OSCILLATIONS OF HEPATIC δ-AMINOLEVULINIC ACID SYNTHETASE PRODUCED IN VIVO BY HEME

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Received July 21, 1966

δ-aminolevulinic acid synthetase (ALA synthetase), the first and rate controlling enzyme in heme biosynthesis is located in mitochondria and is inducible in liver (Granick and Urata, 1963; Tschudy et al., 1964; Marver et al., 1965; Granick, 1966). The induction is inhibited by glucose as effectively as by actinomycin D (Tschudy et al., 1964; Marver et al., 1966a). In the genetic disease acute intermittent porphyria a marked increase in the level of hepatic ALA synthetase has been demonstrated (Tschudy et al., 1965a) as the explanation for the increased porphyrin precursor excretion seen in this disease.

In microorganisms (R. spheroides) data have been presented which suggest control of ALA synthetase by means of both end product (heme) inhibition and repression (Lascelles, 1960; Burnham and Lascelles, 1963). However, it is doubtful that heme is a physiologically significant inhibitor of the enzyme in vivo in liver. Granick found no inhibition of ALA synthetase by heme added to liver mitochondria at concentrations of 2.5×10^{-5} M (Granick, 1966) and Marver et al., found no inhibition of the solubilized enzyme from rat liver until the heme concentration reached 5×10^{-4} M (Marver et al., unpublished). That heme is involved in the repression of hepatic ALA synthetase, however, is evidenced by its inhibition of induction of the enzyme in cultured chick embryo liver cells at concentrations of about 2.5×10^{-6} M (Granick, 1966). Further in vivo evidence of the role

of heme in repression of hepatic ALA synthetase is the fact that administration of tryptophane to rats is followed by induction of hepatic ALA synthetase (Marver et al., 1966b). This is thought to result from the increased binding of "free" or "dissociable" heme to the apoenzyme of tryptophane pyrrolase, making less heme available for repression of ALA synthetase.

The present studies demonstrate directly in vivo a role of heme in the repression of hepatic ALA synthetase as well as a series of oscillations of the hepatic enzyme which follow a single intravenous injection of heme.

Methods

In all experiments 110-130 g. Sprague-Dawley female rats were used. They were given no food for 48 hrs. prior to sacrifice but were given water ad lib. Crystalline bovine hemin (Sigma) was prepared for intravenous administration (Hammel and Bessman, 1965) in a concentration of 2 mg/ml. One ml was given intravenously over a 60-90 second interval. ALA synthetase was determined in liver homogenates (Tschudy et al., 1965; Marver et al., 1966c) and mitochondria (Tschudy et al., 1964) ALA and aminoacetone were measured by column chromatographic methods (Marver et al., 1966d) and homogeneity of ALA and aminoacetone fractions was further checked by paper chromatography of their pyrroles (Mauzerall and Granick, 1956). Allylisopropylacetamide (AIA)* was administered subcutaneously in isotonic saline 5 hrs. prior to sacrifice in a dose of 300 mg/kg. In animals not given AIA, ALA synthetase was measured by using twice the usual amount of homogenate for incubation and twice the usual amount for aminoketone analysis. Actinomycin D, 1.5 mg/kg was given subcutaneously in isotonic saline. All values in the figures were determined from the pooled liver homogenates of 4-7 animals.

Results and Discussion

The effect on hepatic ALA synthetase levels of a single intravenous injection of 2 mg hemin into normal rats is seen in Fig. 1. The irregular

*Provided by Hoffmann LaRoche

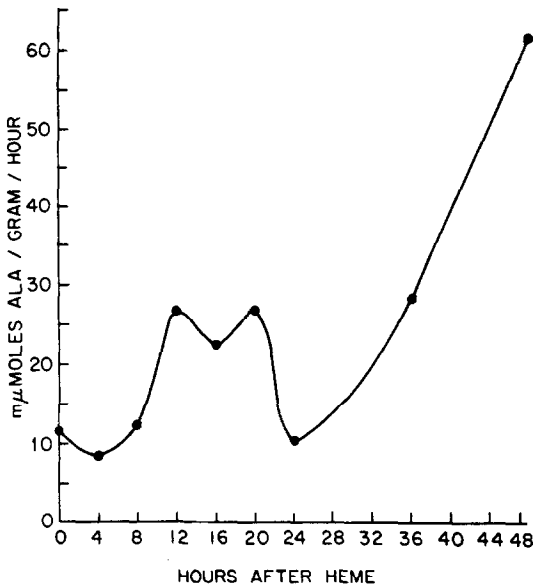

Fig. 1. Oscillations of hepatic ALA synthetase produced by intravenous administration of hemin. Each time point represents the ALA synthetase activity of a pooled liver homogenate from 4-7 rats. All animals were given 2 mg. hemin intravenously and sacrificed at the times designated above.

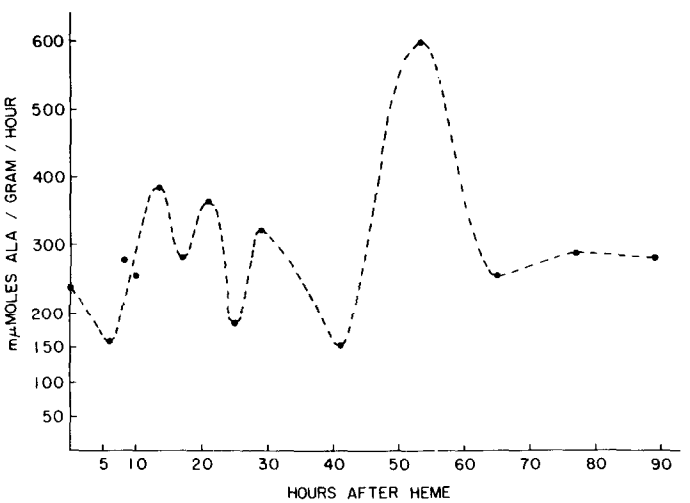

Fig. 2. The curve of hemin mediated hepatic ALA synthetase oscillations amplified by administration of allylisopropylacetamide (AIA). The method for obtaining individual time points is identical to that in Fig. 1. Amplification of the curve was obtained by administration of AIA (300 mg/kg) subcutaneously five hours prior to sacrifice.

oscillations continue for more than 48 hrs. Since the normal enzyme level is extremely low it is difficult to assess the significance of decreases below the normal level. Therefore the curve was "amplified" by administration of AIA 5 hrs. before sacrifice. This compound is known to induce hepatic ALA synthetase (Tschudy et al., 1964; Tschudy et al., 1965b; Marver et al., 1965; Granick 1966) and the shape of the induction curve is such that 5 hours is near the point of the maximum rate of rise of the enzyme (Tschudy et al., 1965b). The "amplified" curve of hepatic ALA synthetase activity following a single intravenous injection of hemin shows irregular oscillations which continue for 3 days (Fig. 2). Although minor variations in the shape of this curve were seen in repeat experiments, the notch in the curve following the first peak and the high rise occurring early on the third day were seen repeatedly.

A number of experiments were performed to demonstrate that the curve presented in Fig. 2 actually represents oscillations in the amount of ALA synthetase rather than various artifacts or changes in enzyme activation. The enzyme is measured in liver homogenates which generate optimal amounts of succinyl Co A for ALA formation (Tschudy et al., 1965a; Marver et al., 1966c). The identity and homogeneity of the ALA measured as the product of the enzyme was demonstrated by isolation of its pyrrole (2-methyl-3-acetyl-4-propionic acid pyrrole) derived from reaction with acetylacetone by both column (Marver et al., 1966d) and paper (using the butanol-ammonia system) chromatography (Mauzerall and Granick, 1956). The fact that the oscillations of ALA synthetase were seen when the enzyme was measured in isolated washed mitochondria by direct addition of the substrates glycine and succinyl Co A shows that variations in availability of substrates do not account for the findings in the homogenate system. Moreover the addition of ATP (10 mM) and succinyl Co A (25 mM) did not significantly affect ALA production in liver homogenates of hemin treated animals. The initial decline in ALA synthetase activity following intravenous hemin administration is not re-

lated to inhibition of the enzyme since the total amount of hemin given could not approach a concentration in the liver which is necessary to inhibit the enzyme in rat liver (Granick 1966; Marver et al., unpublished). Furthermore, no evidence of an inhibitor was obtained by measuring the enzyme activity of various mixtures of homogenates from high and low oscillation points as well as controls (Table 1).

Table 1

Inhibition Studies

Homogenate: Time after hemin	Predicted mean muMoles ALA/g/hr	Observed value muMoles ALA/g/hr
Control + 8 hrs	141	140
Control + 17 hrs	216	234
8 hrs + 17 hrs	180	153

ALA synthetase activity was determined in individual homogenates from the times indicated and from mixtures composed of equal parts of homogenates as indicated in the table. The observed value of the mixture is compared with the calculated mean of the two components of each mixture.

Actinomycin is known to inhibit the synthesis of hepatic ALA synthetase during induction by AIA (Marver et al., 1965, Marver et al., 1966a; Narisawa and Kikuchi, 1965). The fact that it prevents the hemin mediated increases when hemin is given alone or in combination with AIA is shown in Figs. 3 and 4. This implies that the increases of ALA synthetase which occur after hemin administration are related to increases in the rate of synthesis of the enzyme mediated via increased synthesis of its m RNA.

The above data suggest that a single intravenous injection of heme is followed by irregular oscillations in the amount of hepatic ALA synthetase. The findings are most readily explained by the assumption of a closed negative feedback system of the type presented in Fig. 5. Hemin is presumed to form a repressor which inhibits the synthesis of ALA synthetase. For the

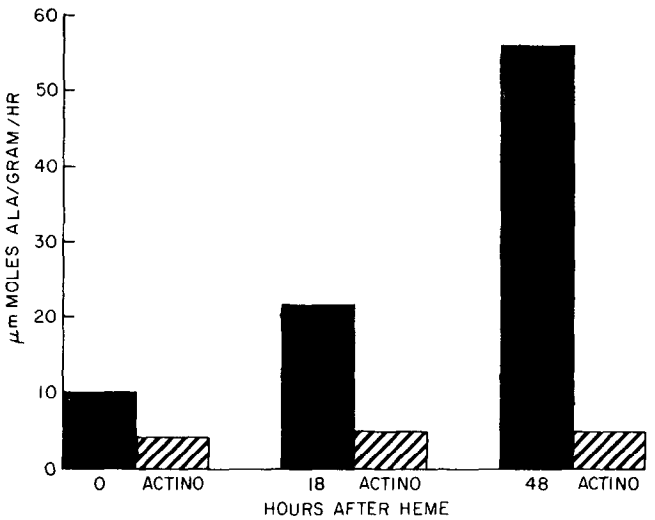

Fig. 3. The effect of actinomycin D on increases of hepatic ALA synthetase which follow hemin administration. Solid bars represent the ALA synthetase activities of pooled liver homogenate following the intravenous administration of 2 mg hemin at the times designated prior to sacrifice. The lined bars represent ALA synthetase activity of comparable groups given actinomycin D (1.5 mg/kg) subcutaneously 6 hours prior to sacrifice in addition to the hemin.

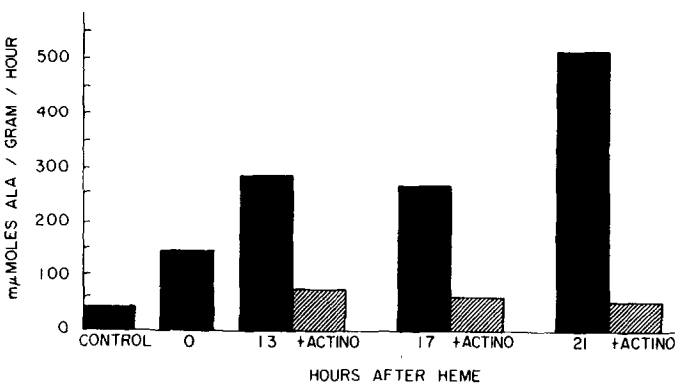

Fig. 4. The effect of actinomycin D on the AIA amplified increases of hepatic ALA synthetase which follow intravenous hemin administration. The bar representation is similar to Fig. 3. Amplification was obtained by subcutaneous administration of AIA (300 mg/kg) five hours prior to sacrifice in all groups except "control." "Control" represents the baseline hepatic ALA synthetase level of a pooled homogenate of seven animals who did not receive AIA or hemin. "0" represents the hepatic ALA synthetase seen in animals given only AIA prior to sacrifice.

present purposes it is immaterial whether the inhibition is exercised at the genetic or ribosomal level. The injected hemin enters liver cells and shuts off the synthesis of ALA synthetase, accounting for the initial decline in enzyme level seen in Figs. 1 and 2. The enlarged intrahepatic heme pool is

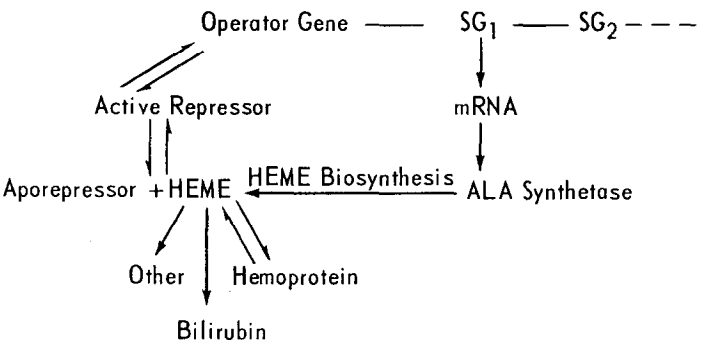

Fig. 5. Closed feedback representation of heme acting in the repression of ALA synthetase. SG: structural gene. mRNA: messenger RNA.

metabolized rapidly to bilirubin, etc., and the intrahepatic "repressor heme" concentration then falls to levels below normal because the lowered level of ALA synthetase prevents its replacement by synthesis. This allows the rate of synthesis of ALA synthetase to then increase until it has produced enough heme to again curtail its own synthesis. The kinetic parameters which allow oscillations to occur in this system are related to 1) the turnover rate of ALA synthetase, its messenger RNA and "repressor heme" and 2) the delay or lag time of changes in the levels of "repressor heme" and changes in the levels of ALA synthetase which result from this. The half life of rat hepatic ALA synthetase and its messenger RNA have been estimated as about 1 hr., making it a short lived enzyme with short lived messenger RNA (Tschudy et al., 1965b).

Direct measurements of the other kinetic parameters of this system are not available and hence detailed analysis of the oscillatory curve is

impossible at present. The complexity of the system is indicated by the complicated nature of the wave form seen in Figs. 1 and 2.

Oscillations at the enzymatic level have previously been observed in glycolyzing preparations from yeast (Chance et al., 1965: Pye and Chance, 1966) and beef heart (Frenkel, 1965). However, the observed oscillations of NADH in these systems differ from the present one in that they do not represent changes in the synthesis of an enzyme, but reflect variations in oxidation-reduction states with time. The fact that major oscillations of hepatic ALA synthetase in vivo can continue for at least 3 days after a single perturbation of the intrahepatic heme level suggests that this enzyme may be involved in biological clock mechanisms. The enzyme is inducible and a number of different type compounds have been implicated as inducers (Granick, 1966). If one assumes a mechanism of control as outlined in Fig. 5, it can be seen that oscillations will not occur while an inducer is present in significant quantity. Failure of AIA to produce oscillations within 48 hrs. after its administration (Tschudy et al., Marver et al., 1966a) is presumably related to a slow metabolism of this compound. Inducers which are present at the proper levels for a shorter period of time (perhaps 4-12 hrs.) might initiate relatively high amplitude oscillations in the enzyme level which would be observed within the first two days.

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