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Control of L-amino acid oxidase in *Neurospora crassa* by different regulatory circuits

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Abstract. L-Amino acid oxidase is synthesized in *Neurospora crassa* in response to three different physiological stimuli: (i) starvation in phosphate buffer, (ii) mating, and (iii) nitrogen derepression in the presence of amino acids. During starvation in phosphate buffer, or after mating, L-amino acid oxidase synthesis occurred in parallel with that of tyrosinase. Exogenous sulfate repressed the formation of the two enzymes in starved cultures, but not in mated cultures. Sulfate repression was relieved by protein synthesis inhibitors, suggesting that the effect of sulfate required the synthesis of a metabolically unstable protein repressor. With amino acids as the sole nitrogen source only L-amino acid oxidase was produced. Under these conditions enzyme synthesis was repressed by ammonium and was insensitive to sulfate. Biochemical evidence suggested that the L-amino acid oxidase formed under the three different conditions was the same protein. Therefore, the expression of L-amino acid oxidase appeared to be under the control of least two regulatory circuits. One, also controlling tyrosinase, seems to respond to developmental signals related to sexual morphogenesis. The other, controlling other enzymes of the nitrogen catabolic system, is used by the organism to obtain nitrogen from alternative sources such as proteins and amino acids.

Key words: L-Amino acid oxidase – Tyrosinase – Enzyme regulation – Fungi – *Neurospora crassa*

Neurospora crassa possesses D- and L-amino acid oxidase (L-amino acid: O_2 oxidoreductase – deaminating – EC 1.4.3.2) activities, which convert several amino acids to the corresponding keto acid (Ohnishi et al. 1962; Thayer and Horowitz 1951). Synthesis of L-amino acid oxidase has been reported for cultures undergoing sexual differentiation, cultures submitted to starvation in phosphate buffer, and cultures treated with protein synthesis inhibitors (Horowitz 1965). The same authors demonstrated that under these conditions L-amino acid oxidase synthesis occurred simultaneously with that of tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1), and that the female sterile *ty-1* mutant is unable to produce both enzymes under starvation. Horowitz (1965) suggested that L-amino acid oxidase and tyrosinase are subject to a common control mechanism, probably related to sexual morphogenesis. On the other hand, L-amino acid oxidase is also produced in biotin deprived cultures and its level is increased by amino acid supplementation (Thayer and Horowitz 1951). In a recent study (Sikora and Marzluf 1982) it is shown that L-amino acid oxidase is synthesized de-novo in nitrogen starved cultures, in response to the addition of amino acids. In the same study it is shown that the *nit-2* regulatory mutant is unable to form L-amino acid oxidase under conditions that permit the enzyme's induction in the wild type and *nit-2* revertants. The authors concluded that L-amino acid oxidase expression is regulated at the level of transcription as a member of the nitrogen catabolic family.

In the present work we studied the control of L-amino acid oxidase formation under three different physiological conditions, namely: (i) starvation in phosphate buffer, (ii) sexual development, and (iii) adaptation to the use of amino acids as the sole nitrogen source. Our results show that for each condition the enzyme synthesis was affected by different environmental factors. We also present biochemical evidence suggesting that the L-amino acid oxidase formed under the three different treatments represented the same protein species.

Material and methods

Neurospora strains and culture conditions. The following strains were used: St.L 74 A (wild type), and the extra fertile strains BAT 9-4 A (*cot-1, nic-3*) and BAT 9-5 a (*cot-1, nic-3*). The extra fertile strains were used when a comparison between enzyme synthesis under conditions of mating, starvation in phosphate buffer, and nitrogen limitation, was required. The mating treatment failed to produce in the wild type the biochemical responses which it elicits in the extra fertile strains (Cruz and Terenzi 1981). Otherwise, the responses of the wild type and the extra fertile strains toward starvation in phosphate buffer, or nitrogen derepression in the presence of amino acids, was essentially the same.

Strains were maintained on slants of solid Vogel (1956) medium supplemented with 2% sucrose and 50 μg/ml nicotinic acid, when required. Liquid cultures were started with an aliquot of a conidial suspension sufficient to give 5×10^6 cells/ml of culture medium.

Derepression of L-amino acid oxidase and tyrosinase by starvation in phosphate buffer or by treatment with protein synthesis inhibitors. These procedures were essentially similar to those used by other workers (i.e. Horowitz et al. 1970). The cultures were prepared in standard Petri dishes containing 15 ml of liquid Vogel medium, and incubated at

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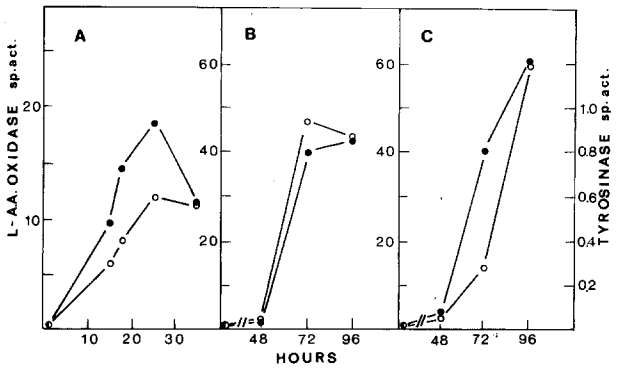

Fig. 1A–C. Coordinated synthesis of L-amino acid oxidase and tyrosinase in the extra fertile strain, under different physiological conditions. **A** Mating. **B** Starvation in phosphate buffer. **C** Treatment with cycloheximide (0.8 µg/ml). Details of these procedures are given in Materials and methods. Symbols: ○ L-amino acid oxidase, ● tyrosinase. Activity is expressed in units per mg protein

25° C in the dark for 72 h. Then the culture medium was removed and the mycelium resuspended in 10 ml of phosphate buffer 0.1 M, pH 6.0, or in Vogel medium without a carbon source, but supplemented with 0.8 µg/ml cycloheximide or 10 µg/ml D-tyrosine. The mycelia were reincubated as before for different periods of time, as indicated for each experiment, before being processed for the enzymatic determinations.

Derepression of L-amino acid oxidase and tyrosinase by sexual stimulation. This procedure has been described elsewhere (Cruz and Terenzi 1981; Prade and Terenzi 1982).

Synthesis of L-amino acid oxidase under nitrogen derepression and amino acid induction. Conidia were inoculated in 500 ml Erlenmeyer flasks containing 100 ml of Vogel liquid medium without ammonium nitrate and with 0.5 mg/ml L-leucine. The cultures were incubated for 24 h at 30° C with agitation.

Preparation of crude mycelial extracts. Mycelia were harvested by filtration on a Buchner funnel, rinsed with chilled distilled water, blotted and stored at −25° C until use. Mycelial samples were ground in a porcelain mortar with the aid of glass beads, and extracted with phosphate buffer 0.1 M, pH 6.0. All operations were carried out at 4° C. The 15,000 × *g* supernatant fraction of the crude extracts was dialysed overnight against phosphate buffer, and was used for the enzymatic determinations.

Enzymatic determinations. Tyrosinase was assayed according to Horowitz et al. (1960) using DL-dihydroxyphenylalanine (DL-DOPA, Sigma Chemical Co, St. Louis, MO, USA) as substrate. The increase in absorbance at 475 nm was monitored spectrophotometrically. The rate of increase in absorbance expressed as protein units, was converted into Enyzme Commission Units using the factor given by Fling et al. (1963).

L-Amino acid oxidase was assayed measuring the amount of keto acid formed in the reaction, according to Friedemann and Haugen (1943) and Friedemann (1957), with some modifications (Sikora and Marzluf 1982). The incubation mixture contained 2.0 mM leucine in 200 mM sodium phosphate buffer pH 7.6, and 400 units of beef liver catalase (Sigma). The reaction was initiated with the enzyme. Final volume was 1.0 ml. Incubation was carried out for

Table 1. Synthesis of tyrosinase and L-amino acid oxidase, and effect of sulfate, in mycelium of the wild type strain submitted to starvation in phosphate buffer

Treatments[a]	Enzyme activity (units per mg protein)			
	Tyrosinase		L-Amino acid oxidase	
	Control	With sulfate	Control	With sulfate
Before starvation	–		0.99	
Starvation	0.28	0.04	16.73	3.11
Starvation with cycloheximide	0.14	0.17	13.57	20.36
Starvation with D-tyrosine	0.45	0.25	17.74	11.29

[a] 72 h-old mycelia of the wild type strain grown in Vogel medium were submitted to starvation for 96 h in phosphate buffer 0.1 M, pH 6.0, supplemented with magnesium sulfate (0.5 mg/ml), cycloheximide (0.8 µg/ml) or D-tyrosine (10 µg/ml), as indicated

60 min at 37° C, and the reaction was stopped by addition of 1.0 ml of 2,4-dinitrophenol hydrazine 0.1% (W/V) in 2.0 N HCl. After further 2 min of incubation 2.0 ml of absolute ethanol was added with stirring followed by the addition of 5.0 ml of 2.5 N NaOH. The absorbance at 550 nm was converted in µmol of keto acid using pyruvic acid (Sigma) as standard.

DEAE cellulose chromatography of L-amino acid oxidase. Before use the crude extracts were centrifuged for 1 h at 100,000 × *g*. Twenty milliliter of the supernatant fraction (about 80 mg protein) were loaded onto a 1.6 × 30.0 cm column of DEAE cellulose (Sigma) equilibrated with phosphate buffer 0.05 M, pH 6.0. The bed volume was 153 ml. The column was washed with the equilibrium buffer until no absorbance at 230 nm was detected in the eluate. After that, 200 ml of a linear gradient of 0 to 0.3 M KCl in the same buffer was applied at a flow rate of 20 ml/h. Fractions of 2.0 ml were collected and assayed for L-amino acid oxidase activity.

Gel filtration of L-amino acid oxidase. Before use the crude extracts were centrifuged for 1 h at 100,000 × *g*. An aliquot of the supernatant fraction (3.5 ml) was applied to a 1.6 × 80.0 cm column of Sephacryl S-300 Superfine (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with sodium phosphate buffer 0.1 M, pH 6.0. The parameters of the column were: bed volume 160 ml, void volume 33 ml, and exclusion volume 153 ml. Protein was eluted with 200 ml of the equilibrium buffer at a flow rate of 20 ml/h. Fractions of 2.0 ml were collected and assayed for L-amino acid oxidase activity.

Polyacrylamide gel electrophoresis of L-amino acid oxidase. Electrophoresis was performed in 7% (W/V) polyacrylamide gels with 0.3 M Tris-HCl, pH 8.3, as the gel buffer. The running buffer contained 5 mM Tris-HCl with 0.04 mM glycine pH 8.3. The gel was run at 30 mA, 100 V, at room temperature until the dye front reached the base of the gel. The gel was stained by incubation in the dark in a solution containing 4 mg tetrazolium blue, 0.4 mg of phenazine methosulfate and 1.5 mg of leucine in 24 ml of Tris-HCl 0.1 M, pH 7.5.

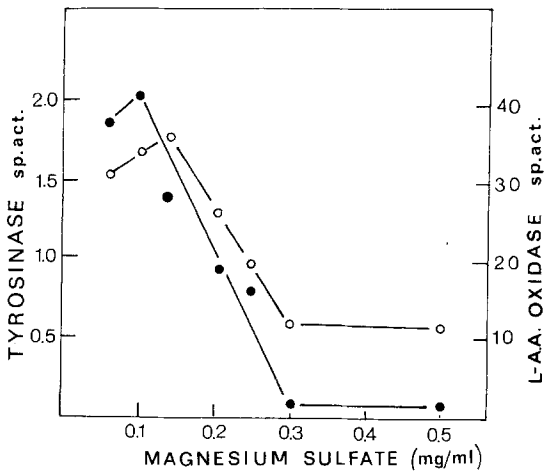

Fig. 2. Dose effect of exogenous sulfate concentration on the activity of tyrosinase and L-amino acid oxidase in mycelia of the extra fertile strain submitted to starvation. Mycelia from 72 h-old cultures were submitted to starvation in phosphate buffer supplemented with magnesium sulfate as indicated. Enzymatic activities were determined after 96 h. Symbols: ○ L-amino acid oxidase, ● tyrosinase

Protein determination. Protein was measured with the method of Lowry et al. (1951) with bovine serum albumin as standard.

Results

Simultaneous derepression of L-amino acid oxidase and tyrosinase under conditions of mating, starvation in phosphate buffer or inhibition of protein synthesis

As shown in Fig.1 the activity levels of L-amino acid oxidase and tyrosinase increased in mycelia stimulated by mating (Fig. 1A), submitted to starvation in phosphate buffer (Fig. 1B) or treated with a low dose of cycloheximide (Fig. 1C). A striking parallelism can be noted in the time-course of derepression of the two enzymes, for the three different treatments. For instance, in mycelia submitted to starvation or in those treated with cycloheximide, about 48 h elapsed before the enzymatic activities would be detected. On the other hand, in cultures stimulated by mating there was a shorter lag, of about 10 to 15 h. In the starved cultures the production of the two enzymes ceased around 72 h, whereas in cultures treated with cycloheximide their level was still increasing at this time. The observed parallelism in L-amino acid oxidase and tyrosinase synthesis appears to be more than simply a coincidence and was probably indicating the existence of a common control mechanism.

Table 1 shows that the formation of L-amino acid oxidase and tyrosinase under starvation conditions was repressed by exogenous sulfate, and that the effect of sulfate required protein synthesis because it was abolished by the inhibitors cycloheximide and D-tyrosine. Figure 2 shows the extent of repression on the production of L-amino acid oxidase and tyrosinase effected by increasing sulfate concentrations. L-Amino acid oxidase was somewhat less sensitive to sulfate repression, and a significant basal activity was present even at high sulfate concentrations. Nevertheless, the parallelism in the responses of both enzymes to this environmental factor was still evident. On the other hand in mycelia stimulated by mating the synthesis of L-amino acid oxidase and tyrosinase was insensitive to sulfate repression, again suggesting the existence of a common control mechanism for the two enzymes.

Table 2. Induction of L-amino acid oxidase by amino acids. Repression by ammonia and absence of repression by sulfate

Culture conditions[a]	Enzyme activity (units per mg protein)			
	No additions	Leucine	Tyrosine	Phenyl-alanine
Vogel minimal medium	1.95	1.38	0.99	0.95
Vogel without nitrogen and sulfate	n.g.[b]	21.05	40.97	18.31
Vogel without nitrogen	n.g.	36.53	36.04	23.39

[a] Conidia of the wild type strain were inoculated in Vogel minimal medium with omission of inorganic nutrients and addition of amino acids, as indicated. When present, nitrogen and sulfate were added at the concentration of standard Vogel medium. Amino acids were added at a concentration of 0.5 mg per ml. The cultures were incubated with shaking at 30 °C for 24 h before the enzymatic determinations

[b] n.g. = absence of mycelial growth

Table 3. Derepression of tyrosinase and L-amino acid oxidase in mycelium of the wild type strain submitted to starvation. Effect of sulfate, ammonia, and nitrate

Starvation conditions[a]	Enzyme activity (units per mg protein)	
	Tyrosinase	L-Amino acid oxidase
Without additions	0.17	34.37
With $MgSO_4$ (0.5 mg/ml)	–	7.36
With NH_4Cl (1.6 mg/ml)	0.33	54.16
With KNO_3 (1.4 mg/ml)	1.03	143.43

[a] 72 h-old mycelia of the wild type strain grown in Vogel medium were submitted to starvation for 96 h in phosphate buffer, supplemented as indicated

Induction of L-amino acid oxidase by amino acids in nitrogen derepressed cultures

Table 2 shows the synthesis of L-amino acid oxidase as an adaptative response to nitrogen limitation. Under these conditions tyrosinase activity was not detected. The cultures were made in nitrogen deficient medium supplemented with amino acids, and with or without sulfate. No growth was observed in the absence of a nitrogen source. The growth which occurred in sulfate deficient medium was probably due to traces of sulfate present in the Vogel microelement solution. From these results it was clear that the formation of L-amino acid oxidase required nitrogen derepression and the presence of an amino acid as inducer, in agreement with the results of Sikora and Marzluf (1982). It was also clear that under these conditions the presence or absence of sulfate did not affect the level of L-amino acid oxidase.

In view of these results it was decided to test the effect of nitrogen sources on the formation of L-amino acid oxidase

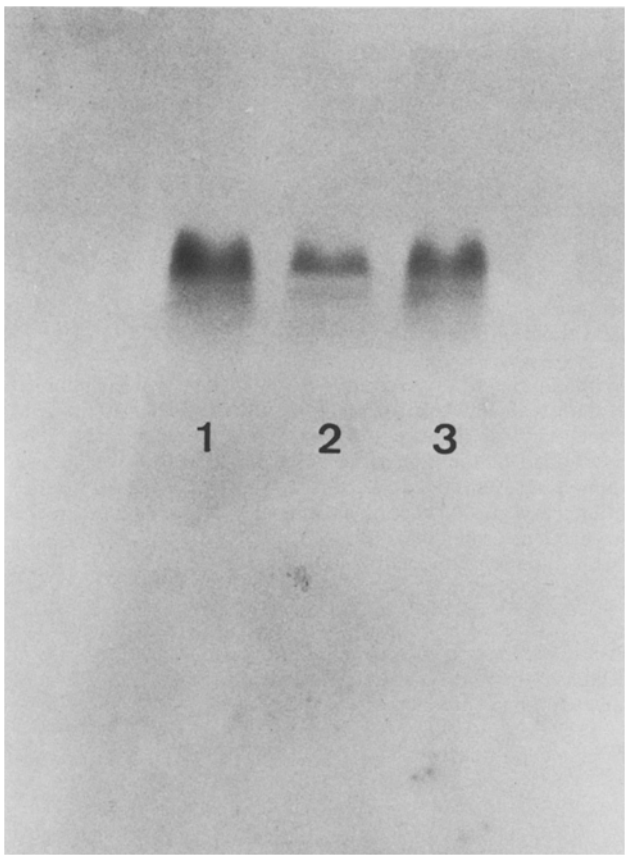

Fig. 3. Gel electrophoresis of L-amino acid oxidase in crude extracts produced by the extra fertile strain under different physiological conditions. *1* L-amino acid oxidase formed in mated cultures: *2* L-amino acid oxidase formed in cultures submitted to starvation in phosphate buffer; *3* L-amino acid oxidase formed under conditions of nitrogen derepression in the presence of leucine. The gel was stained for L-amino acid oxidase activity with leucine as a substrate. Other details are given in Materials and methods

in cultures starved in phosphate buffer. The results, shown in Table 3, demonstrate that in this case L-amino acid oxidase synthesis, as well as that of tyrosinase was resistant to ammonium repression, but sensitive to the presence of sulfate, as previously shown (Table 1 and Fig. 2). Interestingly, the addition of nitrate strongly enhanced the activity level of the two enzymes.

Biochemical characterization of L-amino acid oxidase synthesized under different physiological conditions

The results described above suggested that L-amino acid oxidase was regulated in a different fashion under conditions of mating, starvation, and nitrogen limitation. This could be interpreted either as an indication of the existence of more than one enzyme, independently controlled, or a single enzyme under the control of multiple regulatory circuits. To help to clarify this matter we decided to examine some biochemical properties of the L-amino acid oxidase formed under the three different situations.

When crude extracts from mycelia derepressed for L-amino acid oxidase by starvation in phosphate buffer, nitrogen limitation or mating, were submitted to electrophoresis in polyacrylamide gel (Fig. 3), and stained to detect

Table 4. Relative activity toward different amino acids of DEAE-cellulose purified L-amino acid oxidase produced under different physiological conditions, by the extra fertile strain

Substrate	Percent specific activity[a]		
	Mating	Starvation in phosphate buffer	Nitrogen limitation
Leucine	100	100	100
Tyrosine	100.7	50.0	19.3
Phenylalanine	201.4	36.3	69.3
Tryptophan	18.0	25.0	26.9
Asparagine	45.3	27.0	69.0
Glutamic acid	91.4	35.1	122.8
Alanine	82.7	122.0	153.8
Cysteine	45.3	104.2	57.6
Glycine	100.0	50.6	61.4

[a] Percent activity was calculated relative to that with leucine. All the amino acids were tested at 2.0 mM concentration, in the standard procedure

L-amino acid oxidase activity, a single band at identical positions in the gel was detected for each sample. Single peaks of activity of L-amino acid oxidase were also found when the crude extracts were submitted to gel filtration in a Sephacryl S-300 column (app. MW 450.000) or to DEAE-cellulose chromatography, eluting at 0.22 M KCl (not shown). Table 4 shows the relative specificity of the DEAE-purified L-amino acid oxidases toward several amino acids. It can be noted that despite some differences, the properties of the enzymes formed under the three different conditions were quite similar.

Discussion

In a previous study related to tyrosinase regulation in *Neurospora crassa* (Prade et al. 1984) we demonstrated that during the vegetative growth phase this enzyme seems to be under negative control by a mechanism which requires the presence of exogenous sulfate and sustained protein synthesis. These results were in agreement with a previously postulated model of control of tyrosinase by a metabolically unstable protein repressor (Horowitz et al. 1970). Horowitz (1965) earlier suggested the existence of a common control mechanism for tyrosinase and L-amino acid oxidase. This view was strengthened and extended in the present study, which confirmed the striking parallelism in the response of the two enzymes to the stimuli promoting their synthesis, and in their sensitivity to environmental factors.

Tyrosinase does not have any nutritional function in *Neurospora*. Actually, constitutive synthesis of this enzyme appears to hinder mycelial growth (Schaeffer 1953; Prade et al. 1984). In contrast, L-amino acid oxidase does accomplish a nutritional role, participating in the catabolism of amino acids under conditions of nitrogen limitation (Sikora and Marzluf 1982).

In the present work it is shown that mycelia conditioned to synthesize L-amino acid oxidase in response to different physiological stimuli appeared to produce a single enzyme species, on the basis of several separative criteria. Furthermore, these criteria failed to show significant differences among the L-amino acid oxidase produced under starvation in phosphate buffer, sexual differentiation or nitrogen

limitation. Thus, it is possible that they are the product of the same structural gene. If this assumption is correct, then, during the vegetative growth phase L-amino acid oxidase and tyrosinase could be negatively affected by the product of a still unknown control gene which utilizes sulfate as a corepressor. This would explain the derepression of the two enzymes during starvation in phosphate buffer or by partial inhibition of protein synthesis. L-Amino acid oxidase and tyrosinase also appear simultaneously during sexual differentiation, and are affected in the same manner by the *ty-1* mutation (Horowitz 1965). In this case the synthesis of the two enzymes is triggered by mating in a fashion which resembles an inductive stimulus. The biochemical basis of this process is unclear at the present time.

L-Amino acid oxidase is also synthesized in response to nitrogen starvation in the presence of amino acids. Under these conditions the enzyme makes part of the nitrogen catabolic circuit which is under positive control by the product of the regulatory gene *nit-2*, which under conditions of catabolic repression is inactivated by glutamine, a metabolite derived from exogenous ammonia (Grove and Marzluf 1981; Hanson and Marzluf 1975; Wang and Marzluf 1979).

Additional evidence is necessary to prove that the L-amino acid oxidase produced under the different situations is the product of the same structural gene, and that its control occurs at the level of transcription by different regulatory signals. This may demonstrate that *N. crassa* L-amino acid oxidase is one more example of the convergence of multiple regulatory circuits in a single structural gene, similar to that of alkaline protease (Hanson and Marzluf 1975) and the ribonuclease N4 (Lindberg and Drucker 1984a, b), in the same organism. It has been postulated (Marzluf 1981) that these genes may possess a complex regulatory region, containing independent sites for the recognition of positive and negative signals. Structural genes sharing these features may be common in eukaryotic organisms and probably represent the basic element to integrate gene expression during the processes of differentiation and morphogenesis.

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