Studies on the efflux of metalloporphyrin from isolated rat liver mitochondria

Effect of respiratory substrates and metabolic inhibitors

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Intramitochondrially synthesized Co-deuteroporphyrin is released to the incubation medium at a rate inversely correlated to the energy state of the mitochondria: i.e. the rate of efflux increases when substrate is depleted, respiration inhibited or the mitochondria are uncoupled. The efflux of Co-deuteroporphyrin from mitochondria remains low as long as the residual membrane potential is above one-third that of maximally energized mitochondria. Globin enhances the efflux of Co-deuteroporphyrin not only from mitochondria depleted of substrates [Husby & Romslo (1980) *Biochem. J.* **188**, 459–465], but also from maximally energized mitochondria. The results provide further evidence for a co-operative mechanism between the mitochondria and their surroundings for the mobilization of metalloporphyrin from mitochondria.

In the liver about 80% of the total amount of haem synthesized by the mitochondria is incorporated into extramitochondrial haem proteins, in particular cytochrome *P*-450 and catalase (Granick & Beale, 1978).

The mechanism by which haem reaches its appropriate apoproteins is largely unknown. From studies on mitochondria isolated from liver and reticulocytes it has been shown that the efflux of haem is enhanced in the presence of a suitable haem-binding ligand (Yoda & Israels, 1972; Ponka *et al.*, 1973; Israels *et al.*, 1975; Taketani & Tokunaga, 1980; Husby & Romslo, 1980; Husby *et al.*, 1980, 1981) and a low ionic strength of the incubation medium (Husby & Romslo, 1981).

By analogy with the ADP/ATP transport across the mitochondrial inner membrane (Klingenberg & Rottenberg, 1977) it has recently been suggested that the uptake of porphyrins and iron and the efflux of haem occurs by a coupled symport/antiport mechanism (Husby & Romslo, 1981). If so, it would not be unexpected if the efflux of metalloporphyrin from the mitochondria were regulated by the mitochondrial energy state, as has been found for the uptake of porphyrins (Koller & Romslo, 1978, 1980) and iron ions (Flatmark & Romslo, 1975).

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

The present study deals with the effect of respiratory substrates and metabolic inhibitors on the efflux of metalloporphyrin from isolated rat liver mitochondria.

Materials and methods

Materials

Rat liver mitochondria were prepared as described previously (Romslo & Flatmark, 1973). The functional integrity of the preparations was tested by measuring the respiratory-control ratio with ADP, with succinate as substrate. Only mitochondria with this ratio greater than 4 at the start of the experiments were used.

Structural intactness of the mitochondria was measured from leakage of malate dehydrogenase into the incubation medium. Less than 1% of the total malate dehydrogenase activity of the mitochondria leaked to the incubation medium during a 60 min incubation.

Synthesis and efflux of Co-deuteroporphyrin

Mitochondria (about 2 mg of protein/ml) were loaded with deuteroporphyrin IX by pre-incubation for 1 min at 30°C in a medium containing in a volume of 26 ml: 0.25 M-sucrose, 5 mM-Hepes buffer, pH 7.40 (adjusted with KOH) and 2 µM-deuteroporphyrin added from a stock solution containing 2.5 mM-deuteroporphyrin IX in 0.01 M-KOH. At this

concentration (about 1 nmol/mg of protein) deuteroporphyrin is not detrimental to the mitochondria (Koller & Romslo, 1978), and it is completely taken up by the mitochondria (Koller & Romslo, 1978). Further additions were made as described in the legends to the Figures and Table 1. The metalloporphyrin synthesis was initiated by adding $8 \mu\text{M}$ - $^{57}\text{CoCl}_2$ (30 000 c.p.m./nmol). To secure aerobiosis during the experiments the incubation medium was stirred and continuously flushed with O_2 .

The functional intactness of the mitochondria was followed during the experiments by measuring the respiratory-control ratio with ADP, with succinate as substrate. The mitochondria remained coupled (the respiratory-control ratio was greater than 2) for periods varying from 15 min when substrate was omitted to at least 60 min when exogenous substrate was added.

Synthesis and efflux of metalloporphyrin were determined by extraction into cyclohexanone (Husby & Romslo, 1980).

Other analytical procedures

Mitochondrial respiration rates were determined as described by Husby & Romslo (1980).

The CCCP-dependent spectral shift of safranin was determined by adding $20 \mu\text{l}$ of 4 M-safranin to 1 ml of the incubation mixture from the Co-deuteroporphyrin synthesis experiment plus 2 ml of 5 mM-Hepes buffer, pH 7.40, containing 0.25 M-sucrose. After having reached a steady-state condition, the $\Delta(A_{524} - A_{554})$, after addition of CCCP at a final concentration of $1 \mu\text{M}$, was recorded on an Aminco DW-2 UV/VIS spectrophotometer at a temperature of 30°C (Åkerman & Wikström, 1976; Koller & Romslo, 1978; Zanotti & Azzone, 1980).

Protein was determined by Folin & Ciocalteu reagent (Flatmark *et al.*, 1971).

Malate dehydrogenase (EC 1.1.1.37) was determined by the method described by Bergmeyer (1974).

Chemicals

ADP, Hepes (A-grade), rotenone, succinate, malate, glutamate, CCCP, malonate, β -hydroxybutyrate, antimycin A and HOQNO were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Deuteroporphyrin IX was obtained from Porphyrin Products (Logan, UT, U.S.A.). $^{57}\text{CoCl}_2$ was from The Radiochemical Centre (Amersham, Bucks., U.K.). Globin was the product of ICN Pharmaceuticals (Cleveland, OH, U.S.A.). Other chemicals were of the highest purity commercially available.

Results

Recently we reported (Husby & Romslo, 1980) that Co-deuteroporphyrin synthesized by isolated

rat liver mitochondria in the absence of externally added substrate is released to the incubation medium in an inverse correlation to the respiratory control ratio of the particles, i.e. in tightly coupled mitochondria the efflux remained at about 10% of the total amounts of metalloporphyrin synthesized, whereas in uncoupled mitochondria the efflux amounted to 30–40% (Husby & Romslo, 1980).

Fig. 1. Time-progress curves for the synthesis of Co-deuteroporphyrin (a), release of Co-deuteroporphyrin to the supernatant (b) and the CCCP-dependent wavelength shift of safranin (c)

Experimental conditions were as described in the Materials and methods section. ∇ , $\circ$ and $\diamond$, Incubation medium alone; $\blacktriangledown$, $\bullet$, and $\blacktriangleleft$, incubation medium supplemented with 0.1 mM-succinate; $\blacktriangledown$, $\bullet$ and $\blacktriangleleft$, incubation medium supplemented with 1 mM-succinate.

The ability of isolated rat liver mitochondria to synthesize Co-deuteroporphyrin is essentially the same in the absence as in the presence of respiratory substrate (Fig. 1a). However, the ability of the mitochondria to retain Co-deuteroporphyrin is markedly enhanced when substrate is added; with 1 mM-succinate the efflux of Co-deuteroporphyrin remains low, at about 10% during 60 min incubation (Fig. 1b). By comparison, in the absence of succinate the efflux of Co-deuteroporphyrin increases from 10% at 10 min to about 30% at 60 min (Fig. 1b).

β -Hydroxybutyrate or glutamate/malate behave essentially as succinate, but higher concentrations are required (Table 1).

The effect of succinate on the efflux of Co-deuteroporphyrin can be abolished by blocking the influx of succinate by phenylsuccinate, inhibiting its oxidation by malonate, antimycin A or HOQNO, or de-energizing the mitochondria by CCCP, but not by adding oligomycin (Table 1).

Together with our previous findings (Husby & Romslo, 1980) the results of Table 1 provide strong evidence that the efflux of Co-deuteroporphyrin is regulated also by the energy state of the mitochondria. This contention is further supported by the

results presented in Figs. 2–4. In the absence of respiratory substrates, increased efflux of Co-deuteroporphyrin coincides with a decline in the CCCP-dependent wavelength shift of safranine, and the same is found, but after a delay, when low concentrations of succinate are included (Figs. 1b and 1c). Apparently the mitochondria effectively retain Co-deuteroporphyrin only as long as the energy potential is above that corresponding to an absorbancy change of safranine near 0.04 (Fig. 2).

It could be speculated that the time-dependent release of Co-deuteroporphyrin from mitochondria merely reflects leakage from damaged particles. If so, it would be expected that succinate, added after the time-dependent deterioration of the mitochondria has been initiated, should have no effect on the release of Co-deuteroporphyrin. However, succinate added after 12 min of incubation (Fig. 3) enhances the CCCP-dependent wavelength shift of safranine and at the same time slows down the efflux of Co-deuteroporphyrin.

In substrate-depleted mitochondria globin added to the incubation medium almost totally removes Co-deuteroporphyrin from the particles, and this

Table 1. Effect of respiratory substrates and metabolic inhibitors on the release of Co-deuteroporphyrin from isolated rat liver mitochondria

Mitochondria (about 2 mg of protein/ml) were incubated as described in the Materials and methods section. The percentage release of Co-deuteroporphyrin to the supernatant was determined at 10 and 60 min. The additions were: succinate, 1 mM; phenylsuccinate, 5 mM; malonate, 5 mM; antimycin A, 0.2 μ g/mg of protein; HOQNO, 5 mM; oligomycin, 5 μ g/mg of protein; CCCP, 5 μ M; glutamate, 5 mM; malate, 5 mM; β -hydroxybutyrate, 5 μ M; rotenone, 8 μ M. The amount of Co-deuteroporphyrin present in the assay at 10 and 60 min was 270 pmol/mg of protein and 610 pmol/mg of protein respectively.

Addition	Co-deuteroporphyrin released (%) at:	
	10 min	60 min
None (control)	9	34
+CCCP	28	38
+Succinate	7.5	12.5
+Succinate + phenylsuccinate	10	34
+Succinate + malonate	27	34
+Succinate + antimycin A	14	30
+Succinate + HOQNO	18	34
+Succinate + CCCP	29	35
+Succinate + oligomycin	8	13
+ β -Hydroxybutyrate	10	10
+Glutamate/malate	7	8
+Glutamate/malate + rotenone	23	35

Fig. 2. Relationship between CCCP-dependent wavelength shift of safranine and Co-deuteroporphyrin release to the supernatant (%)

Experimental conditions were as described in the Materials and methods section. Each series of symbols represent experiments on separate mitochondria.

Fig. 3. Time-progress curves for the release of Co-deuteroporphyrin to the supernatant (a) and CCCP-dependent wavelength shift of safranine (b)

Experimental conditions were as described in the Materials and methods section. $\circ$ and $\diamond$, Control experiment; $\bullet$ and $\blacklozenge$, experiment where succinate (1 mM final concentration) was added to the medium at the time interval indicated (arrow).

Fig. 4. Time-progress curves for release of Co-deuteroporphyrin to the supernatant

Experimental conditions were as described in the Materials and methods section. $\circ$, Control without globin and succinate; $\triangle$, with 4 μ M-globin; $\bullet$, with 1 mM-succinate; $\blacktriangle$, with 1 mM-succinate and 4 μ M-globin.

effect bears no relationship to the energy coupling of the particles (Husby & Romslo, 1980). Globin also effectively empties Co-deuteroporphyrin from mitochondria supplemented with succinate (Fig. 4).

Discussion

The results of the present study confirm the assumption that metalloporphyrins are actively retained within the mitochondria (Husby & Romslo, 1980).

Cations, in particular Ca^{2+} , Mg^{2+} and K^{+} , depress the efflux of Co-deuteroporphyrin and Co-protoporphyrin from coupled as well as from uncoupled mitochondria (Husby & Romslo, 1981). This, however, cannot explain the effect of the respiratory substrates on the release of Co-deuteroporphyrin, because the concentration of cations (i.e. K^{+}) was the same in the control and in the succinate experiments, and secondly succinate was only effective as long as it could be oxidized (Table 1) and the oxidative energy conserved in the mitochondria (Figs. 1 and 2). The effects of the substrates could not be explained by a lower gradient of Co-deuteroporphyrin between the mitochondria and the medium, because the amount of Co-deuteroporphyrin synthesized was the same in the absence as well as in the presence of a respiratory substrate (Fig. 1a). Therefore we conclude that the effect of the respiratory substrates (Fig. 1b and Table 1) is through generation of a high-energy state of the mitochondria.

The CCCP-dependent wavelength shift of safranine has been widely used to assess the mitochondrial membrane potential (Åkerman & Wikström, 1976; Zanotti & Azzone, 1980). Under conditions of small variations in the transmembrane pH-gradient, the wavelength shift of safranine closely parallels the magnitude of the transmembrane electrochemical potential gradient (Åkerman & Wikström, 1976), although, however, the slope of the correlation lines differs depending on the dye/mitochondrial protein ratios (Zanotti & Azzone, 1980). As shown here (Figs. 1c and 2) Co-deuteroporphyrin is retained by the mitochondria as long as the CCCP-dependent wavelength shift of safranine remains above 0.04–0.05 absorbance units. According to the work of Zanotti & Azzone (1980) this corresponds to a $\Delta\psi$ of about 60–80 mV, i.e. the retention of metalloporphyrins operates at an energy level about one-third that of maximally energized mitochondria (Mitchell & Moyle, 1969; Nicholls, 1974). Thus the influx of iron (Flatmark & Romslo, 1975) and protoporphyrin (Koller & Romslo, 1980), as well as the efflux of metalloporphyrin, are all under the influence of metabolic energy. Whether or not these are coupled processes mediated by a common carrier is so far unknown. It remains also to be determined what is the significance of the

metabolic requirement for the transport processes to overall haem synthesis.

The energy-dependent retention of metalloporphyrins accounts for only a minor fraction of the total amount of metalloporphyrin synthesized by the mitochondria; in uncoupled mitochondria, 60–70% of the metalloporphyrin synthesized still resides in the mitochondria (Fig. 1b). One explanation of this could be that metalloporphyrins are more soluble in the lipoprotein mitochondrial inner membrane than in the salt/sucrose-buffered medium, in particular at high ionic strength of the medium (Husby & Romslo, 1981), and would therefore tend to concentrate in the mitochondria.

Globin markedly enhances the efflux of metalloporphyrins from substrate-depleted mitochondria (Husby & Romslo, 1980), as shown in the present paper from tightly coupled and highly energized mitochondria as well (Fig. 4). As yet, we do not know the mechanism by which globin removes metalloporphyrin from the mitochondria, be it through binding within the inner membrane, at the interphase inner-membrane/intermembrane space or at the level of the outer membrane. Since albumin does not penetrate to the inner membrane (Rottenberg, 1973) nor does it have any effect on the efflux of metalloporphyrins from tightly coupled mitochondria (Husby & Romslo, 1980) it would be tempting to suggest that globin (Fig. 4; see also Husby & Romslo, 1980, 1981), and perhaps also haemopexin (Husby *et al.*, 1980) and ligandin (Husby *et al.*, 1981), exert their effects adjacent to the inner membrane. The results with globin, being relevant in immature red cells, should, however, be considered only as models for the more likely physiological haem-binding ligand of hepatocytes, apo-cytochrome P-450 (Levin, 1973). According to the results of Meyer & Meier (1977), in the liver the rough endoplasmic reticulum is tightly associated with the mitochondria and thereby allows for a direct transfer of haem from mitochondria to apo-cytochrome P-450. Recent studies by Pickett *et al.* (1980, 1981), have largely confirmed this contention. Thus a role for haem-binding ligands other than the appropriate apo-cytochromes (Yoda & Israels, 1972; Israels *et al.*, 1975; Schulman, 1974; Taketani & Tokunaga, 1980) seems superfluous, at least to account for passage of haem from the mitochondria to apo-cytochrome P-450.

The finding of an energy-dependent retention of metalloporphyrins within the mitochondria may be of relevance to explain the inhibitory effect of haemin on the ferrochelatase (Jones & Jones, 1970; Koller & Romslo, 1977; Koller, 1979) and on the uptake of protoporphyrin (Koller & Romslo, 1980), iron (Koller *et al.*, 1976; Ponka *et al.*, 1977) and δ -aminolaevulinic acid synthase (Yamauchi *et al.*, 1980).

In fact, it could be that the so-called cellular free haem (Granick & Beale, 1978) is localized to the mitochondrial inner membrane. The answer to this question remains to be resolved.

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