EVIDENCE FOR THE LOCATION OF FERROCHELATASE ON THE INNER MEMBRANE OF RAT LIVER MITOCHONDRIA

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Ferrochelatase (protohaem ferrolyase) catalyses the incorporation of ferrous iron into porphyrins to form haems. It is found not only in avian erythrocytes and bone marrow, where it is concerned in haemoglobin formation, but also in liver mitochondria (Nishida & Labbe, 1959), microorganisms and chloroplasts where it presumably functions in the biosynthesis of the prosthetic groups of cytochromes. The protein of cytochrome c is believed to be synthesised on the microsomes and not in the mitochondria (Roodyn, Suttie & Work, 1962; Gonzalez-Cadavid & Campbell, 1967). Preliminary experiments with rat liver homogenates showed that microsomes contained no ferrochelatase; activity appeared to be restricted to the mitochondria and so the structural relationship of the haem synthesising system and the protein synthesising systems was of great interest. It was possible that ferrochelatase might be located on the outer mitochondrial membrane, in proximity to the ribosomes or on the inner membrane where it might be associated with the completed electron transport system. (Previous studies had shown that ferrochelatase was not a soluble mitochondrial enzyme).

In our investigations we have prepared membrane fractions using a modification of the swelling - shrinking - sonication method of Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967). Instead of carrying out the shrinking stage in sucrose (0.45M) we have used KCl (0.12M), which allows much greater contraction of the inner membrane on adding ATP, (Chappell &

Greville, 1958), permitting more efficient separation of inner and outer membranes in subsequent steps. Using this technique of membrane preparation and two sensitive assays for ferrochelatase we have found that ferrochelatase is associated with the inner mitochondrial membrane. (We are grateful to Professor J.B. Chappell for suggesting the use of KCl contraction media).

METHODS

Mitochondria were prepared from rat liver in 0.25M sucrose containing 1mM ethylene glycol di(aminoethyl) tetra acetic acid (EGTA) and 4mM Tris-Cl, pH 7.2. They were subsequently washed twice in a sucrose-Tris medium lacking EGTA.

Preparation of membrane fractions

Mitochondria (about 200 mg. protein) were allowed to swell at 0^{o} for 7 min. in 30ml. Tris-phosphate (10mM, pH 7.5); 10ml of a solution containing 0.48 M KCl, 2mM ATP, 2mM $MgSO_4$ adjusted to pH 7.4 with Tris. was added and after 5 minutes, the suspension was sonicated in aliquots of 5ml. (10 sec., 1.5 amp.; MSE 60 W sonicator) to free outer membranes from the contracted inner membranes. Aliquots (20ml.) were layered on to a discontinuous gradient comprising 1.32M sucrose (25ml.) and 0.76M sucrose (12ml.) and centrifuged for $3\frac{1}{2}$ hr. at 24,00 r.p.m. in the SW 25 rotor of a Beckman-Spinco preparative centrifuge. The distribution of fractions resulting from this procedure is shown in Fig. 1.

Membrane fractions were also prepared by the method of Sottocasa et al (1967) and labelled as in Fig. 1.

Assay of marker enzymes

Malate dehydrogenase was assayed by the method of Ochoa (1955); monoamine oxidase was assayed as described by Schnaitman, Erwin & Greenwalt (1967), cytochrome b5 was assayed at 30^{o} in a dual wavelength spectrophotometer by measuring optical density change at 557 mμ minus 575mμ following the addition of NADH (0.1mM) to a suspension of the fraction in air-saturated phosphate buffer (75mM, pH 7.5) containing rotenone (0.25μg/ml.), using an Σ mM of

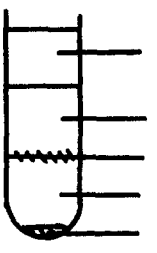


Fig.1 Distribution of submitochondrial fractions following gradient centrifugation. M_1 is at interface of 1.32M sucrose and 0.76M sucrose layers.

$20cm^{-1}$; cytochrome a + a_3 was assayed in the same medium from the change in optical density at 605mμ minus 630mμ following the addition of dithionite (Σ mM of $16cm^{-1}$).

Assay of ferrochelatase

Two types of assay of this enzyme were carried out. Incorporation of ^{59}Fe into porphyrins was measured using the method developed by Jones (1968) for the determination of chloroplast ferrochelatase. This method, which depends upon the extraction of [^{59}Fe] haem into cyclohexanone at the end of the incubation gave less satisfactory results with mitochondria. When concentrations of mitochondria in excess of about 1mg. mitochondrial protein/4ml. were used the counts in the cyclohexanone layer fell sharply, possibly as a result of non-selective binding of ^{59}Fe by mitochondrial protein. To avoid such problems a dual wavelength spectrophotometric assay was used that measured the rate of porphyrin disappearance. The assay used Co^{2+} in place of Fe^{2+}, since with Co^{2+} it was not necessary to exclude air from the cuvette and preliminary experiments had shown that the same enzyme catalysed the incorporation of either Co^{2+} or Fe^{2+}. The incubation mixture at 37° contained 50μM mesoporphyrin in 2ml. of 100mM Tris-Cl, pH 8.2 containing 1% Tween 80, together with the fraction to be investigated (2 or 3mg. mitochondrial protein gave satisfactory rates) and the reaction was started by adding 25μM Co^{2+}. Co-mesoporphyrin formation was measured at 498 minus 511 mμ using an Σ mM = $7.5cm^{-1}$.

TABLE I

Comparison of the distribution of protein and marker enzymes in mitochondrial fractions prepared by swelling, shrinking in sucrose, followed by mild sonication (Sottocasa et al, 1967), or by swelling, shrinking in KCl, followed by mild sonication.

Proportion of activity recovered (%)

Fraction	Total Protein		Cytochrome b5		Cytochrome a/a3		Monoamine oxidase		Malic dehydrogenase	
	sucrose shrunk	KCl shrunk	sucrose shrunk	KCl shrunk	sucrose shrunk	KCl shrunk	sucrose shrunk	KCl shrunk	sucrose shrunk	KCl shrunk
Soluble (S_1)	21	22.1	0	0	0	0	0	0	33	35.5
Light subfraction ($M_1 + S_2$)	22.7	20.0	31.0	76.7	0	0	23	86.0	18.7	11.0
Heavy subfraction	48	54	17	16	82	91	53	20.2	53	60

TABLE 2

Distrubution of ferrochelatase and Co^{2+} chelatase in mitochondrial fractions. The fractions used were those shown in Table I.

Proportion of activity recovered

Fraction	^{59}Fe mesohaem formation	Co-mesoporphyrin formation	
	KCl shrunk	sucrose shrunk	KCl shrunk
Soluble (S_1)	0	0	0
Light subfraction ($S_2 + M_1$)	9.1	0	0
Heavy subfraction (M_2)	67	70	96.4

RESULTS

A comparison of the separation of marker enzymes by the two different methods is given in Table 1. Monoamine oxidase, which was not detectable in microsomes, was used as a marker for the outer mitochondrial membrane and cytochrome $\underline{a}/\underline{a}_3$ for the inner membrane. It can be seen that with the use of the KCl medium for shrinking the recovery of monoamine oxidase was rather higher and that M_1 and the associated S_2 region were very considerably enriched in this enzyme. The distribution of cytochrome $\underline{b}5$ was similar. Following sucrose shrinking, longer sonication released more monoamine oxidase from M_2, but also caused the contamination of M_1 with cytochrome $\underline{a}/\underline{a}_3$. There was obviously much less contamination of the inner by outer membrane fractions when KCl was used as the shrinking medium, 86% of the monoamine oxidase was found in the light fraction and cytochrome $\underline{a}/\underline{a}_3$ was found only in M_2. Using either method about 40% of the malic dehydrogenase was released from the matrix of the inner membrane into the soluble S_1 fraction.

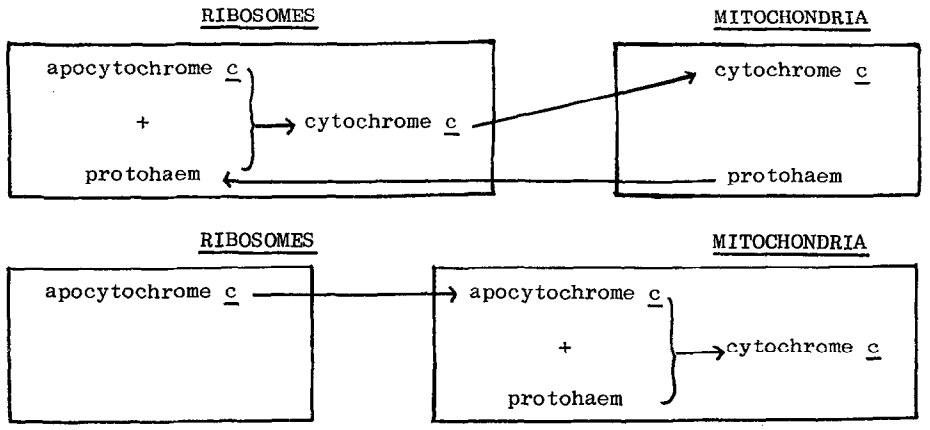
The distribution of ferrochelatase and Co chelatase in these separated membrane fractions was determined (Table 2). In every case the distribution of activity very closely followed that of cytochrome oxidase, i.e. ferrochelatase is an enzyme associated with the inner mitochondrial membrane.

DISCUSSION

In our hands the preparation of mitochondrial fractions by the method of Sottocasa *et al* (1967) has yielded variable results. Although the recovery of enzyme activities was sometimes better than that shown in Table 1, the separation of marker enzymes was rarely satisfactory. This is probably because sucrose inhibits the contraction of the mitochondrial inner membrane and a more vigorous sonication is necessary to free it from the still attached outer membrane. By using a KCl shrinking medium very consistent separations of membranes have been achieved.

The association of ferrochelatase with the inner membrane fraction of mitochondria indicates that it cannot be directly involved with the

synthesis of apocytochromes by the ribosomes. Two alternative routes of synthesis of cytochrome c can be envisaged, shown diagramatically below.



Our results do not enable us to distinguish between these possibilities, although analogy with haemoglobin formation, where globin synthesis appears to be regulated by availability of haem, might support Scheme I.

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