Identification of amino acid changes affecting yeast uroporphyrinogen decarboxylase activity by sequence analysis of *hem12* mutant alleles

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The molecular basis of the uroporphyrinogen decarboxylase defect in eleven yeast 'uroporphyrin' mutants was investigated. Uroporphyrinogen decarboxylase, an enzyme of the haem-biosynthetic pathway, catalyses the decarboxylation of uroporphyrinogen to coproporphyrinogen and is encoded by the *HEM12* gene in the yeast *Saccharomyces cerevisiae*. The mutations were identified by sequencing the mutant *hem12* alleles amplified *in vitro* from genomic DNA extracted from the mutant strains. Four mutations leading to the absence of enzyme protein were found: one mutation caused the substitution of the translation initiator Met to Ile, a two-base deletion created a frameshift at codon 247 and two nonsense mutations were found at codons 50 and 263. Four different point mutations were identified in seven 'leaky' mutants with residual modified uroporphyrinogen decarboxylase activity; each of three mutations was found in two independently isolated mutants. The nucleotide transitions resulted in the amino acid substitutions Ser-59 to Phe, Thr-62 to Ile, Leu-107 to Ser, or Ser-215 to Asn, all located in or near highly conserved regions. The results suggest that there is a single active centre in uroporphyrinogen decarboxylase, the geometry of which is affected in the mutant enzymes.

INTRODUCTION

Uroporphyrinogen decarboxylase (EC 4.1.1.37), an enzyme of the haem-biosynthetic pathway, catalyses the sequential decarboxylation of the four acetyl side chains of uroporphyrinogen to produce coproporphyrinogen and intermediate hepta-, hexa- and penta-carboxyporphyrinogens. Uroporphyrinogen decarboxylase isolated from human (DeVerneuil *et al.*, 1983; Elder *et al.*, 1983) and chicken (Kawanishi *et al.*, 1983) erythrocytes, bovine liver (Straka & Kushner, 1983) and the yeast *Saccharomyces cerevisiae* (Felix & Brouillet, 1990) are very similar with respect to molecular mass, functional properties, the absence of cofactor requirement and sensitivity to thiol-blocking reagents. The mechanism of the enzyme's action and the number of catalytic sites are unknown, but models have proposed one to four active sites (references cited above and DeVerneuil *et al.*, 1980). Uroporphyrinogen decarboxylase cDNA or gene has been cloned and sequenced from rat (Romeo *et al.*, 1984; Romana *et al.*, 1987), human (Romeo *et al.*, 1986) and yeast (Garey *et al.*, 1992) and the proteins from these three species are identical in 47% of the derived amino acid sequence.

Inherited and acquired defects of uroporphyrinogen decarboxylase activity are the cause of porphyria cutanea tarda. This disease of humans is characterized by dermal photosensitivity and excessive hepatic accumulation and urinary excretion of uroporphyrin. Point mutations in the uroporphyrinogen decarboxylase gene resulting in a change of amino acid Gly-281 to Glu (DeVerneuil *et al.*, 1986) or Val (Garey *et al.*, 1989) have been identified in pedigrees with porphyria. These mutations produced a catalytically active, but unstable, protein. A splice site mutation in the human uroporphyrinogen decarboxylase gene has also been identified

which leads to deletion of exon 6 and failure to produce an active protein (Garey *et al.*, 1990). In the yeast *Saccharomyces cerevisiae*, mutants have been obtained at the *HEM12* locus encoding uroporphyrinogen decarboxylase, leading to a pattern of porphyrin accumulation mimicking that seen in porphyria cutanea tarda (Rytka *et al.*, 1984; Kurlandzka *et al.*, 1988). Sequencing of two mutant *hem12* alleles identified two point mutations corresponding to amino acid changes Gly-33 to Asp and Gly-300 to Asp, located in two evolutionarily conserved regions. These mutations led to totally inactive proteins unable to bind substrate (Garey *et al.*, 1992).

Here we report the characterization of additional point mutations in the yeast *HEM12* gene which result in abnormal function of the mutant enzymes. The results, discussed as tentative structure–function relationships, do not favour the presence of multiple active sites in the enzyme.

MATERIALS AND METHODS

Saccharomyces cerevisiae hem12 mutant strains

Yeast strains carrying mutations at the *HEM12* locus are listed in Table 1. The Sm1, Sm2, and Sm3 mutants were isolated from the parental strain SP4 (Rytka *et al.*, 1984). The Sm40 mutant derived from strain DCT26-1C (Kurlandzka & Rytka, 1985) and the Cat9-4C mutant from strain M/S2-1 (Labbe-Bois *et al.*, 1977). The G121 (Urban-Grimal & Labbe-Bois, 1981) and the G227 (Kurlandzka *et al.*, 1988) mutants were isolated from parental strains FL100 and FL200 respectively. The Sm59 and Sm60 mutants were obtained after mutagenesis of strain Sm39/23-C (T. Zoladek, unpublished work). Mutant strains GL11 (Gollub *et al.*, 1977; Arrese *et al.*, 1982) and BJ4627 (Pringle *et al.*, 1989) were kindly provided by J. R. Mattoon

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(University of Colorado, Colorado Springs, CO, U.S.A.) and R. A. Preston (Carnegie Mellon University, Pittsburgh, PA, U.S.A.) respectively. Cells were grown in complete medium (1% yeast extract/1% bacto-peptone) supplemented with 2% (v/v) ethanol for the leaky mutants, or with 2% glucose + 0.2% Tween 80 + 15 mg of ergosterol/litre for the completely blocked haem-deficient mutants. Standard procedures were used for crossing, sporulation and tetrad analysis (Sherman *et al.*, 1986).

Isolation and sequencing of the *hem12* mutant alleles

Genomic DNA was prepared from mutant strains as described (Sherman *et al.*, 1986) and 1 µg was used as a template for the amplification of *hem12* alleles by the PCR employing the GeneAmp kit (Perkin-Elmer/Cetus). The two oligonucleotides used, 5'-CCGGAATTCTAACATAGAGTGATCGATAGG and 5'-CCCAAGCTTCACTTATAACTATAAGAAT, allowed amplification of a 1.27 kb DNA fragment encompassing the entire *HEM12* coding region plus 107 and 13 nucleotides of the 5'- and 3'-flanking regions respectively (Garey *et al.*, 1992). The reaction was carried out with 30 cycles of 1 min denaturation at 90 °C, 1 min annealing at 55 °C, and 3 min extension at 70 °C. The amplified product was cloned in pBluescript (Stratagene). Plasmid DNA isolated from 8–12 individual transformants for each mutant was combined to average-out possible errors introduced by *Taq* polymerase. Both strands were sequenced using the Sequenase kit (United States Biochemicals), [α -³⁵S]dATP (Amersham International), and ten *HEM12*-specific oligonucleotide primers spaced approx. every 250 nucleotides along *HEM12* on both strands.

Biochemical analysis

Total RNA was isolated as described by Schmitt *et al.* (1990). RNA (20 µg) was fractionated by electrophoresis on formaldehyde/agarose gels, transferred to nylon membranes and hybridized to the ³²P-labelled 1.27 kb amplified *HEM12* DNA by following standard protocols (Sambrook *et al.*, 1989). The actin mRNA level, detected after stripping off the *HEM12* probe, was used as an internal control for the amount of RNA loaded on to the gels.

Uroporphyrinogen decarboxylase protein was immunodetected by Western blotting of whole-cell extracts (approx. 40 µg of protein) using a yeast uroporphyrinogen decarboxylase antiserum and revelation with an alkaline phosphatase-coupled secondary antibody (Promega) (Felix & Brouillet, 1990). The amount of protein loaded on to the gel was verified by Coomassie Blue staining of a parallel gel and by direct staining of the blot membranes with 0.2% Ponceau Red in 3% (w/v) trichloroacetic acid, followed by destaining with distilled water before incubation with the antiserum.

Uroporphyrinogen decarboxylase activity was measured as described previously (Rytka *et al.*, 1984), by using h.p.l.c. to quantify the decarboxylation products of uroporphyrinogen I or III incubated anaerobically with cell-free extracts. The specific activity of the enzyme was estimated as the sum of the hepta-, hexa-, penta- and tetra-carboxyporphyrins formed (nmol)/h per mg of protein.

The content of haem in whole cells was determined spectrophotometrically (Rytka *et al.*, 1984). Porphyrins accumulated in the cells and excreted in the growth medium were analysed as described by Rytka *et al.* (1984).

RESULTS

Genetic and phenotypic characteristics of the mutants

Most of the mutants listed in Table 1 have been described previously (Rytka *et al.*, 1984; Kurlandzka *et al.*, 1988). They all

carried recessive mutations at the *HEM12* locus encoding uroporphyrinogen decarboxylase. Three mutants, BJ4627, G121 and G227, were totally haem-deficient and therefore could not grow on a non-fermentable carbon source such as ethanol. These three mutants cells accumulated large amounts of Zn-uroporphyrin, were pink in colour, fluoresced red–orange under u.v. light and were sensitive to light. The other mutants were only partially defective in haem synthesis, were also pink, red-fluorescent and sensitive to light, but could grow on ethanol. They accumulated large amounts of Zn-porphyrins, comprising uroporphyrin and its decarboxylation products, and excreted mainly pentacarboxyporphyrin. Exceptions were mutants Sm1 and Cat9-4C, which excreted mainly dehydroisocoproporphyrin. Mutant Cat9-4C was unable to grow on ethanol, although it had residual uroporphyrinogen decarboxylase activity (Kurlandzka *et al.*, 1988), but was in the ρ^- state (deletion in the mitochondrial genome) causing a non-respiring phenotype.

Genetic analysis revealed that, in addition to the *hem12* mutation, four mutants carried a second extragenic mutation which affected the phenotypic expression of the *hem12* mutation. GL11 carried an extragenic suppressor which partially suppressed the effects of the *hem12-15* mutation that caused complete uroporphyrinogen decarboxylase deficiency and failure to synthesize haem. A mutation named *ipal* (increased porphyrin accumulation) was present in the parental strain of mutants Sm59 and Sm60. A mutation named *dut1* (decreased uroporphyrinogen decarboxylase transcript) was found in mutant Sm40. Details of these mutations and the analysis of mutants Sm6 (Kurlandzka *et al.*, 1988) and Sm39 (Labbe-Bois *et al.*, 1986) carrying no mutation in the *HEM12* gene are available from R.L.-B. on request (T. Zoladek, A. Chelstowska, R. Labbe-Bois & J. Rytka, unpublished work).

Biochemical characteristics of the mutants

The steady-state level of *HEM12* mRNA in the different mutants was estimated by Northern-blot analysis (Fig. 1). The amount of *HEM12* transcript normalized to the amount of (control) actin mRNA was roughly the same in the mutants as in the wild-type strain grown under the same conditions (glucose or ethanol), with the exception of Sm40, which made 2–3 times less *HEM12* transcript because of the presence of the extragenic *dut1* mutation. The presence of a normal amount of transcript in mutants G121 and G227 was surprising, since the *hem12* alleles carried frameshift and nonsense mutations (see below), causing premature termination of translation. Nonsense mutations are often associated with reduced stability of mutant mRNAs resulting in decreased steady-state mRNA levels (Losson & Lacroute, 1979). A slight increase (50–80%) in *HEM12* expression has consistently been observed when wild-type cells are grown in ethanol medium as compared with glucose medium (compare lanes 9 and 10, Fig. 1). This correlates well with the higher (about 70%) enzyme activity measured in ethanol-grown cells versus glucose-grown cells (Rytka *et al.*, 1984). The physiological significance of this small increase, however, is questionable, and most likely reflects differences in the metabolic status of the cells (Labbe-Bois, 1990).

The steady-state amount of uroporphyrinogen decarboxylase protein detected by immunoblotting varied between mutants, as illustrated in Fig. 2. From different experiments we could estimate that, compared with the wild-type strain SP4, the amount of enzyme was increased approx. 1.5–2-fold in mutants Sm1 and Cat9-4C, decreased 2–4-fold in mutants Sm2 and Sm40, and decreased 1.5–2-fold in mutant Sm3. No protein was immunodetected in mutants BJ4627 (Fig. 2, lane 8), G121 and G227 (results not shown). A faint band was observed with mutant

Table 1. Identification of the *hem12* mutations in the different mutants and their effects on the uroporphyrinogen decarboxylase activity and the haem content of whole cells

The underlined nucleotide changes of the *hem12* alleles were determined by sequencing PCR-amplified DNA of the mutants. Values for uroporphyrinogen decarboxylase activity measured in cell-free extracts and for haem content of whole cells were taken from Rytka *et al.* (1984) and Kurlandzka *et al.* (1988), except for those of BJ4627 and GL11 mutants, which were measured in the present study. The enzyme activities of the mutants are reported as percentage of the activities of the wild-type strain SP4 grown in glucose (for mutants BJ4627, Cat9-4C, G121, and G227 growing only in glucose) or ethanol medium (for the other mutants ethanol-grown). The enzyme activity of SP4, estimated as the sum (nmol) of hepta-, hexa-, penta-, and tetra-carboxyporphyrins formed/h per mg of protein, was 0.193 and 0.277 with uroporphyrinogenI (uroI) and 0.255 and 0.365 with uroporphyrinogenIII (uroIII), when grown in glucose and ethanol respectively. –, not determined.

Strain	<i>hem12</i> allele	Codon no.	Nucleotide change	Amino acid change	Uroporphyrinogen decarboxylase activity (%)		Haem (nmol/g dry wt.)
					UroI	UroIII	
SP4	<i>HEM12</i>				100	100	222
BJ4627	<i>hem12-14</i>	1	ATG→ATA	M→I	0	0	0
GL11	<i>hem12-15</i>	50	CAA→TAA	Q→stop	30	10	47
Sm40	<i>hem12-6</i>	59	TCT→TTT	S→F	13	18	43
Sm59	<i>hem12-12</i>	59	TCT→TTT	S→F	–	–	–
Sm3	<i>hem12-4</i>	61	ATT→ATA	I→I	61	35	46
		62	ACT→ATT	T→I			
Sm2	<i>hem12-3</i>	107	TTA→TCA	L→S	35	28	129
Sm60	<i>hem12-13</i>	107	TTA→TCA	L→S	–	–	–
Sm1	<i>hem12-2</i>	215	AGT→AAT	S→N	18	29	65
Cat9-4C	<i>hem12-11</i>	215	AGT→AAT	S→N	31	38	< 5
G121	<i>hem12-1</i>	247	TTA→A	Frameshift	0	0	0
G227	<i>hem12-7</i>	263	TGG→TAG	W→stop	0	0	0

Fig. 1. *HEM12* mRNA level in the different mutants estimated by Northern-blot analysis

Cells were grown in ethanol medium, except those in lanes 5, 7, 10, 11 and 12, which were grown in glucose medium (g). Total RNA (20 µg) was fractionated on a formaldehyde/agarose gel and transferred on to nylon membranes. The membranes were hybridized first with a 1.27 kb *HEM12* radiolabelled probe, then with an actin probe to monitor RNA loading of the gel.

Fig. 2. Uroporphyrinogen decarboxylase protein levels in the different mutants detected by immunoblotting

Total proteins [40 µg, except for the mutant Cat9-4C (lane 7), for which 10 µg were used] were resolved by SDS/10% PAGE and transferred on to nitrocellulose filters. The filters were incubated with anti-(yeast uroporphyrinogen decarboxylase) antibodies, then treated with alkaline phosphatase-coupled secondary antibody. Purified yeast enzyme (lane 1) and extract of cells overproducing the enzyme (due to the presence of a high-copy-number plasmid bearing the *HEM12* gene) (lane 9) were used as markers. x, additional bands due to unidentified proteins that cross-react with the antiserum.

GL11 after longer incubation with the alkaline phosphatase-coupled detection system (result not shown).

Uroporphyrinogen decarboxylase activity in the mutants, measured in cell-free extracts, is shown in Table 1. An estimate of the function of mutant enzymes *in vivo* was provided by measurement of the haem content of whole cells (Table 1). Mutants Sm59 and Sm60 were not analysed in detail, since they carried the same *hem12* mutations as mutants Sm40 and Sm2 respectively (see below).

Identification of the mutations in the *hem12* alleles

Mutant alleles of *HEM12* were amplified *in vitro* from genomic DNA extracted from the mutant strains. The amplified fragments were cloned and sequenced. They contained the entire coding region plus 107 and 13 nucleotides of the 5'- and 3'-flanking regions respectively. A single nucleotide substitution was found in all but two mutants (Table 1). In Sm3, two substitutions were

found in adjacent codons 61 and 62; the transversion T to A at codon 61 (Ile to Ile) was due to a mutagenic event and did not represent a silent polymorphism, since it was not found in mutants Sm1 and Sm2, which are derived from the same parental strain SP4. A two-base (TT) deletion at codon 247 occurred in mutant G121, which altered the reading frame for 27 codons before generating an early stop codon (TGA).

Two mutations generated stop codons, at codon 50 in the mutant GL11 and at codon 263 in the mutant G227. Extragenic suppressor(s) of the nonsense mutation in mutant GL11 appeared spontaneously with a high frequency, causing a leaky mutant phenotype with residual uroporphyrinogen decarboxylase activity *in vitro* and reduced haem synthesis *in vivo* (Table 1).

The other mutations resulted in non-conservative amino acid substitutions at codons 1, 59, 62, 107 and 215 (Table 1), located at or near evolutionarily conserved regions of the enzyme (Fig.

		*				*		*		*	*	*	*	*										
Syne.	47	F	R	E	R	S	E	T	P	E	L	A	I	E	I	S	L	Q	P	F	R	A	F	K
Human	55	F	F	S	T	C	R	S	P	E	A	C	C	E	L	T	L	Q	P	L	R	R	F	P
Yeast	48	F	F	Q	T	C	R	D	A	E	I	A	S	E	I	T	I	Q	P	V	R	R	Y	R

Fig. 3. Amino acid changes of the yeast mutant uroporphyrinogen decarboxylases are located in evolutionarily conserved regions

The sequence of the human enzyme is taken from Romeo *et al.* (1986). The N-terminal sequence of a putative uroporphyrinogen decarboxylase from *Synechococcus* sp. (Syne.) is taken from Kiel *et al.* (1990). Identical amino acids are marked by an asterisk (*) and conservative ones by a bold dot above the sequences. Mutations are indicated by the arrows.

3). Interestingly, the same mutation was found in two independently isolated mutants for codons 59, 107 and 215, suggesting that the amino acids at these positions are critical for proper functioning of the enzyme. The same situation has been described in humans, where mutations changing the same codon (Gly-281) to Glu (DeVerneuil *et al.*, 1986) or to Val (Garey *et al.*, 1989) were identified in unrelated patients with inherited uroporphyrinogen decarboxylase defects.

The order of mutations along the *HEM12* gene established here differed from their tentative order determined by the frequency of meiotic recombination between different *hem12* alleles (Kurlandzka *et al.*, 1988). Such lack of correspondence between physical distances and recombination frequencies has been discussed in detail in the case of the yeast *CYC1* gene encoding iso-1-cytochrome *c* (Moore *et al.*, 1988).

DISCUSSION

The mechanism(s) of uroporphyrinogen decarboxylation is poorly understood. A mechanism for the catalytic removal of the carboxy moiety from the acetyl side chains of uroporphyrinogen has been proposed (Barnard & Akhtar, 1979), but the nature of the amino acid residue(s) involved in the catalytic domain(s) and those involved in substrate binding are unknown. It is also not known whether the four sequential decarboxylations occur in a single or multiple catalytic sites, or in an orderly and clockwise or random fashion (DeVerneuil *et al.*, 1980, 1983; Elder *et al.*, 1983; Kawanishi *et al.*, 1983; Straka & Kushner, 1983; Felix & Brouillet, 1990; Luo & Lim, 1990; Lash, 1991).

Our aim in studying 'uroporphyrin' yeast mutants was to use the power of yeast genetics to decipher the structure-function relationships in uroporphyrinogen decarboxylase. In particular, we hoped to isolate specific mutants accumulating either uroporphyrin, hepta-, hexa- or penta-carboxyporphyrin. Such mutants would indicate a defect at a specific decarboxylation step, thus suggesting the presence of more than one catalytic site in uroporphyrinogen decarboxylase. No such mutants were

found. None of the 26 stable 'uroporphyrin' mutants isolated so far in different laboratories has accumulated a specific intermediate decarboxylation product. For one mutant, *pop1*, no allelism test with *HEM12* has been reported (Pretlow & Sherman, 1967; Arrese *et al.*, 1982). Genetic analyses of 11 other mutants revealed mutations at loci different from *HEM12*, and all accumulated uroporphyrin and the three partial decarboxylation products *in vivo* (Arrese *et al.*, 1982; T. Zoladek, A. Chelstowska, R. Labbe-Bois & J. Rytka, unpublished work).

Among the 14 mutants allelic to the *HEM12* locus, only one, *pop3*, has not been analysed at the molecular level. It also accumulated a mixture of uroporphyrin and its decarboxylation products (Arrese *et al.*, 1982). The 13 other mutants are described by Garey *et al.* (1992) and in the present paper. Five *hem12* mutants accumulated exclusively uroporphyrin and were totally uroporphyrinogen decarboxylase- and haem-deficient. Mutants G121 and G227 had a deletion and a nonsense mutation respectively, preventing the formation of any immunodetectable protein. Mutant BJ4627 also lacked any immunodetectable protein, owing to a change of the translation initiator from Met to Ile. This indicates that either translation could not initiate at the next AUG (codon 29), or, if it did, a truncate protein was produced which was rapidly degraded. Mutants G229 and G230 made normal amounts of enzyme, but the amino acid substitutions (Gly-33 to Asp and Gly-300 to Asp respectively) resulted in a product totally unable to decarboxylate uroporphyrinogen I and III or hepta- and penta-carboxyporphyrinogen I and incapable of binding the substrate (Garey *et al.*, 1992).

The last eight mutants were leaky and accumulated uroporphyrin plus its intermediate decarboxylation products in various proportions. Although the mutation in strain GL11 created an early stop codon, a partially active enzyme was synthesized, owing to the presence of an extragenic suppressor. Since the nature and the efficiency of the suppression are not known, it is not possible to define the effect of the amino acid substitution on enzymic activity.

The substitution Thr-62 to Ile in mutant Sm3 was located in a highly conserved region, and the substitution Ser-59 to Phe in mutant 40 was just adjoining this region. Both mutants had less immunodetectable protein than normal, partly due to decreased transcription in the case of mutant Sm40. However, the decrease of enzymic activity observed *in vivo* and *in vitro* in these two mutants was not simply due to a lower level of transcript or to protein instability. Each substitution caused a catalytically defective enzyme, as assayed both *in vitro* and *in vivo* (Fig. 2 and 4 in Kurlandzka *et al.*, 1988). The pattern of accumulation of the different decarboxylation products catalysed by cell-free extracts from these two mutants was very similar with uroporphyrinogen III as substrate and indicated impairment in all steps. However, when uroporphyrinogen I was used as substrate, the enzyme of mutant Sm40 appeared to be greatly impaired, mainly in the first decarboxylation step (uroporphyrinogen I to heptacarboxyporphyrinogen I), whereas the enzyme of mutant Sm3 was mainly abnormal in the last decarboxylation step (penta-carboxyporphyrinogen I to coproporphyrinogen I). This suggests that the region of the protein containing amino acids 59 and 62 can discriminate between uroporphyrinogen I and III. A discrimination between the two isomers has been shown to occur with the normal enzyme, principally at the first decarboxylation step, but also at the last one (DeVerneuil *et al.*, 1980; Felix & Brouillet, 1990, and references cited therein).

The amount of immunodetectable enzyme in mutant Sm2 was 3–4-fold less than normal, suggesting some instability of the protein. The amino acid substitution Leu-107 to Ser, located in a conserved region, resulted in diminished relative rates of all

decarboxylations, especially the last one (Kurlandzka *et al.*, 1988). However, enough coproporphyrinogen III was made *in vivo* to allow haem synthesis to occur at 60% of normal. It is important to stress that normal amounts of haem are synthesized in strains with wild-type *HEM12*, but which carry extragenic mutations affecting *HEM12* expression, resulting in less than 20% of normal enzyme synthesis (T. Zoladek, A. Chelstowska, R. Labbe-Bois & J. Rytka, unpublished work).

The same mutation, a Ser-215-to-Asn substitution, was found in mutants Sm1 and Cat9-4C. This was surprising, since different phenotypes had been described for the two mutants (Rytka *et al.*, 1984; Kurlandzka *et al.*, 1988). In fact, genetic analysis revealed that Cat9-4C was in the ρ^- state. In the ρ^+ state, Cat9-4C grows on ethanol and synthesizes haem in the same proportion as Sm1. The behaviour of uroporphyrinogen decarboxylase in cell-free extracts of Cat9-4C and Sm1 is very similar, with barely detectable formation of penta- and tetra-carboxyporphyrins. These two mutants shared two other traits unique among all the mutants studied so far. First, they had significantly higher amounts of enzyme protein, perhaps indicative of a more stable protein. Second, they excreted mainly dehydroisocoproporphyrin in place of pentacarboxyporphyrin excreted by the other mutants. Dehydroisocoproporphyrinogen is derived from pentacarboxyporphyrinogen III, and its formation is catalysed by coproporphyrinogen oxidase, the next enzyme of the haem pathway (Elder & Evans, 1978; Kurlandzka *et al.*, 1991). When the pattern of the different porphyrins and haem made *in vivo* by all the leaky mutants (Rytka *et al.*, 1984; Kurlandzka *et al.*, 1988, 1991) was compared, we noticed that the lower the ratio of pentacarboxyporphyrin to coproporphyrin, the higher the relative amount of dehydroisocoproporphyrin excreted. This is unexpected, and differs from findings in hepatocytes (Elder & Evans, 1978). These differences might be explained by: (1) differences in the kinetic parameters and localization of yeast and mammalian coproporphyrinogen oxidase (cytosol in yeast, mitochondria in mammal); (2) differences in the metabolism of dehydroisocoproporphyrinogen to harderoporphyrinogen by coproporphyrinogen oxidase, and (3) differences in the utilization of harderoporphyrinogen by coproporphyrinogen oxidase to yield protoporphyrinogen.

Our analysis of mutant yeast uroporphyrinogen decarboxylases suggests that there is a single active centre in the enzyme. The amino acid residues constituting the catalytic domain are probably required for proper binding of uroporphyrinogen and the partially decarboxylated intermediate reaction products which also serve as substrates. We find no evidence supporting the presence of multiple catalytic sites specific for individual decarboxylation steps. Many of the amino acid substitutions we have identified in mutant yeast enzymes do not totally ablate enzymic activity. Substitutions which permit some residual activity probably affect the tertiary structure of uroporphyrinogen decarboxylase through local or at distance structural rearrangements, but do not represent residues directly involved in catalysis or substrate(s) binding. The interpretation of effects of amino acid substitutions on enzyme activity remains speculative in the absence of any structural information about this versatile and peculiar decarboxylase.

A.C., T.Z. and J.R. acknowledge gratefully the fellowships as 'Boursier de la Convention d'Echanges C.N.R.S.-Polish Academy of Sciences' during their stays in Paris. This work was supported in part by the Centre National de la Recherche Scientifique (France), by the

Polish Academy of Sciences (Poland) and by grant (no. DK20503) from the National Institutes of Health (U.S.A.).

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