Ferrochelatase and δ -Aminolaevulate Synthetase in Brain, Heart, Kidney and Liver of Normal and Porphyric Rats

THE INDUCTION OF δ -AMINOLAEVULATE SYNTHETASE IN KIDNEY CYTOSOL AND MITOCHONDRIA BY ALLYLISOPROPYLACETAMIDE

By R. BARNES, M. S. JONES*, O. T. G. JONES AND R. J. PORRA†

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(Received 17 May 1971)

1. δ -Aminolaevulate synthetase was detected in liver and kidney mitochondria prepared from normal rats. 2. The administration of allylisopropylacetamide induced an increase in δ -aminolaevulate synthetase in both liver and kidney mitochondria and the enzyme also appeared in the cytosol fraction of both tissues. Comparison with the distribution of glutamate dehydrogenase indicated that this soluble kidney δ -aminolaevulate synthetase was truly of cytosol origin and did not arise from disrupted mitochondria. The kidney cytosol enzyme was inhibited by 50% by 50 μ M-protohaem. 3. δ -Aminolaevulate synthetase could not be detected in mitochondria or cytosol from heart or brain from normal or porphyric rats. 4. The administration of allylisopropylacetamide caused little or no increase in ferrochelatase or cytochrome content of liver, kidney, heart or brain mitochondria.

The enzyme δ -aminolaevulate synthetase (succinyl-CoA-glycine succinyltransferase) is generally believed to be the first enzyme of haem biosynthesis. Since this first step is rate-limiting (Granick, 1966), the enzyme plays a key role in the regulation of haem biosynthesis in mammalian cells. Thus the intracellular concentration of δ -aminolaevulate synthetase increases in mammalian liver and chicken embryo cells after the administration of drugs that stimulate the overproduction and excretion of porphyrins (Granick & Urata, 1963; Granick, 1966). Beattie & Stuchell (1970) suggested that δ -aminolaevulate synthetase may regulate the cytochrome content of mammalian mitochondria, since the haemoprotein content of liver mitochondria, as well as δ -aminolaevulate synthetase activity, increased after the administration of allylisopropylacetamide. Such a suggestion would be supported if it could be shown that δ -aminolaevulate synthetase was present in mitochondria from normal non-erythropoietic tissues, other than liver; we have not found published reports of such activity. In liver this enzyme may be involved in the synthesis of the haem, cytochrome *P*-450, that is a component of the microsomal drug-detoxification system: it has been stated (cf. Sassa & Granick,

1970; Granick, 1966) that induction of δ -aminolaevulate synthetase by drugs occurs only in liver.

In this paper we report measurements of δ -aminolaevulate synthetase in mitochondria prepared from liver, kidney, heart and brain of normal and porphyric rats. We also compare the intracellular concentration of δ -aminolaevulate synthetase in preparations from various normal and porphyric tissues with those of ferrochelatase and cytochrome oxidase: these two enzymes are membrane-bound components of the mitochondrion and ferrochelatase catalyses the insertion of iron into protoporphyrin, forming protohaem, which may regulate δ -aminolaevulate synthetase activity by feedback inhibition (cf. Burnham & Lascelles, 1963).

EXPERIMENTAL

Induction of porphyria. Except where otherwise stated in the text, rats (wt. approx. 200 g) were starved for 24 h, then given three intraperitoneal injections of allylisopropylacetamide (each of 225 mg/kg body wt.) dissolved in 0.9% NaCl at 12 h intervals and killed 3 h after the last injection.

Preparation of mitochondria and cytosol. Standard methods (Chappell & Hansford, 1969) were used to isolate mitochondria from heart, kidney, brain and liver. The supernatant fractions obtained after sedimentation of mitochondria were re-centrifuged at 110000g for 1 h to obtain a cytosol free of mitochondrial fragments and microsomal fraction. Respiratory-control ratios (Chance & Williams, 1956) for the mitochondria were measured

* Present address: Department of Chemical Pathology, St Mary's Hospital Medical School, London W.2, U.K.

† Permanent address: Commonwealth Scientific and Industrial Research Organisation Division of Plant Industry, Canberra, A.C.T. 2601, Australia.

Table 1. Comparison of the activities of ferrochelatase and the concentration of cytochrome oxidase in mitochondria of various tissues of normal and porphyric rats

Deuteroporphyrin (100 nmol) and mitochondria (20–50 mg of protein/ml) in 3.0 ml of 100 mM-tris-HCl buffer, pH 8.2, containing 0.1% Tween 80, were incubated at 37°C. The reaction was started by addition of 100 nmol of CoSO₄, and the change in extinction at 498 minus 511 nm was recorded. Metalloporphyrin formation was calculated by using $\Delta\epsilon_{\text{mM}}$ 10 (Jones & Jones, 1969). Cytochrome *a*+*a*₃ concentrations were assayed in the same medium by recording the change in extinction at 605 minus 630 nm after the addition of dithionite: $\Delta\epsilon_{\text{mM}}$ 16 was used (see Chance, 1957).

Source of mitochondria	Ferrochelatase (nmol of metalloporphyrin/min per mg of protein)		Cytochrome oxidase (nmol of <i>a</i> + <i>a</i> ₃ /mg of protein)	
	Normal	Porphyric	Normal	Porphyric
Liver	0.71 ± 0.14	0.64 ± 0.07	0.110 ± 0.011	0.114 ± 0.007
Kidney	0.342 ± 0.03	0.340 ± 0.04	0.203 ± 0.003	0.226 ± 0.015
Heart	0.332 ± 0.04	0.29 ± 0.065	0.46 ± 0.05	0.45 ± 0.03
Brain	0.396 ± 0.09	0.43 ± 0.04	0.110 ± 0.019	0.121 ± 0.021

polarographically with glutamate+malate as substrate: these ratios were generally greater than 3, even for brain, which gave the lowest ratios.

Assays. Ferrochelatase was determined by the method of Jones & Jones (1969), with deuteroporphyrin and Co²⁺ as substrates (see Table 1). Glutamate dehydrogenase was assayed by following the oxidation of NADH at 340 nm during the conversion of oxoglutarate and NH₄Cl into glutamate (see Table 4). Cytochromes *a*+*a*₃ were determined in a dual-wavelength spectrophotometer from the change in extinction at 605 minus 630 nm after reduction with dithionite (Chance, 1957). The cytochrome *a*+*a*₃ concentration was used to indicate the cytochrome content of mitochondria. However, cytochromes *a*, *b* and *c* were determined in all types of mitochondria to ensure that the oxidase was not behaving atypically. Samples of mitochondria were suspended in buffer in two cuvettes of a split-beam spectrophotometer, glutamate+malate was added to the reference cuvette and difference spectra were recorded from 500 to 630 nm after this cuvette had become anaerobic. Cytochrome concentrations were determined by using the constants given by Chance (1957).

δ -Aminolaevulate synthetase was determined in early experiments by the colorimetric method of Mauzerall & Granick (1956); in subsequent experiments the more sensitive and specific radiochemical method of Irving & Elliott (1969) was used. Formation of δ -aminolaevulate was confirmed by t.l.c. as described by Irving & Elliott (1969). Succinyl-CoA synthetase (EC 6.2.1.5) was prepared by the method of Scholnick, Hammaker & Marver (1969) from δ -aminolaevulate-requiring mutant H5 of *Rhodospseudomonas spheroides*, a gift from Dr June Lascelles, Department of Bacteriology, University of California, Los Angeles, Calif., U.S.A., and assayed by the method of Kaufman (1955). The absence of δ -aminolaevulate synthetase in the succinyl-CoA synthetase extract was confirmed by the radiochemical assay.

Proteins were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin as standard.

Chemicals. Crystalline protohaem was prepared from whole blood by the method of Labbe & Nishida (1957). Allylisopropylacetamide was generously given by Roche

Products Ltd., Welwyn Garden City, Herts., U.K. Nagarse was purchased from Teikoku Chemical Industry Co. Ltd., Osaka, Japan. [1,4-¹⁴C₂]Succinate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

RESULTS

The concentrations of the two insoluble enzymes, ferrochelatase and cytochrome oxidase, in mitochondria prepared from normal and porphyric rats are given in Table 1. There was no significant change in ferrochelatase activity after treatment with allylisopropylacetamide: activities in brain, kidney and heart mitochondria were very similar to those of controls but the liver activity was nearly twice as high. Cytochrome *a*+*a*₃ contents were significantly higher in heart mitochondria than in those from other tissues.

Beattie & Stuchell (1970) have measured the cytochrome content of mitochondria obtained from porphyric and normal rats, and have found that within 1 h of administration of allylisopropylacetamide the content of cytochromes *a*+*a*₃, *b* and *c* increased by 30–50%. The experiments described in Table 1 showed possible small increases in cytochrome *a*+*a*₃ in kidney and liver mitochondria after treatment with allylisopropylacetamide; more rigorously controlled experiments (Table 2), in which control animals were injected with 0.9% sodium chloride in place of allylisopropylacetamide, suggested that even these increases may be of doubtful significance. The cytochrome *a*+*a*₃ content of heart and brain mitochondria did not change significantly during porphyria.

Colorimetric assays indicated that δ -aminolaevulate synthetase was present at low concentration in mitochondria from kidney, liver and possibly heart of normal animals. Since the colorimetric

Table 2. Comparison of the cytochrome concentrations in kidney and liver mitochondria of normal and porphyric rats

Six male rats (wt. 205g) were starved and injected as described in the Experimental section and killed 2½ h after the last injections. Six identical rats used as controls were injected with 0.9% NaCl. Cytochrome *a*+*a*₃ content was assayed as described in Table 1. Cytochrome *b* was assayed from the change in extinction at 560 minus 572nm and cytochrome *c* by the change at 550 minus 540nm after addition of dithionite. Concentrations of cytochromes *b* and *c* were calculated by using $\Delta\epsilon_{\text{mM}}$ 22 for cytochrome *b* and $\Delta\epsilon_{\text{mM}}$ 19.1 for cytochrome *c* (see Chance, 1957).

Source of mitochondria	State	Cytochrome <i>a</i> + <i>a</i> ₃ (nmol/mg of protein)	Cytochrome <i>b</i> (nmol/mg of protein)	Cytochrome <i>c</i> (nmol/mg of protein)
Liver	Porphyric	0.138	0.089	0.326
	Normal	0.124	0.097	0.352
Kidney	Porphyric	0.21	0.067	0.53
	Normal	0.20	0.066	0.48

Table 3. Effect of porphyria on the δ -aminolaevulate synthetase in cytosol and mitochondria of various tissues

Cytosol or mitochondria (6mg of protein) were incubated with a reaction mixture containing tris-HCl buffer, pH 7.4 (25 μ mol), MgCl₂ (10 μ mol), glycine (50 μ mol), ATP (12.5 μ mol), coenzyme A (0.25 μ mol), GSH (1 μ mol), pyridoxal phosphate (0.5 μ mol), antimycin A (1.25 μ g), sodium arsenite (2.5 μ mol), sodium malate (2.5 μ mol), sodium malonate (5.0 μ mol), sodium succinate (5 μ mol), [1,4-¹⁴C₂]succinate (2.5 μ Ci; 0.3 μ mol), EDTA (10 μ mol) and succinyl-CoA synthetase preparation from *R. sphaeroides* H5 mutant (sufficient to generate 1.2 μ mol of succinyl-CoA/h). Total volume was 1.3 ml and the mixture was incubated at 37°C. For the kidney and heart experiments the amount of [1,4-¹⁴C₂]succinate was increased fivefold to 12.5 μ Ci. T, extracts where trace incorporation was measured but was too low for satisfactory chromatographic identification.

		δ -Aminolaevulate synthetase activities (nmol of δ -aminolaevulate formed/h per mg of protein)	
		Normal	Porphyric
Liver	Mitochondria	0.124 $\pm$ 0.01	3.95 $\pm$ 0.45
	Cytosol	T	1.35 $\pm$ 0.9
Kidney	Mitochondria	0.045 $\pm$ 0.015	0.50 $\pm$ 0.175
	Cytosol	T	0.108 $\pm$ 0.035
Heart	Mitochondria	0	0
	Cytosol	0	0
Brain	Mitochondria	0	0
	Cytosol	0	0

assay can give falsely high positive results by failing to discriminate between δ -aminolaevulate and aminoacetone, we adopted the more specific and sensitive radiochemical assay (Irving & Elliott, 1969). Low activities were found in mitochondria from liver and kidney of normal rats and traces were detected in cytosol fractions from normal liver (Table 3); however, no activity was detected in heart mitochondria even when the specific radioactivity of labelled succinate was increased fivefold.

After treatment with allylisopropylacetamide, increased δ -aminolaevulate synthetase activity was found in liver and kidney mitochondria, and the proportion of the total activity in the cytosol fraction from the two tissues was now considerable. The specific activities in kidney mitochondria and cytosol were always lower than those of liver. No activity was found in mitochondria from brain or

heart of porphyric animals or in brain cytosols; assays were not attempted on heart cytosol because this contained the proteolytic enzyme Nagarse, added during the preparation of the heart mitochondria. In every case where δ -aminolaevulate synthetase activity was detected the formation of δ -aminolaevulate was confirmed by t.l.c. (see the Experimental section).

Considerable variation in δ -aminolaevulate synthetase activities were found in kidney mitochondria and cytosol, possibly because these activities are very sensitive to the dosage of allylisopropylacetamide. In several experiments with the normal dose of allylisopropylacetamide (200mg/kg body wt.) low activities were found (Table 3). Higher doses (400mg/kg body wt.) induced high activities (Table 4). Induction of the enzyme in liver was less sensitive to dosage.

Table 4. *Comparison of the activities of glutamate dehydrogenase and δ -aminolaevulate synthetase in cytosol and mitochondria of porphyric rat kidney*

Six male rats were starved for 24 h, then given intraperitoneal injections of allylisopropylacetamide (400 mg/kg body wt.) and killed 2 h later. δ -Aminolaevulate synthetase was assayed as described in Table 2. Glutamate dehydrogenase was assayed at 35°C by following the oxidation of NADH at 340 nm by using a Unicam SP.1800 spectrophotometer ($\Delta\epsilon_{\text{mM}}$ 6.4). The incubation medium contained Triton X-100 (0.04%), NADH (0.2 mM), oxoglutarate (8 mM), KCN (10 mM), enzyme preparation (1–2 mg) and 75-mM potassium phosphate, pH 7.5. The final volume was 2.5 ml. The reaction was started by the addition of NH_4Cl to a concentration of 20 mM.

	Glutamate dehydrogenase		δ -Aminolaevulate synthetase	
	Activity (nmol of NADH oxidized/min per mg of protein)	% of total activity	Activity (nmol/h per mg of protein)	% of total activity
Mitochondria	46.5	92	1.0	48
Cytosol	1.1	8	0.3	52

Table 5. *Inhibition of δ -aminolaevulate activity of kidney cytosol by protohaem*

Six rats were made porphyric by four intraperitoneal injections of allylisopropylacetamide (200 mg/kg body wt.) at 0, 12, 24 and 48 h and killed 2 h after the final injection. δ -Aminolaevulate synthetase was assayed as described in Table 3, in the presence of the protohaem concentrations indicated.

Concn. of protohaem (μM)	δ -Aminolaevulate synthetase activity (nmol of δ -aminolaevulate formed/h per mg of protein)	Inhibition (%)
0	0.282	0
30	0.186	34
60	0.114	59

Hayashi, Yoda & Kikuchi (1969) found that allylisopropylacetamide induced the formation of δ -aminolaevulate synthetase in the soluble fraction of rat liver and this has subsequently been confirmed (Scholnick *et al.* 1969; Beattie & Stuchell, 1970). The permeability properties of liver mitochondria appear unchanged by treatment with allylisopropylacetamide (Jones & Jones, 1970) and repeated freezing and thawing does not solubilize significant amounts of δ -aminolaevulate synthetase from these mitochondria (Scholnick *et al.* 1969). However, there was no information available about the fragility of kidney mitochondria in animals with induced porphyria. Since it was possible that the kidney cytosol enzyme arose from disrupted mitochondria, despite the reasonable respiratory control exhibited by these mitochondria, we compared the distribution of glutamate dehydrogenase, a freely soluble enzyme of the mitochondrial matrix, with that of δ -aminolaevulate synthetase (Table 4). The proportion of glutamate dehydrogenase found in the cytosol was always less than that of δ -aminolaevulate synthetase, indicating that the latter is a truly soluble enzyme of the cytosol.

Haem and some related compounds were shown to inhibit activity of the soluble δ -aminolaevulate synthetase of liver induced by allylisopropylacetamide (Scholnick *et al.* 1969). In Table 5 we

show a similar effect of protohaem on the cytosol enzyme from kidneys of porphyric rats.

DISCUSSION

δ -Aminolaevulate synthetase in normal tissues. δ -Aminolaevulate synthetase has been detected in cell-free preparations from normal liver (Marver, Tschudy, Perlroth & Collins, 1966), spleen (Ebert, Tschudy, Choudhry & Chirigos, 1970) and marrow (Bottomley & Smithee, 1968). All these tissues are possibly concerned in haemoglobin synthesis and the enzyme does not appear to have been previously detected in non-erythropoietic organs. The presence of δ -aminolaevulate synthetase in kidney mitochondria of normal animals (Table 3) suggests that it is a component of mitochondria even in tissues not generally associated with haemopoiesis; presumably the enzyme is involved in the synthesis of haemoproteins of the kidney cell. If this enzyme is associated with the synthesis of mitochondrial cytochromes in the kidney, it might be expected that its activity would be proportional to the turnover time of the mitochondria or to their haem content. This does not appear to be the case. From the turnover times of mitochondria from various tissues (cf. Gross, Getz & Rabinowitz, 1969) it would appear that heart mitochondria have the

shortest half-life as well as the highest cytochrome oxidase content (Table 1); yet in heart mitochondria δ -aminolaevulate synthetase could not be detected (Table 3). Similarly ferrochelatase activity (Table 1) was unrelated to mitochondrial turnover time or to oxidase content: the relatively high activities of ferrochelatase (Table 1) and δ -aminolaevulate synthetase (Table 3) in liver mitochondria suggests that this tissue may be important in the synthesis and supply of haems not only for the mitochondrial cytochromes itself but for other extra-mitochondrial haemoproteins.

δ -Aminolaevulate synthetase in tissues of porphyric rats. The induction of δ -aminolaevulate synthetase in kidney mitochondria after treatment with allylisopropylacetamide clearly demonstrates that the effect of this drug is not specific to liver as claimed by Sassa & Granick (1970). It does not, however, remove the possibility that the induction of δ -aminolaevulate synthetase is associated with drug detoxification: kidney, like liver, is well known for its participation in detoxification of some drugs. Some detoxification mechanisms are known to involve reactions catalysed by the haemoprotein cytochrome P-450 (Gillette & Gram, 1969), which is induced by the administration of certain drugs; however, a preliminary report (Marver, 1969) suggests that allylisopropylacetamide does not increase concentrations of hepatic cytochrome P-450.

No induction was found in brain or heart mitochondria. A number of explanations are possible for this: induction may be obligatorily associated with detoxification capacity; the enzyme may be induced but still at activity too low for detection; the inducer, allylisopropylacetamide, which is administered intraperitoneally, may not reach either the heart or the brain in concentrations high enough to cause induction, although its anaesthetic effect on the rats was always observed.

The process of induction in kidney mitochondria appears very similar to that in liver mitochondria: it is accompanied by a rise in δ -aminolaevulate synthetase activity in the cytosol, which may be newly formed by cytoplasmic ribosomes before incorporation into the mitochondria matrix (cf. Hayashi, Yoda & Kikuchi, 1970). Like the cytosol enzyme of porphyric liver (Scholnick *et al.* 1969) it is inhibited by protohaem: 50% inhibition is achieved in the presence of 50 μ M-protohaem.

We have been unable to demonstrate the large increase in the cytochrome content of liver mitochondria after the administration of allylisopropylacetamide reported by Beattie & Stuchell (1970), so we cannot confirm their suggestion that increased mitochondrial content of cytochromes is associated with increased intracellular concentration of δ -aminolaevulate synthetase.

We are grateful to the Science Research Council for financial support and to the Medical Research Council and Science Research Council for studentships awarded to R. B. and M. S. J.; R. J. P. thanks the Science Research Council for a Senior Visiting Fellowship. We are very grateful to Dr C. W. Jones for his collaboration in the treatment of experimental animals.

REFERENCES

- Beattie, D. S. & Stuchell, R. N. (1970). *Archs Biochem. Biophys.* **139**, 291.
- Bottomley, S. S. & Smithee, G. A. (1968). *Biochim. biophys. Acta*, **159**, 27.
- Burnham, B. F. & Lascelles, J. (1963). *Biochem. J.* **87**, 462.
- Chance, B. (1957). In *Methods in Enzymology*, vol. 4, p. 273. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Chance, B. & Williams, G. R. (1956). *Adv. Enzymol.* **17**, 65.
- Chappell, J. B. & Hansford, R. G. (1969). In *Subcellular Components, Separation and Fractionation*, p. 45. Ed. by Birnie, G. D. & Fox, S. M. London: Butterworths.
- Ebert, P. S., Tschudy, D. P., Choudhry, J. N. & Chirigos, M. A. (1970). *Biochim. biophys. Acta*, **208**, 236.
- Gillette, J. R. & Gram, J. E. (1969). In *Microsomes and Drug Oxidations*, p. 133. Ed. by Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R. & Mannering, G. J. New York and London: Academic Press.
- Granick, S. (1966). *J. biol. Chem.* **241**, 1359.
- Granick, S. & Urata, G. (1963). *J. biol. Chem.* **238**, 821.
- Gross, N. J., Getz, G. S. & Rabinowitz, M. (1969). *J. biol. Chem.* **244**, 1552.
- Hayashi, N., Yoda, B. & Kikuchi, G. (1969). *Archs Biochem. Biophys.* **131**, 83.
- Hayashi, N., Yoda, B. & Kikuchi, G. (1970). *J. Biochem., Tokyo*, **67**, 83.
- 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. biophys. Res. Commun.* **41**, 1072.
- Kaufman, S. (1955). In *Methods in Enzymology*, vol. 1, p. 719. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Labbe, R. F. & Nishida, G. (1957). *Biochim. biophys. Acta*, **26**, 437.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Marver, H. S. (1969). In *Microsomes and Drug Oxidations*, p. 513. Ed. by Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R. & Mannering, G. J. New York and London: Academic Press.
- Marver, H. S., Tschudy, D. P., Perloff, M. G. & Collins, A. (1966). *J. biol. Chem.* **241**, 2803.
- Mauzerall, D. & Granick, S. (1956). *J. biol. Chem.* **219**, 435.
- Sassa, S. & Granick, S. (1970). *Proc. natn. Acad. Sci. U.S.A.* **67**, 517.
- Scholnick, P. L., Hammaker, L. E. & Marver, H. S. (1969). *Proc. natn. Acad. Sci. U.S.A.* **63**, 65.