Modification of the targeting presequence of the bovine cytochrome *P*-450scc precursor lifting tissue-specific restrictions on its mitochondrial import

L.A. Novikova[a], A.S. Savel'ev[a], R.A. Zvyagil'skaya[b], V.N. Luzikov[a,*]

[a]*A.N. Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University, Moscow 119899, Russian Federation*
[b]*Bakh Institute of Biochemistry, Russian Academy of Sciences, Moscow 117071, Russian Federation*

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Abstract It has been found that a recombinant cytochrome *P*-450scc precursor supplemented with an extra MRGSH$_6$GIR sequence at the NH$_2$-terminus (6His-pP450scc) is imported into isolated rat liver and heart mitochondria as well as into yeast mitochondria. The import is coupled with proteolytic processing of the precursor resulting in the mature size form of cytochrome *P*-450scc. Modification of the targeting presequence responsible for its increased positive charge is supposed to lift the previously suggested tissue-specific restrictions on the pP450scc import into mitochondria.

Key words: Cytochrome *P*-450scc; Mitochondrial import; Addressing presequence

1. Introduction

The mechanisms of import of cytoplasmically made mitochondrial proteins into mitochondria and those of their proteolytic processing inside the organelles were intensively studied over the last 20 years. According to the data obtained, such proteins are usually synthesized as precursors supplied with a specific amino acid sequence at the NH$_2$-terminus which provides their targeting to mitochondria and location in one or another compartment of the organelle [1,2]. These targeting presequences were considered to be universal, i.e. operating with no tissue or species specificity [3–5]. In a few cases, however, heterologous import of protein precursors into mitochondria did not occur. This is particularly true of the precursor of cytochrome *P*-450scc, an integral protein of the inner membrane of adrenal cortex mitochondria. The above protein is a member of the cholesterol hydroxylating system specific to mitochondria of mammalian steroidogenic tissues. It has been shown that bovine import-competent heart mitochondria can bind the cytochrome *P*-450scc precursor but fail to import and process the protein [6,7]. Establishing the nature of the tissue specificity of the above processes help one to achieve further progress in understanding their mechanisms.

This study demonstrates that modification of the bovine cytochrome *P*-450scc precursor by linking the MetArgGlySer-His$_6$GlyIleArg sequence to its NH$_2$-terminus results in the import of the recombinant protein into rat heart mitochondria, the precursor being processed to the mature size form therewith.

Import and processing of 6His-pP450scc were also observed in the experiments on rat liver and yeast mitochondria. The data obtained suggest that tissue-specific restrictions on these processes can be lifted by modification of the targeting presequence of the cytochrome *P*-450scc precursor.

2. Materials and methods

The QIAexpress system for synthesis and isolation of recombinant proteins (QIAgen, Germany) was used in this work. The system includes the pQE plasmid and *E. coli* cells, strain SG13009[pREP4] and Ni-NTA agarose. The expressing vector was constructed by ligation of the pQE plasmid, restricted at the *SphI*/*KpnI* site in the polylinker region, and a *SphI*/*KpnI* fragment of the pCD plasmid containing cDNA for cytochrome *P*-450scc [8]. The His$_6$ block was located at the NH$_2$-terminus of the recombinant pP450scc. Expression of cDNA encoding 6His-pP450scc was induced by isopropyl β-D-thiogalactopyranoside (final concentration 1 mM) and continued for 5 h at 30°C. Cell fractionation as well as purification on a column containing Ni-NTA agarose and renaturation of 6His-pP450scc were performed as in [9].

Import of the purified 6His-pP450scc into isolated mitochondria was carried out according to [10] with minor modifications. The samples for import (200 μl) contained 10 mM MOPS (pH 7.4), 0.25 M mannitol, 50 mM KCl, 2 mM ATP, 2 mM GTP, 10 mM ADP, 3 mM K_2HPO_4, 1 mM DTT, 2 mM magnesium acetate, 5 mM sodium succinate, 1% (m/v) BSA, rabbit reticulocyte lysate (5%, v/v), isolated mitochondria (20 μg) and 6His-pP450scc (8 μg). The import was continued for 30 min, then the sample was divided in two and one of the aliquotes was treated with pronase K (100 μg/ml, 30 min, 0°C) afterwards. The action of pronase K was interrupted by adding phenylmethylsulfonyl fluoride (final concentration 2 mM). Mitochondria were sedimented by centrifugation (15,000 × *g*, 20 min) and the pellets were analyzed by immunoblotting.

The protein concentration was measured after Lowry [11] using BSA as a standard. SDS-PAGE was performed according to Laemmli [12] in 10–12.5% PAG. Immunoblotting was carried out as described in [13] using consecutively the IgG fraction of anti-P450scc antiserum, a conjugate of anti-rabbit antibodies with horse-radish peroxidase, and a peroxidase substrate (*o*-dianisidine).

Cytochrome *P*-450scc from bovine adrenal cortex and anti-cytochrome *P*-450scc antibodies were a generous gift of Dr. S.A. Usanov (Institute of Bioorganic Chemistry, Academy of Sciences of Belarus, Minsk). The rabbit reticulocyte lysate was supplied by Dr. O.N. Denisenko (Institute of Protein Research, Pushchino). The conjugate of anti-rabbit antibodies with horse-radish peroxidase was purchased from Agro-Bio (Moscow). Yeast mitochondria were isolated from *Candida valida* cells according to [14].

*Corresponding author. Fax: (7) (095) 939 31 81.

Abbreviations: pP450scc, precursor of cytochrome *P*-450scc; 6His-pP450scc, modified form of the cytochrome *P*-450scc precursor expressed in *Escherichia coli*; Ni-NTA agarose, agarose linked with Ni^{2+} nitrilo-tri-acetate.

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3. Results and discussion

Fig. 1 shows targeting presequence of a modified form of the bovine cytochrome *P*-450scc precursor. The presence of the histidine block at the NH_2-terminus of 6His-pP450scc allows one to isolate and to deal selectively with a protein containing a modified presequence.

It has earlier been shown [15] that pP450scc can be imported into isolated rat liver mitochondria taking part in steroid metabolism. To verify whether extra amino acid residues in the NH_2-presequence of 6His-pP450scc are capable of affecting its targeting properties, a similar experiment was carried out with the recombinant protein. As follows from Fig. 2, incubation of 6His-pP450scc (55 kDa) with rat liver mitochondria results in the import of the protein precursor into organelles and in the mature size form of cytochrome *P*-450scc (molecular mass of 51 kDa). Newly formed mature *P*-450scc detectable by immunoblotting has nearly the same molecular mass as cytochrome *P*-450 isolated from bovine adrenal cortex (Fig. 2, lanes 3 and 5). The intramitochondrial location of the mature *P*-450scc is evidenced by its insensitivity to exogenous pronase K while its precursor (i.e. 6His-pP450scc) suffers proteolysis (Fig.2, lanes 2 and 3). Thus, the above results suggest that the extra MetArgGlySerHis_6GlyIleArg sequence at the NH_2-terminus of the polypeptide chain does not impede the import and processing of the cytochrome *P*-450scc precursor.

It follows from the literature data that import of a variety of protein precursors into mitochondria and their processing are not tissue- and species-specific. However, taking into account the fact that bovine heart mitochondria fail to import the in vitro synthesized precursor of cytochrome *P*-450scc normally located in adrenal cortex mitochondria [7,8], it has been suggested that pP450scc can be imported only into mitochondria of steroidogenic tissues. As steroid hydroxylation system including cytochrome *P*-450scc exists in selected tissues only, it was not impossible that mitochondria of specialized steroidogenic organs have a unique translocation apparatus and enzymes (maturases) that are necessary for import and processing of pP450scc.

In this context it was of interest to find out whether the contention that the import of pP450scc is tissue-specific has some relation to its modified form, i.e. to 6His-pP450scc. We studied the heterologous import of 6His-pP450scc into isolated rat heart and yeast mitochondria. As Fig. 3 shows, 6His-pP450scc is imported by both heterologous organelles which is evidenced by the appearance of a mature size form of the protein and by insensitivity of the latter to added pronase K (Fig. 3a,b, lanes 3). As in the experiments with rat liver mitochondria, the electrophoretic mobility of processed P450scc

Met Arg Gly Ser His His His His His His Gly Ile Arg ▼ Met Leu Ala

Arg Gly Leu Pro Leu Arg Ser Ala Leu Val Lys Ala Cys Pro Pro Ile

Leu Ser Thr Val Gly Glu Gly Trp Gly His His Arg Val Gly Thr Gly

Glu Gly Ala Gly | P450scc |

Fig. 1. Primary structure of the modified presequence of the recombinant cytochrome *P*-450scc precursor. The arrow shows the beginning of the native presequence. The sequence of mature cytochrome *P*-450scc is boxed.

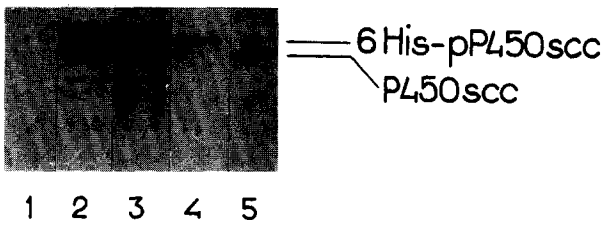

Fig. 2. Import of 6His-pP450scc into rat liver mitochondria. The samples were analyzed by immunoblotting: (1) control sample, without 6His-pP450scc; (2,3) imported protein prior to (2) and after (3) treatment with pronase K; (4) 6His-pP450scc isolated from *E. coli* cells; (5) cytochrome *P*-450scc from bovine adrenal cortex mitochondria.

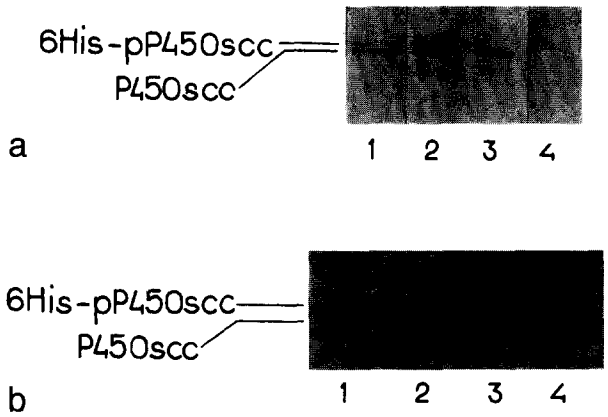

Fig. 3. Import of 6His-pP450scc into rat heart (a) and yeast (b) mitochondria. The samples were analyzed by immunoblotting: (1) 6His-pP450scc isolated from *E. coli* and mature *P*-450scc from bovine adrenal cortex; (2,3) import of 6His-pP450scc, the samples prior to (2) and after (3) the treatment with pronase K; (4) control sample without 6His-pP450scc.

corresponded to that of cytochrome *P*-450scc prepared from bovine adrenal cortex (Fig. 3a,b, lanes 1 and 3). One should emphasize that the import into rat heart mitochondria was almost as effective as that into rat liver and yeast mitochondria. Consequently, the point that the import of pP450scc into mitochondria is tissue-specific bears no relation to its modified form 6His-pP450scc.

The mitochondrial targeting presequence is known to have a high content of positively charged amino acid residues [16]. Perhaps, attachment of the MetArgGlySerHis_6GlyIleArg fragment, enriched in such residues, to the NH_2-terminus of the polypeptide chain could facilitate the penetration of the modified presequence into mitochondria. In other words, a selective barrier for protein precursors realized in mitochondria of steroidogenic organs due to putative tissue-specific receptors or the unique lipid composition of mitochondrial membranes, can be surmounted by modification of the targeting presequence of a precursor to be imported.

By and large, our experiments testify that 6His-pP450scc is capable of penetrating into heterologous mitochondria of various organs (rat heart and rat liver) and into yeast mitochondria. The imported protein suffers proteolytic processing in these mitochondria just as in homologous mitochondria. It has ear-

lier been shown that nonmodified bovine pP450scc synthesized in an in vitro transcription-translation system is imported into isolated mitochondria of soybean cotyledons [17]. All these results suggest that the capacity for import and processing of pP450scc is not inherent exclusively in mitochondria of steroidogenic organs. Crucial moments in these processes are hardly a unique mechanism of transmembrane translocation and the presence of a unique maturase. Both import and processing are rather predetermined by the structure of a protein precursor and specifically by the targeting presequence.

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