SITE OF ACTION OF IRON IN THE INDUCTION OF δ-AMINOLEVULINATE DEHYDRATASE IN NEUROSPORA CRASSA

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SUMMARY: δ-Aminolevulinate (ALA) dehydratase, the second and rate limiting enzyme of the heme biosynthetic pathway in the mold Neurospora crassa is induced maximally in 30 min by the addition of iron to iron-deficient cultures. The induction of the enzyme is blocked by cycloheximide, protoporphyrin, 8-azaguanine and cordycepin. Iron also brings about an increase in poly(A)-containing RNA synthesis under conditions of induction. The iron dependent increase in poly(A)-containing RNA synthesis is blocked by protoporphyrin. It is suggested that at the time intervals examined, bulk of the messenger RNA synthesized in response to iron addition represents the messenger for ALA dehydratase.

δ-Aminolevulinate (ALA) dehydratase, the second enzyme of the heme-biosynthetic pathway catalizing the conversion of ALA to porphobilinogen (PBG), was shown to be the rate-limiting enzyme of the pathway in the mold Neurospora crassa (Muthukrishnan et al., 1968, 1969). In mammalian liver the enzyme is present in 80-fold excess of ALA synthetase, the latter being the first and rate-limiting enzyme of the heme-biosynthetic pathway in a variety of systems (Tschudy et al., 1965).

The properties of ALA dehydratase and features of its induction in N. crassa have clearly indicated its regulatory function. In addition, the enzyme activity is almost undetectable in iron-deficient cultures and is strikingly induced after the addition of

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iron. The induction of the enzyme is blocked by cycloheximide as well as by protoporphyrin, which is the penultimate product of the heme-biosynthetic pathway. The enzyme is also feed-back inhibited by coproporphyrinogen III. A protein inhibitor and a small molecule activator for the enzyme are also present in N. crassa extracts (Muthukrishnan et al., 1968, 1969, 1972; Satyanarayana Rao et al., 1970).

The exact site of action of iron in the induction process, especially the mediation of RNA synthesis could not be clearly elucidated because of permeability problems faced with classical transcription inhibitors such as actinomycin D in N. crassa. In the present study this aspect has been examined with use of inhibitors such as 8-azaguanine and cordycepin as well as direct measurement of RNA synthesis.

EXPERIMENTAL

N. crassa Em 5297a (wild) was grown in stationary cultures in 250 ml flasks containing 25 ml of the basal medium under normal and iron-deficient conditions as described by Padmanaban and Sarma (1965). After 72 hr growth, the cultures received appropriate treatments and identically treated mycelia from three cultures were pooled and processed for measurement of enzyme activity and RNA synthesis.

The iron-deficient cultures were pretreated for 2 hr with 8-azaguanine (200 µg/ml medium) and for 10 min with cordycepin (40 µg/ml medium) before the addition of iron. Protoporphyrin (2 µg/ml medium) and cycloheximide (1 µg/ml medium) were added 5 min before addition of iron. The cultures were shaken during this period and the enzyme was induced by the addition of ferric citrate (1 µg iron/ml medium) and the mycelia were pooled 30 min after the addition of the inducer. ALA dehydratase was assayed in the 0-0.4 ammonium sulphate fraction as described by Muthukrishnan et al. (1969).

For measurement of RNA synthesis during enzyme induction, [^{3}H]-uridine or ^{32}P (carrier free) was added to the iron-deficient cultures 5 min after the addition of iron. The mycelia were washed and removed 10 min after the addition of the tracers. RNA was extracted using the phenol-chloroform extraction procedure (Penmann, 1966) both from the mycelial homogenates prepared in NETS buffer

Table 1: Effect of cycloheximide, protoporphyrin, 8-azaguanine and cordycepin on the induction of ALA dehydratase in Neurospora crassa by iron.

Treatment	ALA dehydratase (nmoles PBG/ mg protein)
Nil (Iron-deficient culture)	0.76
Iron	9.68
Iron + Cycloheximide	1.50
Iron + Protoporphyrin	1.62
Iron + 8-Azaguanine	2.59
Iron + Cordycepin	4.10

ALA dehydratase was induced in 72 hr old iron-deficient cultures of N. crassa by the addition of iron as described in the text. The different compounds were added at concentrations described in the text. The enzyme activity was assayed in the 0-0.4 ammonium sulphate fraction and the assay mixture contained in a total volume of 2 ml: ALA, 2.5 µmol; potassium phosphate buffer (pH 8.0), 100 µmol; GSH, 7.5 µmol; $ZnSO_4.7H_2O$, 0.2 µmol; enzyme protein. The incubatio was carried out at 37°C for 2 hr. The reaction was stopped with 1 ml of trichloroacetic acid-$HgCl_2$ solution [0.1M $HgCl_2$ in 12.5% (w/v) tri chloroacetic acid] and the PBG formed was determined with modified Ehrlich reagent.

(0.1M NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4 and 0.1% SDS containing 0.1% diethyl pyrocarbonate and 200 µg/ml of heparin) as well as post mitochondrial supernatant prepared in 0.44M sucrose and diluted with an equal volume of 2 x NETS. Poly(A)-containing RNA was isolated from a known quantity of total RNA using nitrocellulose filter (Rosenfeld et al., 1972) as well as poly-U Sepharose (Padmanaban et al., 1975). RNA content was estimated by measuring the absorbanc at 260 nm. The total nucleotide pool was isolated from perchloric acid supernatants of mycelial homogenates using activated charcoal for absorption and 50% (v/v) alcohol containing 0.3N ammonium hydroxide for elution (Sardana and Padmanaban, 1975). An aliquot of the eluate was used for radioactivity and A_{260} measurements. Total solu protein synthesis was followed using [^{3}H]-leucine as the precursor. 5 Min after the addition of iron to iron-deficient cultures, [^{3}H]-leucine was added and the mycelia were washed and removed after anot 25 min. Pooled mycelia were ground with 0.1M phosphate buffer (pH 8 and the specific radioactivity of the hot trichloroacetic acid precipitable material of the buffer soluble proteins was measured. Protei was estimated by the method of Lowry et al. (1951) using BSA as the standard. Radioactivity measurements were made in a Beckman LS-100 liquid scintillation counter using 0.5% PPO (w/v) in toluene. Sample were applied on to filter discs dried and counted.

Table 2: Effect of iron on [^{3}H]-uridine incorporation into total and poly(A)-containing RNA in iron-deficient culture of Neurospora crassa.

Treatment	[^{3}H]-uridine incorporation					
	Total RNA (cpm/E_{260} unit)		Poly(A)-containing RNA (cpm/E_{260} unit of total RNA)			
			Poly(U)-Sepharose		Nitrocellulose filters	
	I	II	I	II	I	II
Nil (Iron-deficient culture)	3732	4080	82.7	95.7	44.5	48.7
Iron	4147	4400	195.1	213.6	66.2	103.5

^{3}H-Uridine (50 μC/flask) was added to the iron-deficient cultures 5 min after the addition of iron. The mycelia were removed after another 10 min and total RNA was isolated from mycelia pooled from three flasks. Total RNA (50.0 E_{260} units) was then processed for the isolation of poly(A)-containing RNA using nitrocellulose filters as well as poly(U)-Sepharose columns. The results obtained in two independent experiments are presented.

RESULTS

The results presented in Table 1 indicate that the induction of ALA dehydratase by iron in iron-deficient cultures is not only blocked by cycloheximide and protoporphyrin, but also by 8-azaguanine and cordycepin.

Since 8-azaguanine and cordycepin could have exerted their effects by preventing the formation of a functional messenger, studies on RNA synthesis under conditions of induction were made. The results presented in Table 2 indicate that [^{3}H]-uridine incorporation into total RNA from mycelial homogenates is not significantly affected under conditions of induction. However, there is a two-fold increase in the labeling of poly(A)-containing RNA under these conditions. In

Table 3: Effect of iron and protoporphyrin on RNA and protein synthesis in iron-deficient cultures of Neurospora crassa.

Treatment	^{32}P incorporation			[^{3}H]-leucine incorporation
	Total RNA (cpm/E_{260} unit)	Poly(A)-containing RNA (cpm/E_{260} unit of total RNA)	Nucleotide pool (cpm/E_{260} unit)	(cpm/mg protein)
Nil (iron-deficient culture)	662.2	71.2	1036	6077
Iron	693.1	201.3	1092	6256
Iron + Protoporphyrin	650.2	110.4	1013	6123
Protoporphyrin	651.2	72.5	1004	6291

The experimental details are as given in Table 2 except that ^{32}P (80 μC/flask) was used as the precursor and RNA was isolated from the post-mitochondrial supernatant. Poly(A)-containing RNA was isolated using poly(U)-Sepharose chromatography. For measurement of protein synthesis, [^{3}H]-leucine (5 μC/flask) was added 5 min after the addition of iron and the mycelia were removed 25 min later. The soluble proteins were extracted from pooled mycelia and radioactivity was determined in the trichloroacetic acid precipitable proteins.

addition, the labeling of cytoplasmic poly(A)-containing RNA was examined under these conditions using ^{32}P as the tracer. The results presented in Table 3 confirm the results obtained with RNA isolated from mycelial homogenates. The results also show that there is no significant change in the nucleotide pool specific radioactivity under these conditions. One other striking feature is that protoporphyrin specifically blocks the iron-dependent increase in the labeling of cytoplasmic poly(A)-containing RNA. Protoporphyrin treatment does not have a significant effect on total RNA synthesis or protein synthesis.

DISCUSSION

Enzyme induction in fungal cultures is usually slow but ALA dehydratase is one of the few examples where the enzyme is maximally induced within a short period after the addition of iron to iron-deficient cultures. The cycloheximide sensitivity of the induction process has indicated the involvement of protein synthesis. A lag period of 15 min has been noticed in the induction of ALA dehydratase and maximal activity is reached in about 30 min after the addition of iron. The lag period is probably associated with the time necessary for the synthesis, processing and accumulation of the specific messenger RNA for ALA dehydratase. The sensitivity of the induction process to 8-azaguanine and cordycepin as well as the direct stimulation of cytoplasmic poly(A)-containing RNA synthesis during induction are indicative of the action of iron at the level of the synthesis of ALA-dehydratase messenger. It is striking to observe that protoporphyrin, the penultimate product of the heme-biosynthetic pathway which 'represses' ALA dehydratase induction, also blocks the iron-dependent increase in poly(A)-containing RNA synthesis. At the time intervals examined neither iron nor protoporphyrin has any effect on total RNA or total soluble protein synthesis when added to iron-deficient cultures. In addition, protoporphyrin has no effect on poly(A)-containing RNA synthesis in the absence of added iron. It thus appears possible that bulk of the newly synthesized poly(A)-containing RNA, at the early time interval examined, in response to added iron may represent the messenger RNA for ALA dehydratase. It is likely that ALA dehydratase could be one of the very early proteins to be synthesized in response to iron. It is felt that this system would be ideal to examine the direct role of a metal such as iron in genetic transcription and post-

transcriptional regulatory processes associated with the synthesis of a specific protein.

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