Identification of sequences required for the import of human protoporphyrinogen oxidase to mitochondria

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Protoporphyrinogen oxidase (PPOX; EC 1.3.3.4), the penultimate enzyme of haem biosynthesis, is a nucleus-encoded flavoprotein strongly associated with the outer surface of the inner mitochondrial membrane. It is attached to this membrane by an unknown mechanism that appears not to involve a membrane-spanning domain. The pathway for its import to mitochondria and insertion into the inner membrane has not been established. We have fused human PPOXs containing N-terminal deletions, C-terminal deletions or missense mutations to yellow fluorescent protein (YFP) and have used these constructs to investigate the mitochondrial import of PPOX in human cells. We show that all the information required for efficient import is contained within the first 250 amino acid residues of human PPOX and that targeting to mitochondria is prevented by fusion of YFP to the N-terminus. Deletion of between 151 and 175 residues from the N-terminus is required to abolish import, whereas shorter deletions impair its efficiency. Fully efficient targeting appears to require both a major targeting signal, the whole or part of which is contained

between residues 151 and 175, and which may be involved in anchoring to the inner mitochondrial membrane, together with interaction between this region and a sequence(s) within the first 150 residues. These features suggest that the mechanism for import of human PPOX to mitochondria differs from those identified for the translocation of nucleus-encoded, membrane-spanning, inner membrane proteins. In addition, a missense mutation outside this region (Val³³⁵ → Gly) prevented targeting to mitochondria and delayed the appearance of YFP fluorescence. This mutation appeared to prevent import by a direct effect on protein folding rather than by altering a sequence required for targeting. It may lead to sequestration of the PPOX–YFP construct in an unfolded conformation, followed by proteolytic degradation, possibly through enhanced binding to a cytosolic chaperone protein.

Key words: human missense mutation, mitochondrial import, protoporphyrinogen oxidase, yellow fluorescent protein.

INTRODUCTION

Protoporphyrinogen oxidase (PPOX; EC 1.3.3.4) is a homodimeric flavoprotein that catalyses the oxygen-dependent aromatization of protoporphyrinogen to protoporphyrin IX, the penultimate reaction in the biosynthesis of both haem and the chlorophyll precursor, magnesium protoporphyrin [1]. The enzyme is the target for diphenyl ether and related herbicides [2], and partial deficiency of PPOX activity is the inherited biochemical abnormality in the human autosomal dominant disorder variegate porphyria [3].

Human PPOX contains 477 amino acids and has a molecular mass of 50.8 kDa [4]. Its sequence shows some similarity to PPOXs from other eukaryotic and aerobic prokaryotic organisms, resemblance being greatest at the N-terminus (residues 5–34), where there is a highly conserved $\beta\alpha\beta$ dinucleotide-binding fold [4–6] of the type found in other FAD-binding proteins [6]. A crystal structure has not yet been reported for any PPOX. Yeast PPOX is tightly folded into a compact form that can be separated into 25 kDa N-terminal and 35 kDa C-terminal domains by treatment with proteases [5]. Only the C-terminal domain reacts specifically with an active-site-directed reagent derived from a diphenyl ether herbicide [5].

Eukaryotic mitochondrial PPOXs are strongly associated with the mitochondrial inner membrane [7–11] and require detergent for solubilization [8–11]. Experiments with a membrane-impermeant PPOX inhibitor suggest that the active site is on the cytosolic surface of the membrane [9]; protoporphyrin is then transferred to the next enzyme, ferrochelatase, which spans the membrane with its active site facing the matrix [12]. Secondary-

structure prediction does not reveal a typical membrane-spanning α -helical structure in any eukaryotic PPOX [13–15]. A conserved region in the N-terminal domain (residues 142–192 in human PPOX) has been proposed as a putative membrane-anchoring domain [5,15]. Anchoring of the yeast enzyme may be enhanced by acylation [15], but there is as yet no evidence for a similar process in other PPOXs.

The mechanisms by which PPOXs are targeted to the inner mitochondrial membrane are largely unknown. Other mitochondrial enzymes of haem synthesis all have cleavable presequences that direct import [16]. Of the mitochondrial PPOXs investigated to date, only those of *Arabidopsis thaliana* and spinach conform to this pattern [17,18]. Human, mouse, yeast and tobacco PPOXs have no cleavable presequence [10,13,14,19] and, like many other inner membrane proteins [20], presumably contain non-cleavable N-terminal or more distal internal targeting signals. The 13 N-terminal residues of yeast PPOX may act as a non-cleavable targeting sequence [21], and the 28 N-terminal residues of the human enzyme have some features characteristic of a presequence, although it does not form an amphipathic α -helix [4,22]. Investigation of the effect of truncating mutations that cause variegate porphyria show that these 28 residues contain an independently functioning signal for mitochondrial targeting [22]. However, deletion of this region from wild-type PPOX does not prevent translocation to mitochondria [22], indicating that a more distal region(s) is required for import and insertion of the whole enzyme into the inner membrane.

In the present study, we have fused a series of deletion and missense mutants of human PPOX to yellow fluorescent protein (YFP) and have used these constructs to identify regions of the

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; Hsp, heat shock protein; PPOX, protoporphyrinogen oxidase; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane; YFP, yellow fluorescent protein.

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Figure 1 Fusion proteins

Diagrammatic representation of fusion proteins obtained by expression of the corresponding plasmid. Open boxes, PPOX, either full-length (a), (b) or partially deleted (c)–(i); hatched boxes, YFP. Fusion proteins with deletions of the first 200, 250 and 300 residues of PPOX were also constructed, but are not shown.

enzyme that are required for its import to mitochondria in human cells. Our experiments suggest that the import pathway may differ from those described for membrane-spanning inner membrane proteins.

EXPERIMENTAL

Construction of plasmids expressing full-length and truncated PPOX–YFP fusion proteins

To obtain plasmids expressing the fusion proteins shown in Figure 1, full-length or truncated PPOX cDNA was amplified from wild-type pHPOX plasmid [14] using BIO-X-ACT DNA polymerase (Bioline, London, U.K.) with 30 cycles of PCR (denaturation at 94 °C for 40 s, annealing at 69 °C for 1 min and extension at 73 °C for 2 min). Oligonucleotides were synthesized by OSWEL DNA Service (Southampton, U.K.). Primer sequences are available from the authors upon request. Amplified DNA was gel-purified using a Qiagen gel extraction kit (Qiagen, Crawley, West Sussex, U.K.), and cycle-sequenced using BigDye™ Terminator Ready Reaction mix and an AB1377 automated sequencer (Applied Biosystems, Warrington, U.K.). Purified DNA was cloned into EYFP-N1 (Clontech, Oxford, U.K.) via pGEMT (Promega, Southampton, U.K.), at restriction sites for *Hind*III and *Bam*HI or, for the YFP–PPOX construct, *Bgl*II and *Sal*I. Positive clones were identified by restriction digestion.

Construction of PPOX missense mutant–YFP fusion proteins

Mutants were generated in the prokaryotic expression plasmid pHPOX [14] using the QuikChange method (Stratagene, La Jolla, CA, U.S.A.); primer sequences are available from the authors upon request. Full-length mutant PPOX cDNAs were amplified as above, gel-purified and cycle-sequenced to confirm that only the specified mutation had been created. Mutant PPOX cDNAs were cloned into EYFP-N1 via pGEMT at restriction sites for *Hind*III and *Bam*HI, and positive clones were identified by restriction digestion.

Cell culture, transfection and fluorescence microscopy

HeLa Ohio and HEK-293T cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM; Invitro-

gen, Glasgow, U.K.) containing 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin and maintained at 37 °C with 5% CO₂. Cells were seeded on to sterile coverslips in six-well culture plates at a concentration of 125 000 cells/ml in complete culture medium, and grown for 18–24 h. For transient expression of YFP, cells were transfected with 2 µg of plasmid/well using LIPOFECTAMINE™ PLUS (Invitrogen) according to the manufacturer's standard procedure. At 4–24 h after transfection, fluorescence was observed in living cells using either an MRC-1024MPR multi-photon confocal microscope (Bio-Rad, Hemel Hempstead, Herts., U.K.) with images collected and correlation coefficients (r) for co-localization of fluorophores determined using Laser Sharp Acquisition software (Bio-Rad), or an Axiovert S100TV fluorescence microscope (Zeiss, Welwyn Garden City, Herts., U.K.) and a C4742-95 camera (Hamamatsu) with images collected using Kinetic Imaging AQMe Orca I software (Kinetic Imaging Ltd, Liverpool, U.K.). To test for mitochondrial location, cells were incubated with MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, U.S.A.) for 10 min at 37 °C with 5% CO₂ before microscopic analysis.

Cell fractionation and fluorimetry

HeLa Ohio cells were seeded at a concentration of 5×10^6 cells in a volume of 10 ml of complete DMEM into 100 mm-diameter dishes and grown for 24 h as described above. Cells were transfected with 4 µg of either wild-type PPOX–YFP or EYFP-N1 plasmid, as described above, and grown at 37 °C with 5% CO₂ for 48 h. Soluble and particulate fractions were prepared from transfected cells using digitonin [23]. Fluorescence was measured in whole or fractionated HeLa cells expressing PPOX–YFP or YFP using a TD700 fluorimeter (Turner Designs, Sunnyvale, CA, U.S.A.) using a 486 nm excitation filter and a 510–700 nm emission filter.

Efficiency of mitochondrial targeting

Efficiency of mitochondrial targeting was assessed by determining the percentage of total cell fluorescence located in mitochondria. Fluorescence was counted in each cell within three cellular compartments: the cytosol, nucleus and mitochondria. The mean fluorescence intensity was measured in each selected region of the cell using Laser Sharp processing software (Bio-Rad). In each case, background fluorescence intensity was determined from a cell-free area of the coverslip and subtracted from each of the cellular mean fluorescence intensity measurements. For each construct, a minimum of 60 cells was counted from at least four separate transfection experiments.

Analysis of mRNA

HeLa Ohio cells were seeded on to sterile coverslips in six-well culture plates in duplicate at a concentration of 125 000 cells/ml in complete culture medium and were grown for 24 h at 37 °C with 5% CO₂. Cells were transfected with 2 µg of either wild-type PPOX–YFP or V335G-PPOX–YFP (containing the Val³³⁵ → Gly mutation) plasmid DNA per well using LIPOFECTAMINE™ PLUS, as described above. Total RNA was harvested from transfected cells at 4, 8, 12 and 24 h, or from untransfected control cells at zero time, using Ultraspec RNA isolation reagent (Biotex Laboratories, Houston, TX, U.S.A.), according to the manufacturers' standard procedure. Total RNA (1 µg) was primed using an oligo(dT) primer and reverse-transcribed using 220 units of M-MLV Reverse Transcriptase (Promega) at 42 °C for 50 min

Figure 2 Effect of PPOX deletions on the distribution of fluorescence in transfected cells

HeLa cells were transfected with plasmids expressing YFP or PPOX-YFP (**A**), fusion proteins with C-terminal deletions of PPOX (**B**) or fusion proteins with N-terminal deletions of PPOX (**C**), as indicated above the panels. The right-hand panels of (**A**) and (**B**) are overlay images showing co-localization of MitoTracker Red (preceding panels) and YFP fluorescence in cells incubated with MitoTracker Red and expressing either PPOX-YFP (**A**) or PPOX(Δ151-477) (**B**).

in a reaction volume of 20 μ l. The reaction was terminated by heating at 95 $^{\circ}$ C for 2 min. A multiplex PCR was performed to amplify a 490 bp fragment of PPOX-YFP or V335G-PPOX-YFP cDNA, using primers that spanned the fusion region, and a 204 bp control fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA using DyNAzyme II DNA polymerase (Flowgen, Ashby-de-la-Zouch, Leics., U.K.) with 28 cycles of PCR (denaturation at 94 $^{\circ}$ C for 40 s, annealing at 60 $^{\circ}$ C for 1 min and extension at 73 $^{\circ}$ C for 2 min). Primer sequences are available from the authors upon request.

RESULTS

Human PPOX-YFP is imported to mitochondria

HeLa cells were transiently transfected with either a YFP or PPOX-YFP expression plasmid and examined *in vivo* by confocal or fluorescence microscopy. Cells showed a yellow/green fluorescence at 4 h after transfection; both the number of fluorescent

cells and the intensity of fluorescence increased progressively to 24 h after transfection. In cells expressing YFP alone, fluorescence was spread diffusely throughout the cytosol and nucleus. In contrast, in cells transfected with the PPOX-YFP expression plasmid, 80 % of the total cellular fluorescence was present in rod-like structures typical of mitochondria (Figure 2). The location of this fluorescence in mitochondria was confirmed by showing that it co-localized with MitoTracker Red ($r = 0.98$), a fluorescent dye that accumulates in active mitochondria (Figure 2). An identical pattern of fluorescence was observed in a human kidney cell line, HEK-293T, indicating that it was not specific to liver-derived HeLa cells.

When cells were disrupted with digitonin 24 h after transfection, 66 % of the total recovered fluorescence remained in the particulate fraction of cells expressing the fusion protein, whereas most (88 %) of the fluorescence in cells containing YFP alone was in the supernatant fraction; a finding consistent with tight association between PPOX-YFP and the inner mitochondrial membrane, as reported for the native enzyme [7-9].

Table 1 Effect of deletions on efficiency of mitochondrial import of PPOX–YFP

Cells were transfected with the plasmids indicated and fluorescence was measured after 24 h as described in the Experimental section. Values are means $\pm$ S.D. Statistical significance of differences between groups are: *not significant or ** $P < 0.01$, compared with the PPOX–YFP group, and †not significant or †† $P < 0.01$, compared with the PPOX(Δ 1–50)–YFP group.

Plasmid	No. of transfected cells examined	Mitochondrial fluorescence (% of total cell fluorescence)	Import efficiency (% mean pPPOX–YFP)
pPPOX–YFP	194	79.8 $\pm$ 11.0	100
pPPOX(Δ 251–477)–YFP	89	78.7 $\pm$ 15.4*	98.6
pPPOX(Δ 151–477)–YFP	66	52.2 $\pm$ 10.8**	65.4
pPPOX(Δ 1–149; Δ 251–477)–YFP	69	48.9 $\pm$ 10.6**	61.3
pPPOX(Δ 1–50)–YFP	79	72.8 $\pm$ 13.3**	91.2
pPPOX(Δ 1–100)–YFP	64	68.9 $\pm$ 21.2**†	86.3
pPPOX(Δ 1–150)–YFP	67	67.8 $\pm$ 18.4**††	85.0

These data confirm that fusion of YFP to the C-terminus of human PPOX creates a protein that is targeted exclusively to mitochondria [22]. To determine whether a free N-terminus is necessary for import, we transfected HeLa cells with a plasmid expressing YFP–PPOX. At 24 h, diffuse fluorescence was observed in the nucleus and diffuse or punctuate fluorescence, which did not co-localize with MitoTracker, in the surrounding cytosol. No import into mitochondria was detected (results not shown).

The N-terminal sequence 1–250 of PPOX targets YFP exclusively to mitochondria

To determine which part of PPOX contains the information required for targeting YFP to mitochondria, we initially constructed a plasmid, PPOX(Δ 251–477)–YFP, that encoded a fusion protein that lacked most of the C-terminal part of the enzyme. This protein was as efficiently targeted to mitochondria as PPOX–YFP (Figure 2; Table 1), co-localizing with MitoTracker ($r = 0.98$) to the same extent. Constructs that contained either the first 150 or the second 100 residues of this N-terminal fragment also directed YFP to mitochondria, but with lower efficiency, as indicated by the accumulation of fluorescence elsewhere in the cell (Figure 2; Table 1). These results show that each of these regions contains a signal(s) that is capable of independently targeting YFP to mitochondria, although with low efficiency (Table 1). For fully efficient targeting, a longer N-terminal sequence of between 151 and 250 residues is required. This longer sequence appears to contain at least two separate, interacting signals.

Deletion of the N-terminal third of human PPOX is required to abolish its targeting to mitochondria

To investigate the location of targeting information between residues 1 and 250 of PPOX in more detail, we transfected HeLa cells with plasmids expressing PPOX–YFP fusion proteins with progressively longer deletions of N-terminal sequence. Figure 2 shows that import was not abolished until 175 amino acid residues had been deleted from the N-terminus; the patterns of fluorescence observed with constructs lacking 175, 200, 250 and 300 residue lengths of N-terminal sequence being indistinguishable from those when only YFP was expressed. Shorter deletions (50, 100 and 150 residues) had a much less marked effect, but did impair targeting to a small, and probably progressive, extent (Figure 2; Table 1). In cells transfected with these constructs, extra-mitochondrial fluorescence was present 4 h after transfection, and its intensity relative to mitochondrial fluorescence did not change over the next 20 h. These experiments show that deletion of a maximum of 24 residues beyond the first 150 N-terminal residues has a much greater effect on import than step-wise removal of 50-residue sequences up to that point.

Substitution of an amino acid residue in the C-terminal domain of human PPOX prevents mitochondrial import

Because missense mutations in defined mitochondrial targeting sequences have been shown to disrupt import [24,25], identification of missense mutations that prevent import of those mitochondrial proteins without known signalling sequences may help to locate such sequences. We therefore examined the effect of 22 missense mutations in the human PPOX gene on translocation of PPOX–YFP to mitochondria. All these mutations have been shown to cause variegate porphyria and decrease PPOX activity, as assessed by expression in *Escherichia coli* [26]. HeLa cells were transfected with plasmids expressing PPOX–YFP fusion proteins in which the following amino acid substitutions had been introduced by site-directed mutagenesis: Leu¹⁵ $\rightarrow$ Phe, Arg³⁸ $\rightarrow$ Pro, Gly⁴⁰ $\rightarrow$ Asp, Leu⁷³ $\rightarrow$ Pro, Val⁸⁴ $\rightarrow$ Gly, Leu⁸⁵ $\rightarrow$ Pro, Glu¹⁴³ $\rightarrow$ Val, Arg¹⁵² $\rightarrow$ Cys, Leu¹⁵⁴ $\rightarrow$ Pro, Val¹⁵⁸ $\rightarrow$ Met, Arg¹⁶⁸ $\rightarrow$ His, Ala¹⁷² $\rightarrow$ Val, Gly²³² $\rightarrow$ Arg, Δ His²⁸¹, Val²⁸² $\rightarrow$ Asp, Val²⁹⁰ $\rightarrow$ Leu, Leu²⁹⁵ $\rightarrow$ Pro, Val³³⁵ $\rightarrow$ Gly, Ser³⁵⁰ $\rightarrow$ Pro, Leu⁴⁴⁴ $\rightarrow$ Pro, Gly⁴⁵³ $\rightarrow$ Arg and Gly⁴⁵³ $\rightarrow$ Val. Cells were examined by fluorescence microscopy 24 h after transfection.

All mutant fusion proteins, apart from V335G-PPOX–YFP, were translocated to mitochondria, giving a pattern of intracellular fluorescence that was indistinguishable from that seen in cells transfected with wild-type PPOX–YFP. The intensity of mitochondrial fluorescence was similar for mutant and wild-type PPOX–YFPs, a finding that suggests that none of the missense mutations significantly decreased protein stability.

In contrast, cells transfected with the plasmid expressing V335G-PPOX–YFP showed the same pattern of fluorescence at 24 h as those expressing YFP alone (Figure 3). Diffuse fluorescence was present throughout the cytosol and nucleus, and no mitochondrial fluorescence was observed. In addition, there was a marked delay in the appearance of fluorescence in cells transfected with the mutant construct, with only very faint fluorescence being visible at 8 h (Figure 3). No such delay was observed in cells expressing deletion constructs that impaired import. Semi-quantitative RT-PCR (reverse transcription-PCR) analysis of mRNAs for V335G-PPOX–YFP and PPOX–YFP from these cells showed that their concentrations were similar throughout the time course (results not shown), indicating that the delay in appearance of fluorescence was caused by an effect of the mutation at or after translation.

DISCUSSION

Several different types and arrangements of targeting and sorting signals have been identified in those mitochondrial proteins that, like PPOX, are encoded by the nuclear genome and synthesized in

Figure 3 Effect of the PPOX mutation, Val¹³⁵ → Gly, on the time course of appearance and distribution of fluorescence in transfected cells

HeLa cells were transfected with plasmids expressing V335G-PPOX-YFP (A), YFP (B) or PPOX-YFP (C), and were examined by fluorescence microscopy at the times indicated above each column.

the cytosol [20,27]. Mammalian PPOXs fall into the group of inner membrane proteins that lack a cleavable presequence. Proteins in this group contain an internal targeting signal preceded by a hydrophobic transmembrane sequence [28] or multiple targeting and membrane-insertion signals distributed throughout the whole protein [20,29] or its C-terminal half [30]. Unlike these proteins, PPOX apparently contains no membrane-spanning region [13–15], and the mechanism that targets it to the inner membrane has yet to be established.

Our results confirm the previous report that human PPOX, fused at its C-terminus to green fluorescent protein (GFP) and overexpressed in human cells, is efficiently imported to mitochondria [22]. In addition, we found that, after disruption of cells using conditions likely to separate the outer mitochondrial membrane from mitoplasts [23], most of the fluorescence was firmly bound to a particulate fraction that sedimented at 12 000 *g*. This finding suggests that most of the fusion protein may, like native PPOX, have become anchored to the inner membrane, but further investigation is required to establish its exact location.

Residues 1–28 of human PPOX are sufficient to target GFP to mitochondria [22]. Structural modelling and site-directed mutagenesis experiments suggest that residues Leu⁸, Ile¹² and Leu¹⁵ within a putative hydrophobic α -helix interact with Tom20, a 20 kDa receptor protein of the translocase of the outer membrane

(TOM) [27]. However, since neither deletion of this region, nor pathological missense mutations (Leu¹² → Thr [22] or Leu¹⁵ → Phe) within it, impair mitochondrial import [22], it may act only as an artificial signal, as has been described for the N-terminal region of the uncoupling protein (UCP1) of the inner mitochondrial membrane [30], and not be required for targeting of full-length PPOX. The present study provides some support for the hypothesis that residues 1–28 contain a signal sequence that, in co-operation with more distal elements, functions within the context of the whole enzyme to direct import. First, deletion of the first 50 residues had a small effect on import efficiency (Figure 2; Table 1); however, it is not clear whether the difference between this observation and the previous report that deletion of the putative signal sequence within the first 28 residues had no effect [24] is explained by the presence of a separate signal sequence in our larger deletion or by different experimental conditions. Secondly, our finding that extension of the N-terminus by fusion to YFP prevents import is more consistent with import in an N-to C-terminus orientation directed by an N-terminal signal than in the loop conformation adopted by most transmembrane inner membrane proteins with solely internal signals [31,32].

Whatever the role of residues 1–50, our experiments provide evidence that the major signal(s) for mitochondrial import of PPOX lies between residues 150 and 250, with the sequence of

Figure 4 Multiple sequence alignment of mitochondrial PPOX proteins

(A) The putative PPOX membrane-anchoring domain [15]. (B) The region around Gly³³⁵. The deduced sequence for the human enzyme (GenBank® accession number XP_042436) was compared with those of mouse (AAH02047), spinach (BAB60710), tobacco (O24164), *Arabidopsis thaliana* (NP_192078), *Myxococcus xanthus* (P56601), *Bacillus subtilis* (NP_388895) and *Saccharomyces cerevisiae* (NP_010930) using Clustal (version 1.8) and BLAST protein alignment programmes. Aligned sequences were shaded using Boxshade (<http://www.ch.embnet.org>). Residues shaded black are identical with the column consensus; those shaded grey are similar. Arrows indicate residues 175 and 335 in the human sequence. Putative helical regions of the human enzyme are underlined.

residues 151–175 containing an important component. Direction of YFP to mitochondria with the same efficiency as PPOX–YFP appears to require fusion with an N-terminal sequence that is between 151 and 250 residues long (Figure 2; Table 1). When N-terminal sequences were deleted sequentially, extension of the deletion from 150 to 175 residues completely abolished targeting, whereas shorter deletions did not (Figure 2). However, although the major import signal appears to be located between residues 150 and 250, interaction with a more proximal N-terminal sequence(s) is required for fully efficient import (Figure 2; Table 1). Residues 1–150 also directed YFP to mitochondria, but with lower efficiency, and deletions within this region also decreased import efficiency.

The sequence between residues 151 and 250 contains a region (residues 143–202) that is similar in all mitochondrial PPOXs whose sequence has been determined (Figure 4). It has been suggested that putative amphipathic helical domains in this region interact with the lipid bilayer to anchor PPOX to the inner membrane [5,15]. Secondary-structure predictions indicate that residue 175 may lie in a fold between two helical structures (residues 158–167 and 177–191). Extension of the N-terminal deletion from 150 to 175 residues may therefore disrupt the membrane-anchoring domain and prevent insertion into the inner membrane. For membrane-spanning proteins of the inner membrane that contain internal targeting signals, targeting and insertion is a co-ordinated process mediated by proteins of the translocase of the inner membrane (TIM) 22 complex [20,27,33]. Further experiments are required to determine whether atypical inner membrane proteins, such as PPOX, are processed by a similar mechanism.

Typical membrane-spanning inner membrane proteins without cleavable presequences contain one or more internal sequences that direct translocation through the outer membrane, often in a loop conformation, and insertion into the inner membrane without the involvement of any sequence at the N-terminus [20,27–32]. Our findings suggest that PPOX does not share all these features. They are more consistent with transfer through the outer membrane in an N- to C-terminus direction, guided by interaction between TOM receptors and sequences both within the first 50 residues and more distally, followed by attachment to the inner membrane by a process for which a sequence between residues 151 and 175 is essential.

Since the experiments discussed above suggest that all the information required to target PPOX to mitochondria is contained within its first 250 residues, it was unexpected to find that the only PPOX mutation (Val¹³⁵ → Gly) that disrupted import was well outside this region. Val¹³⁵ is in a region of human PPOX that shows marked sequence similarity with other mitochondrial PPOXs (Figure 4), but, in the absence of information on the three-dimensional structure of any PPOX, its function is unknown. Its mutation to glycine delayed the appearance of YFP fluorescence and led to the eventual accumulation of YFP fluorescence in the cytosol and nucleus (Figure 3). Since YFP does not fluoresce until it is correctly folded [34], the substitution of glycine for Val¹³⁵ appears to delay folding of the construct either by delaying translocation or by a post-translational effect. In addition, since the construct has a molecular mass (96 kDa) above the exclusion limit of about 40 kDa for passive entry into the nucleus [35], the appearance of fluorescence in this compartment indicates that

some proteolytic degradation may have occurred before YFP folding is complete. Newly synthesized hydrophobic mitochondrial membrane proteins are normally protected from incorrect folding and aggregation in the cytosol by binding to chaperone proteins, such as the heat-shock proteins, Hsp70 and Hsp90, that may also direct interaction with the TOM complex [36,37]. The Val³³⁵ → Gly mutation may prevent folding of the PPOX fusion protein and alter binding to a chaperone, so that formation of the required import-competent conformation is prevented, leading to partial proteolysis and subsequent folding of YFP. Missense mutations that enhance interaction with chaperones and lead to protein degradation have been reported in myeloperoxidase and heparin II cofactor [38,39]. Since the Val³³⁵ → Gly mutation lies in part of the PPOX molecule that is not required for efficient import of wild-type PPOX, it seems probable that it acts primarily through a direct effect on protein folding that prevents adoption of the required conformation at a conformation-dependent stage of translocation.

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