OXYGENASES INVOLVED IN THYMINE AND THYMIDINE METABOLISM IN *NEUROSPORA CRASSA*

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1. Introduction

A number of enzyme-catalyzed oxygenations have been disclosed during the last 15 yr but the reaction mechanisms are still incompletely understood. In the case of the 2-ketoglutarate-dependent oxygenations, available evidence indicates that a peroxide of 2-ketoglutarate and the substrate to be hydroxylated is a reaction intermediate [1, 2]; recent suggestions of a primary formation of a peracid from 2-ketoglutarate followed by attack on the substrate [3] have not been supported by experimental data. The wild type of *Neurospora crassa* contains enzymatic activities which catalyze the conversion of thymidine to thymine riboside [4], of thymine to 5-carboxyuracil [m] with the intermediary formation of 5-hydroxymethyluracil and 5-formyluracil [5–12] [m] and of 1-methyluracil to uracil (unpublished). 2-Ketoglutarate and ferrous ion are cofactors for these oxygenase reactions. It is of great interest to elucidate whether these 5 oxygenations are catalyzed by one and the same enzyme or if several enzymes are involved. McCroskey et al. [13] have reported that the enzymic activity catalyzing the hydroxylation of thymidine is less stable than that involved in thymine hydroxylation. This cannot alone be taken as evidence that thymidine hydroxylase is unrelated to the other hydroxylases, as conformational changes of an enzyme protein might change the catalytic properties to an extent which varies for different substrates. Williams and Mitchell [14] have studied the nutritional requirements of a number of mutant Neurospora strains with different blocks in pyrimidine metabolism. They reported that their *uc*-3 mutant is blocked in the conversion of 5-hydroxymethyluracil to 5-formyluracil whereas it retains the capacity to utilize 5-formyluracil. The mutant was unable to utilize thymine. These observations would indicate the presence of at least 2 different enzymes in the reaction sequence thymine to 5-carboxyuracil.

This communication presents data indicating that thymidine 2′-hydroxylase is, in fact, a separate enzyme, and that the sequential oxygenation of thymine to 5-carboxyuracil is catalyzed by one enzyme.

2. Materials and methods

Thymine and 5-hydroxymethyluracil were obtained from Calbiochem AG, Lucerne, Switzerland, and 1-methyluracil, thymidine, 2-deoxyuridine, 2-deoxycytidine, 2-deoxyadenosine, 2-deoxyguanosine and thymidylic acid from Cyclo Chemical Division, Travenol Laboratories Incorp., Los Angeles, California, USA. 2-Keto-1-$^{14}C_1$-glutaric acid was obtained from New England Nuclear Corp., Frankfurt/Main, Germany. 5-Formyluracil was synthesized as described previously [9].

N. crassa strain STA 4 (FGSC 262 A, Fungal Genetics Stock Center, Humboldt State College, Arcata, California) was cultured as described previously [7]. The *uc*-3 mutant strain of Neurospora was a gift from Dr. L. G. Williams, Div. of Biology, Kansas State University, Manhattan, Kansas 66502. The mold was grown in minimal medium [7]. Mycelia were harvested and homogenized as previously reported [9]. After dialysis of the 100,000 *g* supernatant fraction against 10 mM potassium phos-

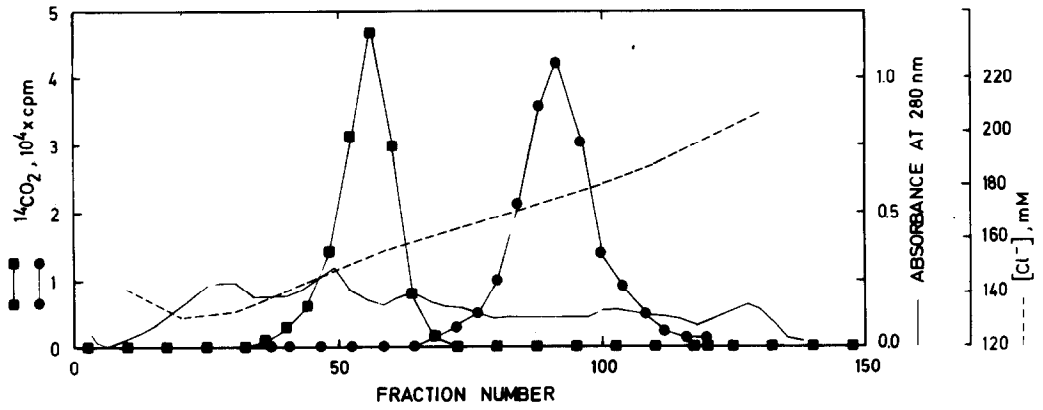

Fig. 1. Chromatography of thymine 7-hydroxylase (▪–▪–▪) and thymidine 2′-hydroxylase (•–•–•) on a column (25 X 125 mm) of DEAE-Sephadex (A-50). The 100,000*g* supernatant fraction from homogenized mycelia of wild strain *N. crassa* had first been fractionated on hydroxylapatite (see fig. 2, upper part). After desalting on Sephadex G-25 (coarse) in 110 mM KCl with 25 mM Tris-HCl and 0.10 M glycine at pH 6.5, the proteins were applied to the column which had been equilibrated with the same salt solution. The column was eluted with a gradient of KCl (110 to 175 mM) in 25 mM Tris-HCl and 0.10 M glycine at pH 6.5. The fraction volume was 4 ml. The enzyme activity was assayed as formation of $^{14}CO_2$ from 2-keto-1-$^{14}C_1$-glutarate as described in Materials and methods.

phate, pH 6.5, the material was fractionated on a hydroxylapatite column (Bio-Rad Laboratories, Richmond, California 94804) as described in the legend to fig. 2. Fractions containing hydroxylase activity were pooled and the proteins concentrated with a 400 ml Diaflo ultrafiltration apparatus (Amicon Corp., Lexington, Mass. 02173). After desalting on a Sephadex G-25 column, the proteins were fractionated on a column of DEAE-Sephadex (A-50, Pharmacia Fine Chemicals AB, Uppsala, Sweden) as described in the legend to fig. 1. All buffers contained 0.10 M glycine.

Assays were based on the finding that 2-ketoglutarate is oxidatively decarboxylated concomitantly with oxygenation of thymine [7], 5-hydroxymethyluracil, 5-formyluracil [9], 1-methyluracil, thymidine, and deoxyuridine (unpublished) The incubation mixture (0.2 ml) had the following composition: 50–300 mg enzyme per l, 0.5 mM pyrimidine or nucleoside substrate, 0.25 mM potassium 2-keto-1-$^{14}C_1$-glutarate (0.25 mCi per l), 5 mM $FeSO_4$, 5 mM sodium ascorbate, and 50 mM potassium phosphate at pH 7.5. Incubations were carried out for 30 min at 37° and then terminated by addition of trichloracetic acid (50 g/l). $^{14}CO_2$ was trapped on a filter paper with Hyamine and counted in a Packard liquid scintillation counter [7].

3. Results and discussion

3.1. *Hydroxylation of thymidine*

The instability of the thymidine hydroxylating enzyme has previously precluded its purification. We have observed that this enzyme is stabilized by glycine and by dithiothreitol and that thymine hydroxylase is stabilized by glycine but inactivated by dithiothreitol. Inclusion of glycine in the buffers made it possible to study the chromatographic and electrophoretic behaviour of these enzymic activities. Chromatography on DEAE-Sephadex separated 2 enzymic activities (fig. 1). The first catalyzed the oxygenation of thymine, 5-hydroxymethyluracil, 5-formyluracil, and 1-methyluracil. The second catalyzed the hydroxylation of thymidine and of deoxyuridine but not of deoxycytidine, deoxyadenosine, deoxyguanosine or thymidylate. This finding establishes thymidine (deoxyuridine) 2′-hydroxylase as a separate enzyme. Isoelectric focusing showed a pI value of 4.6 for thymidine hydroxylase and of 4.9 for thymine 7-hydroxylase. Thymidine 2′-hydroxylase has similar cofactor requirements as thymine 7-hydroxylase, indicating a similar reaction mechanism [9]. The biological role of thymidine 2′-hydroxylase is unknown at present.

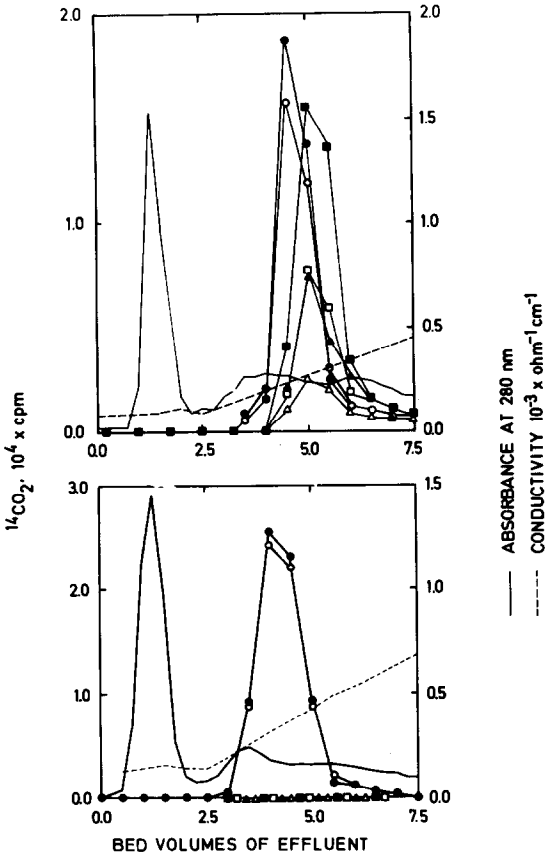

Fig. 2. Fractionation on a column (25 × 200 mm) of hydroxylapatite of the 100,000*g* supernatant fraction of homogenized mycelia from a wild strain (upper part) and from the *uc*-3 mutant strain of *N. crassa* (lower part). The column was eluted with a gradient of 10 to 80 mM potassium phosphate, pH 6.5, containing 0.10 M glycine. The enzyme activity was assayed as formation of $^{14}CO_2$ from 2-keto-1-$^{14}C_1$-glutarate in the presence of Fe^{2+}, ascorbate, catalase and the following substrates: thymine (■–■–■), 5-hydroxymethyluracil (□–□–□), 5-formyluracil (△–△–△), 1-methyluracil (▲–▲–▲), thymidine (●–●–●), and deoxyuridine (○–○–○).

3.2. *Oxygenation of thymine, 5-hydroxymethyluracil, 5-formyluracil and 1-methyluracil*

We have so far purified thymine 7-hydroxylase about 3000 times by a series of chromatographic procedures. During the whole procedure the ratio between the enzymatic activities catalyzing the 3 steps in the oxygenation of thymine to 5-carboxyuracil remained constant. These findings made it of interest to examine the *uc*-3 mutant strain with respect to its enzymatic activities. Extracts of the wild strain and of the *uc*-3 strain were fractionated on columns of hydroxylapatite (fig. 2). Extracts of the wild type catalyzed the hydroxylation of thymine, 5-hydroxymethyluracil, 5-formyluracil, 1-methyluracil, thymidine and deoxyuridine (fig. 2, upper part), whereas extracts of the *uc*-3 mutant catalyzed only the hydroxylation of thymidine and of deoxyuridine (fig. 2, lower part).

Together with results from the enzyme purification studies these findings indicate that thymine 7-hydroxylase catalyzes the 3 sequential oxygenations of thymine to 5-carboxyuracil, each of which is coupled to the degradation of the co-substrate, 2-ketoglutarate. Such an enzyme would be unique from an enzymological point of view. Of further interest is that thymine 7-hydroxylase differs from other 2-ketoglutarate dependent hydroxylases with respect to its sensitivity to different oxidizing agents which makes it particularly suitable for mechanistic studies.

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