Mitochondrial Targeting of Normal and Mutant Protoporphyrinogen Oxidase*

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We have investigated the signal sequence for mitochondrial transport of mutants (I12T, 78insC, IVS2-2a→c, 336G→C, R152C, 470A→C, and L401F) and the wild type protoporphyrinogen oxidase (PPOX), which is the penultimate enzyme in the heme biosynthesis. We constructed the corresponding green fluorescent protein fusion proteins and studied their intracellular localization in COS-1 cells. We showed that 28 amino acids in the amino terminus of PPOX contain an independently functioning signal for mitochondrial targeting. The experiments with amino-terminally truncated green fluorescent protein fusion proteins revealed that amino acids 25–477 of PPOX contained an additional mitochondrial targeting signal(s). We constructed a structural model for the interaction between the amino-terminal end of PPOX and the putative mitochondrial receptor protein Tom20. The model suggests that leucine and isoleucine residues Leu-8, Ile-12, and Leu-15 forming an α-helical hydrophobic motif, LXXIXXXL, were crucial for the recognition of the targeting signal. The validity of the model was tested using mutants L8Q, I12T, and L15Q disrupting the hydrophobic surface of the LXXXIXXL helix. The results from in vitro expression studies and molecular modeling were in accordance supporting the hypothesis that the recognition of the mitochondrial targeting signal is dependent on hydrophobic interactions between the targeting signal and the mitochondrial receptor.

Protoporphyrinogen oxidase (PPOX, EC 1.3.3.4) is the penultimate enzyme in the heme biosynthesis (1). PPOX catalyzes the six-electron oxidation of protoporphyrinogen IX to the planar, fully conjugated macrocycle protoporphyrin IX in the inner membrane of the mitochondrion and requires oxygen for its activity (2). Partial deficiency of PPOX causes a disease, variegate porphyria (VP, OMIM accession number 176200), that is inherited as an autosomal dominant trait displaying incomplete penetrance (3). The biochemical abnormalities found in VP patients include overproduction and increased excretion of porphyrins and porphyrin precursors. VP manifests clinically with photosensitivity and acute attacks, which include various neuropsychiatric symptoms (4, 5).

PPOX is anchored to the inner membrane of mitochondria in eukaryotes (6) with its active site facing the cytosolic side of the membrane (7). The anchoring may involve amphipathic helical domains inserting PPOX into the inner mitochondrial membrane. Alternatively post-translational acylation may facilitate transient or permanent association of PPOX with the membrane (8). The majority of proteins imported to mitochondria contain a signal sequence of 20–60 amino acids in their amino terminus that directs them into mitochondria (9). The mitochondrial targeting signals of different polypeptides show no amino acid sequence identity, but they have characteristic physicochemical properties. They are enriched in positively charged, hydroxylated, and hydrophobic residues; have no acidic residues; and usually are able to form an amphiphilic secondary structure (10, 11). There is evidence that several amino-terminal mitochondrial targeting signals interact with the general import receptor Tom20 (translocase of outer membrane), which is a part of the TOM complex (12, 13) (for a review, see Refs. 14 and 15).

The amino terminus of PPOX contains a characteristic βαβ dinucleotide binding motif (Fig. 1A), which is often found within flavine adenine dinucleotide-binding domains (16). The amino terminus of PPOX is also a putative mitochondrial targeting domain since it contains three basic residues and no acidic residues, and it is capable of forming α-helix (17). Of the more than 110 mutations reported in the PPOX gene worldwide, five are located in this domain and potentially interfere with the mitochondrial transport (18–22).

In this communication we have investigated the mitochondrial transport of seven PPOX mutants by expressing the green fluorescent protein (GFP) fusion proteins in COS-1 cells and studying their intracellular localization. The mitochondrial targeting signal in the amino terminus of PPOX was characterized, and a model for the interaction between the targeting signal and the mitochondrial receptor Tom20 was proposed.

MATERIALS AND METHODS

Seven mutations selected for this study were previously identified from Finnish VP patients and expressed in Escherichia coli and COS-1 cells (Table I) (21, 22).

Normal and Mutant PPOX-GFP Constructs—The normal and mutant PPOX cDNAs were expressed as CT-GFP fusion proteins in COS-1 cells using the pcDNA3.1/CT-GFP vector (Invitrogen). The human PPOX-pUC18 (kindly provided by Prof. S. Taketani, Kyoto Institute of Technology) was amplified by PCR and subcloned into the pcDNA3.1/CT-GFP vector. The resulting plasmid was linearized with Not I and pulse transformed into E. coli DH5α (GIBCO BRL). The selected clone was sequenced. The normal and mutant PPOX-GFP constructs were linearized with Not I and pulse transformed into E. coli DH5α (GIBCO BRL). The selected clone was sequenced.

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§ The abbreviations used are: PPOX, protoporphyrinogen oxidase; GFP, green fluorescent protein; VP, variegate porphyria; Tom, translocase of outer membrane; pALDH, aldehyde dehydrogenase precursor peptide; CT, carboxyl terminus; IVS, intervening sequence.

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Technology, Kyoto, Japan) was digested with EcoRI and XhoI, and the fragment including exons 1–7 was ligated as a cassette into the pcDNA3.1/CYT-GFP vector with T4 DNA ligase (New England Biolabs, Beverly, MA). Exons 7–13 were amplified using PPOX-pUC18 as a template and primers 1 and 2, which abolished the stop codon (Table II). The PCR fragment was digested with XhoI and ligated as a cassette into PPOX-{ex1–7}-pcDNA3.1/CYT-GFP to obtain a full-length PPOX-GFP. For PPOX-(1–28)-L8Q-GFP, exons 2–7 were amplified using PPOX-pUC18 as a template and primers 3 and 11, and PPOX-(1–28)-I12T-GFP was created with primers 3 and 4 together with PPOX-pUC18 as a template (Protein Data Bank entry 1OM2). pALDH-(12–22), as a template (Protein Data Bank entry 1GOS) with 42.7% amino acid identity as a template (Swiss-Model). The model of PPOX-(6–23) bound to Tom20 was constructed by the program Malign (26) in Bodil was used in the coordinates of the NMR structure of rat Tom20 in a complex with an 11-residue recognition peptide from rat aldehyde dehydrogenase, pALDH-(12–22), as a template (Protein Data Bank entry 1OM2). pALDH-(12–22) contains a hydrophobic LXXLL motif in an $\alpha$-helical conformation where leucines are in contact with a hydrophobic groove on the surface of Tom20 (Fig. 2F) (25). Rat Tom20 shows a 98% amino acid identity with human Tom20, and the amino acids in the vicinity of the hydrophobic groove are fully conserved (25).

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<td>CTC TCC ACT AGG TCC TCT AGT ATG GCC TGG CTC</td>
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$^a$ The mutation I12T co-segregated with the polymorphism P256R (767C→G).
$^b$ Refs. 21 and 22.
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Model of the Interaction of PPOX with Tom20—Analysis of the amino-terminal sequence of PPOX using the Swiss-Model and MolMol programs predicts that an 11-residue polypeptide, PPOX-(11–21), forms an $\alpha$-helical structure where hydrophobic residues are clustered together (Fig. 1) (Refs. 23 and 24; www.expasy.org/swissmod/SWISS-MODEL.html, compared with monoamine oxidase-B structure (Protein Data Bank entry 1GOS) with 42.7% amino acid identity as a template).

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important determinant of this comparison was alignment of the ISGLA motif in PPOX-(1–23) with the LSRLL motif in pALDH-(12–22). ISGLA is the only part in PPOX-(1–23) that has a hydrophobic surface in the /H9251-helical conformation similar to the /H9251-helical LSRLL motif in pALDH-(12–22), which is needed for the interaction with Tom20 (25). The model of the 18-residue PPOX-(6–23) was constructed using Sybyl 6.8 (Tripos Inc., St. Louis, MO) (Fig. 2A).

The /H9251-helical model of PPOX-(6–23) was superimposed on the coordinates of pALDH-(12–22) in a complex with Tom20 applying a constraint that the LXXXIL motif in pALDH-(12–22) must be aligned with the LXXXIA motif in PPOX-(6–23). Steric clashes were removed manually by modifying the amino acid side chains using Bodil Modeling Environment. The /H9251-helical structure of pALDH-(12–22) bound to Tom20 ends amino-terminally to Pro-13, whereas in our model of PPOX-(6–23) (VVLGGGISGLASYHLSR) the /H9251-helix (underlined) continues an additional turn to facilitate the contact of Leu-8 with Tom20 (Fig. 2C). Together the residues 8–15 (LXXXIXXL) of PPOX-(6–23) form a continuous hydrophobic surface on one face of the /H9251-helix. (Fig. 2A). The validity of the model was tested using mutants Leu-8, Ile-12, and Leu-15 that disrupt the hydrophobic face of the LXXXIL helix.

The Amino Terminus of PPOX Contains a Mitochondrial Targeting Signal—We investigated the transport of the wild type and seven PPOX mutants to mitochondria by constructing GFP fusion proteins in which GFP was located at the carboxyl-
terminal end of PPOX (Fig. 3). PPOX-GFP and GFP construct alone were used as controls in all experiments. COS-1 cells were transfected with the constructs, and GFP fluorescence was analyzed with confocal laser scanning microscopy to monitor subcellular localization of the polypeptides. The cells expressing the wild type PPOX-GFP demonstrated a typical filamentous mitochondrial pattern (27, 28), which was clearly distinguishable from the cytosolic pattern of GFP construct alone (Fig. 3, A and B). The localization was confirmed by counterstaining with a mitochondrion-specific dye (Fig. 3C). A pattern indistinguishable from PPOX-GFP was observed for each of the seven mutants (Fig. 3D) indicating that transport to mitochondria was not impaired in any of them. Since the truncated PPOX fusion protein corresponding to the mutation 78insC in exon 2 (PPOX-(1–28)-GFP) contained only the first 28 residues of PPOX, these amino acids must contain an independently functioning mitochondrial targeting signal.

The Amino Acids 25–477 of PPOX Contain an Alternative Mitochondrial Targeting Signal(s)—After localization of the mitochondrial targeting signal in the amino terminus of PPOX, two additional constructs were created where this region was removed either totally (PPOX-(Δ5–23)-GFP) or partially (PPOX-(Δ5–11)-GFP) (Fig. 4A). The experiments using fluorescence confocal microscopy demonstrated that these fusion proteins were associated with the mitochondria indicating that the amino acids 25–477 of PPOX must contain an additional mitochondrial targeting signal(s).

To make a hypothesis for the molecular recognition of the amino-terminal mitochondrial targeting signal of PPOX, we constructed an α-helical model of PPOX-(6–23) and a model of the interaction between PPOX-(6–23) and the mitochondrial import receptor Tom20. The model places the LXXXIXL
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Fig. 4. A, PPOX-GFP fusion proteins with modified amino termini and GFP fluorescence of transfected COS-1 cells. The numbers refer to the amino acids of PPOX. B, PPOX-(1–28)-GFP fusion proteins with modified amino termini and GFP fluorescence of transfected COS-1 cells. The mutated residues are shown in a black box. C, solvent-accessible surfaces of the α-helical PPOX-(6–23) models. Hydrophobic and polar regions are colored blue and red, respectively.

(Leu-8, Ile-12, and Leu-15) motif of PPOX-(6–23) in contact with the hydrophobic groove on the Tom20 surface (Fig. 2B). We tested our structural model by mutating the PPOX-(6–23) residues Ile-12 into threonine and residues Leu-8 and Leu-15 into glutamine. These amino acid substitutions disrupt the hydrophobic face of the LXXIILXX motif (Fig. 4C), which should affect the capability of interaction with Tom20. Each mutation caused a disruption of PPOX transport into mitochondria (Fig. 4, B and C) confirming that each of these residues was essential for the PPOX targeting. In the amino terminus of PPOX, the LXXIILXX motif including residues Leu-8, Ile-12, and Leu-15 appeared to be crucial for the putative interaction between PPOX and Tom20.

DISCUSSION

In this communication, we have investigated the signal sequence for the mitochondrial transport of the wild type PPOX and mutants by constructing the corresponding GFP fusion proteins. The intracellular localization of the seven mutants corresponding to clinically manifest VP showed no impairment of mitochondrial targeting. Because of the naturally occurring mutation 78insC, we were able to show that the first 28 amino acids in the amino terminus of PPOX contained sufficient information for transporting a reporter protein into mitochondria.

The predicted secondary structure of the PPOX amino terminus consists of a βββ motif (17) where the α-helix is a common structure found in mitochondrial targeting signals. Our structural model of interaction between the amino terminus of PPOX and Tom20 and the experiments with amino-terminally mutated fusion proteins indicated that the critical residues for the recognition of the PPOX targeting signal include leucine and isoleucine residues that form a hydrophobic motif, LXXIILXX. Our findings support the hypothesis that recognition of the mitochondrial targeting signal is dependent on hydrophobic interactions with the mitochondrial receptor. The NMR structure of rat Tom20 in a complex with the mitochondrial presequence peptide revealed that an amphiphilic α-helical structure of the presequences was important for binding to the receptor (25). Subsequent mutagenesis studies showed that the hydrophobic residues were essential for binding to Tom20, while the hydrophilic residues, including two positively charged arginines in the presequence, were dispensable (25). Consistently, in the amino-terminal recognition sequence of PPOX, replacing a single hydrophobic leucine or isoleucine with a hydrophilic residue of the same size could prevent the mitochondrial transport.

The amino terminus of PPOX contains only three positively charged residues, which is less than in most presequences (29). It has been postulated that the positively charged residues could be involved in subsequent ionic interactions between the targeting signal and Tom22, which is also known to bind amino-terminal recognition sequences, especially their carboxyl-terminal parts, in a salt-sensitive manner (13, 30). In the case of PPOX, two positively charged residues, Arg-23 and Lys-29, which are located in the carboxyl-terminal part of the recognition sequence, could facilitate this interaction.

Surprisingly PPOX derivatives, where the amino-terminal targeting signal was removed either totally or partially, were still located in the mitochondria. This implies that the residual part of PPOX must contain an additional mitochondrial targeting signal(s) (29). PPOX is a further example of mitochondrial proteins whose import is not strictly dependent on the presence of an amino-terminal presequence (29, 31–33). Secondary structure prediction of PPOX reveals several internal leucine-rich α-helical segments with a net positive charge. Such segments can putatively form hairpin-like structures that mimic a typical amphiphilic presequence and function as an internal mitochondrial targeting sequence (34). Without knowledge of the tertiary structure of PPOX it is, however, difficult to predict which of these segments could be accessible to the receptor and serve as an effective targeting signal.

Since the naturally occurring mutation I12T resides in the conserved amino terminus of PPOX, the mutation could inter-
fere with mitochondrial transport and modify the phenotype of the disease. Our study shows that although the I12T substitution is able to disrupt the mitochondrial transport of the truncated PPOX, the corresponding full-length PPOX is transported into mitochondria. In vitro expression studies of the I12T substitution have shown a dramatic loss of the enzyme activity both in prokaryotic and eukaryotic cells (21). A homozygous patient with the I12T substitution has been identified with around 10% residual PPOX activity measured from his lymphocytes (21). It would be intriguing to hypothesize that in this patient the secondary mitochondrial targeting signal(s) could serve as a backup system that directs the polypeptide into mitochondria if the primary signal fails. This transport could disrupt the final steps of the heme biosynthesis in the non-optimal submitochondrial compartmentalization. This may be, however, less specific and efficient (35) and lead to non-optimal sub mitochondrial compartmentalization. This could disrupt the final steps of the heme biosynthesis in the inner mitochondrial membrane especially if the substrate channeling between the last three enzymes of the pathway, namely coproporphyrinogen oxidase, PPOX, and ferrochelatase, occurs through an enzyme complex as suggested by Ferreira et al. (7). Disruption of the enzyme complex would explain the low ferrochelatase activity (10% and 30–40% of normal) measured from the homozygous and heterozygous patients' erythrocytes, respectively.

Acknowledgments—We thank Dr. Marc Baumann for excellent assistance in protein chemistry and Dr. Pekka Lehto vuori for assistance in molecular modeling.

REFERENCES
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