Biochimica et Biophysica Acta, 449 (1976) 480–490

BBA 47213

STUDIES ON THE FERROCHELATASE ACTIVITY OF ISOLATED RAT LIVER MITOCHONDRIA WITH SPECIAL REFERENCE TO THE EFFECT OF OXIDIZABLE SUBSTRATES AND OXYGEN CONCENTRATION

M.-E. KOLLER[a], I. ROMSLO[a] and T. FLATMARK[b]

[a]*Laboratory of Clinical Biochemistry, N-5016 Haukeland Sykehus, and* [b]*Department of Biochemistry, University of Bergen, (Norway)*

(Received July 1st, 1976)

SUMMARY

The mitochondrial ferrochelatase activity has been studied in coupled rat liver mitochondria using deuteroporphyrin IX (incorporated into liposomes of lecithin) and Fe(III) or Co(II) as the substrates.

1. It was found that respiring mitochondria catalyze the insertion of Fe(II) and Co(II) into deuteroporphyrin. When Fe(III) was used as the metal donor, the reaction revealed an absolute requirement for a supply of reducing equivalents supported by the respiratory chain.

2. A close correlation was found between the disappearance of porphyrin and the formation of heme which allows an accurate estimate of the extinction coefficient for the porphyrin to heme conversion. The value $\Delta\varepsilon$ ($mM^{-1} \cdot cm^{-1}$) = 3.5 for the wavelength pair 498 509 nm, is considerably lower than previously reported.

3. The maximal rate of deuteroheme synthesis was found to be approx. 1 $nM \cdot min^{-1} \cdot mg^{-1}$ of protein at 37 °C, pH 7.4 and optimal substrate concentrations, i.e. 75 μM Fe(III) and 50 μM deuteroporphyrin.

4. Provided the mitochondria are supplemented with an oxidizable substrate, the presence of oxygen has no effect on the rate of deuteroheme synthesis.

INTRODUCTION

The final step in the biosynthesis of heme, i.e. the insertion of Fe(II) into the corresponding porphyrin, is catalyzed by the enzyme ferrochelatase (protoheme ferrolyase, EC 4.99.1.1.) which is reported to be firmly bound to the inner phase of the mitochondrial inner membrane [1]. Ferrochelatase activity has been measured in a variety of animal tissues such as bone marrow cells [2], reticulocytes [2] as well as in homogenates of liver, kidney and spleen [4–6]. The ferrochelatase activity has

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).

been mostly determined in whole cells or in disintegrated subcellular particles, and only in a few studies has the ferrochelatase activity been determined in structurally and functionally intact mitochondria [1, 7].

The inhibitory effect of oxygen on the ferrochelatase activity has been repeatedly stressed [5, 8–10], but determination of the rate of heme synthesis as a function of oxygen concentration has not been carried out. Thus, the mechanism(s) of the so-called oxygen effect [9, 11] is not well characterized.

The present paper deals with studies on the mitochondrial ferrochelatase, with particular emphasis on the catalytic activity of this enzyme in intact rat liver mitochondria, the mechanism(s) by which the reducing equivalents are supplied for the reduction of Fe(III) to Fe(II) and the effect of oxygen concentration on the rate of heme formation.

MATERIALS AND METHODS

Preparation of mitochondria

Rat liver mitochondria were prepared in 0.25 M sucrose and 5 mM HEPES buffer, pH 7.40 at a concentration of approx. 40 mg/ml essentially as described [12]. The functional integrity of the preparation was tested by measuring the respiratory control ratio with ADP ($R.C._{ADP}$) [12], using succinate as the substrate. Only mitochondria with $R.C._{ADP}$ values above 3.0 were used.

Incorporation of deuteroporphyrin into liposomes

Deuteroporphyrin IX-dimethylester was hydrolyzed in 7 M HCl at 4 °C overnight [13]. The solution was adjusted to pH 4.0 and the free porphyrin was extracted into ether. Residual deuteroporphyrin IX-dimethylester was removed from the free acid by extracting the ether solution with 0.5 M NH_4OH [14]. Deuteroporphyrin IX was then reextracted into ether. A solution of bovine brain lecithin in chloroform was added, and the ether/chloroform was evaporated under a stream of nitrogen. The residue was suspended in 10 mM HEPES buffer, pH 8.2 to give a final concentration of approx. 2 mM porphyrin and 10–15 mM of lecithin. The suspension was sonicated to clarity using an Insonator S-model 500 Sonifier (Savant Instrument Inc.) operated a meter reading of 100.

Assay of ferrochelatase activity

The ferrochelatase activity was measured by the spectrophotometric procedure described by Jones and Jones [1], using an Amino DW-2 UV/VIS dual-wavelength spectrophotometer with both monochromators calibrated at an accuracy of ± 0.2 nm.

The formation of deuteroheme IX was estimated from the change in absorbance $\Delta A = \Delta(A_{498} - A_{509})$ using the extinction coefficient $\Delta\varepsilon\ (mM^{-1} \cdot cm^{-1}) = 3.5$ which was calculated from the formation of pyridine hemochrome in parallel experiments essentially as described by Jones and Jones [1] (see Results). The extinction coefficient $\varepsilon\ (mM^{-1} \cdot cm^{-1}) = 15.3$ for the reduced minus oxidized difference spectrum at 545 nm minus 530.5 nm of the pyridine hemochrome derivative of deuteroheme IX [15] was used.

The mitochondria (approx. 4 mg of protein) were preincubated at 37 °C for 10 min in a medium containing in a volume of 1 ml: 0.25 M sucrose and 10 mM

HEPES buffer, pH 7.4, 5 mM succinate, 50 μM deuteroporphyrin IX. Further additions or ommissions were as described in legends to figures. The reaction was initiated by the addition of iron (see text), and the change in absorbance $\Delta(A_{498}-A_{509})$ was recorded. The rates of deuteroheme IX formation were calculated from the slope of the linear part of the progress curve (see Figs. 6 and 7). A vibrating platinum electrode (from Aminco) was used for the simultaneous determination of oxygen concentration and consumption.

Chemicals

ADP, antimycin A (type III), rotenone, phosphatidyl choline (L-lecithin, type III from bovine brain), (4-(2-hydroxyethyl)-1-piperazine propane sulphonic acid) (EPPS), *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (HEPES) (A grade), piperazine-*N,N'*-2-bis(2-ethanesulphonic acid) PIPES) were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Deuteroporphyrin IX dimethylester was purchased from Koch-Light Laboratories Ltd. (Colnbrook, England).

Other chemicals were of highest purity commercially available.

RESULTS

It is now well established that submitochondrial particles catalyze the conversion of various porphyrins into the corresponding hemes [1, 7]. In most studies deutero- or meso-porphyrin have been selected as the preferable substrates as the reaction rates are higher than those obtained with protoporphyrin, the presumed natural substrate, and they are also more soluble in the incubation medium used [1, 5, 6, 7, 9, 10]. In the present study deuteroporphyrin was selected as the porphyrin substrate.

From Fig. 1 it is seen that when intact rat liver mitochondria were incubated in

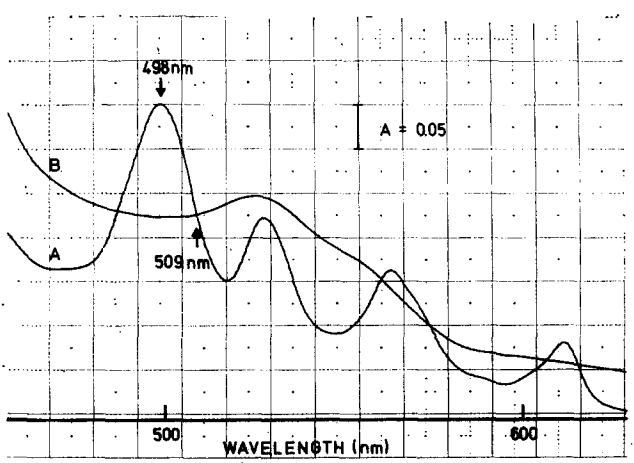

Fig. 1. Spectral changes as a result of the conversion of deuteroporphyrin to deuteroheme in coupled rat liver mitochondria. Experimental conditions were as described in Methods section. The spectra were recorded before (A) and 30 min after the addition of 100 nmol $FeCl_3$ (B).

a medium containing lecithin liposomes loaded with deuteroporphyrin, $FeCl_3$ and oxidizable substrate (succinate), a change in the spectral properties was observed by time. The disappearance of the four-banded porphyrin spectrum is characteristic of a conversion of deuteroporphyrin (Fig. 1, curve A) to deuteroheme (Fig. 1, curve B) [1]. When intact mtiochondria were used as the enzyme source the isosbestic point of the transition was found by repetitive scanning at 509 nm rather than at 511 nm as previously reported by Jones and Jones from experiments with submitochondrial particles [1]. In the following experiments the wavelength pair 498 nm–509 nm was therefore selected for the measurement of porphyrin disappearance.

Relationship between porphyrin disappearance and heme formation

From studies on the ferrochelatase activity of submitochondrial particles it has been repeatedly observed that the formation of heme, measured as ^{59}Fe-labelled heme or as pyridine hemochrome, parallelled the disappearance of porphyrin as measured spectrophotometrically [1, 8]. From Fig. 2 A and B it is seen that following a lag period, the formation of deuteroheme proceeded almost linearly as did the disappearance of deuteroporphyrin. By comparing the spectral change $\Delta(A_{498}-A_{509})$ with the formation of deuteroheme during the linear part of the progress curves, the extinction coefficient was calculated to be $\Delta\varepsilon\,(mM^{-1}\cdot cm^{-1}) = 3.5$. In the following experiments this value has been used in our calculations of the amount of deuteroheme synthesized.

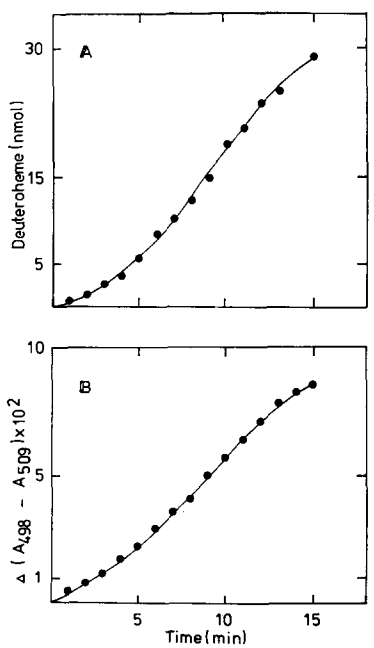

Fig. 2. Time course of the deuteroheme formation (A) and the disappearance of deuteroporphyrin (B). (A) Mitochondria, approx. 3 mg of protein per ml were incubated as described in Methods section. The reaction was initiated by the addition of 100 nmol $FeCl_3$ and the amount of deuteroheme formed was assayed as pyridine deuterohemochrome. (B) In parallel and identical experiments the change in absorbance $\Delta(A_{498}-A_{509})$ was recorded (for details, see Methods section).

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

The assay of ferrochelatase activity in intact mitochondria is complicated by a number of factors which prevent a more detailed kinetic study of the overall reaction. Thus, whereas one of the substrates (deuteroporphyrin) is incorporated into liposomal structures, the other (Fe(III)) is rather insoluble in the medium used and binds to the mitochondrial membranes [16]. Furthermore, evidence has been presented that the ferrochelatase is localized in the inner phase of the inner membrane [1] and hence a permeability barrier exists for both substrates. With these reservations in mind, the effect of pH, temperature and increasing concentrations of substrates and mitochondrial protein have been studied in some detail.

Under standard experimental conditions (see Methods) addition of 100 nmol $FeCl_3$ resulted in a significant rate of deuteroheme formation (Fig. 1). A saturation level was reached at 50–75 μM iron, and half maximal rate was obtained at an iron concentration of 15–20 μM. On the other hand, if the solution of $FeCl_3$ was adjusted to pH 7.4 and then immediately transferred to the measuring cuvette, there was no measurable synthesis of deuteroheme (figure not shown). Thus, as expected from our previous studies on mitochondrial iron accumulation [17], the mitochondria utilize ferric ions only when its precipitation as ferric hydroxides is prevented.

When the rate of deuteroheme formation was measured as a function of the concentration of deuteroporphyrin, maximal rate was observed at 50–75 μM of this substrate. At higher concentrations of deuteroporphyrin the reaction rate was slightly inhibited (Fig. 3), essentially as observed in studies on mesoheme formation by pig liver mitochondria [10], and protoheme formation by bone marrow cells [2]. Furthermore, deuteroporphyrin (in lecithin liposomes) at concentrations of 10–15 nmol/mg mitochondrial protein, had no effect on the State 4 and State 3 respiration rate of tightly coupled rate liver mitochondria. At higher concentrations (> 25–30 nmol/mg protein), however, deuteroporphyrin induced a slight uncoupling (figures not shown).

The rate of deuteroheme formation as a function of the mitochondrial protein concentration was essentially as previously reported by Llambias [10] and Porra and

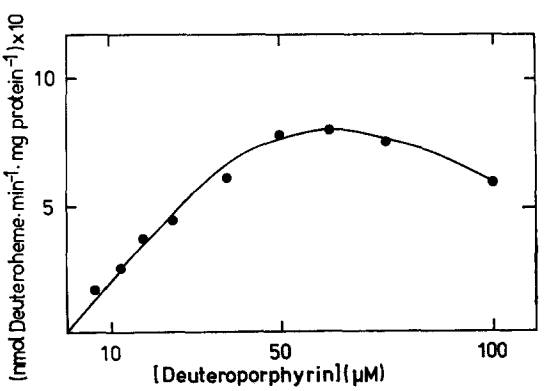

Fig. 3. Effect of increasing deuteroporphyrin concentrations on the rate of deuteroheme formation. Experimental conditions were as described in Methods section. The concentration of $FeCl_3$ was 100 μM. The rates were calculated from the slope of the linear part of the progress curve (see Fig. 6).

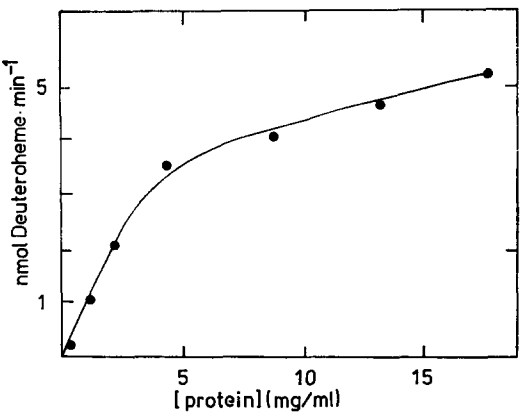

Fig. 4. Effect of mitochondrial protein concentration on the rate of deuteroheme formation. Experimental conditions were as described in Methods section. The concentrations of deuteroporphyrin and $FeCl_3$ were 50 μM and 100 μM, respectively.

Jones [5]; i.e. the rates increased almost linearly with the protein concentration below approx. 5 mg of protein/ml (Fig. 4). No disappearance of deuteroporphyrin was measured in the absence of added mitochondria, irrespective of whether Fe(II) (Fig. 6B) or Co(II) was added as the metal substrate (data not shown).

With intact mitochondria the rate of deuteroheme formation revealed two apparent pH optima, i.e. at pH 7.3–7.5 and at pH 8.0–8.2 (Fig. 5).

The rate of deuteroheme formation was also strongly influenced by the temperature, with an apparent maximum at approx. 50 °C, which is in close agreement with the results from similar experiments reported by Porra and Lascalles [18].

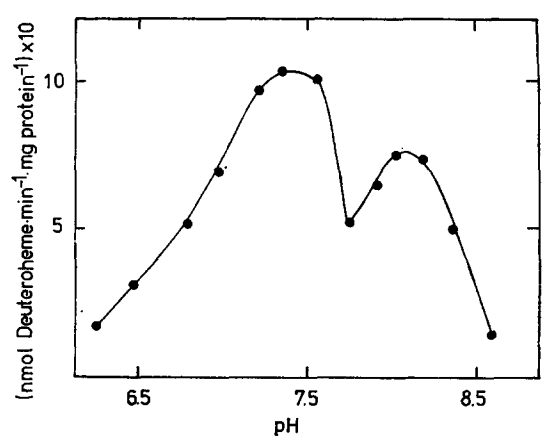

Fig. 5. Effect of pH on the rate of deuteroheme formation. Experimental conditions were as described in Methods section except that the incubation medium was supplemented with 10 mM EPPS and 10 mM PIPES. The reaction was initiated with 100 nmol $FeCl_3$. The pH values refer to the actual pH measured in the reaction medium at the end of incubation period.

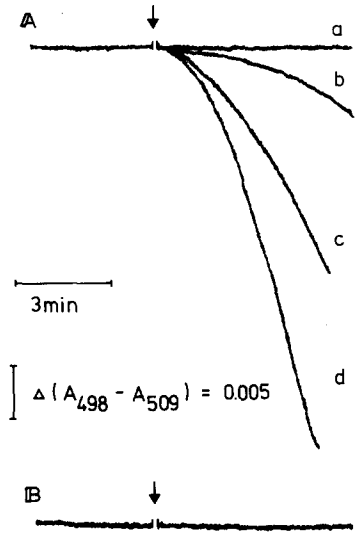

Fig. 6. (A) Effect of respiratory substrates on the formation of deuteroheme. Experimental conditions were as described in Methods section except that succinate was omitted (a, b and d). 100 nmol $FeCl_3$ was added as indicated by the arrows. The progress curves were obtained (a) in the presence of 4 μg antimycin A, 5 μM rotenone and 5 mM NaN_3; (b) in the presence of endogenous respiration; (c) with 5 mM succinate; (d) with 5 mM pyruvate plus 5 mM malate. (B) Experimental conditions as in (A), (c) except that the mitochondria were omitted, and the reaction was initiated with 100 nmol $FeCl_2$.

Effect of oxidizable substrates, respiratory inhibitors and oxygen concentration

When $FeCl_3$ is used as the iron donor, the formation of heme in submitochondrial particles has an absolute requirement for a supply of reducing equivalents [6] and experimental evidence has been presented that the rate of heme synthesis is markedly accellerated by anaerobic conditions [1]. So far, however, no experiments have been reported where the oxygen concentration of the incubation medium and the rate of heme synthesis have been determined simultaneously.

As seen from Fig. 6A, trace a, the respiratory inhibitors rotenone, antimycin A and NaN_3 prevented the formation of deuteroheme by rat liver mitochondria almost completely. Furthermore, when mitochondria respiring on endogenous substrates were added $FeCl_3$, a lag period was observed before the heme synthesis reached the maximal rate (Fig. 6A, trace b). On the other hand, in the presence of exogenous substrates, the lag period was markedly shortened, and the maximal rate of deuteroheme formation was increased by a factor of 2–3 as compared to that obtained with endogenous substrates only. This difference was most clearly seen when pyruvate plus malate were used as the substrates (Fig. 6A, trace d).

It should also be mentioned that in respiratory inhibited mitochondria neither CoASH nor reduced glutathione, cystein and NADH (data not shown) were able to initiate the formation of deuteroheme.

From Fig. 7 it is seen that the effect of oxidizable substrates is not related to the onset of anaerobiosis as previously suggested in submitochondrial particles [6]. Thus, in rotenone-antimycin A inhibited mitochondria, the addition of ascorbate was

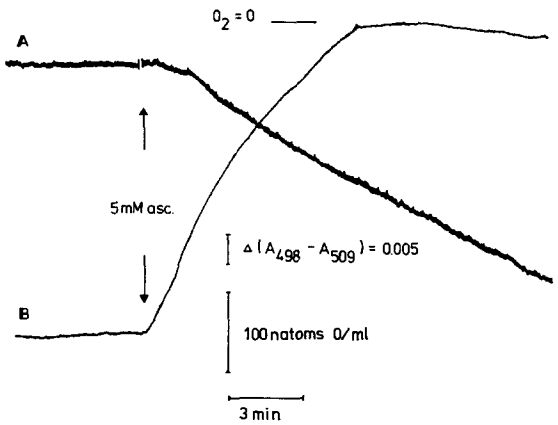

Fig. 7. Effect of oxygen tension on the rate of formation of deuteroheme. The mitochondria, approx. 10 mg of protein were incubated as described in Fig. 6A, (a) except that NaN_3 was omitted; the concentration of iron was 100 μM. The heme synthesis was initiated by 5 mM ascorbate. Oxygen consumption was followed by a vibrating platinum electrode inserted into the cuvette of the spectrophotometer.

followed by a burst in the oxygen consumption (Fig. 7, trace b) as well as in the deuteroheme formation (Fig. 7, trace a), the maximal rate of which was reached long before the suspension had reached anaerobiosis. Furthermore, when anaerobiosis was obtained, no change in the rate of deuteroheme synthesis was measured. It therefore appears that when $FeCl_3$ is the metal donor, the rate of deuteroheme formation is limited by the supply of reducing equivalents supported by the respiratory chain. This conclusion is also supported by the finding what when Co(II) is used as the metal donor, neither addition of oxidizable substrates nor anaerobiosis had any effect on the synthesis of Co(II)-deuteroporphyrin (Fig. 8).

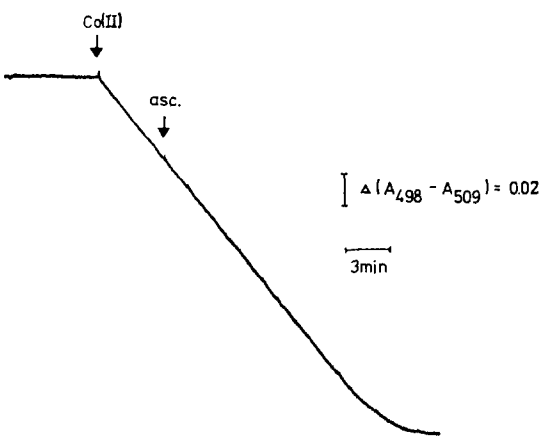

Fig. 8. Effect of respiratory inhibitors and reducing substrates on the formation of Co(II)-deuteroporphyrin. Experimental conditions were as described in Fig. 7. At the point indicated Co(II) (100 nmol) and ascorbate (5 μmol) were added.

DISCUSSION

Although the formation of heme occurs non-enzymically under certain conditions [19], mitochondria play an important role in the incorporation of ferrous iron into porphyrin under physiological conditions [1, 6, 11, 20]. The enzymic nature of this reaction is confirmed in the present study.

Of the many methods used for the determination of ferrochelatase activity [2, 3, 8, 9, 21], the measurement of substrate (porphyrin) disappearance by a dual-wavelength spectrophotometer is one of the most widely used and reliable methods [1, 11]. The major advantages of this assay are its simplicity and speed and that it is not affected by the turbidity of the sample or by the subsequent degradation of heme which may occur in a crude system [5]. A disadvantage of the method is, however, that the porphyrin disappearance may not always be synchronous with heme formation. Thus, it has been reported that e.g. in pig liver mitochondria the disappearance of mesoporphyrin apparently preceded the formation of mesoheme by as much as 30 min [10]. However, as shown by Jones and Jones [1], and confirmed in the present study (Fig. 2) the deuteroporphyrin disappearance closely parallelled the formation of deuteroheme.

A main problem when measuring ferrochelatase activity in isolated mitochondria concerns the very limited solubility of both substrates in the incubation medium. To overcome one of the problems, Jones and Jones [1] incorporated the porphyrin into liposomes of lecithin. This approach was successfully used in the present study using intact and coupled mitochondria as the source of the enzyme without any detrimental effects on the functional integrity of the organelles.

Although Fe(III) ions are extremely insoluble at neutral pH, it has been repeatedly observed that mitochondria utilize ferric iron in the synthesis of heme when added as $FeCl_3$ [1, 10, 11] which is in good agreement with our recent findings of an effective mechanism of iron accumulation by mitochondria [12, 16, 17, 22, 23]. The primary events of this process are, however, not yet well characterized.

In previously studied in vitro systems, working with biological materials, the ferrochelatase activity has been assayed at a pH ranging from 7.2 to 8.2 [1, 9, 10, 11, 24]. Utilizing a solubilized pig liver extract as the enzyme source, Porra and Jones [5], found an apparent pH optimum at pH 7.8 with a shoulder at around pH 9.0, essentially as reported for the formation of proto- and meso-heme in spinach chloroplasts [24]. Similarly, in rat liver mitochondria prepared from frozen livers (i.e. uncoupled mitochondria) a pH optimum of 8.1–8.4 has been reported [10]. In contrast the maximal rate of deuteroheme synthesis was found at pH 7.3–7.5 using tightly coupled rat liver mitochondria as the enzyme source (Fig. 5). This difference in pH optimum between the ferrochelatase activity of coupled and uncoupled mitochondria is of considerable interest in view of the difference in proton permeability [25, 26], the transmembrane pH-gradient between the two types of mitochondria [27], and the proposed localization of the ferrochelatase to the inner phase of the inner membrane [1]. It is tempting to suggest that the apparent pH optimum at 7.3–7.5 shown in Fig. 5 reflects the pH dependence partial reactions, e.g. the transport of substrate(s) across the inner membrane rather than the pH dependence of the ferrochelatase reaction. Thus, the pH optimum for the energy-dependent transport of iron across the inner membrane is 7.4 [12]. The existence of a permeability barrier to the substrate(s) of

the ferrochelatase reaction is also supported by the temperature dependency. A pronounced increase in the rate of deuteroheme synthesis was observed at temperatures above 40 °C, reaching a maximal value at approx. 50 °C; concomittantly there is a disruption of the permeability barrier of the mitochondrial inner membrane.

As previously shown [1], there is no requirement either of reducing equivalents or of anaerobiosis for the ferrochelatase reaction to proceed when Co(II) is used as the metal substrate. On the other hand, with iron as the metal substrate Barnes et al. [11] could find no heme synthesis in submitochondrial particles when respiratory substrates were absent. Working with higher instrumental sensitivity (approx. 20-fold) than Barnes et al. [11], a significant rate of heme formation was observed in intact mitochondria respiring on endogenous substrates using $FeCl_3$ as the metal substrate (Fig. 6A, trace b).

In the presence of exogenously added oxidizable substrates, an initial lag period was observed in the time course of the deuteroheme synthesis (Fig. 6A, traces c and d and ref. 11). The increase in the reaction rate with time has been considered to be related to and actually coincide with the onset of anaerobiosis [11]. However, as seen from Fig. 7 anaerobisosis was not followed by any changes in the rate of heme synthesis provided the mitochondria were supplemented with an oxidizable substrate.

Another point of considerable importance concerns the finding that in rotenone plus antimycin A supplemented mitochondria Fe(III) could not be reduced to Fe(II) utilizable in the ferrochelatase reaction either by CoASH, reduced glutathione, cysteine or NADH. Thus, using iron as the metal substrate, the rate of heme formation is largely determined by the rate at which reducing equivalents are supplied by the respiratory chain and the oxygen concentration does not have any measurable effect on the rate of the reaction.

In conclusion, our studies point to important functions of the mitochondrial membranes in the overall ferrochelatase reaction. Thus, these membranes have the ability to bind iron ions in a rather specific manner as well as to reduce Fe(III) to Fe(II) [23]. Furthermore the inner membrane represents a permeability barrier through which iron and porphyrin have to cross in order to reach the ferrochelatase [1]. In this way the mitochondrial membranes should be considered of great importance in the regulation of heme biosynthesis, and subsequent studies are aimed at a more close study of this problem.

ACKNOWLEDGEMENTS

The technical assistance of Mrs. K. Williams is greatly acknowledged. The study was supported in part by the Norwegian Research Council for Science and the Humanities.

REFERENCES

1 Jones, M. S. and Jones, O. T. G. (1969) Biochem. J. 113, 507–514
2 Bottomley, S. S. (1968) Blood 31, 314–322
3 Karibian, D. and London, I. M. (1965) Biochem. Biophys. Res. Commun. 18, 243–249
4 Lochhead, A. C. and Goldberg, A. (1961) Biochem. J. 78, 146–150
5 Porra, R. J. and Jones, O. T. G. (1963) Biochem. J. 87, 181–185

6 Barnes, R., Jones, M. S., Jones, O. T. G. and Porra, R. J. (1971) Biochem. J. 124, 633–637
7 McKay, R., Druyan, R., Getz, G. S. and Rabinowitz, M. (1969) Biochem. J. 114, 455–461
8 Porra, R. J., Vitols, K. S., Labbe, R. F. and Newton, N. A. (1967) Biochem. J. 104, 321–327
9 Porra, R. J. (1975) Anal. Biochem. 68, 289–298
10 Llambias, E. B. C. (1976) Int. J. Biochem. 7, 33–40
11 Barnes, R., Connelly, J. L. and Jones, O. T. G. (1972) Biochem. J. 128, 1043–1055
12 Romslo, I. and Flatmark, T. (1973) Biochem. Biophys. Acta 325, 38–46
13 Fuhrhop, J. H. and Smith, K. M. (1975) in Laboratory Methods in Porphyrin and Metalloporphyrin Research (Fuhrhop, J. H. and Smith, K. M., eds.), pp. 80–81, Elsevier Scientific Plblishing Company, Amsterdam
14 Falk, J. E. (1964) in Porphyrins and Metalloporphyrins (Falk, J. E., ed.), p. 118, Elsevier Publishing Company, Amsterdam
15 Falk, J. E. (1964) in Porphyrins and Metalloporphyrins (Falk, J. E., ed.), p. 241, Elsevier Publishing Company, Amsterdam
16 Romslo, I. and Flatmark, T. (1974) Biochim. Biophys. Acta 347, 160–167
17 Romslo, I. and Flatmark, T. (1973) Biochim. Biophys. Acta 305, 29–40
18 Porra, R. J. and Lascelles, J. (1968) Biochem. J. 108, 343–348
19 Kassner, R. J. and Walchak, H. (1973) Biochim. Biophys. Acta 304, 294–303
20 Sano, S., Inoue, S., Tanabe, Y., Sumiya, C. and Koike, S. (1959) Science 129, 275–276
21 de Goeij, A. F. P. M., Christiansen, K. and van Steveninck, J. (1975) Eur. J. Clin. Invest. 5, 397–400
22 Romslo, I. (1975) Biochim. Biophys. Acta 387, 69–79
23 Flatmark, T. and Romslo, I. (1975) J. Biol. Chem. 250, 6433–6438
24 Jones, O. T. G. (1968) Biochem. J. 107, 113–119
25 Mitchell, P. and Moyle, J. (1967) Biochem. J. 104, 588–600
26 Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 7, 471–484
27 Rottenberg, H. (1975) J. Bioenergetics 7, 61–74