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Isolation and Characterization of Low-Kynureninase Mutants of *Neurospora crassa*

STEVEN C. SCHLITT, GABRIEL LESTER,[1] AND PETER J. RUSSELL
Biology Department, Reed College, Portland, Oregon 97202

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Two kynureninase activities are known in *Neurospora crassa*, one of which (kynureninase I) is inducible, the other (kynureninase II) being constitutive. A method is described for the isolation of low-kynureninase mutants of *N. crassa*. When grown on an inducer, the mutants show significantly less kynureninase I activity compared with wild type, whereas constitutive kynureninase II activity is unaffected. Since a low level of kynureninase I activity remains in the mutants examined, the mutations may be in a regulatory gene or genes. Other experiments are described concerning the molecular weights of the two enzymes and the intracellular localization and specificity of kynureninase II.

Kynureninase (L-kynurenine-hydrolase, EC 3.7.1.3) catalyzes three reactions in *Neurospora crassa*, namely the conversion of L-kynurenine to anthranilic acid, L-3-hydroxykynurenine to 3-hydroxyanthranilate, and *N*-formyl-L-kynurenine to *N*-formylanthranilate (4). Enzyme activity may be induced by growth of a culture on indole, tryptophan, or kynurenine (9). Turner and Drucker (7) have demonstrated that induced cultures have two kynureninase activities (kynureninase I and kynureninase II) separable by diethylaminoethyl (DEAE)-cellulose column chromatography. The two activities were shown to differ in their kinetic properties and in their reaction to the cofactor pyridoxal phosphate. They presented evidence that uninduced cultures have very little kynureninase I, with no detectable kynureninase II. More recently Gaertner et al. (3) demonstrated that one of the enzymes, that eluting first from their DEAE-cellulose column (kynureninase I), was inducible, whereas the second enzyme eluted (kynureninase II) was not. From kinetic experiments, they concluded that the first enzyme is a kynureninase which preferentially catalyzes the kynurenine-to-anthranilate reaction and is induced over 400-fold by tryptophan. They believe that the second enzyme is a constitutive hydroxykynureninase, noninducible by tryptophan, which preferentially catalyzes the reaction of L-3-hydroxykynurenine to 3-hydroxyanthranilate. In this paper we describe a method for the isolation and characterization of low-kynureninase mutants of *N. crassa*. In addition, we present evidence regarding enzyme molecular weight, specificity, and localization.

[1] Deceased November 1972. This manuscript was prepared by P. J. Russell.

MATERIALS AND METHODS

Organism. All strains used were derived from the *N. crassa* wild-type strain 74A.

Mutagenesis. Conidia from a *leu*$^-$ strain were brought to a concentration of 1.5×10^8/ml in saline. The mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in saline was added to a final concentration of 60 μg/ml, and incubation was carried out at room temperature for 40 min, at which time the conidia were harvested by centrifugation and the mutagen was washed out with saline. Under these conditions a 99% kill was routinely obtained.

Selection for kynureninase mutants. When grown on media containing high concentrations of inducer (about 1 μmol of L-tryptophan per ml), wild-type strains produce a marked fluorescence which may be attributed to anthranilate, the product of the kynureninase-catalyzed reaction. When the strains are grown on low levels of inducer, very little fluorescence results. The accumulation of anthranilate in the presence of high tryptophan is consonant with the fact that tryptophan induces kynureninase. Kynureninase mutants were, therefore, sought in the following manner, the basic medium throughout containing Vogel minimal salts (8), 1% sucrose, 0.2 μmol of L-leucine per ml, 0.01 μmol of nicotinamide per ml, and 1% Casamino Acids, supplemented as indicated. Mutagenized conidia were plated onto low-tryptophan medium (basic medium ⊕ 0.1 μmol of L-tryptophan per ml), and the resulting colonies were isolated into culture tubes containing high-tryptophan liquid broth (basic medium ⊕ 0.8 μmol of L-tryptophan per ml) and incubated for 72 h at 30 C. Isolates were made on slants of low-tryptophan medium from those culture tubes showing low fluorescence under ultraviolet illumination. These were tested for growth on

low-tryptophan medium (as above) and on low-kynurenine medium (basic medium ⊕ 0.15 μmol of L-kynurenine/ml) by incubation for 72 h at 30 C. Those isolates that grew on tryptophan but not on kynurenine were the potential kynureninase mutants and were assayed for kynureninase activity. By this scheme, other tryptophan or niacin mutants should not be obtained since only kynureninase mutants should exhibit low fluorescence.

Growth and harvesting conditions. Harvested conidia were inoculated to a final concentration of 10^7/ml into flasks containing 200 ml of Vogel minimal medium with or without the inducer L-tryptophan. The flasks were shaken overnight at 28 C. The resulting germinated conidia were used to inoculate flasks, containing 800 ml of the same medium, which were shaken for 72 h at 28 C. The mycelia were harvested on filter paper, washed with distilled water, and lyophilized.

Enzyme extraction. The lyophilized mycelia, ground to a fine powder by using a 40-mesh screen in a Wiley Mill, were extracted by stirring for 30 min in 0.1 M potassium phosphate buffer (pH 7.8) containing 10^{-3} M ethylenediaminetetraacetate (EDTA) with 25 ml of buffer per g dry weight. After centrifugation for 30 min at 35,000 × *g*, the supernatant liquid was extracted by stirring for 10 min with 1.5% protamine sulfate (pH 6.5), using 2 ml/g of mycelia extracted and centrifuged for 10 min at 15,000 × *g*. The supernatant liquid was subjected to ammonium sulfate fractionation in 0.1 M potassium phosphate buffer, pH 7.8. The fraction was clarified by centrifugation.

Column chromatography. A 5-ml sample of a 48 to 68% ammonium sulfate fraction was introduced into a column (2.2 by 20 cm) containing a 50-ml bed of DEAE-cellulose. The column was eluted with 450 ml of 0.065 M potassium phosphate buffer (pH 7.8; containing 10^{-3} M EDTA) to isolate kynureninase I activity, and then with 400 ml of 0.2 M potassium phosphate buffer (pH 7.8; containing 10^{-3} M EDTA) to isolate kynureninase II activity. The flow rate was 0.75 ml/min.

Kynureninase assay. The enzyme was assayed essentially as described by Jakoby and Bonner (4). Enzyme activity was determined in a volume of 1.0 ml containing 0.5 ml of enzyme preparation, 50 μmol of 0.2 M $MgSO_4$, 0.5 μmol of L-kynurenine or L-3-hydroxykynurenine (omitted in blank), and 7.5 μliters of pyridoxal phosphate (400 μg/ml). The reaction mixture was incubated for 30 min at 37 C and then stopped by chilling and adding 0.5 ml of 1.0 M acetate buffer, pH 4.0. Five milliliters of ethyl acetate was added and the mixture was extracted on a Vortex mixer. The solution was clarified by centrifugation, and the product of the reaction was determined fluorometrically with an Aminco Bowman spectrophotofluorometer. When L-kynurenine was used as substrate, excitation was at 340 nm; fluorescence emission was monitored at 402 nm. The wavelengths were 340 and 420 nm, respectively, when L-3-hydroxykynurenine was substrate. One micromole of anthranilic acid in 5 ml of ethyl acetate was determined to give 640 relative fluorescence units (RFU) at the given wavelengths. Specific activity is defined as RFU per milligram of protein under the described assay conditions. Protein was determined by the method of Lowry et al. (6).

Assay for the presence of mitochondria. Mitochondria were isolated by differential centrifugation and washed with isotonic sucrose (0.25 M) as described by Koke et al. (5). An assay for succinic dehydrogenase was used to identify the presence of mitochondrial membrane (1).

RESULTS

Figure 1 presents the DEAE-cellulose column profile of kynureninase from a *kyn*$^+$ strain grown in the absence (Fig. 1A) and in the presence (Fig. 1B) of the inducer L-tryptophan. As has been demonstrated by other workers, the first eluting activity (kynureninase I) is inducible by L-tryptophan, whereas the second is constitutive, showing relatively little increase in activity in the presence of inducer. The two activities were shown to be pure by rechromatographing the eluted peak fractions separately. In both cases only one peak was observed eluting with the expected buffer concentration.

Compared with wild type, kynureninase I activity is induced to a relatively lesser degree

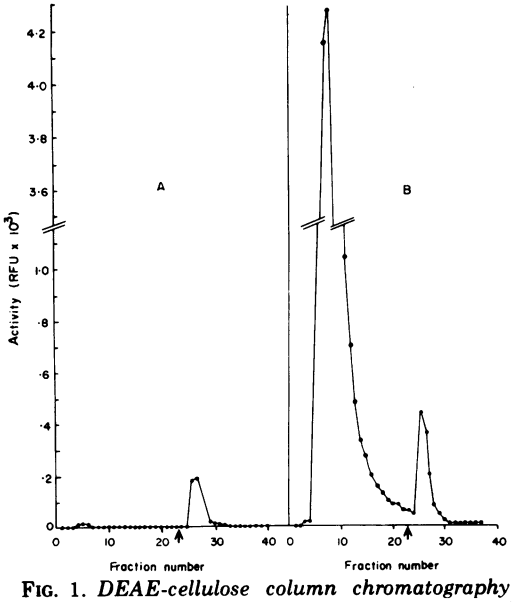

FIG. 1. *DEAE-cellulose column chromatography of the 48 to 68% ammonium sulfate fraction of extracts prepared from a kyn*$^+$ *strain. Conditions of chromatography are described in Materials and Methods. In each case the sample applied to the column contained 160 mg of protein. The point at which the buffer was changed is indicated by an arrow. (A) Kynureninase activity profile of a kyn*$^+$ *strain grown in the absence of L-tryptophan. (B) Kynureninase activity profile of a kyn*$^+$ *strain grown in the presence of 0.8 μmol of L-tryptophan per ml.*

(100-fold versus 700-fold) in a low-kynureninase mutant strain that we have designated *kyn-1* (Fig. 2). The constitutive kynureninase II activity in the presence or absence of inducer is not significantly different in the two strains. Other low-kynureninase mutants isolated both by the procedure described and by another method were also examined for kynureninase activities. They were shown to display essentially the same biochemical properties as the mutant described.

For further studies, peak column fractions were routinely collected and concentrated, by using Union Carbide Carbowax 20,000, and rechromatographed. Combined peak fractions from this step constituted the purified enzyme preparations.

Molecular weight determination. A 55-ml bed column of Sephadex G-100, (1 cm in diameter), calibrated with standard proteins (bovine serum albumin, ovalbumin, human gamma globulin), was used to determine the molecular weights of purified kynureninase I and kynureninase II. The column was eluted with 0.05 M ammonium acetate buffer, pH 8.3. We determined that the molecular weights are 90,000 and 70,000 for kynureninase I and kynureninase II, respectively.

Enzyme localization. Gaertner et al. (3) have presented evidence, based on kinetic experiments, that kynureninase I preferentially catalyzes the reaction L-kynurenine to anthranilic acid, whereas kynureninase II preferentially catalyzes the reaction L-3-hydroxykynurenine to 3-hydroxyanthranilic acid. Therefore, they term the constitutive enzyme a hydroxykynureninase. Cassady and Wagner (2) have shown that the enzyme L-kynurenine-3-hydroxylase, which catalyzes the formation of 3-hydroxykynurenine from L-kynurenine, is localized on the outer membrane of *Neurospora* mitochondria. Therefore, we reasoned that, if kynureninase II is indeed a hydroxykynureninase, we should be able to detect kynureninase activity in a mitochondrial fraction. Table 1 presents the results of a typical assay for kynureninase activity in mitochondria compared with total kynureninase activity present in the crude extract. The cultures used were grown in the absence of the inducer L-tryptophan and, therefore, only kynureninase II activity is present. The results show that negligible kynureninase II activity is associated with the mitochondria and that no significant difference in activity is apparent when L-kynurenine and L-3-hydroxykynurenine are used as substrates for the reaction.

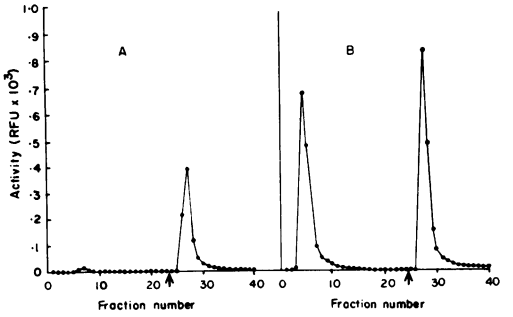

FIG. 2. *DEAE-cellulose column chromatography of the 48 to 68% ammonium sulfate fraction of extracts prepared from a kyn-1 mutant strain. Conditions of chromatography are described in Materials and Methods. In each case the sample applied to the column contained 155 mg of protein. The point at which the buffer was changed is indicated by an arrow. (A) Kynureninase activity profile of kyn-1 grown in the absence of L-tryptophan. (B) Kynureninase activity profile of kyn-1 grown in the presence of 0.8 μmol of L-tryptophan per ml.*

DISCUSSION

Our results concur with the conclusions of other workers (3, 7) that the two kynureninase activities of *N. crassa* may be differentiated in that kynureninase I is inducible and kynureninase II is constitutive. When wild type is induced, kynureninase I activity increases 700-fold and kynureninase II activity increases twofold over uninduced levels. In an attempt to obtain an understanding of the regulation of kynureninase synthesis, we devised a selection scheme to isolate mutants with low-kynureninase activity. One of these mutants, *kyn-1*, is described in detail in this paper. In contrast to wild type, this mutant, when induced, shows a 100-fold increase in kynureninase I activity and a twofold increase in kynureninase II activity over uninduced levels. Two conclusions may be drawn from these results. Firstly, the action of the inducer L-tryptophan is not confined to kynureninase I activity since a significant in-

TABLE 1. *Assay for kynureninase in mitochondria of Neurospora*

Fraction	Kynureninase II activity[a] with substrate:	
	L-Kynurenine	L-3-Hydroxy-kynurenine
Crude fraction	462	453
Mitochondrial fraction	2.1	2.0

[a] Expressed as total RFU in fraction.

crease in kynureninase II activity is observed, and to the same degree, both in wild type and the mutant *kyn-1* strain. Secondly, since kynureninase I activity is not completely abolished in induced cultures of any of the low-kynureninase mutants identified, it is possible that the mutations are in a locus or loci involved with the regulation of kynureninase I synthesis rather than in the structural gene itself. In addition, according to our results, the mutations do not show any effect on the induction of kynureninase II activity by L-tryptophan.

The question of specificities of the two kynureninase enzymes found in *N. crassa* has been considered by Gaertner et al. (3). They presented evidence that kynureninase II is a hydroxykynureninase which preferentially catalyzes L-3-hydroxykynurenine to 3-hydroxyanthranilate. The enzyme catalyzing the formation of L-3-hydroxykynurenine, kynurenine hydroxylase, is found on the outer membrane of the mitochondria of *N. crassa* according to Cassady and Wagner (2). Thus, one would expect kynureninase II activity to be associated with the mitochondrial fraction if kynureninase II were indeed a hydroxykynureninase. Our results indicate that negligible kynureninase II activity is present in *Neurospora* mitochondria. And, since no significant difference in activity is apparent when L-kynurenine and L-3-hydroxykynurenine are used as substrates in the reaction, we consider that the designation of kynureninase II as a hydroxykynureninase is still open to question and warrants further investigation.

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