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# Evidence that the Coproporphyrinogen Oxidase Activity of Rat Liver is Situated in the Intermembrane Space of Mitochondria

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Preferential rupture of the outer membrane of mitochondria from rat liver releases coproporphyrinogen oxidase in parallel with components of the intermembrane space. Coproporphyrinogen III enters the mitochondrion through the freely-permeable outer membrane. Either protoporphyrinogen IX or protoporphyrin IX must then cross the inner membrane before haem synthesis can be completed.

The first reaction and the final stages of haem biosynthesis take place in the mitochondria in eukaryotic cells. The final mitochondrial reactions are the oxidative decarboxylation of coproporphyrinogen III by coproporphyrinogen oxidase (EC 1.3.3.3) (Sano & Granick, 1961), the oxidation of protoporphyrinogen IX by protoporphyrinogen oxidase (Poulson & Polglase, 1975; Poulson, 1976) and the insertion of ferrous iron into protoporphyrin IX to form protohaem, which is catalysed by ferrochelatase. Ferrochelatase is located in the inner mitochondrial membrane (Jones & Jones, 1969; McKay *et al.*, 1969) and protohaem is formed inside this membrane (Jones & Jones, 1969; Barnes *et al.*, 1972). Either coproporphyrinogen III, protoporphyrinogen IX or protoporphyrin IX must therefore cross the inner membrane.

The exact locations of protoporphyrinogen oxidase and coproporphyrinogen oxidase within the mitochondrion are unknown. Protoporphyrinogen oxidase has been purified from a mitochondrial membrane fraction (Poulson, 1976). Coproporphyrinogen oxidase is readily solubilized without the use of detergents (Sano & Granick, 1961; Battle *et al.*, 1965; Poulson & Polglase, 1974) and does not appear to be membrane-bound.

In certain types of hepatic porphyria, pentacarboxylic porphyrinogen III, which is formed in the cytosol, competes with coproporphyrinogen III for decarboxylation by coproporphyrinogen oxidase (Elder, 1972; Elder & Evans, 1978). To investigate the accessibility of these substrates to coproporphyrinogen oxidase, we have examined the location of this enzyme in the mitochondrion. Our results indicate that it is situated outside the inner membrane, probably in the intermembrane space.

## Methods

### *Preparation and subfractionation of mitochondria*

All procedures were carried out at 4°C. Mitochondria were prepared from rat liver as described by Greenawalt (1974), except that the liver was homogenized by six strokes of a motor-driven (800 rev./min) Teflon pestle (clearance 75 $\mu$m) in a glass mortar. Mitochondria were subfractionated by exposure to digitonin (Greenawalt, 1974) or hypo-osmotic buffer, followed by centrifugation at $144000g_{av.}$ for 60 min in the 10×10 ml angle rotor of an MSE automatic Superspeed 50 centrifuge to give a compact pellet and a clear supernatant. Treatment with hypo-osmotic buffer was carried out as follows. Mitochondria (approx. 150 mg of protein) were suspended in 9.0 ml of 0.01 M-potassium phosphate buffer, pH 7.4, and stirred gently for 15 min before 3.0 ml of 1.0 M-sucrose was added to raise the osmolality.

### *Enzyme measurements*

Mitochondria and submitochondrial fractions were treated with 0.3 mg of Lubrol PX (ICI, Manchester M60 7JT, U.K.)/mg of protein before enzyme activities were measured (Schnaitman & Greenawalt, 1968). Coproporphyrinogen oxidase was assayed by a radiochemical method (Elder & Evans, 1978) and sulphite oxidase by following the reduction of potassium ferricyanide spectrophotometrically (Cohen & Fridovich, 1971). The following assay methods were used: adenylate kinase, Schnaitman & Greenawalt (1968); citrate synthase, Srere (1969); malate dehydrogenase, Mehler *et al.* (1948); monoamine oxidase, Tabor *et al.* (1954); cytochrome oxidase, Cooperstein & Lazarow (1951).

### *Protein measurements*

Protein concentrations were determined as described by Lowry *et al.* (1951), except during subfraction experiments, when they were calculated from the difference between the $A_{280}$ and $A_{310}$ of detergent-solubilized samples (Clarke, 1976).

## Results and Discussion

Previous reports have shown that at least 80% of the total coproporphyrinogen oxidase activity of guinea pig and rat liver is present in the mitochondrial fraction (Sano & Granick, 1961; Batlle *et al.*, 1965). We found that 54% (mean of two experiments) of the coproporphyrinogen oxidase activity of the homogenate was sedimented by centrifugation at $660g_{av.}$. The high activity in this pellet appeared to be due to incomplete disruption of the cells by the homogenization procedure, since it contained similar percentages of two mitochondrial enzymes assayed as markers, citrate synthase (45%) and sulphite oxidase (55%). Most of the coproporphyrinogen oxidase activity (76–79%) in the supernatant from this centrifugation was present in the final mitochondrial preparation at a specific activity, which was approx. 4 times that of the liver homogenate.

Table 1 shows that a high percentage of the total coproporphyrinogen oxidase activity of mitochondria is released into the supernatant subfraction after exposure to digitonin (0.1 mg/mg of protein) or hypo-osmotic buffer, with an approx. 7-fold increase in specific activity. Both procedures rupture the outer mitochondrial membrane selectively (Sottocasa *et al.*, 1967; Schnaitman & Greenawalt, 1968). The supernatant is similarly enriched by adenylate kinase and sulphite oxidase, both of which are located in the intermembrane space of rat liver mitochondria (Schnaitman & Greenawalt, 1968; Wattiaux-De Conninck & Wattiaux, 1971). Most of the activity of the matrix markers, citrate synthase and malate dehydrogenase (Table 1), and all that of the outer and inner membrane markers, monoamine and cytochrome oxidases, remains in the pellet. When mitochondria are exposed to increasing concentrations of digitonin, enzymes located in the intermembrane space are released before other components (Schnaitman & Greenawalt, 1968;

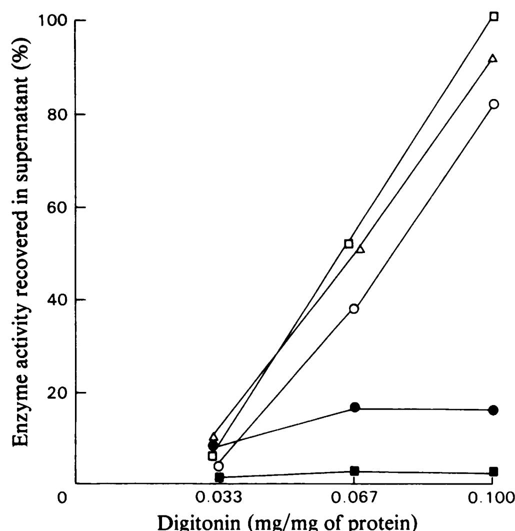


Fig. 1. *Release of enzymes from mitochondria by digitonin*
○, Coproporphyrinogen oxidase; □, adenylate kinase; △, sulphite oxidase; ●, citrate synthase; ■, malate dehydrogenase. Recoveries of enzyme activities in the supernatant fraction are expressed as a percentage of the total activity of the mitochondrial preparation before subfractionation. The supernatant fraction was obtained as described in the Methods section. Mean percentage recoveries, with ranges in parentheses, for each enzyme were: coproporphyrinogen oxidase, 95 (90–103); adenylate kinase, 82 (76–93); sulphite oxidase, 103 (96–112); citrate synthase, 86 (75–108); malate dehydrogenase, 76 (67–83).

Table 1. *Distribution of enzymes in mitochondrial subfractions*
The enzyme activity recovered in each fraction is shown as a percentage of the total activity in the mitochondrial preparation, with specific activities in parentheses. Specific activities are expressed as nmol of substrate consumed or product formed/min per mg of protein or, for coproporphyrinogen oxidase only, as pmol of $CO_2$/min per mg of protein. No cytochrome oxidase or monoamine oxidase activity was detected in the supernatant. Recovery of monoamine oxidase activity in the pellet was 94%. Mitochondria were subfractionated and enzyme activities assayed as described in the Methods section.

| | | | Percentage of enzyme activity in subfraction | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Fraction | Protein recovery (%) | Coproporphyrinogen oxidase | Adenylate kinase | Sulphite oxidase | Citrate synthase | Malate dehydrogenase |
| Digitonin (0.1 mg/mg of protein) | Mitochondria | 100 | 100 (123) | 100 (305) | 100 (35.7) | 100 (6.1) | 100 (4225) |
| | Supernatant | 13 | 84 (787) | 93 (2211) | 102 (279) | 16 (7.5) | 1 (414) |
| | Pellet | 82 | 19 (28) | 0 | 10 (4.3) | 92 (6.9) | 66 (3469) |
| | Recovery | 95 | 103 | 93 | 112 | 108 | 67 |
| Hypo-osmotic buffer | Mitochondria | 100 | 100 (122) | 100 (341) | 100 (38.3) | 100 (6.1) | 100 (4186) |
| | Supernatant | 11 | 75 (842) | 93 (2933) | 103 (364) | 19 (6.2) | 2 (773) |
| | Pellet | 75 | 17 (28) | 0 | 9 (4.6) | 102 (6.0) | 90 (4994) |
| | Recovery | 86 | 92 | 93 | 112 | 121 | 92 |

Wattiaux-De Conninck & Wattiaux, 1971). Fig. 1 shows that coproporphyrinogen oxidase and the intermembrane space marker enzymes are released in parallel as the digitonin:protein ratio increases. These results suggest that coproporphyrinogen oxidase is situated in the intermembrane space of rat liver mitochondria. However, it is not released as completely as adenylate kinase or sulphite oxidase either by digitonin or by hypo-osmotic buffer (Table 1; Fig. 1). This may indicate that, in intact mitochondria, coproporphyrinogen oxidase is loosely attached to one of the membrame surfaces surrounding the intermembrane space; although location of a minor fraction elsewhere within the mitochondrion cannot be excluded.

The assignment of coproporphyrinogen oxidase to a location that is outside the inner membrane has a number of implications. First, it indicates that coproporphyrinogen III probably enters the mitochondrion by passive diffusion, since the outer membrane appears to be freely permeable to compounds with molecular weights of several thousands. Similarly, there may be no permeability barrier to prevent utilization of the alternative substrate, pentacarboxylic porphyrinogen III, when this accumulates in the cytosol (Elder & Evans, 1978), or the subsequent conversion of dehydroisocoproporphyrinogen into protoporphyrinogen IX (Elder, 1972).

Secondly, it implies that either protoporphyrinogen IX or protoporphyrin IX must cross the inner membrane, since protohaem is formed inside this membrane. The site of protoporphyrinogen oxidation has not been established and it is not clear which of these compounds crosses the membrane. Intact mitochondria can form haem from protoporphyrin IX (Yoda & Israels, 1972), but the possibility that they may also be able to use protoporphyrinogen IX does not appear to have been investigated. Whichever substrate crosses the membrane under physiological conditions, it is unlikely that an anion of this size will penetrate the inner membrane unless it is transported by a specific process.

Finally, it is known that even gentle homogenization procedures liberate components of the mitochondrial intermembrane space (Cohen *et al.*, 1972). Saez de Cordova (1977) showed that particle-free cytosol fractions of rat liver can synthesize haem from 5-aminolaevulinate and suggested that coproporphyrinogen oxidase may be partly located in this fraction. We found that a cytosol fraction prepared by centrifuging the post-mitochondrial supernatant at $100000g_{av.}$ for 60min contained less coproporphyrinogen oxidase than sulphite oxidase, the activities being approx. 8 and 20% of the total activity in the pre-mitochondrial supernatant respectively. Thus it seems unlikely that any coproporphyrinogen oxidase is located in the cytosol compartment *in vivo*, although, since it is in the intermembrane space, there may be no permeability barrier to prevent participation in reaction sequences otherwise taking place outside the mitochondrion.

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