Role of sulfhydryl compounds in the control of tyrosinase activity in Neurospora crassa

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Role of Sulfhydryl Compounds in the Control of Tyrosinase Activity in *Neurospora crassa*[1]

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It is known that Neurospora crassa *mycelia cultured in standard concentrations (76 to 190 μg/ml) of sulfate accumulate a low molecular weight inhibitor of tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidorenductase; EC 1.14.1.18.1.). This is not observed in cultures grown under sulfate-limiting conditions. The chemical nature of tyrosinase inhibition was investigated. It was shown to be due to the low molecular weight sulfhydryl fraction of the extracts, in which glutathione is predominant. The concentration of low molecular weight sulfhydryl compounds decreased sharply in mycelia submitted to various treatments which also derepressed tyrosinase, such as (i) starvation in phosphate buffer, (ii) treatment with cycloheximide, and (iii) mating. These results suggest that the concentration of sulfhydryl compounds may be of physiological significance in the control of tyrosinase activity in* N. crassa.

KEY WORDS: tyrosinase; low molecular weight sulfhydryl; glutathione; fungi; enzyme regulation; sexual morphogenesis.

INTRODUCTION

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidorenductase; EC 1.14.1.18.1.). activity in *Neurospora crassa* is associated with the

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biochemical events of sexual morphogenesis and participates in the formation of the melanic pigment which accumulates in maturing perithecia (Hirsch, 1954). The vegetative mycelium formed in liquid cultures lacks tyrosinase, but the enzyme rapidly accumulates after the addition of conidia of the opposite mating type (Cruz and Terenzi, 1981). In the absence of mating, tyrosinase also accumulates when the mycelium is starved in phosphate buffer (Horowitz *et al.*, 1961) or treated with protein synthesis inhibitors (Horowitz *et al.*, 1970a, b). Tyrosinase regulation has been a matter of interest for many years; nevertheless, we still know little about the genetic and biochemical mechanisms which control this enzyme activity in *Neurospora crassa*. On the basis of results obtained using protein synthesis inhibitors, Horowitz and co-workers (1970a, b) proposed that tyrosinase synthesis was under the control of a repressor protein of rapid turnover.

Among the environmental factors which influence tyrosinase, there have been reports on the effects of light (Schaeffer, 1953) temperature, and the level of sulfate in the medium (Horowitz and Shen, 1952). The effect of sulfate over tyrosinase activity is interesting. Crude extracts from mycelia grown under sulfate-limiting conditions showed considerable tyrosinase activity, in contrast to crude extracts from mycelia grown in standard sulfate concentrations (76 to 190 μg/ml), which had little, if any, tyrosinase activity (Horowitz and Shen, 1952). In the latter, however, tyrosinase activity increased after dialysis, but it was much lower than that in the extracts of mycelia grown in low-sulfate medium. In view of these results, Horowitz and Shen (1952) suggested that sulfate was affecting tyrosinase in a double manner: (i) causing repression of synthesis of the enzyme and (ii) participating in the formation of a low molecular weight metabolite which acted as an inhibitor of the enzyme. The chemical nature of the natural inhibitor of tyrosinase was never clarified. On the other hand, it has been well established that sulfur-containing compounds, which, like cysteine, may form copper complexes, strongly inhibit tyrosinase activity (Sussman, 1961).

In the present study we show that tyrosinase inhibition is caused by low molecular weight sulfhydryl compounds and that reduced glutathione is the major component of the inhibitor fraction. We also present physiological evidence demonstrating that under conditions of tyrosinase derepression, the increase in enzyme activity is associated with a decrease in the concentration of the low molecular weight sulfhydryl compounds in the mycelial extracts.

MATERIALS AND METHODS

Neurospora Strains and Culture Conditions

Wild-type ST L 74 A and two mutant strains—BAT 9-4, A, *cot-1, nic-3*, and BAT 9-5 a, *cot-1, nic-3*—were employed throughout this work. The latter

were selected on the basis of high fertility (Cruz and Terenzi, 1981). Cultures were maintained by transfers on slants of solid Vogel's (1956) medium supplemented with 2% sucrose and, when required, 50 μg/ml of nicotinic acid. Liquid cultures were carried out in standard petri dishes containing 15 ml of Vogel's or Westergaard and Mitchell's (1947) crossing medium, as indicated for each experiment. The cultures were incubated in the dark, at 25°C, without agitation, for variable time periods before the experimental treatments.

Derepression of Tyrosinase

Derepression in Phosphate Buffer or by Treatment with Cycloheximide. These procedures were essentially similar to those used by other workers (Horowitz *et al.*, 1970a). Seventy-two-hour cultures were used. The culture medium was removed by aspiration and the mycelial mat was washed with sterile distilled water. The mycelium was then resuspended in 10 ml of 0.1 M sodium phosphate buffer, *p*H 6.0, (phosphate buffer), or a Vogel salts solution without sugar, supplemented with 0.8 μg/ml of cycloheximide.

Derepression by Sexual Stimulation. This procedure has been described elsewhere (Cruz and Terenzi, 1981). Briefly, it consists in growing a "female receptor" mycelium in Westergaard and Mitchell's (1947) crossing medium for 7 days. After this period the mycelial mat is evenly covered with 2 ml of a heavy conidial suspension (10^7/ml) of the opposite mating-type strain. Undisturbed cultures and cultures receiving conidia from the same mating type were used as controls. For these experiments, only the high-fertility strains were used. These strains showed a rapid and dramatic increase in tyrosinase activity after mating. Our wild-type strain did not respond well to this treatment.

Enzymatic Determinations

Mycelial samples were ground with glass beads in a porcelain mortar and extracted with phosphate buffer. The 15,000*g* supernatant fraction of the crude extract was used for the enzymatic measurements. Tyrosinase activity was assayed according to Horowitz *et al.* (1960) using DL-DOPA[3] as substrate. The increase in absorbance at 475 nm was recorded continuously with a Beckman ACTA III spectrophotometer. The rate of increase in absorbance, expressed as protein units, was converted into Enzyme Commission Units using the factor given by Fling *et al.* (1963).

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[3] Abbreviations used: PCMB, *p*-hydroxymercuribenzoate; GSH, reduced glutathione; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); ATP, adenosine-5′-triphosphate; DL-DOPA, DL-β-3,4-dihydroxyphenylalanine.

Preparation of the Inhibitor Fraction

Aliquots of the crude extract of mycelium grown in standard sulfate medium were applied to a 2 × 20-cm G-25 Sephadex column and eluted with phosphate buffer. Two-milliliter fractions were collected, and the amount of protein and sulfydryl material was determined. The fractions eluting after the protein peak, which contained practically all the low molecular weight sulfhydryl material (inhibitor fraction), were pooled and concentrated.

Gel Chromatography of the Inhibitor Fraction

The concentrated inhibitor fraction was applied to a 2.2 × 40-cm G-10 Sephadex column, previously calibrated with ATP (MW 551.15), reduced glutathione (GSH; MW 307.33), and cysteine (MW 82.6). Elution was performed with phosphate buffer, and 1.2-ml fractions were collected and assayed for the presence of sulfydryl material.

Chemical Determinations

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The sulfydryl concentration was determined with Ellman's (1959) reagent, DTNB, with GSH as standard. The presence of GSH in the inhibitor fraction was determined with a colorimetric test (Patterson and Lazarow, 1955).

Chemicals

Sephadex, GSH, cysteine, ATP, and PCMB were purchased from Sigma Chemical Co. All other reagents were of the best purity available.

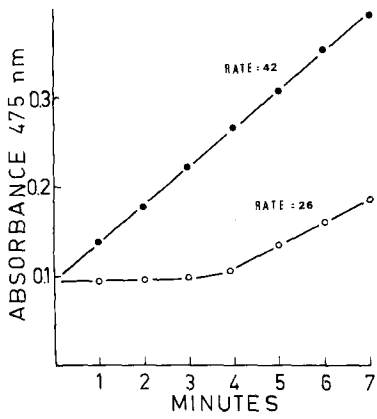

Fig. 1. Effect of gel filtration on the activity of tyrosinase. A crude extract was prepared from a 72-hr mycelium of the wild-type stain, ST L 74 A, after being starved for 48 hr in phosphate buffer. One aliquot of the crude extract was assayed directly for tyrosinase activity (O). Another aliquot was passed through a 0.7 × 10-cm G-25 Sephadex column before the enzymatic assay (●). The amount of protein in the reaction mixtures was adjusted to be the same.

RESULTS AND DISCUSSION

The existence of an inhibitor of tyrosinase activity could be noticed readily by following the course of the enzymatic reaction (Fig. 1) with a crude extract. A lag period was observed first, after which the reaction proceeded at a linear rate. When the crude extract was filtered through a G-25 Sephadex column in order to remove low molecular weight metabolites, the lag was abolished and the reaction rate also increased. A similar observation was made earlier by Schaeffer (1953). The possibility that the inhibitory compound could be a sulfhydryl metabolite was considered. In that case, an SH reagent such as PCMB might overcome the inhibitory effect. It has been shown that PCMB does not interfere with tyrosinase activity (Fling *et al.*, 1963). Table I shows that when 0.4 mM PCMB was added to the reaction mixture prepared with the crude extract which contained the endogenous inhibitor, there was an activation of tyrosinase similar to that observed after gel filtration. It is also shown in Table I that the concentrated pool of low molecular weight sulfhydryl material obtained from the crude extract inhibited tyrosinase activity, and that this inhibition was overcome by PCMB. Moreover, sulfhydryl compounds such as cysteine and GSH produced an inhibitory effect on tyrosinase activity comparable to that of the natural inhibitor. These results suggested that the low molecular weight endogenous tyrosinase inhibitor was indeed a sulfhydryl metabolite.

Table I. Inhibition of Tyrosinase by Sulfhydryl Compounds and Reactivation by *p*-Hydroxy Mercuribenzoic Acid

Enzyme sample[a]	Addition	Lag (min)	Percentage activity[b]
Crude extract	None	2.5	70
Crude extract	0.4 mM PCMB	—	96
G-25 Sephadex-filtered extract	None	—	100
G-25 Sephadex-filtered extract	Inhibitor[c] (0.2 mM)	4	48
G-25 Sephadex-filtered extract	0.2 mM cysteine	11	25
G-25 Sephadex-filtered extract	0.2 mM GSH	4.5	44
G-25 Sephadex-filtered extract	Inhibitor (0.2 mM) plus 0.4 mM PCMB	—	102
G-25 Sephadex-filtered extract	0.2 mM GSH plus 0.4 mM PCMB	—	95

[a] Mycelia were obtained from 72-hr cultures submitted to starvation for 50 hr in 0.1 M sodium phosphate buffer, *p*H 6.0. Extracts were prepared as described under Materials and Methods.
[b] Relative to the activity of the G-25 Sephadex-filtered extract.
[c] Concentrated pool of DTNB-positive material eluting after the protein peak of an extract of 72-hr mycelium, filtered through a column of G-25 Sephadex, as described under Materials and Methods.

Characterization of the Natural Inhibitor

A sample of the concentrated pool of low molecular weight sulfhydryl of the crude extract was chromatographed in a G-10 Sephadex column, as described under Materials and Methods. As shown in Fig. 2, all DTNB-positive material eluted as a single peak, in a volume equal to that of the GSH standard. When the colorimetric test was applied to this material, it reacted as authentic glutathione (data not shown). Therefore, we must conclude that this substance is the major component of the tyrosinase inhibitor fraction. This finding was not unexpected, since it has been demonstrated that in *Neurospora crassa* (Fahey *et al.*, 1975), as well as in most living cells, the GSH concentration is elevated (2 to 5 mM) and is the most predominant low molecular weight thiol present.

In vivo Correlation Between Tyrosinase Activity and Low Molecular Weight Sulfhydryl Concentration

The possible involvement of sulfhydryl compounds in mammalian skin pigmentation was suggested early by Flesch and Rothman (1948). Therefore, we decided to measure the concentration of low molecular weight sulfhydryl compounds in mycelia under conditions of derepression of tyrosinase, in order to detect any possible correlation between enzyme activity and sulfhydryl content.

Mycelial samples were submitted to the different treatments for dere-

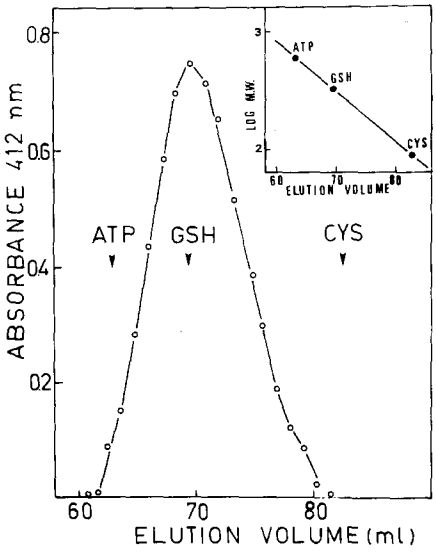

Fig. 2. Gel filtration of the tyrosinase inhibitor fraction. A concentrated sample of the low molecular weight sulfhydryl fraction of a crude extract was passed through a 40 × 2.2-cm column of Sephadex G-10 equilibrated with 0.1 M sodium phosphate buffer, *p*H 6.0. Elution was performed with the same solution, and 1.8-ml fractions were collected. The concentration of sulfhydryl in each fraction was measured with DTNB as described under Materials and Methods. ATP, GSH, and cysteine served as molecular weight standards.

pression of tyrosinase described under Materials and Methods. Cultures were sampled at various time intervals, and the activity of tyrosinase and the low molecular weight sulfhydryl concentration were measured. The results are shown in Figs. 3A and B, which show the profiles of tyrosinase activity and sulfhydryl concentration as a function of time, in mycelia starved in phosphate buffer (Fig. 3A) or mated (Fig. 3B). The results obtained with the mycelia treated with cycloheximide were essentially similar to those with the starved mycelia and are not included. It can be seen that in the mycelia submitted to starvation in phosphate buffer (Fig. 3A), tyrosinase accumulated rapidly after a lag period of 72 hr. The sulfhydryl concentration decreased during starvation and attained its lowest value at the onset of tyrosinase derepression. The results shown in Fig. 3A were obtained using the wild-type strain, St L 74 A, but the response of the other strains, BAT 9-4 and BAT 9-5, to this treatment (not shown) was the same. An inverse correlation was observed between the changes in tyrosinase activity and the sulfhydryl concentration. However, this may be just a coincidence, due to the drastic unphysiological conditions to

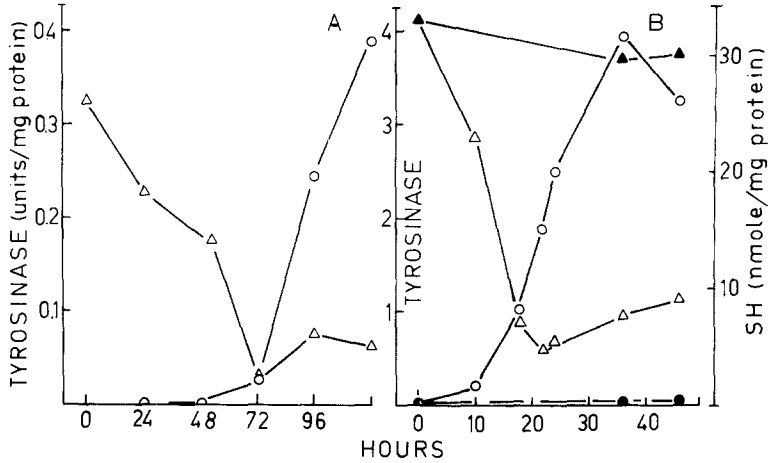

Fig. 3. Tyrosinase activity and low molecular weight sulfhydryl concentration in extracts from mycelia of the wild-type strain, ST L 74 A, submitted to starvation in phosphate buffer (A) and of BAT 9-4 A after being treated with conidia from BAT 9-5 A. Treatments were performed as described under Materials and Methods. Samples of the mycelial extracts were passed through 0.7 × 10-cm columns of G-25 Sephadex equilibrated with 0.1 M sodium phosphate buffer, *p*H 6.0, and eluted with the same solution. Tyrosinase activity was assayed in the protein fraction, and sulfhydryl concentration in the fraction eluting after the protein peak was measured with DTNB using GSH as standard. Other details were as specified under Materials and Methods. (△) Sulfhydryl concentration (nmol/mg protein) in starved (A) and mated cultures; (▲) sulfhydryl concentration in control cultures (unmated or "mated" with conidia from the same mating type); (○) tyrosinase activity (units/mg protein) in starved (A) and mated (B) cultures; (●) tyrosinase activity in control cultures.

which the mycelia were submitted, for instance, the lack of sulfur, energy source, etc., under starvation, or the decay of the sulfate transport system when it was treated with cycloheximide (Marzluf, 1972). On the other hand, the same phenomenon was observed in the mated mycelium of strain BAT 9-4 (Fig. 3B). In this case tyrosinase derepression occurred much earlier, and its activity level was 10 times higher than under starvation conditions. The sulfhydryl concentration decreased equally rapidly. In this case the two phenomena seem to be part of a sex-specific response, because they were elicited only when conidia of the opposite mating type were added to the "female receptor" mycelium. Thus, it seems that under physiological conditions, the decrease in the level of the low molecular weight sulfhydryl fraction, in which glutathione is predominant, is associated with tyrosinase derepression.

More work is needed to explain these phenomena in biochemical terms, for instance, to study the participation of the γ-glutamyl cycle enzymes (Meister, 1974), and to clarify if the decrease in sulfhydryl concentration is due to release to the external medium or to a change in the thiol-disulfide status of GSH. We are at present studying these problems concerning the metabolism of the natural inhibitor of *Neurospora* tyrosinase.

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