Vol. 100, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1981 Pages 651-659

EFFECT OF LIGANDIN ON THE EFFLUX OF Co-DEUTEROPORPHYRIN FROM ISOLATED RAT LIVER MITOCHONDRIA

Paul Husby[a], Kaila Singh Srai[b], Brian Ketterer[b] and Inge Romslo[a]

a : Laboratory of Clinical Biochemistry, University of Bergen, N-5016 Haukeland Sykehus, Norway.

b : Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London W1P 7PN, U.K.

Received March 31, 1981

SUMMARY

Ligandin markedly accelerated the efflux of Co-deuteroporphyrin from tightly coupled mitochondria. This was associated with an increase in the rate of Co-deuteroporphyrin synthesis. The addition of 0.1 M KCl to the medium reduced but did not abolish the effect on efflux.

In the absence of Co^{2+}, ligandin inhibited deuteroporphyrin uptake by mitochondria.

When added to sonicated mitochondria ligandin inhibited Co-deuteroporphyrin synthesis.

These results suggest that ligandin plays an important part in the transfer of heme from mitochondria to its other sites of utilization. The effect of ligandin on heme release requires intact functional mitochondrial membranes and overcomes the tendency to deplete the porphyrin substrate for which it has affinity.

INTRODUCTION

In the hepatocyte a major proportion of the heme synthesized in mitochondria is utilized as the prosthetic group for such enzymes as the cytochrome P_{450} mixed function oxygenases, tryptophan oxygenase and catalase (1). The means whereby heme is transferred to these extra-mitochondrial sites is under debate.

One proposal is that heme is transferred via rough endoplasmic reticulum at occasional points of contact with mitochondria which have been observed in electron micrographs (2,3).

An alternative hypothesis allows for transfer to heme-binding proteins in the cytosol. This mechanism is attractive because the cytosol has contact with the entire surface of the mitochondrion and access to all cytoplasmic organelles. This route was first proposed by Yoda & Israels (4,5) who

0006-291X/81/100651-09$01.00/0

noted that both albumin and liver soluble supernatant fraction increased the rate of heme release from isolated mitochondria and attributed the effect of the liver soluble supernatant to the presence of heme binding proteins (4,5). The interpretation of this work suffers from lack of information concerning the structural and functional integrity of the mitochondria which were used.

Romslo & Husby (6) extended the work of Yoda & Israels by studying the effect of globin and albumin on mitochondria the energy coupling of which was monitored. Iron and protoporphyrin were replaced by Co^{2+} and deuteroporphyrin since Co-deuteroporphyrin is a good model compound for heme and many of the difficulties associated with the use of iron salts and protoporphyrin are minimized (see Discussion in ref.6). It was shown that in the absence of proteins, the efflux of Co-deuteroporphyrin varied inversely with the respiratory coupling of the mitochondria, being very little at high levels of coupling. If globin was introduced at high levels of coupling the efflux of Co-deuteroporphyrin was strikingly increased. Albumin on the other hand only affected efflux from mitochondria which had been standing, and as a result were less tightly coupled and already had increased efflux. Subsequently hemopexin was also studied and found both to increase both the rate of synthesis and the rate of efflux of Co-deuteroporphyrin in tightly coupled mitochondria (7).

However, none of these proteins are present in significant quantities in normal hepatic cytosol and therefore they are unlikely to implement heme transfer in the hepatocyte.

In the present paper ligandin is investigated. It is present in hepatic cytosol in approximately 0.1 mM concentrations (8),when isolated binds heme with an association constant of 10^7 M^{-1} (9) and also binds heme *in vivo* in experiments in which labelled precursor 5-aminolaevulinic acid is administered (10). Fore these various reasons it is very likely to play a role in intrahepatocytic heme transfer. It is shown that ligandin, like hemopexin stimulates both the rate of synthesis and efflux of Co-deuteroporphyrin from tightly coupled mitochondria.

MATERIAL AND METHODS

Mitochondria

Rat liver mitochondria were prepared as described (6). The functional integrity of these preparations was tested by measuring the respiratory control ratio with ADP using succinate as the substrate. Only mitochondria with this ratio greater than 4 at the start of the experiments were used.

Deterioration of the mitochondria during incubation was determined from the leakage of malate dehydrogenase and the decrease in the respiratory control ratio (6). Less than 1% of the malate dehydrogenase activity of the mitochondria leaked to the incubation medium during 60 min incubation, and the mitochondria remained coupled for about 20 min, in the absence as well as in the presence of K^+.

Submitochondrial particles

Submitochondrial particles were prepared by sonicating the mitochondrial suspension at 4°C for 30 s x 3 using an MSE 150 W ultrasonic disintegrator model MK 2 operated with 9.5 mm diameter end probe and an amplitude reading of 18 µm.

Uptake of deuteroporphyrin

Samples of mitochondria containing about 1.5 mg protein were incubated with mixing at 30°C in 1 ml of solution 0.25 M in sucrose, 5 mM in GSH and 5 mM in HEPES buffer at pH 7.4. Further additions were as described in the Figures. The reaction was initiated by adding deuteroporphyrin. After 30s the reaction was stopped by adding 0.5 ml of ice-cooled buffer, followed immediately by centrifugation in an Eppendorf microcentrifuge (Type 3200) for 2 min. Deuteroporphyrin in the supernatant was estimated by fluorescence using a Jasco FP-4 fluorescence spectrophotometer and excitation and emmision wavelengths of 393 and 593 nm respectively. The amount accumulated in the mitochondria was estimated by difference.

Synthesis and efflux of ^{57}Co-deuteroporphyrin

Synthesis and efflux of Co-deuteroporphyrin from mitochondria were determined as described (6,11) except that the medium was supplemented with 5 mM GSH.

Other analytical procedures

The mitochondrial respiration rates were determined as previously described (6).

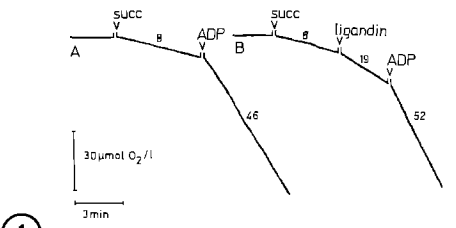
Protein was determined according to Lowry et al.,(12).

Chemicals

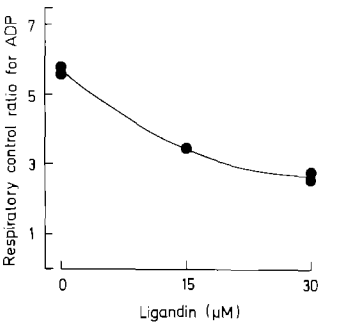
ADP,HEPES (A-grade) and rotenone were the products of Sigma Chemical Co.(St. Louis,MO.USA), deuteroporphyrin IX was from Porphyrin Products (Logan,UT.,USA) and ^{57}Co was obtained from The Radiochemical Centre (Amersham,Bucks.,U.K.). Other chemicals were of analytical grade whereever possible. Water, double destilled from quartz, was used throughout.

Ligandin

Ligandin (glutathione transferase B) was prepared by the application of the method of Tipping et al.,(13) to the fraction of rat liver soluble liver supernatant precipitating between 60% and 80% satured ammonium sulfate.



①



②

Fig.1 Effect of Ligandin on the respiratory rate of rat liver mitochondria. The mitochondria, about 1.5 mg protein, were incubated at 30°C in a medium containing in a final volume of 3 ml 50 mM glucose, 175 mM sucrose, 10 mM HEPES buffer at pH 7.40, $MgCl_2$ 5 mM, P_i 5mM and GSH 5mM. At the points indicated 3 mM succinate, 30 µM ligandin and 1 mM ADP were added. The numbers immediately above each trace represent the respiratory rate (nmole O_2/min/mg protein) in the time interval indicated.

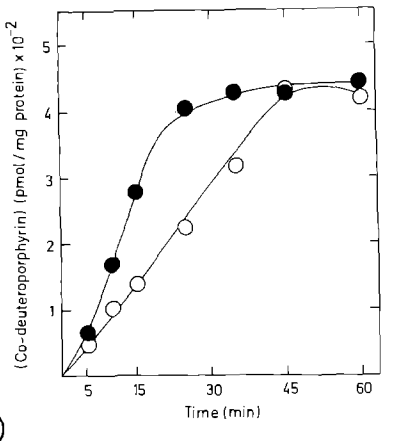
Fig.2 Effect of Ligandin on the energy-coupling of rat liver mitochondria. The mitochondria, about 1.5 mg protein, were incubated as indicated in Fig.1, in the presence of increasing concentrations of ligandin.

RESULTS

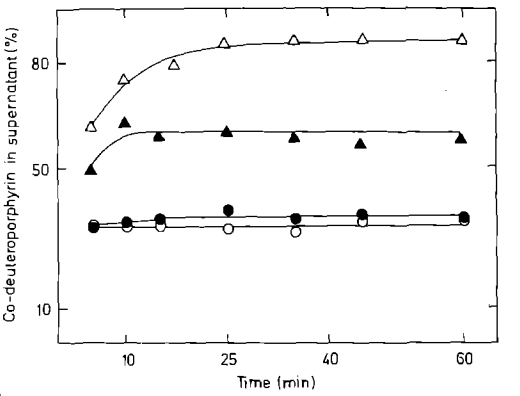
Ligandin (unlike globin, albumin or hemopexin), when added to freshly prepared mitochondria, depressed their respiratory control ratio for ADP which is an index of the tightness of their respiratory coupling (Fig.1). This depression was marked above 15 µM (Fig.2). The nature of this effect is being investigated. In the meantime 5 µM ligandin which gives respiratory control ratios for ADP greater than 4 has been used in these experiments to obtain data relating to the effect of ligandin on Co-deuteroporphyrin synthesis and release.

Fig.3 shows that the addition of 5 µM ligandin to the medium increased the rate of Co-deuteroporphyrin synthesis without affecting the total amount synthesized. Fig. 4 shows that ligandin also brings about a much enhanced Co-deuteroporphyrin efflux which could be reduced but not prevented by 0.1 M KCl.

If Co^{2+} is absent from the incubation medium ligandin was found to inhibit the mitochondrial uptake of deuteroporphyrin



3



4

Fig.3 The time-progress curves for synthesis of Co-deuteroporphyrin. The experimental conditions were as described in the Materials and Methods section.(O), control without ligandin; (●), with 5 μM ligandin.

Fig.4 Time-progress curves for the release of Co-deuteroporphyrin from rat liver mitochondria. Mitochondria,about 2 mg protein/ml, were incubated as described in the Materials and Methods section. The release of Co-deuteroporphyrin was determined at the time intervals indicated. The percent release was determined in a K^+-depleted medium (Δ,O), and in a medium supplemented with 100 mM KCl (▲,●) in the absence (●,O) and presence of ligandin(5μM) (▲,Δ).

in a concentration dependent fashion. Analysis of the inhibition using a Dixon plot (Fig.5) is consistent with some depletion of available deuteroporphyrin by binding to ligandin.

While 5 μM ligandin increases the rate but not the extent of Co-deuteroporphyrin synthesis by tightly coupled mitochondria it reduces both the rate and the extent when mitochondria are sonicated (Fig.6), a procedure which externalizes much of the ferrochelatase. The effects are greater with 15 μM ligandin and they are interpreted to be the effects of substrate depletion by ligandin on the ferrochelatase reaction.

DISCUSSION

Ligandin markedly stimulates the release from mitochondria of Co-deuteroporphyrin which would otherwise be retained by an energy dependent process (6). At high concentrations ligandin inhibits mitochondrial uptake of the precursor

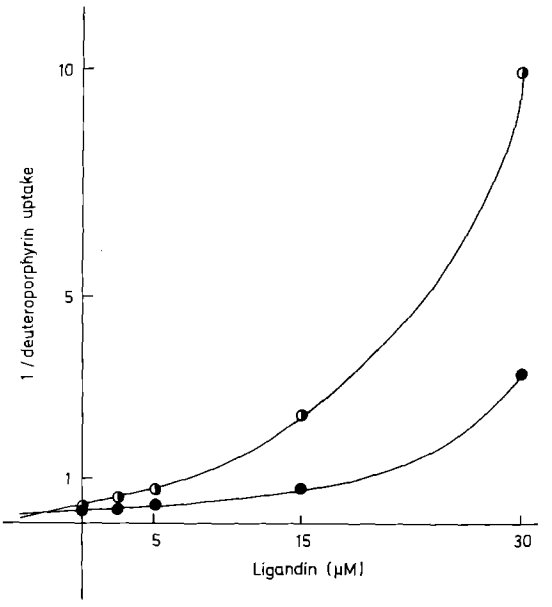


Fig.5 Effect of Ligandin on the uptake of deuteroporphyrin by isolated rat liver mitochondria. The mitochondria were incubated as described in the Materials and Methods section. The concnetration of deuteroporphyrin was 7.5 μM (◑) or 12 μM (●).

deuteroporphyrin, but at 5 μM concentration this effect is small and, as shown in Fig.3 does not prevent an overall effect of increasing the rate of Co-deuteroporphyrin synthesis. The

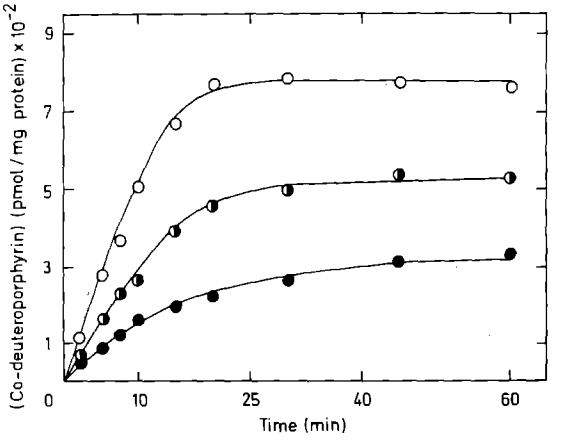


Fig.6 Time-progress curves for the synthesis of Co-deuteroporphyrin by submitochondrial particles. Submitochondrial particles, about 2 mg protein/ml, were incubated as described in the Materials and Methods section.(○), control without ligandin; (◑), with 5 μM ligandin; (●), with 15 μM ligandin.

increased rate of synthesis may be due to reduced product inhibition of ferrochelatase (6) by Co-deuteroporphyrin because of its enhanced efflux.

The mechanism of the releasing action of ligandin on mitochondrial Co-deuteroporphyrin is not known. Its effectiveness with tightly coupled mitochondria suggests that it crosses the outer mitochondrial membrane and in some way intervenes in the system which links energy coupling to Co-deuteroporphyrin retention. The passage of proteins from the cytosol into the mitochondrion is an established phenomenon at least with respect to the 90 % of mitochondrial protein which originates outside the mitochondria (14). Ligandin may interact with the inner membrane of the mitochondrion but does not cross it since we have evidence that it inhibits ferrochelatase which is on the matrix side of the inner mitochondrial membrane (15).

Ligandin shares this property of releasing Co-deuteroporphyrin from tightly coupled mitochondria with globin (6) and hemopexin (7). Despite its affinity for heme, albumin does not have this property: it presumably does not make the appropriate interaction with mitochondria. By virtue of their affinity for heme all the above proteins should cause a considerable reduction in free Co-deuteroporphyrin levels outside the mitochondria and greatly increase the Co-deuteroporphyrin gradient between the interior and exterior of the mitochondria. However results with albumin show that this is not sufficient to cause release of Co-deuteroporphyrin from tightly coupled mitochondria, but can enhance release when mitochondria become leaky (6).

K^+ markedly depresses the efflux of Co-deuteroporphyrin (Fig.4). The effect is even more pronounced in the absence of metalloporphyrin-binding ligands of the medium (11). The effect of K^+ has been ascribed to an increase in the ionic strenth of the medium with retention of lipophilic metalloporphyrin within the hydrophobic mitochondrial membrane (11).

It is assumed that what is seen to occur with Co-deuteroporphyrin *in vitro* also occurs with heme *in vivo* with the difference that the tetrapyrrole utilized by the mitochondria for heme synthesis is coproporphyrinogen which should bind to

ligandin less strongly than does deuteroporphyrin (16) and which therefore should be less subject to depletion by ligandin with respect to mitochondrial uptake. It is noteworthy that the effects we have observed are at 5 μM levels of ligandin whereas the concentration *in vivo* is near to 100 μM. Although the ligandin preparation used in this work causes uncoupling at concentrations approaching physiological, such an effect would be lethal *in vivo*. Ligandin *in situ* clearly does not have this effect. The possibility that toxic contaminants are present in our preparations of ligandin is under investigation.

Once released from mitochondria, heme will tend to concentrate in phospholipid bilayers of membranes for which it has a high affinity (17). Theoretical concentrations show that the presence of ligandin in cytosol together with the network of phospholipid provided by the endoplasmic reticulum and other organelles should enable the rapid distribution of heme throughout the cell (8) where it will tend to locate preferentially on the high affinity sites of those apoenzymes for which it is a prosthetic group (2,3,18,19,20,21,22).

Although ligandin is the most abundant liver protein which has been shown to bind heme *in vivo*, there is at least one other, which has not been studied in detail (23) which may play an important role.

ACKNOWLEDGEMENT

This work was supported by the Norwegian Research Council for Science and the Humanities and the Cancer Research Campaign of which BK is a Life Fellow.

REFERENCES

1. Granick,S. & Beale,S.I.(1978) Adv.Enzymol.41,33-201.
2. Meier,P.J. & Meyer,U.A.(1976) Z.Physiol.Chem.357,1041.
3. Meyer,U.A. & Meier,P.J.(1977) Experientia,33,807.
4. Yoda,B. & Israels,L.G.(1972) Can.J.Biochem.50,633-637.
5. Israels,L.G.,Yoda,B.& Schachter,B.A.(1975) Ann.N.Y.Acad. Sci.,244,651-661.
7. Husby,P.& Romslo,I.(1980) Biochem.J.,188,459-465.
8. Husby,P.,Müller-Eberhard,U. & Romslo,I.(1980)Biochem. Biophys.Res.Commun.,94,1345-1352.
9. Tipping,E.,Ketterer,B.,Christodoulides,L.& Enderby,G. (1976)Biochem.J.,157,461-467.
10. Ketterer,B.,Srai,K.S. & Christodoulides,L.(1976) Biochim. Biophys.Acta,428,683-689.
11. Husby,P.& Romslo,I. (1981)Biochem.J. (in press).

12. Lowry,O.H.,Rosebrough,N.J.,Farr,A.L. & Randall,R.J.(1951) J.Biol.Chem.,193,265-275.
13. Tipping,E.,Ketterer,B.,Christodoulides,L.&Enderby,G.(1976) Eur.J.Biochem.,67,583-590.
14. Neupert,W. & Schatz,G.(1981)Trends in Biochemical Sciences, January,1-7.
15. Jones,M.S. & Jones,O.T.G.(1969)Biochem.J.,113,507-514.
16. Tipping,E.,Ketterer,B. & Koskelo,P.(1978)Biochem.J.,169, 509-516.
17. Tipping,E.,Ketterer,B. & Christodoulides,L.(1979)Biochem.J., 180,327-337.
18. Feigelson,P. & Greengard,O.(1961)J.Biol.Chem.,236,153-157.
19. Higashi,T.,Kawamata,F. & Sakamoto,T.(1974)J.Biochem.76, 703-708-
20. Legg,P.G. & Wood,R.L.(1970)J.Cell.Biol.,45, 118-129.
21. Lazarow,P.B. & de Duve,C.(1971)Biochem.Biophys.Res. Commun.,45,1198-1204.
22. Redman,C.M., Crab,D.J. & Irukulla,R.(1972) Arch.Biochem. Biophys. 152, 496-501.
23. Srai,K.S. (1978) Ph.D.Thesis,University of London.