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Control of Haem Synthesis by Feedback Inhibition on Human-Erythrocyte δ-Aminolaevulate Dehydratase

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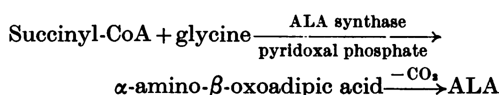
The action of haem, haemoglobin and other haemoproteins on crude and purified δ-aminolaevulate dehydratase of human erythrocyte is described. The results show a feedback inhibition by haem of porphyrin synthesis at the level of erythrocyte δ-aminolaevulate dehydratase. Some kinetic characteristics of this inhibition are described.

Burnham & Lascelles (1963) demonstrated in *Rhodopseudomonas spheroides* preparations an inhibition by haem of ALA* synthase, which catalyses the condensation of glycine with succinyl-CoA. These authors suggested that a negative-feedback control by haem of porphyrin synthesis in *R. spheroides* occurs at the level of ALA synthesis. Karibian & London (1965) studied the effects of haem on the incorporation of [2-^{14}C]glycine and [4-^{14}C]ALA into the haem of rabbit reticulocytes. The results showed a negative-feedback mechanism that involved the conversion of glycine into ALA. According to Karibian & London (1965), in agreement with Burnham & Lascelles (1963), it was likely that the feedback control of haem synthesis was at the level of ALA synthase. They did not exclude the possibility that it might involve the transformation of α-amino-β-oxoadipic acid into ALA:



$$\text{Succinyl-CoA} + \text{glycine} \xrightarrow[\text{pyridoxal phosphate}]{\text{ALA synthase}} \alpha\text{-amino-}\beta\text{-oxoadipic acid} \xrightarrow{-CO_2} \text{ALA}$$

We now report the results of experiments on the effect of haem, haemoglobin and other haemoproteins on crude and purified ALA dehydratase of human erythrocytes. Our results point to another mechanism of feedback control by haem on porphyrin synthesis at the level of ALA dehydratase. Some kinetic characteristics of this inhibition are also described.

EXPERIMENTAL

Materials

Recrystallized haem was supplied by the Eastman Organic Chemicals Department, Division of Eastman Kodak Co., Rochester, N.Y., U.S.A. The solution was prepared by the method of Burnham & Lascelles (1963) and utilized within 2hr. after preparation. The solution was diluted to 5ml. with freshly boiled distilled water. A 0·01M solution was thus obtained, which could be further diluted.

Recrystallized haemoglobin was supplied by Sigma Chemical Co., St Louis, Mo., U.S.A.

Cytochrome *c* and catalase were supplied by Boehringer, Milan, Italy.

Methods

The ALA-dehydratase activity was estimated by the formation of porphobilinogen, which was estimated by the colour developed at 555mμ with *p*-dimethylaminobenzaldehyde in acid solution after 10min. (Treibs & Herrman, 1955). One unit of enzyme is defined as that quantity causing a change in E_{555} of 0·1 unit after 30min. incubation in a mixture made up as described in Table and Figure legends.

Proteins were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Purification of the enzyme by means of ammonium sulphate fractionation

In the purification process described below all centrifugations were carried out for 15min. at 15000*g* for the purpose of discarding or collecting precipitates, all sediments containing enzymic activity were dissolved in water (since cysteine, GSH, mercaptoethanol and other thiol compounds did not stabilize the enzyme better than water) and all procedures were carried out at 2°.

The specific activity and results for each step of the purification are shown in Table 1.

Preparation of the haemolysate. A 200ml. blood sample in acid–citrate–glucose was centrifuged at 2000*g* and the supernatant discarded. The packed cells (175ml.) were washed three times with 2vol. of 0·15M-KCl and then haemolysed by adding water to give a final volume of 425ml. The stroma were not separated.

First ammonium sulphate fractionation. A 500ml. volume of haemolysate was brought to 20% saturation with solid

* Abbreviation: ALA, δ-aminolaevulate.

Table 1. *Purification of ALA dehydratase by ammonium sulphate fractionation*

Experimental details are given in the text.

Fraction	Activity (units/ml.)	Total activity (units)	Yield (%)	Specific activity (units/mg. of protein)	Purification
Haemolysate	5·0	2120	100	0·034	—
First $(NH_4)_2SO_4$ fraction	14·4	1540	73	1·50	44
Second $(NH_4)_2SO_4$ fraction	12·7	1270	60	7·20	210
Third $(NH_4)_2SO_4$ fraction	13·2	1080	51	28·0	820
Fourth $(NH_4)_2SO_4$ fraction	46·2	970	47	41·0	1200

$(NH_4)_2SO_4$ at pH 7 and the precipitate removed after 10 min. at 2° by centrifugation. The supernatant was brought successively to 50% and then 55% saturation with $(NH_4)_2SO_4$ at pH 7. After 10 min. in each case the two precipitates were collected separately by centrifugation, dissolved in water and combined (first fraction, 107 ml.).

Second ammonium sulphate fractionation. The first fraction was brought successively to 45% and then 55% saturation with solid $(NH_4)_2SO_4$ at pH 7. After 10 min. in each case the two precipitates were collected separately by centrifugation as before, dissolved in water and combined (second fraction, 100 ml.).

Third ammonium sulphate fractionation. The second fraction was brought to 30% saturation with solid $(NH_4)_2SO_4$ at pH 7 and the precipitate discarded. The supernatant was brought successively to 40%, 45% and 50% saturation with solid $(NH_4)_2SO_4$ at pH 7. After 10 min. in each case the three precipitates were collected separately by centrifugation, dissolved in water and combined (third fraction, 83 ml.).

Fourth ammonium sulphate fractionation. The third fraction was brought to 30% saturation with solid $(NH_4)_2SO_4$ at pH 7 and the precipitate was discarded. The supernatant was brought successively to 40%, 45% and 50% saturation with solid $(NH_4)_2SO_4$ at pH 7. After 10 min. in each case the three precipitates were collected separately by centrifugation as before, dissolved in water and combined (fourth fraction, 21 ml.).

As shown in Table 1, this procedure gives a purification of about 1200-fold and 47% yield.

RESULTS

In preliminary experiments we observed an inhibition by crude haemolysate on human-erythrocyte ALA dehydratase purified 1200-fold. This fact, together with an increase in the total enzymic activity during the process of purification, could be attributed to an inhibition by the haemoglobin removed during purification. ALA-dehydratase activity in the presence of haemoglobin and other haemoproteins, such as cytochrome *c* and catalase, was studied.

Only haemoglobin inhibits the enzyme (by 30%) at a concentration of 0·01 μmole of haem/ml.; even at a concentration of 0·1 μmole of haem/ml. inhibition by haemoglobin is incomplete (80%), as shown in Table 2.

Table 2. *Effect of haemoproteins on ALA dehydratase*

Incubation mixtures contained: 0·16 mg. of purified enzyme (corresponding to 6·57 units), 125 μmoles of glycylglycine buffer, pH 7·5, 10 μmoles of cysteine, 10 μmoles of ALA and haemoproteins as indicated, in a final volume of 2·0 ml. Incubation was for 30 min. and precipitation was carried out with 1 vol. of 10% (w/v) trichloroacetic acid–$HgCl_2$. The activities are expressed as percentages of the values obtained without haemoproteins.

Haemoprotein added	Concn. of haemoprotein (μmole of haem/ml.)	Activity (% of control)
Cytochrome *c*	0·01	100
Catalase	0·01	100
Haemoglobin	0·10	20
Haemoglobin	0·05	30
Haemoglobin	0·01	70

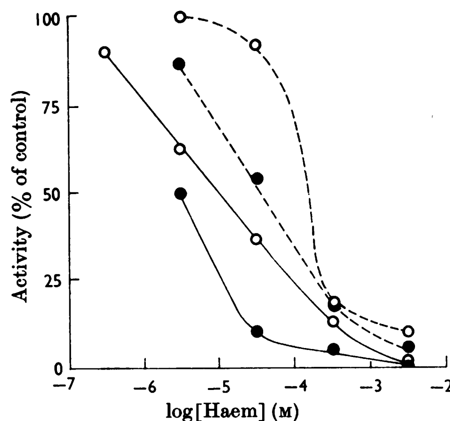


Fig. 1. Action of haem with and without cysteine on crude and purified ALA dehydratase. The incubation mixtures contained: 0·16 mg. of purified enzyme (corresponding to 6·57 units) (——) or 0·2 ml. of crude enzyme (corresponding to 1·0 units) (– – –), 125 μmoles of tris–HCl buffer, pH 7·5, and 10 μmoles of cysteine, where indicated. Haem, prepared as described in the text, was added to the concentrations indicated, in a final volume of 2 ml. Incubation was for 120 min. at 37°. The activities are expressed as percentages of those in similar experiments but without haem. ○, Cysteine present; ●, cysteine absent.

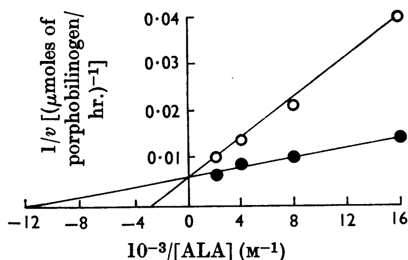


Fig. 2. Affinity of ALA dehydratase for the substrate with and without haem. The purified enzyme was previously incubated for 15 min. with 25 μmoles of cysteine/ml. Then 0·32 mg. of enzyme was added to the mixtures containing: 125 μmoles of glycylglycine buffer, pH 7, ALA as indicated, and 0·02 μmole of haem, in a final volume of 2 ml. Incubation was at 37°. The results were plotted according to the procedure of Lineweaver & Burk (1934). ○, Haem present; ●, haem absent.

The action of haem on ALA-dehydratase activity was studied with the crude and purified enzyme. Fig. 1 shows that haem strongly inhibits both enzyme preparations. The inhibition was greater with the purified enzyme than with the crude enzyme. This may be due to small quantities of haem present in the crude enzyme. However, it is notable that inhibition by haem at 50 μM decreased the enzymic activity by 50% in purified preparations and by 40% in crude preparations.

These experiments were carried out in parallel with and without cysteine. Cysteine may be involved in activating the enzyme, as suggested by experiments with lead acetate and EDTA (Calissano, Cartasegna & Matteini, 1966; Bonsignore, Calissano & Cartasegna, 1965*a*,*b*,*c*). This thiol compound, however, increased inhibition by haem, whereas it reversed the inhibition caused by lead acetate to about 60% of the original activity. It would appear that there are two mechanisms of inhibition, lead acetate acting on the SH groups of the enzyme, but haem acting on another active site of the enzyme.

To ascertain whether inhibition by haem could be removed, at least partially, with high concentrations of substrate, we determined the K_m of the enzyme with and without haem at 10 μM. Fig. 2 shows the inhibition to be competitive and the K_i is $3 \cdot 5 \times 10^{-4}$ M. The K_m for the purified enzyme is $8 \cdot 3 \times 10^{-5}$ M, whereas the K_m for the enzyme with haem is $3 \cdot 2 \times 10^{-4}$ M; this value is equal to the value of the crude enzyme where the presence of free haem or haem bound to haemoglobin may exert an action similar to that of haem on the purified enzyme.

The results of these preliminary experiments demonstrate a negative-feedback inhibition by haem on human-erythrocyte ALA dehydratase. In addition, haem bound to globin, but not haem bound to other proteins, can cause an inhibition of ALA dehydratase that is about one-tenth as effective as inhibition by haem itself. These experiments further emphasize the control of haem on the metabolism of haemoglobin; in fact haem inhibits at least two enzymic reactions involved in its biosynthesis: ALA synthase and ALA dehydratase. On the other hand, it stimulates the formation of globin (Bruns & London, 1965).

REFERENCES

Bonsignore, D., Calissano, P. & Cartasegna, C. (1965*a*). *Med. d. Lavoro*, **56**, 199.
Bonsignore, D., Calissano, P. & Cartasegna, C. (1965*b*). *Lav. Um.* **17**, 493.
Bonsignore, D., Calissano, P. & Cartasegna, C. (1965*c*). *Med. d. Lavoro*, **56**, 727.
Bruns, G. P. & London, I. M. (1965). *Biochem. biophys. Res. Commun.* **18**, 236.
Burnham, B. F. & Lascelles, J. (1963). *Biochem. J.* **87**, 462.
Calissano, P., Cartasegna, C. & Matteini, M. (1966). *Ital. J. Biochem.* **15**, 18.
Karibian, D. & London, I. M. (1965). *Biochem. biophys. Res. Commun.* **18**, 243.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Treibs, A. & Herrman, E. (1955). *Hoppe-Seyl. Z.* **299**, 171.