Met Glu Met Tyr Gly Arg Asn Pro Ala His Tyr Ala Glu Tyr Trp Lys Ala

343  GTG CAG GAG CTG TGG CTG AGG CTC TAC TTG TCC AAC CTG ACC TTG ATA TCT GCC ATC AAT 402
     Val Gln Glu Leu Trp Leu Arg Leu Tyr Leu Ser Asn Leu Thr Leu Ile Ser Ala Ile Asn

                                                                          S①
403  GGC GCC TCT CCA GCT GGA GGC TGC CTC ATG GCT CTC ACC TGT GAC TAC AGG ATA ATG GCG 462
     Gly Ala Ser Pro Ala Gly Gly Cys Leu Met Ala Leu Thr Cys Asp Tyr Arg Ile Met Ala

                                  E①
463  GAC AAC TCC AAG TAT ACC ATA GGA TTG AAT GAG AGC CTG CTG GGC ATT GTT GCC CCC TTC 522
     Asp Asn Ser Lys Tyr Thr Ile Gly Leu Asn Glu Ser Leu Leu Gly Ile Val Ala Pro Phe

523  TGG TTA AAA GAC AAC TAC GTG AAC ACC ATC GGG CAC CGA GCA GCC GAG CGT GCC CTT CAA 582
     Trp Leu Lys Asp Asn Tyr Val Asn Thr Ile Gly His Arg Ala Ala Glu Arg Ala Leu Gln

583  CTG GGG ACG CTT TTC CCA CCA GCA GAG GCC CTC AAG GTG GGT TTG GTG GAT GAG GTG GTA 642
     Leu Gly Thr Leu Phe Pro Pro Ala Glu Ala Leu Lys Val Gly Leu Val Asp Glu Val Val

           S ⑦                      E ⑦
643  CCT GAG GAT CAG GTA CAC AGC AAG GCT CGC TCA GTG ATG GCC AAG TGG TTC ACC ATT CCG 702
     Pro Glu Asp Gln Val His Ser Lys Ala Arg Ser Val Met Ala Lys Trp Phe Thr Ile Pro

703  GAC CAT TCT CGG CAG CTG ACG AAG AGC ATG ATG CGG AAG GCC ACG GCG GAC AAC CTG ATC 762
     Asp His Ser Arg Gln Leu Thr Lys Ser Met Met Arg Lys Ala Thr Ala Asp Asn Leu Ile

763  AAG CAG CGA GAG GCT GAC ATC CAG AAC TTC ACC AGT TTC ATC TCC AGA GAC TCC ATC CAG 822
     Lys Gln Arg Glu Ala Asp Ile Gln Asn Phe Thr Ser Phe Ile Ser Arg Asp Ser Ile Gln

823  AAG TCC CTG CAC GTG TAC TTG GAA AAG CTC AAG CAA AAG AAG GGC TGA CCT AAG GGT GCC 882
     Lys Ser Leu His Val Tyr Leu Glu Lys Leu Lys Gln Lys Lys Gly

883  ACA CGG GTG TTT AGT TAC ACG TGC CTT CTA GGA CCC ATG GTC CCA AGG ATT ATC AAT AAG 942

943  GTA TTT TTC AAT TTA AAA AAA AAA AAA AA                                        971
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Fig. 4. The nucleotide sequence of rat liver mitochondria 3-2*trans*-enoyl-CoA isomerase and its predicted amino-acid sequence. Nucleotides are numbered designating the first nucleotide of the putative initiator methionine codon as +1. From this ATG on, the cDNA includes an open reading frame of 867 nucleotides, coding for 289 amino-acid residues. The internal *Eco* RI site is located at nucleotide 163. The nucleotides 937–941 (AATAA) may serve as a polyadenylation signal. The amino-acid sequences underlined were determined by Edman degradation of cyanogen bromide, trypsin, Lys-C and V8 protease fragments (S: start of peptides; E: end of peptides). The proposed two-step cleaved mitochondrial targeting sequence is double underlined.

If the mitochondria are lysed with the detergent Lubrol PX in the presence of EDTA then subsequently added precursor isomerase is processed to an intermediate form of about 30 kDa (Fig. 7). This may be due to an inhibitory effect of chelating EDTA on the $Zn^{2\oplus}$ ions requiring MIP[12].

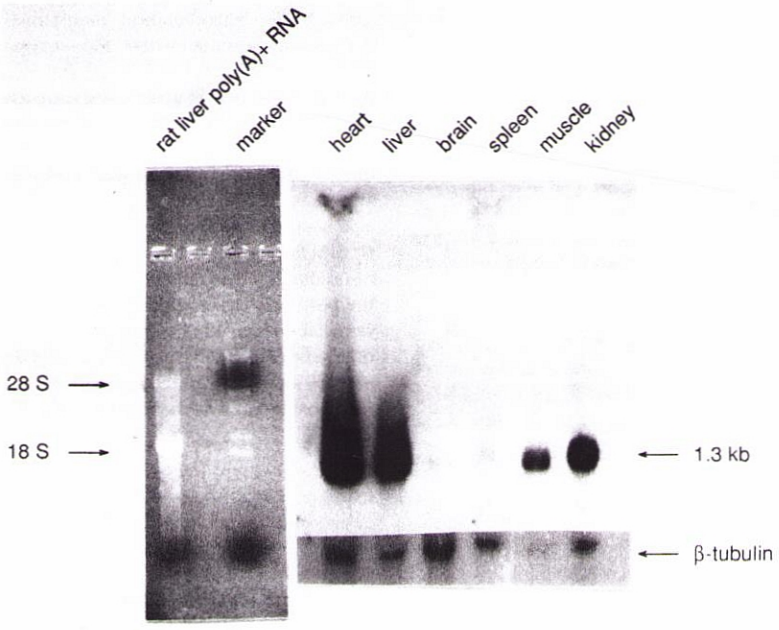


Fig. 5. Northern blot hybridization of rat heart, liver, brain, spleen muscle, and kidney total RNA with a ^{32}P-labelled 808 bp isomerase cDNA and β-tubulin cDNA fragment.

The radioactive probe detects one 1300 bp isomerase transcript in the RNA of all organs.

Fig. 6. SDS PAGE (17.5%) of 3-2*trans*-enoyl-CoA isomerase fluorescence-labelled with I-AEDANS.

Lane 1: isomerase before, lane 2: after DTT reduction, lane 3: pancreatic ribonuclease A after, lane 4: before DDT reduction.

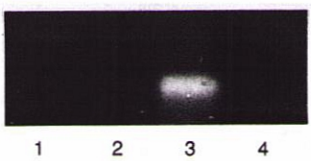
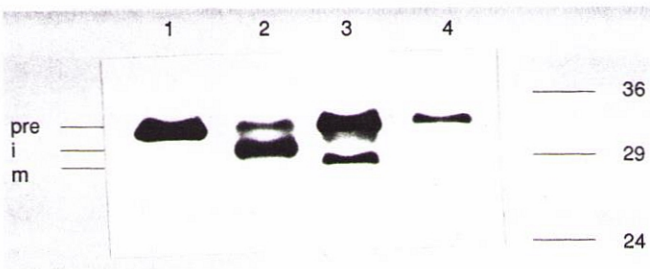


Fig. 7. Two-step processing of isomerase precursors during mitochondrial import.

Lane 1: 32 kDa [^{35}S]methionine labelled precursor (pre) synthesized in reticulocyte lysate. Lane 2: lysed mitochondria (0.3% Lubrol PX, 0.25mM EDTA) processed the precursor to the 30 kDa intermediate form (i). Lane 3: intact mitochondria processed the precursor to the 29 kDa mature form (m). Lane 4: isomerase precursor synthesized in wheat germ extract is not processed by intact mitochondria.



Discussion

3-2*trans*-enoyl-CoA-Isomerase plays a pivotal role in the degradation of mono- and polyunsaturated fatty acids and is the key enzyme linking in the β-oxidation of unsaturated and saturated fatty acids. Here we describe an efficient and simple procedure for the purification of the isomerase from rat liver mitochondria. A synthetic oligodeoxynucleotide designed according to the most suitable amino-acid sequences was used as probe for the screening of rat liver cDNA libraries. A 987 bp cDNA was obtained, which embraces part of the 5′-untranslated region, the complete coding region of 867 nucleotides, and the 3′-untranslated region. The cDNA-deduced amino-acid sequence of the isomerase contains 289 residues. The initiator methionine was assigned to the first ATG-codon for two reasons:

i) because of the functional integrity of the *in vitro* translated preisomerase which is imported into the mitochondria and processed to the mature form and
ii) because of the amino-acid homologies to the related mitochondrial enoyl-CoA-hydratase (see below), Fig. 9.

The isomerase precursor protein is processed in two successive steps probably by the MPP and MIP. The intermediate form is generated by the MPP in the mitochondrial matrix fraction, in which the $Zn^{2\oplus}$-dependend MIP is inhibited by excess chelating EDTA. A common three-amino-acid motif in the presequence of mitochondrial precursor proteins which are cleaved in two successive steps has been proposed: an arginine residue in positions −10 is essential for the cleavage to an intermediate form, from which the mature form is generated by the loss of an octapeptide with a hydrophobic residue (Leu, Ile, Val, or Phe) in position −8 and glycine, serine or threonine in position −5[19]. The proposed presequence of the 3-2*trans* enoyl-CoA isomerase matches the three-amino-acid motif in position −10 (arginine) and −5 (glycine) but fails in position −8 where glycine appears instead of a hydrophobic amino acid. We assume a cleavage of the isomerase preform between leucine 17/glycine 18 to the intermediate form and between glycine 25/alanine 26 to the mature form. The resulting molecular masses of 30475 Da for the intermediate form and 29706 Da for the mature form agree well with the molecular masses determined by gel electrophoresis (29 kDa and 30 kDa, respectively). More elaborate radiosequencing of *in vitro*-processed isomerase precursors are in progress to verify the proposed cleavage sites. The N-terminal leader sequence of 25 amino-acid residues carries all the characteristic features essential for targeting and the passage of the isomerase through the mitochondrial membranes into the matrix compartment where the enzyme has been localized by mitochondrial fractionating studies (unpublished results). In general the known targeting sequences of mitochondrial matrix enzymes are highly variable but share general features: the signal sequence of the isomerase also lacks anionic side chains and shows an abundance of positively charged amino acid residues, mainly of arginine[20,21]. An amphiphatic α-helix is indicated in the helical wheel diagramm of residues 1–25 of the signal sequence. All but one of the positively charged side chains are located on the same face of the α-helix, the nonpolar residues on the other one (Fig. 8). The positively charged surface supposedly interacts with the acidic polar head groups of the phospholipids of the mitochondrial membrane.

In peroxisomes three enzymes carry out the fatty acid oxidation without energy coupling[22]: an acyl-CoA oxidase, enoyl-CoA hydratase:3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme and 3-ketoacyl-CoA-thiolase. All three enzymes have been cloned[23–25]. It has been reported recently that the peroxisomal bifunctional enzyme exhibits an additional 3-2*trans*-enoyl-CoA-isomerase activity and should be regarded as a trifunctional enzyme[26]. In *E. coli* the enzyme activities required for the β-oxidation of saturated and unsaturated fatty acids are assembled as a multienzyme complex with tetrameric structure consisting of two α- and two β-subunits. The

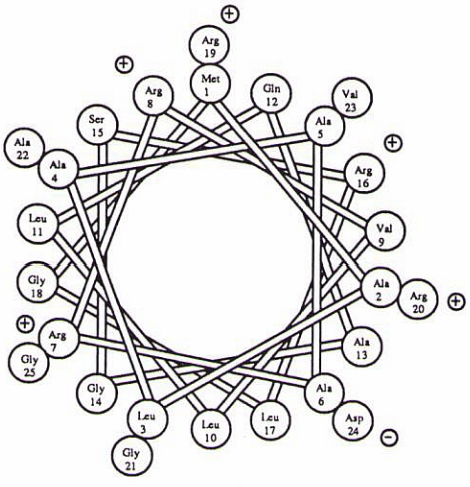


Fig. 8. Presentation of residues 1–25 of the signal sequence of 3-2- *trans*-enoyl-CoA isomerase as helical wheel.

An amphiphatic α-helix with four out of five arginine residues oriented toward the hydrophilic side is indicated.

α-subunit of 79 kDa is encoded within the fad B operon and forms a multifunctional enzyme with the enzyme activities of enoyl-CoA hydratase, L(+)3-hydroxyacyl-CoA dehydrogenase, 3-2*trans*-enoyl-CoA isomerase and D(−)3-hydroxyacyl-CoA epimerase, whereas the two identical small β-subunits, associated with the 3-ketoacyl-CoA-thiolase activity are encoded in the fad A operon[27]. The 3-2*trans*-enoyl-CoA isomerase shows significant homologies to the mitochondrial enoyl-CoA hydratase and to the respective N-terminal portions of the peroxisomal bifunctional enzyme (Exon I–V) and the α-subunit of the *E. coli* β-oxidation complex (Fig. 9). The polypeptide chain of the isomerase contains two cysteine thiole groups in positions 142 and 148 similar to the cysteine residues 143 and 149 of the related mitochondrial enoyl-CoA hydratase. The C-terminal sequence of the peroxisomal bifunctional enzyme (Exon VII), on the other hand, shows homologies to the mitochondrial 3-hydroxyacyl-CoA-dehydrogenase[28,29] and to the C-terminal portion of the α-subunit of the *E. coli* β-oxidation complex[7]. The sequence homology data suggest that the mitochondrial isomerase and hydratase activities separated on distinct

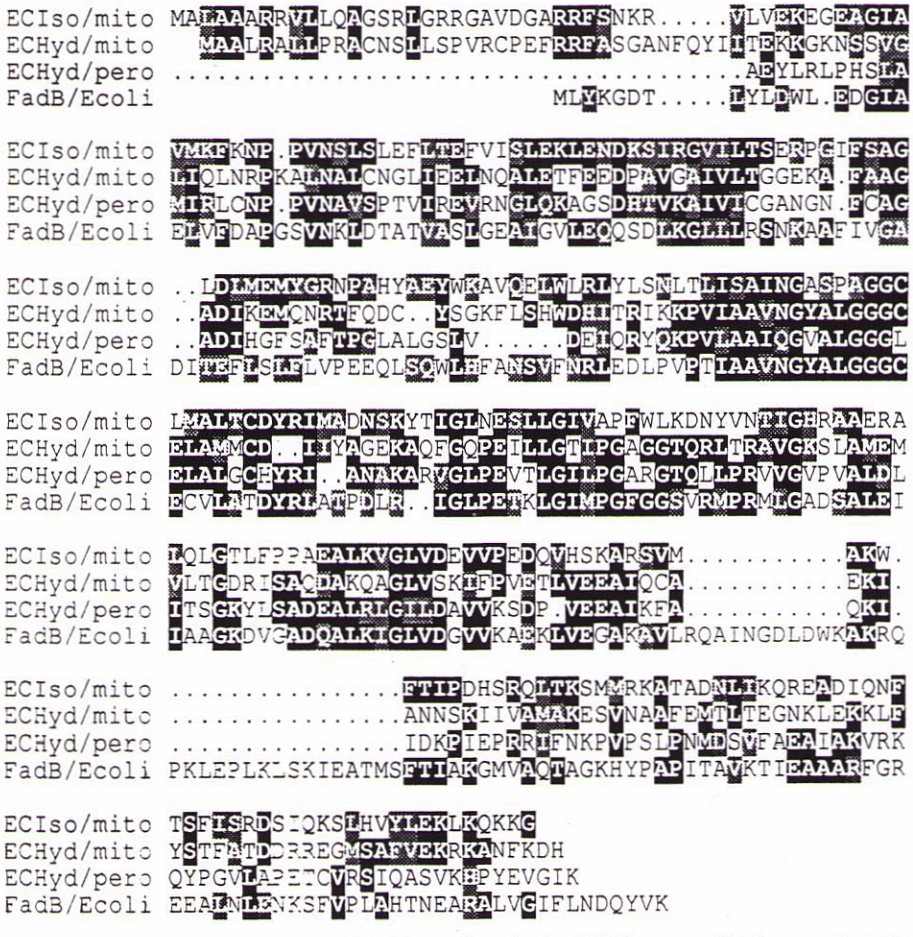


Fig. 9. Comparison of the amino acid sequences. ECIso/mito, rat liver mitochondrial 3-2*trans*-enoyl-CoA isomerase (complete sequence of 289 amino acids); ECHyd/mito, rat liver mitochondrial enoyl-CoA hydratase (complete sequence of 290 amino acids); ECHyd/pero, rat liver peroxisomal enoyl-CoA-hydratase:3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (amino acids 2–249 out of 723 amino acids); fad B/*E. coli*, α-subunit of the *E. coli* fatty acid oxidizing multienzyme complex (amino acids 1–300 out of 729 amino acids). Identical amino acids are printed white on black, similar amino acids white on grey, and other amino acids black on white.

polypeptides are located together on the N-terminal domain of the single polypeptide multifunctional peroxisomal and *E. coli* protein.

One may speculate about the evolutionary relationship of the fad B operon of *E. coli* encoding the enzymic activities of hydratase, dehydrogenase, isomerase and epimerase, of the genes of the 77 kDa peroxisomal bifunctional enzyme, the mitochondrial 32 kDa enoyl-CoA hydratase, and the 32 kDa 3-2-*trans*-enoyl-CoA isomerase. The bifunctional polypeptide in the ancient peroxisomal organelle might have resulted from gene fusion. With the development of the mitochondria carrying out the same reactions of β-oxidation with ATP formation as compared with peroxisomes without energy coupling, gene fission or a partial gene duplication (exons I–V) could have occurred. Peroxisomal proteins are imported directly from the cytosol without requiring an N-terminal targeting sequence, whereas the single activity-single polypeptide of the enoyl-CoA-hydratase and the 3-2-*trans*-enoyl-CoA isomerase acquired targeting sequences for mitochondrial import.

Unravelling the structural organisation of the gene of the mitochondrial 3-2*trans*-enoyl-CoA isomerase will help to understand the evolutionary interrelationship.

Materials and Methods

Dodec-3*cis*-enoyl-CoA was synthesized as described before[2]. Restriction enzymes and DNA modifying enzymes were purchased from Pharmacia LKB, Freiburg, radioactive chemicals from Amersham-Buchler, Braunschweig. Synthetic oligonucleotides were prepared with an automated DNA synthesizer Model 381A (Applied Biosystems, Forster City, California).

The enzyme assay with dodec-3*cis*-enoyl-CoA as substrate has been described previously[1]. Protein was determined with Coomassie Brillant Blue G250[30].

*Purification of 3-2*trans*-enoyl-CoA isomerase from rat liver*

Preparation of mitochondria and heat step

Mitochondria of 10 livers (70 g) of 3-months old Lewis rats were isolated according to established procedures[31]. The mitochondrial pellet was suspended in 100 ml 0.025M phosphate buffer, pH 7.4, and sonified with a Branson sonifier for 2 min in an ice bath. The suspension was heated to 70 °C for 30 s, cooled on ice and centrifuged at 15000 × *g* for 15 min at 4 °C. The mitochondrial lysate was dialysed against 0.025M phosphate buffer, pH 6.0.

CM52 cellulose chromatography

The dialysate was equilibrated with 0.025M phosphate buffer (pH 6.0) and applied to a CM52 cellulose column (bed volume 70 ml). Unbound protein was eluted with the same buffer and the isomerase with 30 ml 0.025M phosphate buffer, pH 7.4.

FPLC chromatography

The CM52 cellulose eluate was dialysed against 20mM Tris/HCl, pH 8.7 and separated on a FPLC Mono Q anion exchange column by gradient elution with buffer A: 20mM Tris/HCl, pH 8.7, and buffer B: 20mM Tris/HCl, pH 8.7, 1M NaCl. The isomerase eluted at 50mM NaCl.

*Fluorescence labeling of 3-2*trans*-enoyl-CoA isomerase*

Aliquots of FPLC purified rat liver isomerase (10 μg) and pancreatic ribonuclease A were treated with an excess of I-AEDANS (5mM, 1 h) immediately or after reduction with DTT (1mM, 2 h, 1% SDS) in 0.1M NH_4HCO_3 and both rechromatographed by FPLC as described before for PAGE (17.5% acrylamide). The bands were visualized and photographed under UV light[18].

Immunological techniques

Anti rat liver 3-2*trans*-enoyl-CoA isomerase antibodies were raised against the FPLC-purified protein in rabbits. The IgG fraction was isolated by protein A Sepharose chromatography. Monospecific antibodies were obtained by affinity chromatography on an isomerase-Sepharose 4B-CL column. The titer was determined with the ELISA technique[13].

Immunoprecipitations were carried out in a volume of 500 μl containing the translation products: 10 μg of affinity-purified monospecific IgG, 25 μl of a formaldehyde-treated *Staphylococcus aureus* (Cowan strain) suspension, 10mM Tris/HCl, pH 8.0, 50mM NaCl, 0.1% Nonidet P40 added and rotated overnight at 4 °C. *S. aureus* were washed five times with the same buffer. sample buffer added and directly used for SDS-PAGE followed by autoradiography.

Western blot analysis

Proteins were electroblotted from the SDS-polyacrylamide gel to nitrocellulose membranes and the bound anti isomerase antibodies visualized with goat anti rabbit IgG coupled with alkaline phosphatase as second antibody and 5-bromo-4-chloroindolylphosphate as substrate[14].

Antibody-enzyme inhibition assay

6 mU isomerase in 30 μl 0.025M phosphate buffer pH 7.4, 0.1% BSA were incubated with 30 μl of increasing dilutions of antiserum and nonimmune serum for one hour at room temperature and the enzymatic activity subsequently assayed under the standard conditions at antiserum dilutions indicated below the abscissa of the diagram in Fig. 2.

Protein sequencing

50 μg purified isomerase protein was subjected to cyanogen bromide cleavage, tryptic, Lys C and V8 protease digestion. The products were separated by high resolution polyacrylamide gel electrophoresis[15]. Polypeptides were eluted from the bromophenol blue-stained polyacrylamide slices for microsequencing as described before[17] or electroblotted to siliconized glass fiber. An Applied Biosystems Model 470A gas phase sequencer with an online PTH analyser was used.

cDNA cloning

Rat liver cDNA libraries were constructed in λgt11 and λZAPII starting from rat liver poly(A)$^+$RNA using oligo(dT) and random hexanucleotide primers. The protocol of the manufacturer (Pharmacia) for the construction of cDNA libraries was followed. They were screened with the 5′-^{32}P-labelled synthetic oligonucleotide corresponding to the partial peptide sequences determined by Edman degradation or with a random-primed[32] 120 bp Eco RI fragment of the isomerase cDNA. Approximately 1×10^6 clones were screened by standard procedures. Four positively hybridizing λgt11 clones and eight positively hybridizing λZAPII clones were iso-

lated, their inserts released by *Eco* RI digestion, separated by 1% agarose electrophoresis, and analysed by Southern blot hybridization. Since the cDNA contained an *Eco* RI site, the intact 987 bp insert of the full-length λZAPII clone was excised with *Sac* I-*Xho* I. Fragments separated by agarose electrophoresis were either transferred by electrophoresis to DEAE membrane[33] or adsorbed to glass milk from the dissolved agarose.

Plasmid construction

Vectors used for *in vitro* transcription and translation were constructed from pGEM3Z. The full length isomerase insert in λZAPII was excised as a *Sac* I-*Xho* I fragment and cloned into the *Sal* I-*Sac* I-linearized pGEM3Z.

RNA preparation and Northern blot analysis

RNA was isolated from liver, heart, muscle, brain, kidney, and spleen with the acid guanidine-thiocyanate phenol/chloroform (AGTPC) procedure[34]. Poly(A)$^+$ RNA of rat liver was purified by repeated affinity chromatography on oligo(dT)$_{12-18}$ cellulose[35]. Poly(A)$^+$ 10 μg of rat liver, heart, brain, skeletal muscle, spleen and kidney RNA were separated by electrophoresis in 1% agarose gels containing 0.2% formaldehyde and transferred to nitrocellulose membranes. The blot was hybridized with a randomly ^{32}P-labelled, 808 bp Eco RI fragment of the isomerase cDNA (specific activity 10^6 cpm/pmol) in 50% formamide at 42 °C, washed in 2 × SSC, 0.1% SDS at 60 °C and exposed to Kodak XAR-5 film using an intensifying screen at −80 °C. Subsequently the blots were washed for 5 min in boiling 1% SDS to remove the hybridization probe and again hybridized to randomly ^{32}P-labelled β-tubulin as internal standard. The autoradiograms were scanned with the Laser densitometer LKB model 220L.

Sequence analysis

Double strand DNA sequencing was performed by the dideoxy chain termination method[36] of the *Eco* RI fragments subcloned into the *Eco* RI-restricted and dephosphorylated pGEM3Z vector. Specific T7 and SP6 primers and oligonucleotides which were synthesized on the basis of established sequences were used. Both strands of the cDNA inserts were sequenced.

Transcription in vitro[37]

1–2 μg of linearized plasmid DNA was transcribed in a total volume of 50 μl reaction mixture which contained 40mM Tris/HCl, pH 7.9, 6mM $MgCl_2$, 2mM spermidine, 10mM DTT, 0.4mM ATP, CTP, UTP each, 0.04mM GTP, 0.5mM (5′)GpppG(5′), 20 U RNAsin* and 10–20 USP6 RNA-polymerase. Reaction was carried out at 37 °C for 60 min, DNA digested with 250 ng DNAse (RNAse-free) for 10 min at 37 °C and stopped with 1 μl 0.5M EDTA. 7 μl 3M Na-acetate, pH 5.5, and 5 μl tRNA (5 ng/ml) of calf liver were added and extracted with phenol/chloroform. RNA was precipitated with ethanol. RNA was dissolved in RNAse-free water and stored at −70 °C.

Translation in vitro

Cell-free translation was carried out using a wheat germ S-23 extract in the presence of [^{35}S]methionine as described by Erickson and Blobel[38]: the incubation mixture of 25 μl contained 1–5 μg of synthetic capped mRNA, 10 μl wheat germ extract, 90mM K-acetate, 3mM Mg-acetate, 38mM Hepes, pH 7.4, 1.25mM ATP, 0.25mM GTP, 0.2mM DTT, 100μM amino-acid mixture (without methionine), 20 μCi [^{35}S]methionine. Incubation at 25 °C for 1 h. Translations with the rabbit reticulocyte lysate were performed according to the procedure described by the supplier. The ^{35}S-labelled translation products were analysed directly or after immunoprecipitation by SDS PAGE.

* RNAsin, RNAse inhibitor (placental) (one unit of inhibitor is the amount required to inhibit by 50% the activity of 5 ng RNAase A).

Mitochondrial import and processing of the isomerase

6 μl translation product was incubated with 3 μl freshly prepared mitochondria (20 mg protein/ml) or lysed mitochondria[39] (10 mg protein/ml, 0.3% Lubrol PX, 0.5mM EDTA) for 40 min at 27 °C. Mitochondria were sedimented by brief centrifugation and analysed by SDS PAGE and fluorography.

Gel electrophoresis

Protein samples were reductively carboxymethylated for SDS-PAGE[40], performed according to Schägger and von Jagow[15] using 12 to 17.5% acrylamide. Gels were stained with Coomassie Blue R-250 (Serva, W-6900 Heidelberg, Germany) or with silver[41].

Fluorography of polyacrylamide gels was carried out according to Bonner and Laskey[42].

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