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The Mitochondrial Localization of Coproporphyrinogen III Oxidase

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The location of coproporphyrinogen III oxidase in mitochondria was studied in rat liver by using the digitonin method or hypo-osmotic media for fractionation. The enzyme was found in the intermembrane space with a fraction loosely bound to the inner membrane. This fraction was released by washing the inner-membrane–matrix complex with alkaline solutions or solutions of high ionic strength. The enzyme in both fractions had the same K_m (0.16 μM) for coproporphyrinogen III. When incubation was performed in a medium that avoided destruction of enzyme membrane binding, a dramatic increase in activity was observed after sonication of whole mitochondria or of the inner-membrane–matrix complex.

The synthesis of haem in mammalian cells involves co-operation between mitochondrial and cytoplasmic compartments. Synthesis of 5-aminolaevulinate is a mitochondrial process, its conversion into coproporphyrinogen occurs in the cytoplasm, and the last steps (from coproporphyrinogen to haem) again take place within the mitochondrion (Elder, 1976). The intramitochondrial location of some enzymes has already been studied and it is now known that 5-aminolaevulinate synthetase is located in the matrix space (Zuyderhoudt *et al.*, 1969); protoporphyrinogen oxidase seems to be strongly bound to the inner membrane (Poulson & Polglase, 1975), whereas ferrochelatase is bound to the inner surface of this membrane (Jones & Jones, 1969; McKay *et al.*, 1968).

The objective of the present study was to determine the mitochondrial localization of the coproporphyrinogen oxidase (EC 1.3.3.3), which catalyses the oxidative decarboxylation and dehydrogenation of the 2- and 4-propionate side chains of coproporphyrinogen III to form protoporphyrinogen IX (Sano & Granick, 1961). It is shown that the enzyme is located in the intermembrane space (with a fraction loosely bound to the outer surface of the inner membrane), as was suggested by Elder (1976).

Materials and Methods

Chemicals

These were obtained from the following sources: 5-amino[4-^{14}C]laevulinate (The Radiochemical Centre, Amersham, Bucks., U.K.); porphobilinogen, coproporphyrin and protoporphyrin (ester) (Sigma Chemical Co., St. Louis, MO, U.S.A.); digitonin [Merck, Darmstadt, Germany; recrystallized before use (Schnaitman & Greenawalt, 1968)]. All other chemicals used were of reagent grade and were obtained from the usual commercial sources.

Mitochondrial preparation and fractionation

Male Wistar rats were killed by cervical fracture. Liver mitochondria were isolated in 220mM-mannitol, 70mM-sucrose, 2mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and 0.05% bovine serum albumin as described by Schnaitman & Greenawalt (1968). The pH of the medium was adjusted to 7.4 with 1M-KOH immediately before use. The mitochondria obtained were washed twice and diluted in isolation medium to a protein concentration of 50mg/ml.

Digitonin method for fractionation of mitochondria (*Levy et al.*, 1967; *Schnaitman & Greenawalt*, 1968). This was used with slight modifications. Portions of ice-cold digitonin (10mg/ml) were diluted in isolation medium and added to the same volume of the suspension of mitochondria. The digitonin/mitochondrial protein ratio was varied as described below (see the Results section). The resulting suspension was gently mixed for 10min at 0°C, diluted with 2vol. of isolation medium, mixed again and centrifuged at 12000*g* for 10min. The supernatant was collected and the pellet (inner-membrane–matrix complex) resuspended in the same volume of isolation medium. Further studies of the inner-membrane–matrix complex included dilution of the pellet in 4mM-Hepes buffer, pH6.8, to a protein concentration of 10mg/ml and sonication in a Bronson B12 sonifier at 3A for a total of 60s while being cooled in an NaCl/ice/water bath at −10°C. The sonicated suspension was centrifuged at 145000*g* for 60min. The resultant pellet (inner membrane) was resuspended in isolation

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medium. The supernatant was designated the matrix fraction. In some experiments (see below) freezing and thawing (twice) of the inner-membrane–matrix complex was used (instead of sonication).

Treatment of mitochondria with hypo-osmotic solution. The method of Pfaff & Schwalbach (1967) was used with some modifications. Mitochondria suspended in isolation medium were diluted 10 times (v/v) in a medium containing 10mM-EDTA, 2mM-Hepes buffer, pH7.4, and 0.5mg of bovine serum albumin/ml. After 5min at 25°C, the incubation was stopped with a hyper-osmotic solution containing 2mM-Hepes buffer, pH7.4, 418mM-mannitol, 133mM-sucrose and 0.5mg of bovine serum albumin/ml so that the final solution was as concentrated as the isolation medium. The mitochondrial suspension was then centrifuged at 12000*g* and supernatant and pellet used as described in the digitonin-fractionation method.

Mitochondrial preparations were frozen and kept at −20°C for enzyme assays, which were performed within the following 2 days.

Enzyme assays

Malate dehydrogenase was assayed as described by Schnaitman & Greenawalt (1968); samples were activated with Triton X-100 (1% final concn.). Succinate dehydrogenase was assayed by the method of King (1967); samples were activated by brief sonication (10s while being cooled in an NaCl/ice/water bath at −10°C). Adenylate kinase was assayed as described by Schnaitman & Greenawalt (1968). Monoamine oxidase was assayed by the method of McEwen (1965), with 3.3mM-benzylamine as substrate; after incubation, the benzaldehyde formed was extracted with cyclohexane.

Coproporphyrinogen III oxidase was assayed with the technique previously described (Grandchamp & Nordmann, 1977); briefly, the standard reaction mixture (2.0ml) contained 100μmol of Tris/HCl buffer, pH7.4, 5mg of bovine serum albumin, 0.2ml of enzyme solution and 10nmol of [$^{14}C_8$]coproporphyrinogen. After incubation under aerobic conditions for 1h at 37°C in the dark, the reaction was terminated by the addition of 4ml of ethyl acetate/acetic acid (3:1, v/v). The amount of labelled protoporphyrinogen synthesized was measured after separation (as methyl ester) from coproporphyrinogen by t.l.c. The labelled substrate was obtained from human erythrocytes incubated with 5-amino[4-^{14}C]-laevulinate (Grandchamp & Nordmann, 1977).

For each enzyme activity, recovery was calculated by using the following formula:

$$\frac{\text{Sum of total activity in each fraction}}{\text{Total activity in unfractionated material}} \times 100$$

Enzyme activity in each fraction was expressed as a percentage of total recovered activity.

Protein concentration was determined by the method of Lowry *et al.* (1951), with human serum albumin as a standard.

Results

Table 1 and Fig. 1 show that, after treatment with 0.2mg of digitonin/mg of protein, the distribution of marker enzymes corresponds to that observed by other investigators (Hoppel & Tomec, 1972; Schnaitman & Greenawalt, 1968). Whereas succinate dehydrogenase and malate dehydrogenase are mostly found in the pellet (inner-membrane–matrix complex) monoamine oxidase and adenylate kinase are found in the supernatant (outer membrane and intermembrane space). Coproporphyrinogen oxidase predominates in the supernatant (76% of activity), but the pellet contains around 24% of the total activity. Treatment of mitochondria with a hypo-osmotic solution allows separation of enzymes located in the intermembrane space (supernatant)

Table 1. *Intramitochondrial distribution of coproporphyrinogen oxidase and some marker enzymes after treatment by digitonin*
The enzymes were assayed as described in the Materials and Methods section. The specific activity for untreated mitochondria is expressed in nmol/min per mg of protein. Fractionation was performed with 0.2mg of digitonin/mg of mitochondrial protein. Supernatant and pellet were obtained after centrifugation at 12000*g*. The results are expressed as means ± S.D.

Enzyme	No. of experiments	Specific activity of untreated mitochondria	Percentage of enzyme activity: Pellet (inner-membrane–matrix complex)	Percentage of enzyme activity: Supernatant (outer membrane and intermembrane space)	Recovery (%)
Malate dehydrogenase	3	2250 ± 550	85 ± 0.6	15 ± 0.6	95 ± 4.6
Succinate dehydrogenase	3	93 ± 24	92 ± 3.6	8 ± 3.6	87 ± 11
Adenylate kinase	3	360 ± 52	2 ± 0.75	98 ± 0.76	102 ± 6
Monoamine oxidase	3	13 ± 3	3 ± 2.3	97 ± 2.3	108 ± 11
Coproporphyrinogen oxidase	5	0.024 ± 0.005	24 ± 4	76 ± 4	104 ± 4

from those located in the outer membrane (pellet), because the outer membrane is not removed (Smoly *et al.*, 1970). Table 2 shows that, whereas monoamine oxidase, the outer-membrane marker, is found exclusively in the pellet, adenylate kinase, the inter-membrane-space marker, is found only in the supernatant. The major fraction (80%) of coproprophyrinogen oxidase is found in the supernatant. However, 20% of the coproporphyrinogen oxidase is still found in the pellet (outer membrane plus inner-membrane–matrix complex).

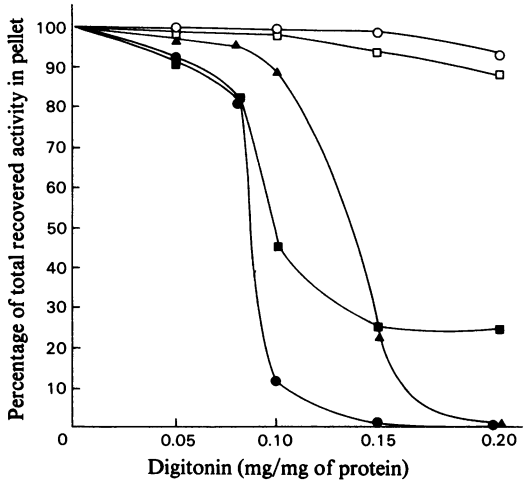

Fig. 1. *Release of mitochondrial enzymes with various digitonin concentrations*

The percentage of the total recovered enzymic activity sedimented by centrifugation at 12000*g* for 10min (see the Materials and Methods section) is plotted against the digitonin concentration. ■, Coproprophyrinogen oxidase; ○, succinate dehydrogenase; □, malate dehydrogenase; ▲, monoamine oxidase; ●, adenylate kinase.

It seems, therefore, that coproporphyrinogen oxidase does not exhibit a distribution similar to that shown by typical marker enzymes. Similar results were obtained by treatment of mitochondria with various concentrations of digitonin and comparing recovery of coproporphyrinogen oxidase activity with that of marker enzymes in the inner-membrane–matrix complex (Fig. 1): when mitochondria were treated with 0.1 mg of digitonin/mg of protein, 90% of adenylate kinase was released in the supernatant as compared with only 55% of coproporphyrinogen oxidase and 10% of monoamine oxidase. Succinate dehydrogenase (inner-membrane marker) and malate dehydrogenase (matrix marker) did not reflect any shift in distribution.

If mitochondria were exposed to 0.15mg of digitonin/mg of protein, approximately 75% of monoamine oxidase (outer-membrane marker) and coproporphyrinogen oxidase were released, but around 25% of coproporphyrinogen oxidase activity still remained in the inner-membrane–matrix complex. The results obtained with 0.2mg of digitonin are described above (see Table 1).

Coproporphyrinogen oxidase seems to be distributed in two fractions: the major one is recovered from the intermembrane space and the minor one is associated with the inner-membrane–matrix complex. This atypical distribution could be explained either by the existence of two distinct coproporphyrinogen oxidases, or by a single coproporphyrinogen oxidase in the intermembrane space, loosely bound to the inner membrane.

Another line of investigation was followed to help clarify this atypical distribution pattern.

Releasing effect of media with various pH values or ionic strengths

After digitonin fractionation, the activity of the inner-membrane–matrix complex was studied. Table 3 shows that in an acid medium (pH6.8) copro-

Table 2. *Intramitochondrial distribution of coproporphyrinogen oxidase and some marker enzymes after treatment by hypo-osmotic solution*

The mitochondria were treated with a hypo-osmotic solution as described in the Materials and Methods section. For the other details see Table 1.

Enzyme	No. of experiments	Percentage of enzyme activity: Pellet: outer membrane and inner-membrane–matrix complex	Percentage of enzyme activity: Supernatant: intermembrane space	Recovery (%)
Malate dehydrogenase	3	95±3	5±3	102±2
Succinate dehydrogenase	3	97±2	3±2	92±4
Adenylate kinase	3	0	100	105±3
Monoamine oxidase	3	100	0	94±3
Coproporphyrinogen oxidase	4	20±7	80±7	103±11

Table 3. *Influence of pH and ionic concentration on the release of coproporphyrinogen oxidase from the inner-membrane–matrix complex*

The inner-membrane–matrix complex was isolated by digitonin fractionation (0.15 mg of digitonin/mg of mitochondrial protein) as described in the Material and Methods section. The complex was resuspended in different washing media (10 mg of protein/ml), incubated for 10 min at 0°C and centrifuged at 12000*g*. Washing media were identical with the isolation medium except that pH was modified or KCl was added as indicated. The results are expressed as means ± S.D.

	No. of experiments	Coproporphyrinogen oxidase activity (%)			Malate dehydrogenase activity (%)		
		Pellet	Supernatant	Recovery (%)	Pellet	Supernatant	Recovery (%)
Isolation medium							
pH 7.4	3	70±9	30±9	95±6	99±0.5	1±0.5	102±3
pH 6.8	3	78±6	22±6	95±13	99±0.6	1±0.4	98±9
pH 8.1	3	29±4	71±4	102±10	98±0.6	2±0.4	102±2
Isolation medium +KCl (pH 7.4)							
0.1 M	3	20±4	80±4	105±9	98±0.6	2±0.4	105±6
0.2 M	3	22±3	78±3	105±3	98±0.5	2±0.5	101±7

Table 4. *Coproporphyrinogen oxidase and some marker enzymes in the inner-membrane–matrix complex*

The inner-membrane–matrix complex obtained from digitonin fractionation (0.2 mg of digitonin/mg of protein) was diluted in 4 mM-Hepes buffer, pH 6.8, sonicated and centrifuged as described in the Materials and Methods section. Enzymes were assayed in sonicated mitochondria, 'high-speed' pellet and 'high-speed' supernatant. The results are expressed as means ± S.D.

Enzyme	No. of experiments	Percentage of activity: 'High-speed' pellet (inner membrane)	Percentage of activity: 'High-speed' supernatant (matrix)	Recovery (%)
Coproporphyrinogen oxidase	3	18±10	81±10	112±18
Malate dehydrogenase	3	20±13	80±13	113±11
Succinate dehydrogenase	3	98±0.6	3±0.6	107±3

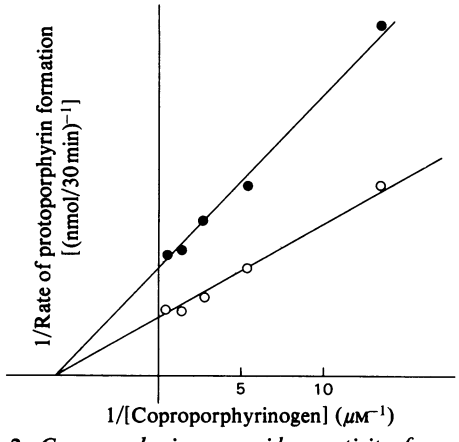

Fig. 2. *Coproporphyrinogen oxidase activity for various substrate concentrations (Lineweaver–Burk plot)*

The enzymic preparations were obtained by digitonin fractionation (0.15 mg of digitonin/mg of mitochondrial protein). After centrifugation at 12000*g*, the supernatant was used for measurement of intermembrane-space enzyme activity. The pellet (inner-membrane–matrix complex) was sonicated at 3 A for 3×10 s, then centrifuged at 145000*g* for 1 h. The supernatant was used for enzyme assay. ○, Intermembrane-space enzyme; ●, inner-membrane enzyme. The K_m value is 0.16 μM.

porphyrinogen oxidase stays firmly bound to the pellet. On the contrary, in an alkaline medium (pH 8.1) or one with increased ionic strength, much less enzyme activity is found in the pellet. These results suggest that the coproporphyrinogen oxidase fraction that is not released with the enzymes of the intermembrane space is bound to the external face of the inner membrane mostly by ionic bonds.

Coproporphyrinogen oxidase activity of the inner-membrane–matrix complex

This was investigated further; the complex was fractionated into an inner-membrane and a matrix fraction (see the Materials and Methods section). Table 4 shows that coproporphyrinogen oxidase does not parallel succinate dehydrogenase (the inner-membrane marker), but is recovered in the 'high-speed' supernatant, as is malate dehydrogenase (the matrix marker). This suggests that sonication releases the enzyme loosely bound to the inner membrane. Similar results were obtained when the inner-membrane–matrix complex was treated by freezing and thawing twice.

Activity of coproporphyrinogen oxidase (a) released by digitonin fractionation and (b) released from the inner-membrane–matrix complex

The enzyme was assayed in the presence of various

Table 5. *Coproporphyrinogen oxidase in mitochondria: effect of sonication and fractionation*
With the incubation conditions used, coproporphyrinogen oxidase activity is expressed as pmol of protoporphyrin/20min per 10mg of protein in whole mitochondria at 30°C. The incubation mixture contained 0.5μM-[^{14}C]coproporphyrinogen, 0.01M-NaCl and 0.001M-KCl in an isolation medium. Digitonin fractionation was performed with 0.12mg of digitonin/mg of mitochondrial protein. The results are expressed as means±S.D. (three experiments).

	Coproporphyrinogen oxidase activity		
	After sonication	Without sonication	Increase of activity (%)
Whole mitochondria	1084±70	275±107	347±216
Pellet (inner-membrane–matrix complex)	432±205	123±79	372±73
Supernatant	789±255	780±245	—
Recovery after digitonin method fractionation (%)	112±3	348±106	

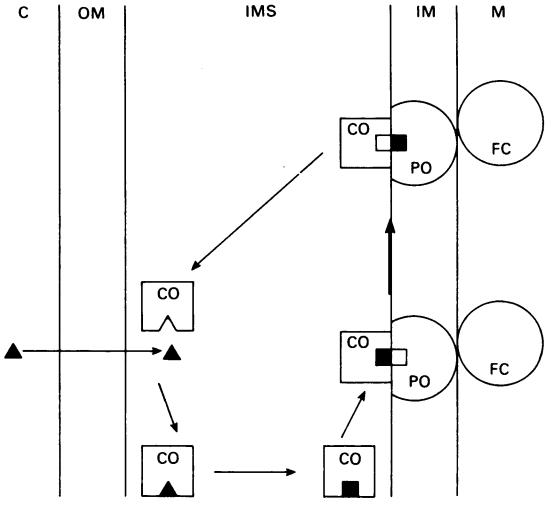

Fig. 3. *Possible role of coproporphyrinogen oxidase in the transfer of coproporphyrinogen from cytoplasm to mitochondrial inner membrane*
Abbreviations used: C, cytoplasm; M, matrix; OM, outer membrane; IM, inner membrane, IMS, intermembrane space; CO, coproporphyrinogen oxidase; PO, protoporphyrinogen oxidase; FC, ferrochelatase. ▲, Coproporphyrinogen; ■, protoporphyrinogen; □, unoccupied active site.

concentrations of substrate. For both fractions the same Michaelis constant (K_m) of 0.16μM was calculated from a Lineweaver–Burk plot (Fig. 2); this finding supports the concept of only a single coproporphyrinogen oxidase.

Effect of sonication on coproporphyrinogen oxidase activity

Such effects in whole mitochondria and in supernatant and pellet obtained after digitonin fractionation are shown in Table 5. Particular care was taken so avoid destroying the enzyme binding to the inner membrane. Coproporphyrinogen oxidase activity was measured immediately after sample preparation; the incubation medium was iso-osmotic and low ionic concentrations were used (see the legend to Table 5). Incubation was performed at 30°C for only 20min. After sonication, whole mitochondria showed a 347% increase in specific activity, whereas the increase of inner-membrane–matrix-complex activity was as high as 372%. It can also be seen that fractionation of non-sonicated mitochondria sharply increased the activity of pellet plus supernatant (recovery 348%), whereas this increase was not observed when fractions were sonicated before coproporphyrinogen oxidase measurement. These data suggest that the larger the amount of bound enzyme released (by sonication and/or by fractionation), the greater the increase in activity. Results similar to those obtained by sonication were observed with more concentrated ionic solutions in the incubation mixture (for instance 0.1M-sodium phosphate, pH7.4, used as buffer instead of 0.002M-Hepes).

Discussion

Our results show that, when mitochondria are treated by digitonin or by a hypo-osmotic solution, the major portion (75%) of coproporphyrinogen oxidase is recovered in the soluble fraction containing enzymes of the intermembrane space. The release of coproporphyrinogen oxidase activity does not exactly parallel that of adenylate kinase, the enzyme assayed as a marker for the intermembrane space (Tables 1 and 2, Fig. 1). A smaller fraction (25%) was found to be associated with the inner-membrane–matrix complex. An identical K_m (0.16μM; see Fig. 2) was found for both fractions, a finding that does not support the concept of two different enzymes. This value of K_m is very low when compared with that found by Elder & Evans (1978), namely 1.2μM. The reasons for this discrepancy remain to be clarified. Anyway, the same value of K_m (0.16μM) has been found for whole homogenate of rat liver, human liver,

human blood lymphocytes (0.17 μM) and human skin fibroblasts (0.15 μM) (the results given are means for three experiments; B. Grandchamp & Y. Nordmann, unpublished work).

The pattern of coproporphyrinogen oxidase activity release from the inner-membrane–matrix complex by washing with alkaline solutions or solutions of high ionic strength (Table 3) suggests that coproporphyrinogen oxidase may be loosely bound to the external face of the inner membrane by ionic bonds. The integrity of the inner membrane during these experiments is illustrated by the fact that malate dehydrogenase was never found in the supernatant (Table 3).

It was not possible to demonstrate binding of coproporphyrinogen oxidase to the isolated inner membrane (see Table 4), no matter how much care was taken (in particular, the inner-membrane–matrix complex was resuspended in a low-ionic-concentration medium, 4mM-Hepes, pH 6.8). Every procedure used to open the inner membrane (sonication, freezing and thawing) might release not only matrix enzymes, but also enzymes loosely bound to the inner membrane (Hoppel & Tomec, 1972; Zuyderhoudt *et al.*, 1969), and it appears that only tightly bound enzymes (such as succinate dehydrogenase) are not released.

When binding to the inner membrane is preserved, at least during incubation (see Table 5), sonication and/or fractionation are followed by a striking increase of activity. In contrast, high activity is obtained without sonication when the incubation mixture is made with a more concentrated ionic solution. These data could be explained by a correlation between high activity and the release of enzyme. The active site might be hindered by the inner membrane (see Fig. 3).

The mitochondrial localization of the other enzymes involved in haem synthesis has already been described (Jones & Jones, 1969; Poulson & Polglase, 1975; Zuyderhoudt *et al.*, 1969). Like coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase exhibit variable binding strength to the inner membrane. Each reaction product is a substrate for the next enzyme. The neighbourhood of these enzymes and their connection with the inner membrane could allow the non-water-soluble protoporphyrinogen to be transferred directly from one enzyme to the next. On the basis of the present results, Fig. 3 shows a detailed illustration of a possible role of coproporphyrinogen oxidase in haem synthesis; initially, the substrate coproporphyrinogen, synthesized in the cytoplasm, crosses the outer membrane; the enzyme–substrate complex is formed and the enzyme is then bound to the inner membrane near the protoporphyrinogen oxidase, allowing protoporphyrinogen to be transferred to protoporphyrinogen oxidase.

However, our data do not rule out the possibility that, *in vivo*, all the coproporphyrinogen oxidase is bound to the external face of the inner membrane, some more loosely than the other.

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