PERMEABILITY PROPERTIES OF MITOCHONDRIAL MEMBRANES AND THE REGULATION OF HAEM BIOSYNTHESIS

M.S. Jones and O.T.G. Jones.

Department of Biochemistry, University of Bristol, BRISTOL, BS8 1TD, England.

Received October 19, 1970

SUMMARY

Mitochondrial ferrochelatase was over 50% inhibited by 16 uM protohaem and the ALA-synthetase of porphyric mitochondria was 40% inhibited by 10uM protohaem. The matrix space of rat liver mitochondria was readily penetrated by glycine (a substrate for ALA-synthetase) but not by ALA, which penetrated only the intermembrane space. When ALA was synthesized within the matrix of porphyric mitochondria an efflux of ALA was found, although the mechanism for this was uncertain. An endogenous inhibitor of ALA-synthetase was present in the particulate fraction of porphyric mitochondria.

The enzymes concerned in the biosynthesis of protohaem are distributed within the liver cell between the mitochondrion and the cytosol and it has frequently been suggested that this distribution may be important in the regulation of haem synthesis (cf Sano & Granick, 1961). The possibility that the permeability properties of the mitochondrial membranes to biosynthetic intermediates is important in regulation of haem and consequently cytochrome synthesis has become more likely with the finding that the first enzyme of the sequence, ALA synthetase, is located within the matrix space (McKay, Druyan, Getz & Rabinowitz, 1969; M.S. Jones & O.T.G. Jones, unpublished work) and that the last enzyme, ferro-

chelatase, is located on the inside of the inner mitochondrial membrane (Jones & Jones, 1969). This distribution means that both glycine and ALA must pass across the inner mitochondrial membrane where their movements may be regulated by specific carriers and that protohaem is formed within the matrix compartment where its local high concentration could affect the activity of the biosynthetic enzymes ALA synthetase and ferrochelatase within the compartment. In this communication we report that the matrix space is readily penetrated by glycine but not by ALA and that both ALA synthetase and ferrochelatase are inhibited by protohaem.

EXPERIMENTAL PROCEDURE

Rats were made porphyric by intra-peritoneal injections of a 1.5% solution of allyl isopropyl acetamide in 0.9% NaCl. Two injections were given, separated by a period of 24 hours; the doses were 300mg. per kg. body weight.

Mitochondria were prepared from rat livers by the method described by Chappell & Hansford (1969).

Determination of mitochondrial spaces. Mitochondria (about 30mg. protein) were suspended in 10ml centrifuge tubes containing 3ml of a medium of 80mM KCl, 20mM Tris-HCl, pH 7.2, 20mM sucrose and tritiated water (2uCi/ml). [^{14}C] sucrose, [^{14}C] glycine or [^{14}C] ALA were added to 0.5u Ci/ml, together with carrier at the concentrations specified. After incubation at 20^{o}C for 10 min the tubes were centrifuged at 20,000g for 5min., the surface of the pellet washed with isotonic KCl-Tris buffer and both supernatant and pellet treated with 5% (w/v) perchloric acid. Protein precipitates were removed by centrifuging and the potassium perchlorate that precipitated when the extracts were adjusted to pH 6.8 was also removed by centrifuging. The volumes of both extracts were recorded and their contents of tritium and ^{14}C determined

in a Nuclear Chicago scintillation counter following the addition of aliquots to 10ml of scintillator dissolved in dioxan. The space penetrated by each compound was calculated using the formula

$$\text{Permeable space} = \frac{B \times Vi \times Vp}{A \times Vs} \text{ ml}$$

where B = Conc. in pellet extract ; A = Conc. in supernatant extract; Vi = initial incubation vol., Vp = vol. of pellet extract. Vs = vol. of supernatant extract.

Sucrose was also assayed colorimetrically (Kulka, 1955) and ALA was also assayed by the method of Manzerall & Granick (1956).

Assay of ferrochelatase. Co- or Fe porphyrin formations was measured on a dual wavelength spectrophotometer (Jones & Jones, 1969) Fe^{2+} was added to mitochondrial suspensions that had become anaerobic following the addition of 1mM NADH. To avoid permeability effects the mitochondria were sonicated before use.

Assay of ALA-synthetase. The method of Manzerall & Granick (1956) was used, using frozen and thawed porphyric mitochondria.

RESULTS

Previous work (Jones & Jones, 1970) has shown that the ferrochelatase of photosynthetic bacteria is inhibited by protohaem. Such an inhibition was therefore sought in mitochondria, and in addition the effect of protohaem upon the ALA-synthetase of mitochondria was determined (Table 1). It was found that haem formation was at least as sensitive to protohaem inhibition as ALA-synthetase. The activity of ferrochelatase in mitochondria is lower than that in photosynthetic bacteria, where in detailed kinetic studies it was possible to show that Co^{2+} incorporation was inhibited by protohaem non-competitively with respect to both metal substrate and porphyrin substrate (Jones & Jones, 1970), but furbher work is in progress in attempts to determine the nature of the inhibition in the mammalian system.

TABLE 1

Effect of added protohaem on the ferrochelatase activity of rat liver mitochondria and on the ALA-synthetase activity of mitochondria from livers of porphyric rats. Ferrochelatase was assayed by measurement of rates of incorporation of either Co^{2+} or Fe^{2+} in deuteroporphyrin or protoporphyrin.

Activity measured	Rate (nmoles/min/mg protein)	Effect of protohaem.
Co-deuteroporphyrin formation	0.59	34% inhibition at 32uM
Co-protoporphyrin formation	0.15	50% inhibition at 32uM
Deuterohaem formation	0.51	65% inhibition at 16uM
Protohaem formation	0.12	60% inhibition at 16uM
ALA synthetase	25.3×10^{-3}	40% inhibition at 10uM

TABLE 2

Effect of sonication upon the ALA-synthetase activity of porphyric rat liver mitochondria.

Fraction	ALA-synthetase activity (pmoles/min/mg protein)	Yield(%)
Intact mitochondria	25.3	100
sonicated mitochondria	4.23	17
High speed pellet (A)	12.8	20.5
High speed supt. (B)	26.0	61.3
A + B (equal protein aliquots)	12.8	-

Sonication of intact mitochondria reduced the activity of ALA-synthetase to about 17% of that found in intact mitochondria, but there

was a good recovery of activity in the soluble fraction following centrifugation.

The activity of the soluble fraction was inhibited by the addition of the pellet fraction (Table 2).

Mitochondria may be considered as freely permeable to tritiated water, whereas sucrose does not penetrate the inner mitochondrial membrane. We have found that glycine behaves like tritiated water whereas ALA did not penetrate the matrix space (Table 3). Glycine penetration did not require the addition of any exchangeable ion to the medium nor was it inhibited by N-ethylmaleimide, which has been reported to inhibit leucine penetration of mitochondria (Buchanan, Popovitch & Tapley, 1969). ALA penetration was not activated by the addition of possible exchangeable penetrants such as malate, glutamate, glycine or phosphate, nor by the

TABLE 3

Penetration of normal and porphyric rat liver mitochondrial membranes by glycine and ALA.

Type of mitochondria	Penetrant	Tritiated water space penetrated (%)
Normal	^3H_2O	100
	Sucrose	58.3
	ALA (2mM)	62.7
Porphyric	^3H_2O	100
	Sucrose	48.6
	ALA (2mM)	43.2
Normal	^3H_2O	100
	Sucrose	71
	glycine (5mM)	97

addition of ATP. No ALA permease was activated in porphyria or by added allylisopropyl acetamide. When porphyric mitochondria were supplied with ATP, glycine and succinate, to promote ALA synthesis within the matrix space an efflux of ALA was detected, although the concentration of ALA within the matrix was higher than outside. (Table 4). This result might suggest the presence of a uni-directional porter system for ALA, but control experiments have shown that mitochondria can become more permeable to ALA during incubation at 37°C, whilst remaining impermeable to sucrose and interpretation of this efflux of ALA must be cautious.

TABLE 4.

Efflux of ALA from porphyric rat liver mitochondria. Mitochondria (31.5 mg protein) were incubated for 20min at 37°C in 80mM KCl, 20mM Tris-Cl. pH7.2, 4mM ATP, 20mM succinate, 20mM glycine, 0.2mM pyridoxal phosphate, 6mM $MgCl_2$, ^3H_2O and $[^{14}C]$ sucrose in a final volume of 3ml. The tubes were then cooled to 0°C and spaces determined. The specific activity of the ALA-synthetase was 78.6 pmoles/min/mg protein.

Space	Conc.ALA (μM)	% Total ALA recovered in space.
Extramitochondrial	13.8	86.5
Matrix	132	13.5

DISCUSSION

The results of the experiments described in this paper support the view that the subcellular distribution of enzymes may be important in the regulation of haem biosynthesis. Protohaem is formed within the matrix space where the first and last members of the biosynthetic sequence are located, and its local concentration may become quite high. Both enzymes are inhibited by relatively low concentrations of protohaem. The inhibition

of ALA-synthetase by protohaem, first described by Burnham & Lascelles (1963) in the photosynthetic bacterium Rhodopseudomonas spheroides, is probably of great significance in regulating tetrapyrrole formation in this organism, and although there has been a lack of agreement in the literature about the effect of protohaem on ALA-synthetase in higher organisms the inhibitions we have found are in agreement with those noted by Irving & Elliott (1969) and Scholnick Hagmaker & Marver (1969). The inhibition of ferrochelatase by protohaem may explain why porphyrins and not haems are excreted in porphyria, a condition characterised by a large increase in the level of ALA-synthetase, normally the rate-limiting step in haem biosynthesis. The maximum inhibition of ALA-synthetase by protohaem that we have found in mitochondria is about 50% and the sensitivity of ferrochelatase to protohaem would be an additional safeguard against overproduction of haem and depletion of mitochondrial iron reserves.

Mitochondria are not freely permeable to charged molecules and it appeared possible that specific carriers may be necessary for the transport of glycine into mitochondria and ALA outwards. The ready penetration of glycine (Table 3) suggests that this process could not limit haem synthesis but the impermeability of the inner membrane to the inward movement of ALA means that some special mechanism would be necessary for rapid transport of this molecule across the membrane. The limitations of the "space" technique do not enable the mechanism of ALA efflux to be decided, but it is possible to say that a barrier to the movement of ALA exists, that could be important in regulating haem synthesis and that the permeability of mitochondria to ALA is not affected by porphyria.

The significance of the endogenous inhibitor of ALA-synthetase present in the particulate fraction of mitochondria is as yet unknown. Its existence must be considered in quantitative studies on ALA-synthetase.

ACKNOWLEDGEMENTS

We are grateful to Roche Products Ltd. for a supply of allylisopropyl acetamide The work was supported by grants from the Science Research Council.

REFERENCES

Buchanan, J.B., Popovitch, J.R. & Tapley, D.F. (1969). Biochim. Biophys. Acta. 173, 532.

Burnham, B.F. & Lascelles, J. (1963). Biochem. J. 87, 462.

Chappell, J.B. & Hansford, R.G. (1969). In Subcellular Components, Separation and Fractionation, p.43. Ed. by Birnie, G.D. & Falc, S.M. London: Butterworths.

Irving, E.A. & Elliott, W.H. (1969). J. biol. Chem. 244, 60.

Jones, M.S. & Jones, O.T.G. (1969). Biochem. J. 113, 507.

Jones, M.S. & Jones, O.T.G. (1970). Biochem. J. In press.

Kulka, R.G. (1956). Biochem. J. 63, 542.

Mauzerall, D. & Granick, S. (1956). J. biol. Chem. 219, 435.

McKay, R. Druyan, R., Getz, G.S. & Rabinowitz, M. (1969). Biochem. J. 114, 455.

Sano, S. & Granick, S. (1961). J. biol. Chem. 236, 1173.

Scholnick, P.L., Hammacker, L.E. & Marver, H.S. (1969). Proc. Natn. Acad. Sci., U.S.A. 63, 65.