STUDIES ON THE UPTAKE OF PORPHYRIN BY ISOLATED RAT LIVER MITOCHONDRIA WITH PARTICULAR EMPHASIS ON THE EFFECT OF HEMIN

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1. Introduction

The last three reactions in the biosynthesis of heme are catalyzed by enzymes confined to the mitochondria. Thus, coproporphyrinogen III which is synthesized in the cytosol, is assimilated by the mitochondria where it is decarboxylated, oxidized and chelated to ferrous iron [1–4]. The metalloporphyrin thus formed is united with its apoprotein either within the mitochondria [5] or in the cytosol [6].

Until recently the mechanism(s) by means of which the mitochondria accumulate porphyrin(ogen)s was largely unknown. Deuteroporphyrin IX [7] as well as protoporphyrin IX [8] have been shown to be accumulated by mitochondria by mechanism(s) similar in many aspects to carrier-mediated transport, and in part depend on an energized inner membrane. Furthermore, from studies of the ferrochelatase reaction of intact and sonicated mitochondria, it has been suggested that uncommitted extramitochondrial hemin may control the uptake of porphyrins [9].

Here, isolated rat liver mitochondria is shown to possess two classes of porphyrin-binding sites. One class (high-affinity binding sites) binds ~13 nmol deuteroporphyrin IX/mg protein with K'_m 22 μmol/l. These binding sites are inhibited by CCCP + valinomycin. A second class (of lower affinity) binds ~22 nmol deuteroporphyrin IX/mg protein with K'_m 110 μmol/l. Only porphyrins accumulated by the high-affinity binding sites are accessible to the ferrochelatase of intact mitochondria.

Hemin inhibits the binding of deuteroporphyrin IX non-competitively in the absence of CCCP + valinomycin (K_i 100 μmol/l), whereas in the presence of CCCP + valinomycin a competitive type of inhibition is found, K'_i 50 μmol/l. Globin and albumin are competitive inhibitors of both total and low-affinity binding. Hemoglobin has only negligible effects, both on the total and the low-affinity binding of deuteroporphyrin IX.

2. Materials and methods

Rat liver mitochondria were prepared as in [7]. The functional integrity of the preparations was tested by measuring the respiratory control ratio with ADP, using succinate as the substrate. Only mitochondria with respiratory control ratios >4 were used.

Deuteroporphyrin IX was dissolved in 1 mmol/l NaOH. The purity of the preparation was determined by thin-layer chromatography on silica gel after esterification [7]. The chromatograms were scanned on a Shimadzu dual-wavelength thin-layer chromatography scanner, Model CS-900 equipped with a fluorescence accessory. About 90% of the fluorescent material was recovered as a single spot (R_F 0.74).

Mitochondria, ~2 mg protein were preincubated in 1 ml final vol. for 2 min at 25°C with 0.25 mol/l sucrose, 10 mol/l Hepes adjusted to pH 7.4 with Tris-base and, where indicated, CCCP + valinomycin. Further additions or omissions were as described in the figure legends and table. The reaction was initiated by adding deuteroporphyrin IX. After 30 s the reaction was terminated by adding 2 vol. ice-cooled incubation buffer (see above) immediately followed by centrifugation in an Eppendorf microcentrifuge (type 3200). The amount of deuteroporphyrin IX

accumulated was determined from the decrease in the fluorescence of the supernatant following centrifugation as in [7]. To correct for possible fluorescence quenching a control was included in which the compound tested (hemin, globin, albumin or hemoglobin) was added to the supernatant after centrifugation.

Hemoglobin and globin were prepared from anticoagulated human blood. After removal of plasma and buffy coat, the red cells were washed 5 times with 10 vol 155 mmol/l NaCl and lyzed by freezing and thawing. The ghosts were sedimented (50 000 × *g* for 1 h) and hemoglobin was purified from the supernatant by chromatography (Ultrogel AcA 44, column 2.6 × 90 cm, elution buffer 10 mmol/l Hepes buffer (pH 7.4).

Globin was prepared from hemoglobin by precipitation with excess acetone at −20°C [10]. The precipitate was washed twice with cold acetone, and dissolved in 10 mmol/l Hepes buffer, pH 7.4 at 1.5 mg/ml. On SDS–polyacrylamide gel, electrophoresis, hemoglobin and globin revealed only one stained protein band.

Protein was determined by the Folin-Ciocalteau reagent [11].

2.1. *Chemicals*

N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) (A grade), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), hemin (bovine, type I) and valinomycin were obtained from the Sigma Chemical Co. (St Louis, MO). Deuteroporphyrin IX was purchased from Porphyrin Products (Logan, UT). Ultrogel AcA 44 was the product of Industrie Biologique Francaise.

Other chemicals were of the highest purity commercially available. Double quartz-distilled water was used throughout.

3. Results

Rat liver mitochondria were examined for their capacity to accumulate deuteroporphyrin IX in the absence and presence of CCCP + valinomycin. Figure 1 shows the results presented as Scatchard plot. In the absence of metabolism inhibitors the Scatchard plot is biphasic. In the presence of CCCP + valinomycin, however, a monophasic plot is obtained. By analogy

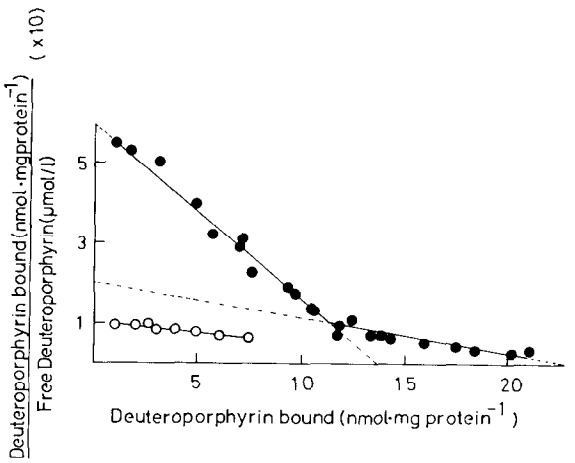

Fig.1. Scatchard plot of deuteroporphyrin IX accumulation by isolated rat liver mitochondria. Mitochondria, ~2 mg protein/ml were incubated as in section 2 in the absence (•) and presence (○) of 5 μmol/l CCCP + 1 μg valinomycin (0.5 μg/mg protein). Increasing concentrations of deuteroporphyrin IX were added and the amount of deuteroporphyrin IX accumulated was determined as in section 2.

to the mitochondrial uptake of divalent cations [12], the horizontal rectilinear leg corresponds to low-affinity binding sites and the vertical leg to high-affinity binding sites. The apparent number of binding sites as obtained from the extrapolated intercepts are 13 nmol/mg protein and 22 nmol/mg protein, half-saturation is obtained at ~22 μmol/l and 110 μmol/l and the stability constants are 5×10^4 l/mol and 9×10^3 l/mol for the high- and the low-affinity binding, respectively. These calculations are based on the assumption that the amount of free deuteroporphyrin IX equals the amount of deuteroporphyrin IX not taken up by the mitochondria.

Hemin inhibits the ferrochelatase reaction of intact mitochondria and of submitochondrial particles [9]. In submitochondrial particles hemin functions as a competitive inhibitor of the ferrochelatase, whereas in intact mitochondria a more complex type of inhibition is observed [9]. As shown in fig.2 hemin decreases the uptake of deuteroporphyrin IX and at the same time increases the K_m of the reaction, typically of a non-competitive type of inhibition. A most simple explanation could be that hemin functions as an uncoupler of oxidative phosphorylation. However, hemin at <50 μmol/l neither increases the

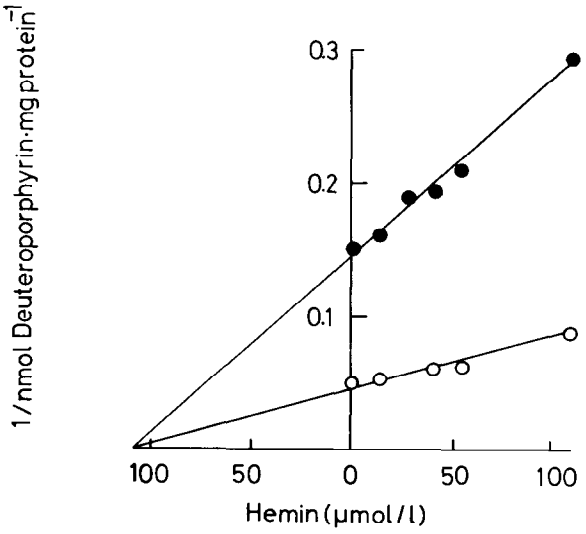

Fig.2. Effect of hemin on the accumulation of deuteroporphyrin IX by isolated rat liver mitochondria. Mitochondria, ~2 mg protein/ml were incubated as in section 2. Hemin was added prior to 12.5 (•) or 17.5 μmol/l deuteroporphyrin IX (○).

state 3 respiration rate of tightly coupled rat liver mitochondria [13] nor increases the state 4 respiration rate (data not shown). Hence, the effect of hemin on the uptake of deuteroporphyrin IX cannot be ascribed to loose coupling and dissipiation of the mitochondrial energy potential.

In the presence of CCCP + valinomycin the effect of hemin on the uptake of deuteroporphyin IX changes to a competitive type of inhibition (fig.3B).

Hemoglobin has only negligible effects both on the total and the CCCP + valinomycin-insensitive binding of deuteroporphyrin IX (table 1). On the other hand, globin and albumin are competitive inhibitors both of the total and the inhibitor-insensitive binding of deuteroporphyrin IX (table 1) in agreement with their high affinity for the binding of porphyrins [14–16].

4. Discussion

At pH 7.4 deuteroporphyrin IX is slightly negatively charged. Compared to binding data of metabolite anions like ADP and ATP [17], the results obtained with deuteroporphyrin IX differ both with respect to the relatively small difference in the number of low- and high-affinity binding sites and the rather low affinity of the high-affinity binding sites (fig.1). On the other hand, deuteroporphyrin IX behaves very similar to iron, both in affinity and in the number of binding sites [18]. The low affinity of mitochondria to deuteroporphyrin IX (and iron [18]) could be more apparent than real; we do not know their real, free concentration [19].

From the localization of ferrochelatase to the M-side of the inner membrane [4] and coproporphyrinogen oxidase to the intermembrane space [1,2] a mechanism for the transfer of porphyrins across the inner membrane would be expected. That the high-affinity binding described here represents deuteroporphyrin IX destined for deuteroheme synthesis within the inner membrane is supported by the findings that the metabolic inhibitors CCCP + valinomycin abolish

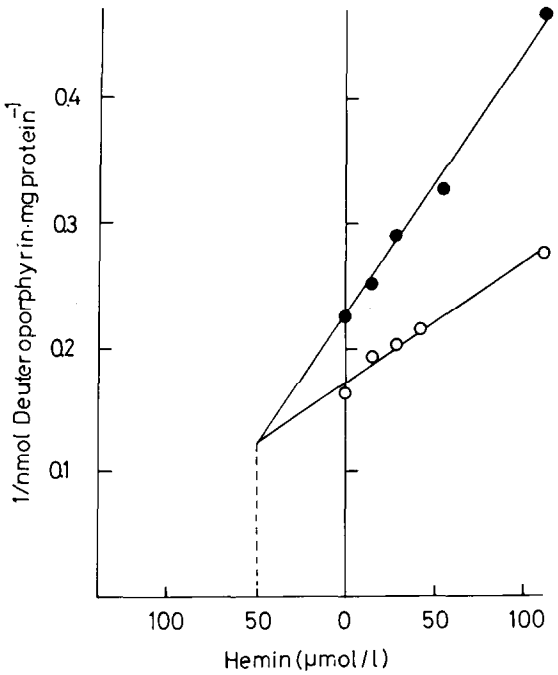

Fig.3. Effect of hemin on the accumulation of deuteroporphyrin IX by de-energized mitochondria. Mitochondria, ~2 mg protein/ml were incubated as in section 2 with 5 μmol/ml CCCP + 1 μg valinomycin (0.5 μg/mg protein). Hemin was added prior to 12.5 (•) or 17.5 μmol/l deuteroporphyrin IX (○).

Table 1
Effect of hemin, hemoglobin, globin and albumin on the uptake of deuteroporphyrin IX by isolated rat liver mitochondria

	− CCCP/valinomycin			+ CCCP/valinomycin		
	Uptake (% of control)	Type of inhibition	K'_i (μmol/l)	Uptake (% of control)	Type of inhibition	K'_i (μmol/l)
Hemin	55.0 (47.7– 65.0)	non-competitive	110	46.0 (40.2–48.4)	competitive	50
Hemoglobin	93.8 (91.2–102.3)	–	–	96.5 (92.5–99.4)	–	–
Globin	45.5 (35.7– 50.5)	competitive	16	48.4 (35.0–49.9)	competitive	16
Albumin	8.0 (0 – 10.5)	competitive	2	5.0 (0 – 9.0)	competitive	2

Mitochondria, ~2 mg protein/ml were incubated as in section 2 in the absence and presence of 5 μmol/l CCCP + 1 μg valinomycin (0.5 μg/mg protein). Hemin, hemoglobin, globin and albumin (100, 27, 28 and 24 μmol/l, respectively) were added just prior to the addition of 12.5 μmol/l deuteroporphyrin IX. The results, which are given as % of the uptake in the absence of inhibitor, are the means and the ranges (in parentheses) from 4 separate experiments. The mean 100% values were: in the absence of CCCP/valinomycin 5.9 nmol/mg protein, in the presence of CCCP/valinomycin 2.4 nmol/mg protein. The type of inhibition and corresponding K'_i-values have been derived from Dixon plots of experiments analogous to those reported in fig.2,3

high-affinity binding, and at the same time they inhibit the ferrochelatase activity of intact mitochondria without having any effect on the ferrochelatase of sonicated mitochondria [7–9].

From the observation that deuteroporphyrin IX accumulated in the presence of CCCP + valinomycin cannot be paved into deuteroheme synthesis by intact mitochondria [7] and that low-affinity binding sites are blocked by hemin at half the concentration necessary to abolish high-affinity binding (fig.2,3) we believe that low-affinity binding is not an obligatory step to precede transport of deuteroporphyrin IX to the ferrochelatase.

The results of fig.2 indicate a novel regulatory function of hemin, that of controlling the binding of porphyrin to the mitochondria. The effect of hemin may result from an inhibition of the transport of deuteroporphyrin IX to within the mitochondria, or hemin may change the binding properties of a hypothetical porphyrin carrier. Interestingly, a similar type of inhibition has been reported for carboxyatractyloside on the adenine nucleotide translocator [17] and for 2-oxoglutarate and citrate on the dicarboxylate carrier [20].

An additional effect of hemin is to compete with deuteroporphyrin IX for the passive binding to the outer compartment (fig.3).

Albumin and globin reduce the binding of deuteroporphyrin IX presumably by trapping deuteroporphyrin IX [14–16] making it unavailable for the binding to the mitochondria. Two more conclusions emerge from this finding:

(i) Mitochondria apparently do not assimilate protein-bound porphyrins, or at least with far less avidity than the free-porphyrin base,

(ii) The affinity of the mitochondria to bind deuteroporphyrin IX compared to that of albumin (or globin) is of the order 1:10 on a protein basis (table 1).

These figures should be compared to the stability constant reported for the porphyrin–albumin complex (K_d 10^{-6} mol/l) and the stability constant of the high-affinity binding reported in fig.1 (K_d 5×10^4 l/mol).

The lack of inhibitory effect of hemoglobin support the suggestion that globin binds deuteroporphyrin IX at vacant heme-binding sites [21].

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