EVIDENCE FOR A NEGATIVE FEEDBACK SYSTEM IN THE CONTROL OF PORPHYRIN BIOSYNTHESIS

Bruce F. Burnham*
Microbiology Unit, Department of Biochemistry,
University of Oxford, Oxford, England

Received January 16, 1962

Previous investigations upon the control of porphyrin biosynthesis have been carried out in this laboratory by Lascelles (1956, 1960). The photosynthetic bacterium Rhodopseudomonas spheroides when cultured anaerobically in the light under iron deficient conditions accumulated large quantities of porphyrin in the medium; addition of iron resulted in a decreased production of porphyrin. The effect seemed to be catalytic and not the result of formation of an equivalent amount of hemin, as demonstrated with C. diphtheriae (Pappenheimer, 1947). In R. spheroides the synthesis of two enzymes required for porphyrin biosynthesis, δ-aminolevulinic acid (ALA) synthetase, and ALA dehydrase, was repressed by oxygen, ALA, and hemin (Lascelles, 1960). Hemin was also found to inhibit the activity of ALA synthetase in crude sonic extracts of R. spheroides (Lascelles, unpublished experiments). Similar results have also been reported by Gibson et al. (1961).

The present investigation was undertaken to examine the possibility that iron added to deficient cultures exerted its apparent catalytic influence upon porphyrin formation after being incorporated into hemin, which, in turn, inhibited the activity of an enzyme concerned in porphyrin synthesis through a negative

*Fellow of The Jane Coffin Childs Memorial Fund for Medical Research

feedback mechanism. In other negative feedback systems, the final product in a metabolic sequence inhibits the first enzymatic step that leads directly and exclusively to the end product.

The present results indicate that such a negative feedback system may operate as one of the mechanisms for control of tetrapyrrole biosynthesis.

Experimental

R. spheroides were cultured in the light on malate-glutamate medium, harvested and sonicated as described by Lascelles (1956, 1960).

All tetrapyrrole compounds tested in the enzyme reactions were prepared within one hour of use.

ALA was determined by the method of Mauzerall and Granick (1956). ALA synthetase activity was measured in a standard assay mixture containing (μ moles): glycine 100; succinate 100; CoA 0.58; pyridoxal phosphate 0.25; ATP 7.5; $MgCl_2$ 10; tris buffer pH 7.8, 50; EDTA 1.0; succinic thiokinase, inhibitors, water and ALA synthetase to 1.0 ml. Specific activity is expressed as mμ moles ALA/hr/mg protein at 37°. Succinic thiokinase, assayed according to the method of Lipmann and Tuttle (1945), was added to the system as a cyclic generator of succinyl CoA. The quantity of succinic thiokinase per reaction was sufficient to generate 1.6 μ mole of succinyl CoA or roughly 2-fold in excess of the ALA formed.

Purification of ALA synthetase -- The crude sonic extract of R. spheroides was centrifuged at 34,000 x g. for 20 min. The supernatant was added to 7 volumes of acetone at -20° and stood for 10 min. The precipitate was collected by centrifugation, and was washed with one volume of cold acetone. The precipitate was extracted twice with $\frac{1}{2}$ volume of 0.1 M tris buffer pH 7.5,

containing 0.01 M β mercapto ethanol, 0.001 M EDTA, and 0.0005 M o-phenanthroline. An equal volume of saturated $(NH_4)_2SO_4$ was added to these combined supernatants. After standing at 1° for 30 min., the precipitate was removed by centrifugation. The precipitate was extracted twice with 1/6 volumes of 0.1 M tris buffer as above. The combined supernatants were dialyzed against 0.01 M tris buffer containing 0.01 M β mercapto ethanol, and 0.001 M EDTA for several hours.

The partially purified ALA synthetase had a specific activity of about 2,000 mμ mole ALA/hr/mg, representing a 20-fold purification with an 80% yield. Such a preparation was stable for several days at 1° without appreciable loss of activity.

Preparation of succinic thiokinase -- The crude sonic extract of R. spheroides was centrifuged to remove cell debris. The supernatant was treated batchwise with protamine sulfate, following which it was fractionated with $(NH_4)_2SO_4$. The fraction which precipitated between 40 and 60% saturation contained most of the succinic thiokinase as was noted by Kikuchi et al. (1958). This material was further purified on DEAE cellulose, yielding a product with a specific activity of 560 μ mole/hr/mg.

Results and Discussion

Protohemin IX was a relatively potent inhibitor of ALA synthetase (Table 1). This inhibition was examined as a function of glycine concentration, and it was found to be non-competative with glycine. The specificity of the inhibition was examined by measuring the activity of the enzyme in the presence of several porphyrins and their metal complexes. The inhibition (Table II) is quite specific for protohemin IX. Iron hematoporphyrin, cobalt protoporphyrin, manganese protoporphyrin, and magnesium protoporphyrin had only slight inhibitory

Table I

Inhibition of ALA Synthetase by Protohemin IX

Additions to standard assay	Enzyme activity mμ mole ALA/hr/mg
None (control)	1,990
Hemin (10^{-4} M)*	490
Hemin (10^{-5} M)	1,120
Hemin (10^{-6} M)	1,480
None (control)	1,900

*Final concentration in assay mixture

Table II

Specificity of Inhibition by Tetrapyrrole Compounds

Additions to standard assay*	Enzyme activity mμ mole ALA/hr/mg
None (control)	1,900
Mn Protoporphyrin IX	1,835
Co Protoporphyrin IX	1,660
Mg Protoporphyrin IX	1,820
Fe Protoporphyrin IX	820
Protoporphyrin IX	1,690
Fe Hematoporphyrin	1,625
Hematoporphyrin	1,740
None (control)	1,860

*All additions to give 2 x 10^{-5} M final concentration

action. The corresponding free porphyrins caused only slight inhibition. The inhibition caused by protohemin IX was reversible (Table III). Enzyme was preincubated 5 min at 0° with 10^{-5} M hemin, then diluted by a factor of 5 in the assay. The activity of such enzyme corresponded to that in systems which had 2 x 10^{-6} M hemin added to the reaction mixture.

Several metals were examined as possible inhibitors of ALA synthetase. Of those tested, only Fe^{++} was found to have any appreciable effect at a concentration of 10^{-4} M (Table IV). The inhibition by Fe^{++} could be overcome by o-phenanthroline or by increasing the concentration of pyridoxal phosphate.

Table III

Concentration of hemin preincubated with enzyme	Concentration of hemin in assay mixture	Enzyme activity mμ mole ALA/hr/mg
0	0	1,800
0	10^{-5} M	910
0	2×10^{-6} M	1,370
10^{-5} M	2×10^{-6} M	1,320
10^{-5} M	10^{-6} M	1,640

Table IV

Effect of Metal Ions upon ALA Synthetase*

Addition to standard assay	Enzyme activity - % of control
None (control)	100
Mg^{++} (10^{-3} M)	100
Mn^{++} (10^{-4} M)	105
Zn^{++} (10^{-4} M)	90
Cu^{++} (10^{-5} M)	100
Fe^{++} (10^{-4} M)	45
Fe^{+++} (10^{-4} M)	97
Fe^{++} 10^{-4} M + o-phenanthroline (10^{-3} M)	93
Fe^{++} (10^{-4} M) + pyridoxal phosphate (10^{-3} M)	98

*Crude sonic extracts used in these experiments

All inhibitors were also tested for their effect on succinic thiokinase since the inhibition of the succinyl CoA generating system would cause decreased formation of ALA in the standard assay mixture. This enzyme was not adversely affected by any of the inhibitors at the concentrations employed in the ALA synthetase assay. That the effect of hemin was not connected with the formation of succinyl CoA was further demonstrated by using chemically prepared succinyl CoA (Simon and Shemin, 1953), glycine, and pyridoxal phosphate as substrates for ALA synthetase. Under these assay conditions, 2×10^{-5} M hemin caused 50% inhibition.

The results of this preliminary investigation indicate that the inhibition of ALA synthetase by hemin may be of physiological importance in the control of porphyrin biosynthesis. The fact that this inhibition is apparent at very low concentrations of hemin, that the inhibition is reversible, and that it is specific, all support this possibility. It should be noted, however, that R. spheroides also forms bacteriochlorophyll; a negative feedback by hemin cannot therefore be the only factor controlling tetrapyrrole synthesis. Further work on the purification of ALA synthetase and succinic thiokinase is in progress. These results, and more detailed experiments on the nature of the inhibition by ferrous iron and hemin will be published subsequently.

This work was aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research. The author wishes to especially thank Dr June Lascelles for her help and encouragement in this investigation.

References

Gibson, K.D., Mathew, M., Neuberger, A. and Tait, G.H., Nature, 192, 204, (1961).

Kikuchi, G., Kumar, A., Talmage, P., and Shemin, D., J. Biol. Chem., 233, 1214, (1958).

Lascelles, J., Biochem. J., 62, 78, (1956).

Lascelles, J., J. Gen. Microbiol., 23, 487, (1960).

Lipmann, F., and Tuttle, L.C., J. Biol. Chem., 159, 21, (1945).

Mauzerall, D., and Granick, S., J. Biol. Chem., 219, 435, (1956).

Pappenheimer, A.M., J. Biol. Chem., 167, 251, (1947).

Simon, E.J., and Shemin, D., J. Am. Chem. Soc., 75, 2520, (1956).