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# STUDIES ON THE FERROCHELATASE ACTIVITY OF MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES WITH SPECIAL REFERENCE TO THE REGULATORY FUNCTION OF THE MITOCHONDRIAL INNER MEMBRANE

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SUMMARY

Mitochondrial ferrochelatase activity has been studied in coupled rat liver mitochondria and in ultrasonically treated rat liver mitochondria using deuteroporphyrin IX and Fe(III) as the substrates.

1. There were significant differences between the ferrochelatase reaction of intact mitochondria as compared to sonicated mitochondria with respect to pH and temperature optima, changes in substrate and protein concentration, and sensitivity to uncoupler and product inhibition.

2. The translocation of iron and deuteroporphyrin to the ferrochelatase on the M-side of the inner membrane depended on an energized inner membrane, i.e. when the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone was added to a suspension of intact mitochondria prior to either iron or deuteroporphyrin, the ferrochelatase reaction was depressed by approx. 70 %. The uncoupler had no effect on the ferrochelatase reaction of sonicated mitochondria.

3. Hemin inhibited the ferrochelatase reaction of intact mitochondria as well as of sonicated mitochondria, partly by inhibiting the catalytic activity of the ferrochelatase, and partly by interfering with the translocation of the substrate(s).

4. When the mitochondria were incubated in a sucrose medium, endogenously synthesized deuteroheme did not penetrate freely to the outside of the inner membrane.

5. It is concluded that the transport of the substrates as well as the products across the inner membrane serves an important regulatory role in the overall ferrochelatase reaction

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Abbreviations. EPPS, (4-(2-hydroxyethyl)-1-piperazine propane sulphonic acid), HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, PIPES, piperazine-*N,N'*-2-bis(2-ethanesulphonic acid), CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

## INTRODUCTION

The localisation of the ferrochelatase (protoheme ferrolyase EC 4.99.1.1) to the inner phase of the mitochondrial inner membrane [1] implies that the substrates as well as the products, have to penetrate the mitochondrial membranes. This contention raises the question whether the iron ions, the porphyrins and the hemes are freely permeable across the mitochondrial membranes, and if not, what are the mechanism(s) of their transport and to what extent does the transport interfere with the overall ferrochelatase reaction

Findings in patients with congenital hypochromic microcytic anemia suggest that in this disorder there is a defect in the transfer of iron from plasma transferrin to the ferrochelatase inside the mitochondria [2]. Furthermore, in patients with erythropoietic protoporphyria the accumulation of protoporphyrin in the erythroid cells has been ascribed to a normal, but labile ferrochelatase [3]. It has also been suggested that differences in membrane permeability and the existence of energy-mediated transport systems may explain the different porphyrin excretion pattern observed in human porphyria as compared to that of experimentally induced porphyria in rabbits [4].

Experimentally, there is substantial evidence in favour of a regulatory function of the mitochondrial membranes on the ferrochelatase reaction. Thus, the accumulation of iron by mammalian mitochondria is highly dependent on the mitochondrial energy potential [5, 6], the efflux of heme from the mitochondria is regulated by cytosolic proteins [7, 8] and in yeast mitochondria coproporphyrinogenase and protoporphyrinogen oxidase catalyze and simultaneously transfer coproporphyrinogen from the cytosol, to protoporphyrinogen inside the mitochondria [9].

So far, however, more detailed studies of the regulatory functions of the mitochondrial membranes on the ferrochelatase reaction have not been undertaken.

Recently we reported on the ferrochelatase reaction of intact rat liver mitochondria [10]. Compared to the results from experiments with sonicated mitochondria, in tightly coupled mitochondria the ferrochelatase reaction behaved differently to aerobiosis and changes in pH and concentration of substrates [1, 11, 12].

The present study deals with the ferrochelatase activity of sonicated rat liver mitochondria and of tightly coupled rat liver mitochondria with particular emphasis on the effect of factors known to interfere with the structural and functional integrity of the mitochondrial membranes.

The results confirm our previous suggestions of the mitochondrial inner membrane as an important permeability barrier to the reactants of the ferrochelatase reaction [10].

## MATERIALS AND METHODS

*Preparation of mitochondria and submitochondrial particles*

Rat liver mitochondrial were prepared in 0 25 M sucrose/5 mM HEPES buffer, pH 7 40 at a concentration of approx 40 mg/ml essentially as previously described [6].

The functional integrity of the preparations was tested by measuring the respiratory control ratio with ADP ($R.C._{ADP}$) [13], using succinate as substrate. Only mitochondria with $R.C._{ADP}$ values $> 3\ 0$ were used.

Submitochondrial particles were prepared by sonicating the mitochondrial

suspension 30 s ×3 using an Insonator S-model 500 Sonifier (Savant Instruments Inc.) operated at a meter reading of 100. The efficiency of the ultrasonic treatment was estimated from the release of malate dehydrogenase to a supernatant obtained after centrifugation of the sonicated mitochondria for 2 min in an Eppendorf microcentrifuge (Type 3200). Only particles releasing more than 70 % of the total malate dehydrogenase activity were used.

*Preparations of deuteroporphyrin IX*

Deuteroporphyrin IX, final concentration approx. 2 mM, was prepared by hydrolysis of the corresponding dimethyl ester in 7 M HCl [14]. After extraction of the deuteroporphyrin into ether and evaporation of the ether, the free deuteroporphyrin was dissolved in dilute NaOH (pH $\approx$ 10).

*Assay of ferrochelatase activity*

The ferrochelatase activity was measured by the dual-wavelength spectrophotometric procedure as previously described [1, 10], using an Aminco DW-2 UV/VIS spectrophotometer. The formation of deuteroheme was calculated from the change in absorbance $\Delta A = \Delta(A_{498} - A_{509})$ using the extinction coefficient $\Delta\varepsilon$ ($mM^{-1} \cdot cm^{-1}$) = 3.5 [10].

The mitochondria were preincubated at 25 °C for 10 min in a medium containing in a volume of 1.0 ml : 0.25 M sucrose/10 mM HEPES buffer (pH 7.40)/5 mM succinate. Further additions or omissions were as described in the legend to the figures. The reaction was initiated by the addition of iron or deuteroporphyrin, and the change in absorbance was recorded. The rate of deuteroheme formation was calculated from the slope of the linear part of the progress curve [10].

*Determination of the intra mitochondrial concentration of deuteroheme*

The mitochondria approx. 5 mg of protein were incubated as described (see above) and after 20 min incubation (i.e. the time necessary to reach zero-rate deuteroheme formation, Fig. 1), the mitochondrial suspension was pelleted by centrifugation in an Eppendorf microcentrifuge (Type 3200). The concentrations of deuteroporphyrin and iron were 25 $\mu$M and 50 $\mu$M respectively. Pyridine deuterohemochrome was determined in the pellet and in the supernatant [1, 15]. In parallel experiments the dextran-impermeable space of the pellet was calculated from the distribution of $^{14}C$-labeled dextran and tritiated water [16]. Assuming that the extramitochondrial pellet water contained only negligible amounts of deuteroheme (see Table I), the intramitochondrial concentration of deuteroheme was calculated from the amount of pyridine deuterohemochrome of the pellet divided by the calculated matrix volume.

Protein was determined by the Folin-Ciocalteau reagent [17]. Malate dehydrogenase (EC 1.1 1.37) was determined according to Bergmeyer [18].

*Chemicals*

ADP, 4-(2-hydroxyethyl)-1-piperazine propane sulphonic acid (EPPS), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), (A grade), piperazine-*N,N'*-2-bis(2-ethanesulphonic acid) (PIPES), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and hemin (bovine, type I) were obtained from the Sigma Chemical Co. (St. Louis, Mo , U.S.A.). Deuteroporphyrin IX-dimethyl ester was purchased from

Koch-Light Laboratories Ltd. (Colnbrook, England). Dextran carboxyl (*carboxyl-*$^{14}$C), $M_r$ = 75 000, and $^3H_2O$ were the products of the New England Nuclear Corp (Mass. U.S.A.) and the Radiochemical Center (Amersham, England), respectively.

The ionophore X537A was a gift from Professor T. Flatmark, Department of Biochemistry, University of Bergen.

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

## RESULTS

The conclusions to emerge from the present study rest largely on the purity of the enzyme sources, i.e. to what degree there are submitochondrial particles in the mitochondrial preparations and vice versa. As already mentioned (see Materials and Methods) the submitochondrial particles contain at least 70 % disrupted mitochondria, and the mitochondrial system, mitochondria with $R.C._{ADP}$ values > 3 (most R.C. values > 5), and malate dehydrogenase of the supernatant (see Materials and Methods) less than 5 %.

To minimize disruption of the mitochondria during incubation, the temperature of the incubation medium was reduced from 37 °C [10] to 25 °C.

With these reservations in mind, the results from the present study refer to

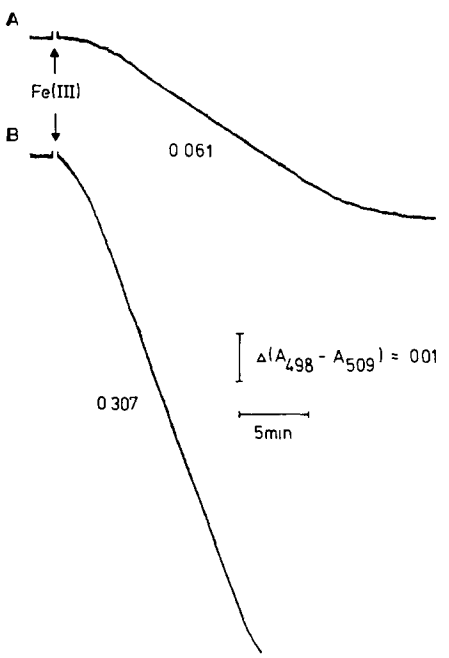


Fig. 1. Time course of deuteroheme formation in intact mitochondria (A) and in submitochondrial particles (B) Mitochondria or submitochondrial particles, approx 8 mg protein/ml, were incubated as described in Materials and Methods The concentration of deuteroporphyrin IX was 40 $\mu$M At the points indicated 100 nmol $FeCl_3$ was added. The numbers below each trace represent the rate of deuteroheme formation (nmol deuteroheme $min^{-1}$ $mg^{-1}$ protein) in the time interval indicated

ferrochelatase activity of tightly coupled rat liver mitochondria and of submitochondrial particles.

*Ferrochelatase activity of tightly coupled mitochondria and of submitochondrial particles*

As seen from Fig. 1 the progress curve of the ferrochelatase activity of intact mitochondria differed in several ways from that of sonicated mitochondria. Thus, in contrast to the almost immediate and maximal response obtained with submitochondrial particles, with intact mitochondria there was a lag phase until the maximal rate of deuteroheme synthesis was reached. Furthermore, the maximal rate of deuteroheme synthesis as well as the total amount of deuteroheme synthesized by intact mitochondria were significantly below that obtained with submitochondrial particles.

*Effect of CCCP on the ferrochelatase activity of tightly coupled mitochondria and of submitochondrial particles*

The uncoupler CCCP inhibits the energy-dependent accumulation of iron by tightly coupled rat liver mitochondria [5, 6]. Once iron is accumulated, however, addition of CCCP has no effect on the release of the intramitochondrial iron [5, 19] Thus the sequence of adding iron and CCCP is of fundamental importance to the results.

The effect of CCCP on the ferrochelatase activity is given in Fig 2. When

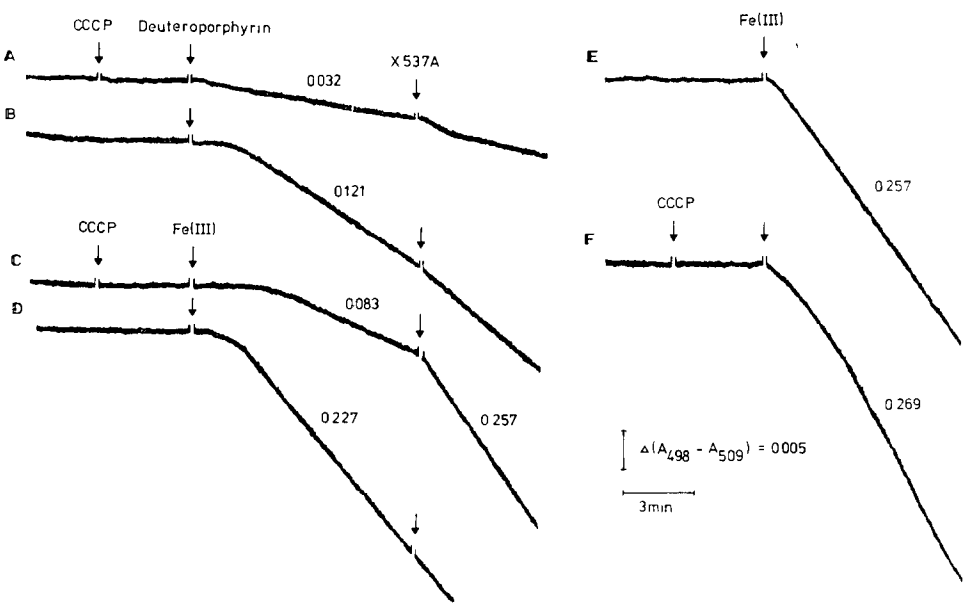


Fig 2 Effect of added CCCP and X537A on the rate of deuteroheme formation in intact mitochondria (A, B, C and D) and in submitochondrial particles (E and F). Mitochondria or submitochondrial particles, approx 4 mg protein, were preincubated for 5 min as described (see Materials and Methods) in the presence of 75 $\mu$M deuteroporphyrin (C, D, E and F) Further additions were 5 nmol CCCP, 75 nmol $FeCl_3$, 40 nmol deuteroporphyrin and 50 $\mu$g X537A (arrows) The numbers above each trace represent the rate of deuteroheme formation (nmol deuteroheme $min^{-1} \cdot mg^{-1}$ protein) in the time interval indicated.

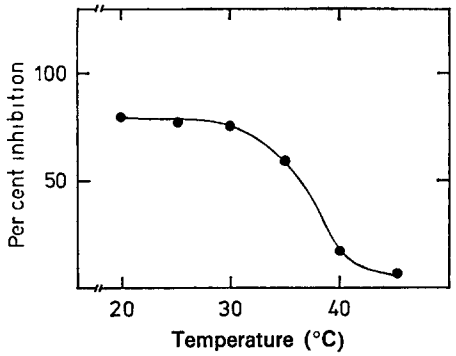


Fig. 3. Effect of temperature on the CCCP-sensitive ferrochelatase activity of intact mitochondria. Mitochondria, approx. 5 mg protein, were incubated as described in Materials and Methods in the presence of 40 $\mu$M deuteroporphyrin. The reaction was initiated by the addition of 75 nmol $FeCl_3$. The figures show the ferrochelatase activity in the absence minus the ferrochelatase activity in the presence of 5 $\mu$M CCCP (added 5 min prior to iron).

tightly coupled mitochondria were preincubated with deuteroporphyrin followed by CCCP and iron at timed intervals, the ferrochelatase activity was reduced by 60–70 % (Fig. 2 traces C and D). Subsequent addition of the cation ionophore X537A [20] to the CCCP-treated mitochondria restored the ferrochelatase activity. Essentially similar results were obtained when the mitochondria were preincubated with iron followed by CCCP prior to deuteroporphyrin (Fig. 2, traces A and B). However, when the reactants were added in this sequence (iron, CCCP, deuteroporphyrin), the ferrochelatase activity could not be restored by X537A.

These findings suggest that CCCP may suppress the translocation of the substrates, or inhibit the enzymic reaction proper. As seen from Fig. 2 (traces E and F), in sonicated mitochondria CCCP had no effect on the ferrochelatase reaction when added prior to iron. Similar results were obtained when CCCP was added prior to deuteroporphyrin (data not shown). The effect of CCCP cannot therefore be explained by an inhibition of the enzymic reaction. A more likely explanation is that CCCP, by dissipating the mitochondrial energy potential, reduced the inward movement of deuteroporphyrin and iron. This suggestion is supported also by the results reported in Fig. 3; i.e. when the reaction was run at increasing temperatures, there was a rapid decline in the CCCP-sensitive ferrochelatase activity, and simultaneously the mitochondria became uncoupled (data not shown). Thus, in agreement with the results from studies of the energy-dependent accumulation of iron [5, 6, 19] in intact mitochondria there was a close correlation between the ferrochelatase activity and the mitochondrial energy potential. It should be noted that the correlation between energy potential and ferrochelatase activity apparently holds not only for the transfer of iron, but for the transfer of deuteroporphyrin as well.

*Effect of hemin on the rate of deuteroheme synthesis*

Hemin is known to interfere with the synthesis of heme [21, 22] as well as of globin and other proteins (for review, see ref. 23). Of particular interest to the present study are the inhibition by hemin of the ferrochelatase reaction in sonicated rat liver mitochondria [21] and the findings of hemin as an inhibitor of the release of iron from

transferrin at the level of the cell surface [24] as well as at the level of the mitochondria [25]. To what degree the latter effect could be extrapolated to iron-donor compounds other than transferrin is not known. Neither is it known what are the effects of hemin on the uptake of porphyrins by mitochondria. The effect of exogenous hemin on the overall ferrochelatase activity of tightly coupled mitochondria and of submitochondrial particles is outlined in Figs. 4, 5 and 6. In submitochondrial particles hemin inhibited the ferrochelatase reaction competitively with respect to deuteroporphyrin (Figs. 4 and 6) and uncompetitively with respect to iron (Fig. 5). In tightly coupled mitochondria hemin inhibited the ferrochelatase competitively with respect to iron (Fig. 5). With respect to deuteroporphyrin, however, at a given concentration of hemin, the per cent inhibition by hemin increased by increasing the concentration of deuteroporphyrin (Figs. 4 and 6).

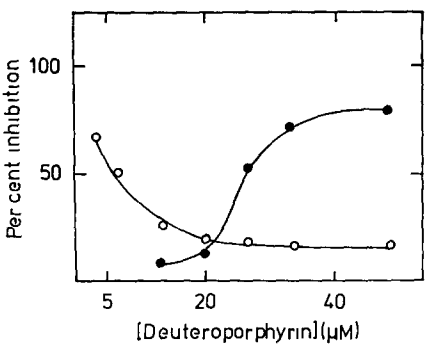


Fig. 4. The effect of hemin on the rate of deuteroheme formation at increasing concentrations of deuteroporphyrin by intact mitochondria (●), and by submitochondrial particles (○). Mitochondria or submitochondrial particles approx. 4 mg protein/ml were incubated as described in Materials and Methods, except that the incubation medium was supplemented with 10 $\mu$g hemin. The reaction was initiated with 75 nmol $FeCl_3$. The per cent inhibition was calculated from the activity measured when hemin was omitted from the incubation mixture.

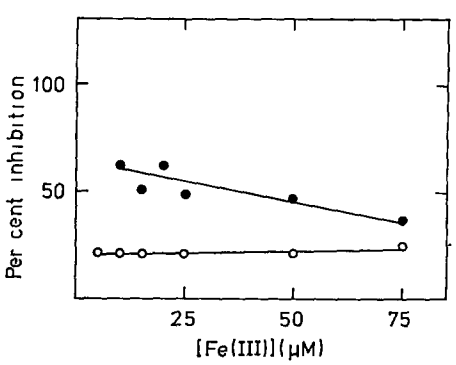


Fig. 5. Effect of hemin on the rate of deuteroheme formation at increasing concentrations of $FeCl_3$ by intact mitochondria (●) and by submitochondrial particles (○). Experimental conditions were as described (see Fig. 4) except that the concentration of deuteroporphyrin was 25 $\mu$M and 5 $\mu$M in the experiments with intact mitochondria and submitochondrial particles, respectively. The per cent inhibition was calculated as described in the legend to Fig 4.

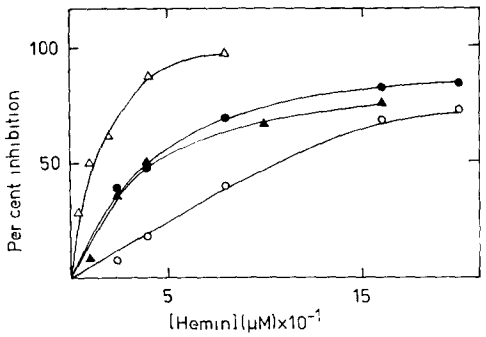


Fig 6 Effect of increasing concentrations of hemin on the rate of deuteroheme formation by intact mitochondria (triangles), and by submitochondrial particles (circles) at 40 $\mu$M (open symbols) and 10$\mu$M (closed symbols) deuteroporphyrin Mitochondria or submitochondrial particles approx. 4 mg/ml were incubated as described in Materials and Methods The reaction was initiated with 75 nmol $FeCl_3$ The per cent inhibition was calculated as described in Fig 4

The inhibitory effect of hemin on the ferrochelatase reaction of submitochondrial particles suggests that the reduction in the total amount of deuteroheme synthesized by intact mitochondria (Fig. 1) may be ascribed to product inhibition, i.e. the intramitochondrial accumulation of deuteroheme to rate limiting concentrations.

As seen from Table I approx. 80 % of the deuteroheme synthesized during 20 min incubation at 25 °C was pelleted together with the mitochondria. By increasing the temperature to 30 °C, the amount of deuteroheme pelleted together with the mitochondria decreased to 63.2 % and when the experiments were run on submitochondrial particles, less than 10 % of the deuteroheme could be pelleted. From the deuteroheme concentration of the mitochondrial pellet and the dextran-impermeable space of the mitochondrial pellet, the intramitochondrial concentration of deuteroheme at zero rate deuteroheme formation was found to be approx 200 $\mu$M ($n = 4$) which was in agreement with the inhibitory concentration of exogenous hemin reported in Fig. 6.

TABLE I

PARTITION OF DEUTEROHEME BETWEEN PELLET AND SUPERNATANT AFTER CENTRIFUGATION OF INTACT MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

Mitochondria or submitochondrial particles, approx 6 mg protein/ml were incubated as described (see Materials and Methods) at the temperature indicated in the presence of 35 $\mu$M deuteroporphyrin The reaction was initiated with 75 nmol $FeCl_3$ After 20 min, the suspension was pelleted in an Eppendorf microcentrifuge (Type 3200) Pyridine deuterohemochrome was determined in the pellet and in the supernatant The data represent the means of four different experiments.

| | Per cent deuteroheme in | |
|---|---|---|
| | pellet | supernatant |
| Intact mitochondria (25 °C)[a] | 79 5 | 20 5 |
| Intact mitochondria (30 °C)[b] | 63 2 | 36 8 |
| Submitochondrial particles (25 °C)[c] | 8 7 | 91 3 |

[a,b,c] The mean 100 % values were 17 1, 25 1, 25 5 nmol deuteroheme respectively

*Effect of changes in substrate concentration, pH, temperature and protein concentration on the reaction rate*

The effect of increasing concentrations of deuteroporphyrin on the ferrochelatase activity is seen in Fig. 7. Under standard experimental conditions (see Materials and Methods) the maximal rate of deuteroheme formation by submitochondrial particles was found at a deuteroporphyrin concentration of 5 $\mu$M and half-maximal rate was obtained at a deuteroporphyrin concentration of approx. 2 $\mu$M. At higher concentrations of deuteroporphyrin, the reaction rate was markedly inhibited

The maximal rate of deuteroheme synthesis by intact mitochondria was found at a substrate concentration of approx. 35 $\mu$M and, as previously shown [10], at higher concentrations deuteroporphyrin markedly depressed the ferrochelatase reaction.

The effect of increasing concentrations of $FeCl_3$ on the ferrochelatase activity is seen in Fig. 8. $K'_m$ obtained for the ferrochelatase of the submitochondrial particles was approx. 30 $\mu$M, and for the intact mitochondria 55 $\mu$M. Pertinently, the observed

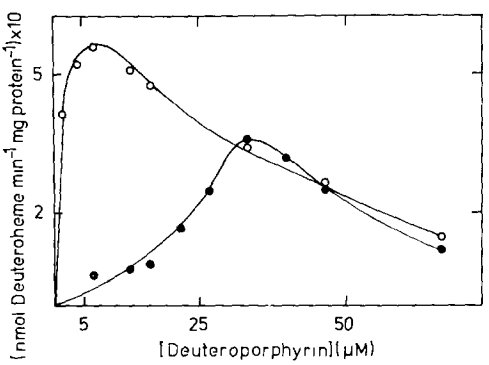


Fig. 7. Effect of increasing concentrations of deuteroporphyrin on the rate of deuteroheme formation by intact mitochondria (●) and by submitochondrial particles (○) Mitochondria or submitochondrial particles approx 4 mg/ml, were incubated as described in Materials and Methods section The reaction was initiated with 75 nmol $FeCl_3$

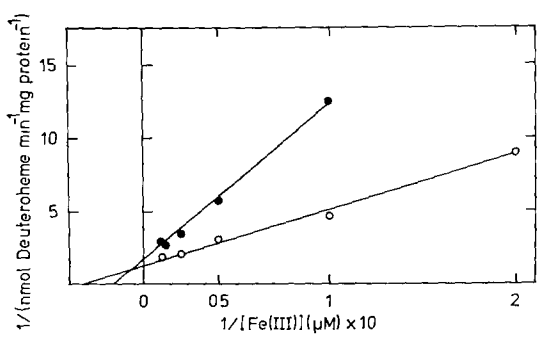


Fig 8 Effect of increasing concentrations of $FeCl_3$ on the rate of deuteroheme formation by intact mitochondria (●) and by submitochondrial particles (○) Mitochondria or submitochondrial particles approx 4 mg/ml were incubated as described in Materials and Methods in the presence of 40 $\mu$M or 5 $\mu$M deuteroporphyrin respectively The reaction was initiated with the addition of $FeCl_3$.

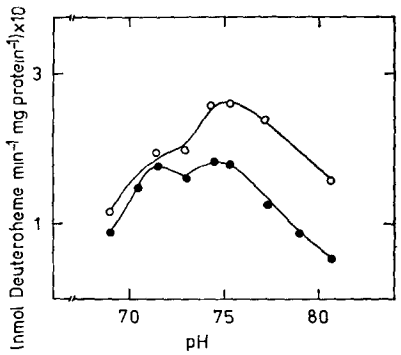


Fig. 9 Effect of pH on the rate of deuteroheme formation in intact mitochondria (●) and in submitochondrial particles (○). Mitochondria or submitochondrial particles, approx. 4 mg/ml were incubated as described in Materials and Methods except that the incubation mixture was supplemented with 10 mM EPPS and 10 mM PIPES buffer. The concentration of deuteroporphyrin was 40 $\mu$M. The reaction was initiated with 75 nmol $FeCl_3$.

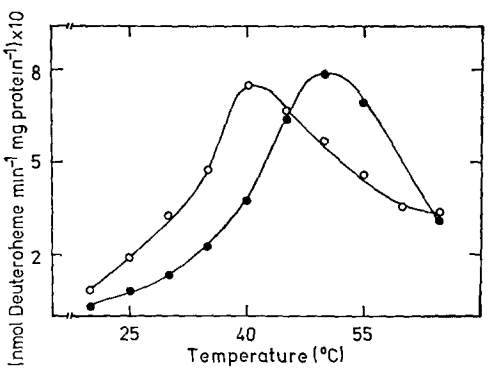


Fig. 10. Effect of temperature on the rate of deuteroheme formation by intact mitochondria (●) and by submitochondrial particles (○). Mitochondria or submitochondrial particles, approx. 6 mg protein /ml, were incubated as described in Materials and Methods. The concentration of deuteroporphyrin was 40 $\mu$M. The reaction was initiated with 75 nmol $FeCl_3$.

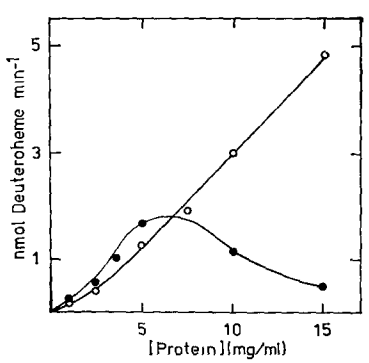


Fig. 11. Effect of increasing protein concentrations on the rate of deuteroheme formation by intact mitochondria (●) and by submitochondrial particles (○). Experimental conditions were as described in Materials and Methods The concentrations of deuteroporphyrin and $FeCl_3$ were 40 $\mu$M and 75 $\mu$M, respectively.

$K'_m$ values were only apparent, because at pH 7.4 the concentration of free ferric ions was far below that given in Fig. 8 [26].

In sonicated mitochondria, the ferrochelatase activity showed a peak at pH approx. 7.5 and a shoulder at pH 7.3. In intact mitochondria an optimum was found at pH 7.2–7.6 (Fig. 9).

The rate of deuteroheme synthesis was greatly influenced by changing the temperature of the incubation medium (Fig. 10). In agreement with previous studies [10] a temperature optimum at approx. 50 °C was found for the ferrochelatase activity of intact mitochondria. In sonicated mitochondria the maximal activity was found at 40–45 °C

The effect of increasing the concentration of protein is shown in Fig. 11. The rate of deuteroheme synthesis increased almost linearly up to a protein concentration of 5 mg/ml in both systems. With intact mitochondria the rate of synthesis declined at protein concentrations above 5 mg/ml. With submitochondrial particles, however, the activity parallelled the protein concentration up to approx. 15 mg/ml.

## DISCUSSION

The present study disclosed significant differences between the ferrochelatase reaction of sonicated mitochondria and of intact mitochondria.

These related to differences in the substrate and protein concentration curves, pH and temperature optima as well as to energy requirement and sensitivity towards product inhibition.

From a functional point of view, a main finding concerns the energy requirement of the ferrochelatase reaction of tightly coupled mitochondria, and the lack of effect of uncoupler on the ferrochelatase reaction of sonicated mitochondria. In agreement with the recent finding of an energy dependent accumulation of iron by tightly coupled mitochondria [5, 6, 19], the ferrochelatase reaction of intact mitochondria was depressed by CCCP added prior to iron (Fig. 2C). That the effect of CCCP reported in Fig. 2C did in fact reflect a depression of the influx of iron was supported by the observation that in de-energized mitochondria, the ferrochelatase activity was restored by the electroneutral cation inophore X537A [27, 28] (Fig. 2C). X537A is known to facilitate the transport of ferrous ions across phospholipid membranes [27], without having any effect on the transport of ferric ions [27]. Thus, the results of Fig. 2C not only substantiated our finding of an energy-dependent transport of iron across the inner membrane [5, 6], but in addition also supported the suggestion that ferric ions were reduced to ferrous ions prior to translocation [19, 29].

The energy requirement for the supply of iron to the ferrochelatase reaction was evident also from the observation of a rapidly decreasing CCCP-sensitive ferrochelatase activity at higher temperatures (Fig. 3).

The results with CCCP, however, not only pointed to iron as an important metabolite to be transported across the inner membrane. The accumulation of deuteroporphyrin as well revealed sensitivity towards the uncoupler (Fig. 2A), notably, however, only in intact mitochondria. Thus, in iron-loaded and uncoupled mitochondria, the ferrochelatase was depressed by 60–70 % compared to that of iron-loaded and tightly coupled mitochondria (Fig. 2, A and B). Pertinently, the effect of CCCP could not be ascribed to a release of iron [5, 19] as shown by the lack of effect

of the ionophore X537A (Fig. 2A compared to Fig. 2C). Jones and Jones [21] have proposed that there is a uni-directional porter system for the outward movement of δ-aminolaevulinic acid, and as shown by Israels et al. [8] and Ponka et al. [24], the efflux of heme from the mitochondria depends on cytosolic proteins, the nature of which has not been conclusively established [8, 24, 30, 31] So far, however, a more detailed analysis of these transport systems is lacking. To our knowledge, the results reported in Fig. 2, A and B are the first direct experimental evidence to indicate that in intact mitochondria, the accumulation of intermediates of heme biosynthesis other than iron depends on metabolic energy. The translocation of iron through the inner membrane rests on an initial reduction of the iron ions from the ferric to the ferrous form by the respiratory chain, followed by an energy-dependent influx by a mechanism essentially as reported for $Ca^{2+}$ [29, 32, 33, 34]. With respect to deuteroporphyrin, virtually nothing is known about the mechanism by which it penetrates the mitochondrial inner membrane. A challenging hypothesis is that deuteroporphyrin, which is a dicarboxylic porphyrin, present predominantly as an anion at pH 7.40 may penetrate by mechanism(s) analogous to that of the anion shuttles of the inner membrane [36]. Notably, tetraphenylboron and *p*-hydroxymercuribenzoate, both highly efficient inhibitors of the uptake of anions by isolated mitochondria [37, 38], inhibited the ferrochelatase activity of intact mitochondria (Koller, M. E., unpublished observations)

From the experiments with hemin (Figs. 4, 5 and 6) the following conclusions emerged; (1) in intact mitochondria hemin interfered with the availability of iron to the ferrochelatase; (2) in sonicated mitochondria hemin inhibited the ferrochelatase competitively with respect to deuteroporphyrin and (3) endogenously synthesized deuteroheme did not freely penetrate to the outside of the inner membrane.

In agreement with previous studies [25] it was found that exogenous hemin markedly reduced the availability of iron to the ferrochelatase in intact mitochondria. This inhibition was not mediated by a reduction of the mitochondrial energy potential [25], nor could it be ascribed to an effect on the release of iron from its donor ligand (i.e. $Cl^-$), as suggested for transferrin [39]. From the uncompetitive nature of the effect of hemin with respect to iron in sonicated mitochondria, and the competitive nature of the effect of hemin on iron in intact mitochondria (Fig. 5), it is suggested that hemin inhibited the transport of iron from the iron-binding ligands of the outer mitochondrial compartment to the ferrochelatase on the M-side of the inner membrane. This interpretation is in agreement with the very recent proposal of Ponka et al. [22] on the effect of hemin on the transferrin-mitochondrial ligand complexes; i e. hemin functions as a feed-back regulator of the release of iron from the donor-receptor compound at the mitochondrial membrane.

The inhibition by hemin of the ferrochelatase (Figs. 1 and 4) supports the data of Jones and Jones [21], but are at variance with the results of Yoda and Israels [7]. However, the figures in their publication [7] show that the progress curves for the synthesis of heme deviated from linearity already after 10 min incubation and the total amount of heme synthesized during 20 min incubation in the sucrose medium were only 60 per cent of that produced in a cell sap medium Therefore their experiments do not rule out an inhibitory effect of endogenously accumulated heme The inhibition of the ferrochelatase reaction by hemin as shown in the present study, is evident from two sets of experiments; (1) in sonicated mitochondria the effect of hemin was competi-

tively abolished by deuteroporphyrin and (2) in intact mitochondria the progress curve for the deuteroheme formation gradually leveled off as the intramitochondrial accumulation of hemin increased (Fig. 1 and Table I).

The finding of a time-dependent intramitochondrial accumulation of hemin (Fig. 1 and Table I) focuses on still another aspect of the regulatory function of the inner membrane, namely the control of the efflux of hemin [8] and the extent of binding of hemin [22, 24] According to Granick et al. [40], the "free hemin" concentration in hepatic parenchyma cells is between 50 and 100 nM. In intact mitochondria, we do not know the concentration of "free hemin", or the nature of the heme-binding structures [8, 22, 24, 30, 31] From the data reported in Table I, however, it appears that the hemin-binding components are relatively soluble matrix and/or membrane structures

In contrast to the findings with porphyrins, the transport of hemin does not depend on an energized inner membrane (Fig 1 and Table I) nor can it be ascribed to a passive diffusion mechanism (Table I and refs. 8 and 24). Furthermore, it seems unlikely that the porphyrin-heme fluxes are linked by a symport-antiport mechanism of the anion-shuttle type [35]. With respect to cytosolic proteins, so far, neither their structure, nor the mechanism(s) of their effect on the efflux of hemin have been conclusively established [8, 24, 30, 31].

Still another effect of hemin should be mentioned; in intact mitochondria, the per cent inhibition of exogenous hemin is linearly related to the concentration of deuteroporphyrin, i.e the inverse of what is found in a solubilized system (Fig. 4).

From the temperature curves it is seen that the latency of the ferrochelatase in intact mitochondria [1] apparently also represented a protection of the enzyme to thermal inactivation (Fig. 10). At 25 °C, however, the differences in the rate of deuteroheme synthesis in intact mitochondria compared to submitochondrial particles (Fig. 1) depended mainly on differences in the optimal deuteroporphyrin/protein ratios of the two systems. In sonicated mitochondria, the optimal deuteroporphyrin/protein ratio was found to be approx. 1 nmol/mg protein, whereas in intact mitochondria the ratio was approx. 9 nmol/mg protein (Fig. 7). Under optimal conditions, however, the $V$ of the ferrochelatase of intact mitochondria was found to be only 60 % of that of submitochondrial particles (Fig. 7).

Thus, compared to studies on the relationship between the influx and metabolism of e.g. succinate [41] or ADP [42], the results of the present study are all consistent with the inner mitochondrial membrane as an important permeability barrier to the reactants of the ferrochelatase reaction.

Future experiments aim at a more detailed study of the mechanism(s) of these transport processes, with particular emphasis on their significance to the rate of synthesis of heme in health and diseases.

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REFERENCES


1 Jones, M S and Jones, O T G (1969) Biochem J 113, 507–514
2 Stavem, P , Soltvedt, E , Elgjo, K. and Rootwelt, K (1973) Scand J Haemat 10, 153–160
3 De Goeij, A F P M , Smit, S and Steveninck, J (1977) Clin Chim Acta 74, 27–31
4 Kramer, S , Viljoen, J D and Becher, D M (1976) in Porphyrins in Human Diseases (Doss, M , ed ), pp. 390–397, S Karger, Basel
5 Romslo, I and Flatmark, T (1973) Biochim Biophys Acta 305, 29–40
6 Romslo, I (1975) Biochim Biophys Acta 387, 69–79
7 Yoda, B and Israels, L G (1972) Canad J Biochem 50, 633–637
8 Israels, L G , Yoda, B and Schachter, B A (1975) Ann N Y Acad Sci 244, 651–661
9 Poulson, R and Polglase, W J (1975) J Biol Chem 250, 1269–1274
10 Koller, M E , Romslo, I and Flatmark, T. (1976) Biochim Biophys Acta 449, 480–490
11 Barnes, R , Connelly, J L and Jones, O T G (1972) Biochem J 128, 1043–1055
12 Llambias, E B C (1976) Int J Biochem 7, 33–40
13 Grav, H J , Pedersen, J I and Christiansen, E N (1970) Eur J Biochem. 12, 11–23
14 Furhop, J. H and Smith, K M. (1975) in Laboratory Methods in Porphyrin and Metalloporphyrin Research (Furhop, J E and Smith, K M , eds ), pp 80–81, Elsevier Scientific Publishing Company, Amsterdam
15 Falk, J E (1974) in Porphyrins and Metalloporphyrins (Falk, J E , ed ), p 241, Elsevier Publishing Company, Amsterdam
16 Addanki, S , Cahill, F D and Sotos, J F (1968) J Biol Chem 243, 2337–2348
17 Flatmark, T , Terland, O. and Helle, K B (1971) Biochim Biophys Acta 226, 9–19
18 Bergmeyer, H U (1974) in Methods of Enzymatic Analysis (Bergmeyer, H U , ed ), pp 613–617, Verlag Chemie Weinheim, Academic Press, Inc New York
19 Flatmark, T and Romslo, I (1975) J Biol Chem 250, 6433–6438
20 Green, D E (1976) Ann. N Y Acad Sci 246, 61–82
21 Jones, M S and Jones, O T. G (1970) Biochem Biophys Res Commun 41, 1072–1079
22 Ponka, P , Neuwirt, J , Borová, J and Fuchs, O (1977) in Symposium on Iron Metabolism (in press), The Ciba Foundation, London
23 London, I M , Clemens, M J , Ranu, R S , Levin, D H , Cherbas, L F and Ernst, V. (1976) Fed Proc 35, 2218–2222
24 Ponka, P , Borová, J and Neuwirt, J (1973) Biochim Biophys Acta 304, 715–718
25 Koller, M E , Prante, P H , Ulvik, R and Romslo, I (1976) Biochem Biophys Res Commun 71, 339–345
26 Saltman, P (1965) J Chem Educ 42, 682–687
27 Young, S , Baker, E , Gomparts, B D and Huehns, E R (1975) in Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crighton, R R , ed ), pp 417–426, North Holland Publishing Company, Amsterdam
28 Pozzan, T and Azzone, G. F. (1976) FEBS Lett 71, 62–66
29 Romslo, I and Flatmark, T (1975) Biochim Biophys Acta 387, 80–94
30 Schulman, H M. (1974) Canad J. Biochem 52, 665–669
31 Ketterer, B , Srai, S K and Christodoulides, L (1976) Biochim Biophys Acta 482, 683–689
32 Reed, K C and Bygrave, F L (1975) Eur J Biochem 55, 497–504
33 Lehninger, A L (1974) Proc. Natl Acad Sci U S 71, 1520–1524
34 Sandri, G , Panfili, E and Sottocasa, G L (1976) 68, 1272–1279
35 Hambright, P (1975) in Porphyrins and Metalloporphyrins (Smith, K M., ed ), pp 233–278, Elsevier Scientific Publishing Company, Amsterdam
36 Williamson, J R (1976) in Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Tager, J M , Soling, H D and Williamson, J R , eds ), pp 79–95, North Holland Publishing Company, Amsterdam
37 Meisner, H (1973) Biochim Biophys Acta 318, 383–389
38 Palmieri, F , Passarella, S , Stipani, I. and Quagliariello, E (1974) Biochim. Biophys Acta 333, 195–208
39 Ponka, P , Neuwirt, J. and Borová, J (1975) in Erythropoiesis (Nakao, K , Fisher, J E and Takaku, F , eds ), pp. 403–411, University Park Press, Baltimore
40 Granick, S , Sinclair, P., Sassa, S and Grieninger, G (1975) J Biol Chem 250, 9215–9225
41 Quagliariello, E and Palmieri, F (1968) Eur J Biochem 4, 20–27
42 Kemp, Jr , A , Groot, G S P and Reitsma, H J (1969) Biochim Biophys Acta 180, 28–34