ROLE OF IRON IN THE REGULATION OF HEME BIOSYNTHESIS IN *NEUROSPORA CRASSA*

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In the photosynthetic bacterium *Rhodopseudomonas spheroides*, heme has been shown to regulate its own synthesis by exerting a repressive as well as a feed-back control on δ-aminolevulinic acid synthetase (ALA synthetase), the first enzyme of the heme biosynthetic pathway (Lascelles, 1960; Burnham and Lascelles, 1963). In iron deficiency this organism accumulates porphyrins, since the control on the first enzyme is released owing to the metal being unavailable for insertion into the porphyrin nucleus. Many bacteria, yeasts and the ciliate *Tetrahymena* accumulate porphyrins in iron deficiency (Lascelles, 1964). The mold *Neurospora crassa* does not accumulate porphyrins in iron deficiency but instead accumulates a sideramine, which is a cyclic trihydroxamate having a profound binding affinity for iron (Padmanaban and Sarma, 1964). Sideramines have been widely detected in fungi (Keller-Schierlein *et al*, 1965). The findings that *N. crassa* fails to accumulate porphyrins in iron deficiency even though heme synthesis is significantly lowered and that the addition of iron to an iron-deficient

culture results in induced synthesis of heme suggest that iron may regulate an enzyme or enzymes of the heme biosynthetic pathway prior to porphyrin formation (Padmanaban et al, 1967). The results obtained in the present investigation indicate that δ-aminolevulinic acid dehydratase (ALA dehydratase), the second enzyme of the pathway may represent a major site of regulation of heme biosynthesis in this organism.

EXPERIMENTAL

N. crassa Em 5297a (wild) was grown in stationary cultures in 50 ml flasks containing 10 ml of the basal medium under normal and iron-deficient conditions as described by Padmanaban and Sarma (1965). After 40 hrs. growth suitable additions were made and the flasks were shaken for 2 hrs (Padmanaban et al, 1967). The mycelia which received identical treatment were then pooled and the crude extract prepared using potassium phosphate buffer (0.05 M, pH 7.5) containing 0.01 M cysteine. ALA dehydratase was too low in the crude extract and hence the enzyme was concentrated by preparing the 25 - 40 $(NH_4)_2SO_4$ fraction, which accounted for all the enzyme activity. The enzyme was assayed essentially according to the method of Burnham and Lascelles (1963). The porphobilinogen formed was estimated using perchloric-Ehrlich reagent after arresting the reaction with TCA-Hg reagent (Granick and Mauzerall, 1958). Total phrphyrins in the mycelia and culture filtrates were estimated after extraction with ethyl acetate: acetic acid (3:1 v/v). The porphyrins were extracted from the ethyl acetate layer using 10% HCl and the optical density measured at the peak of the soret band (Kreimer - Birnbaum et al, 1965). The ALA and

porphobilinogen contents of the mycelia were measured in 10% TCA extracts after column fractionation of these components according to the procedure of Urata and Granick (1963). The experiments were repeated at least four times and the results presented are those obtained in a typical experiment.

RESULTS AND DISCUSSION

The data presented in Table 1 indicate that while there is no accumulation of porphyrins in the iron-deficient cultures of N. crassa, there is a significant accumulation of δ-amino levulinic acid. This suggested that ALA dehydratase, the enzyme catalysing the formation of porphobilinogen from δ-amino levulinic acid might be lowered in iron deficiency.

Table 1. Levels of heme biosynthetic intermediates in iron deficiency in N. crassa

Condition	Level of the intermediates mμ moles/100 mg dry wt		Total porphyrin
	ALA	Porphobilinogen	
Normal	34.0	Nil	14.2
Iron-deficient	114.7	Nil	13.5

The same levels of porphyrins in the normal and deficient cultures despite the accumulation of δ-aminolevulinic acid in the latter might be due to the fact that further utilization of the porphyrins would also be impaired due to the non-availability of the metal.

From the results presented in Table 2, it is clear that ALA dehydratase level is low in iron deficiency. Addition of iron at a level of 1 μg/10 ml of medium followed by incubation

Table 2. Effect of iron on ALA dehydratase in N. crassa

40 hrs. old N. crassa cultures were shaken for 2 hrs. after appropriate additions were made. The enzyme assay mixture in a total volume of 2 ml contained: ALA- 5 µmoles; $MgCl_2$- 10 µmoles; GSH- 15 µmoles; Buffer (pH 7.5) - 25 µmoles: protein - 5 to 7 mg. After 2 hrs. incubation at 37° the reaction was stopped with TCA-Hg reagent and the porphobilinogen formed was estimated.

Condition	Additions to 10 ml medium	ALA dehydratase level (Porphobilinogen formed mµmoles/mg protein)
Normal	Nil	2.32
Normal	Iron (1 µg)	2.32
Normal	Iron (1 µg) + cycloheximide (5 µg)	0.96
Deficient	Nil	0.96
Deficient	Iron (1 µg)	2.50
Deficient	Iron (10 µg)	2.53
Deficient	Iron (1 µg) + cycloheximide (5 µg)	0.50
Deficient	Iron (1 µg) + protoporphyrin (1.8×10^{-6}M)	0.92
Deficient	Iron (10 µg) + protoporphyrin (1.8×10^{-6}M)	1.55
Deficient	Iron (1 µg) + protoporphyrin (9.0×10^{-6}M)	Nil
Deficient	Iron (1 µg) + Hemin (1.8×10^{-6}M)	2.20
Deficient	Iron (1 µg)+ Hemin (9.0×10^{-6}M)	1.64

The zero time ALA dehydratase levels for the normal and iron-deficient mycelia just before shaking were 1.74 and 0.58 respectively.

of the flasks with shaking for 2 hrs. results in a striking increase in the dehydratase level. Even in the absence of added iron there is some increase in the enzyme level when the iron -deficient cultures are put to shaking. Increased aeration may elicit this response and endogenous iron is probably made available for this process by some unknown

mechanism. The increase in dehydratase level due to added iron is completely blocked by cycloheximide, a potent inhibitor of protein synthesis in N. crassa (Pall, 1966). The increase in the enzyme level in normal cultures for the same period of incubation is marginal. Addition of iron to the normal cultures has no effect on this process. However, addition of cycloheximide not only blocks the marginal increase but also results in a considerable decrease over the initial level of the enzyme. This trend is also noticeable in the iron-deficient cultures but to a lesser degree. It appears possible that the turnover of the enzyme in normal mycelia may be greater and in the absence of any synthesis due to the presence of cycloheximide, enzyme breakdown can result in a considerable decrease in its level. Neither cycloheximide nor iron has any effect on the enzyme activity when included in the assay mixture in vitro. These results suggest that iron induces the synthesis of ALA dehydratase when added to iron-deficient cultures.

As a working hypothesis it can be proposed that iron may inactivate a corepressor for the enzyme ALA dehydratase. The possibility whether protoporphyrin can function as the corepressor for this enzyme has been tested. It can be seen from Table 2 that protoporphyrin at a level of 1.8×10^{-6}M represses the ALA dehydratase enzyme by about 61%. Hemin at the same level has very little effect and at five times this concentration represses the enzyme by only 34%. Neither protoporphyrin nor hemin has any effect in vitro. It can thus be explained that when the iron deficiency effects set in, even though iron is unavailable for insertion into

the tetrapyrrole nucleus at the final step of heme synthesis, porphyrins do not accumulate because protoporphyrin represses ALA dehydratase. The iron status of the mycelium should then decide whether protoporphyrin is available for the corepressor function or is fixed as heme in heme proteins. Even in the iron-deficient cultures it can be seen that when the added iron level is increased from 1 μg to 10 μg, there is a partial counteraction of the repressive effect due to protoporphyrin (Table 2).

These results are also striking from the view point that ALA dehydratase may represent at least a major site of regulation of heme biosynthesis in fungi which do not accumulate porphyrins in iron deficiency. This is in contrast to the results obtained in R. spheroides (Lascelles, 1964) and animal systems which also accumulate porphyrins under conditions of drug induced porphyria (Granick, 1962). In all these systems ALA synthetase has been shown to be the regulating enzyme with heme serving the corepressor function, the other enzymes of the pathway being present in non-limiting amounts (Lascelles, 1964; Granick, 1966; Waxman et al, 1966).

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