Thymidine 2'-Hydroxylation in *Neurospora crassa*  

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**SUMMARY**

An enzyme preparation catalyzing the 2'-hydroxylation of thymidine was prepared from mycelial extracts of *Neurospora crassa* strain STA 4 (FGSC 262A) by chromatography on hydroxylapatite and DEAE-Sephadex A-50. The enzyme had an isoelectric point of 4.6 and a molecular weight of 47,000 (determined by gel filtration). The reaction product was identified with thymine riboside on ion exchange and thin layer chromatography. Paper chromatography after reduction and hydrolysis showed that the pentose moiety in the product was ribose. Of the pyrimidine deoxyribonucleosides tested as substrates, only those with oxygen at carbon atoms 2 and 4 were active, *i.e.*, deoxyuridine, thymidine, 5-hydroxymethyldeoxyuridine, 5-bromodeoxyuridine, and 6-azathymidine. Thymidylate was attacked only slowly. Purine deoxyribonucleosides, deoxyribose, and 1-ethyluracil were not substrates. No evidence for control by nucleotides was obtained. The enzyme had an absolute requirement for ferrous ion and 2-ketoglutarate, and ascorbate and catalase were stimulatory. 2-Ketoglutarate was oxidatively decarboxylated during the reaction. Together with the previously demonstrated oxygen requirement (Shaffer, P. M., McCroskey, R. P., Palmater, R. D., Midgett, R. J., and Abbott, M. T. (1998) *Biochem. Biophys. Res. Commun.* 35, 800) the present findings establish that thymidine 2'-hydroxylase belongs to the group of oxygenases which require a 2-keto acid for oxygen reduction (Holm, E., Lindstedt, G., Lindstedt, S., and Toft, M. (1968) *Fed. Eur. Biochem. Soc. Lett.* 2, 29; Lindblad, B., Lindstedt, G., Lindstedt, S., and Toft, M. (1969) *J. Amer. Chem. Soc.* 91, 4604).

The hydroxylation of thymidine was discovered by Shaffer *et al.* (1) during a study of thymines 7-hydroxylation in extracts from a *Neurospora crassa* strain. The reaction required molecular oxygen and was stimulated by 2-ketoglutarate. The product was identified as thymine riboside on the basis of data from chromatography on thin layers of silica gel. The hydroxylating activity was less stable than thymine 7-hydroxylase, indicating that two separate enzymes were involved (2). Recently, conclusive evidence for this assumption was obtained from our laboratory (3), when the two enzymes were separated on an ion exchanger.

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human albumin from Dakopatt A/S, Copenhagen, Denmark. Human β2-microglobulin was prepared according to the method of Berggård and Bearn (5) from urine from uric acid cases with a "tubular" type of proteinuria, and antisera were raised in rabbits.

**Chromatographic Procedures**—Thin layer chromatography of pyrimidines was carried out on glass plates coated with kieselgél GF 254 which had been heated at 105°C for 1 hour. The mobile phase was tert-butyl alcohol-ethylmethyl ketone-water-25% aqueous ammonia (4:3:2:1). The plates were scanned for light absorption at 260 nm in a Zeiss chromatogram scanning photometer M4QIII and for radioactivity with a scanner LB 2722 (Laboratory Professor Dr. Berthold, Wildbad, West Germany). Approximate RF values were: thymine, 0.78; thymidine, 0.72; thymine riboside, 0.57. Descending paper chromatography of chromatography on hydroxylapatite and DEAE-Sephadex, see "Enzyme Preparation." Approximate RF values were: deoxyribose, 0.45; arabinose, 0.19; ribose, 0.28; dihydrothymine, 0.56; dihydrothymidyne, 0.50; dihydrothymine riboside, 0.38.

Separation of succinic and 2-ketoglutaric acids was carried out on a column (1.0 x 17.5 cm) of silicic acid (about 8 g) (6, 7). The stationary phase was 5.0 ml of 0.25 M aqueous sulfuric acid and the mobile phase tert-butyl alcohol-benzene (1:9). The fractions (3 ml) were analyzed for radioactivity and titrated with 0.02 M potassium hydroxide after the addition of 2 ml of 96% ethanol and 2 drops of a 0.1% aqueous solution of phenol red.

Purification of the product after incubating the enzyme preparation with [14C]thymidine was carried out on a column (1.0 x 38 cm) of the anion exchanger Dowex AG 2-X8 (300 to 400 mesh, bicarbonate form) which was eluted with a linear gradient of 0.05 to 0.20 M ammonium carbonate at pH 9.0 (total volume, 1500 ml). Nonlabeled thymidine and thymine riboside were added to the incubation mixture before the chromatography. Light absorption at 254 nm was recorded in the effluent with an Uvicord 2 (LKB-Produkter AB, Stockholm, Sweden).

The isotope content was measured by liquid scintillation counting. The relative amounts of thymidine and thymine riboside were estimated by thin layer chromatography and scanning for light absorption at 260 nm. Fractions containing thymine riboside were pooled and lyophilized. This material contained a catalyst poison which interfered with subsequent catalytic hydrogenation. This could be removed on a small column (0.5 x 2 cm) of Dowex AG 50W-X8 (minus 400 mesh, hydrogen form) which was eluted with 3 bed volumes of water. The eluate was lyophilized.

Iseoelectric focusing was carried out on a 110-ml electrofocusing column (LKB-Produkter AB, Stockholm, Sweden) at 4°C. A pH gradient between 3 and 6 was established with a 1% solution of a carrier ampholyte. The enzyme preparation (20 mg of protein) had been applied in the middle of the column. A potential of about 750 volts was applied for 48 hours and the content was then collected in fractions of 3 ml. Particle size ("molecular weight") determinations were carried out by gel filtration in a column (2.6 x 87 cm) of Sephadex G-100 (superfine) eluted with 50 mM potassium phosphate buffer at pH 6.8 containing 0.1 M glycine. About 60 mg of protein were added 0.5 ml of human serum, 10 mg each of myoglobin and human β2-microglobulin, and 1.6 ng of [57Co]cobalamin (0.1 μCi). Fractions of 6 ml were collected and analyzed for labeled transcobalamin I (mol wt 120,000) with a well-type scintillation counter (Autowell II, Picker Nuclear, Division of Picker X-Ray Co., White Plains, New York), for albumin (mol wt 68,000) with the immunochromatogram scanning photometer M4QIII and for radioactivity with a scanner LB 280 (Laboratory Professor Dr. Berthold, Wildbad, West Germany). Sugars were made visible by spraying with aniline hydrochloride solution (0.93 g of aniline and 0.66 g of phthalic acid in 100 ml of n-butyl alcohol saturated with water) and heated at 100°C for 15 min. Approximate RF values were: deoxyribose, 0.45; arabinose, 0.19; ribose, 0.28; dihydrothymine, 0.56; dihydrothymidyne, 0.50; dihydrothymine riboside, 0.38.

Enzyme Preparation—The purification procedure has been described recently (3, 4), and will be summarized. All buffers contained 0.1 M glycine which has been found to stabilize thymine 7-hydroxylase, and to some extent, thymidine 2'-hydroxylase (3, 4). In N. crassa strain STA 4 (FGSC 262 A, Fungal Genetics Stock Center, Humboldt, State College, Arcata, California) was grown for 4 days at 25°C in 50-liter flasks with an air flow of 1 liter per min. Mycelia (500 g, wet wt) were homogenized in about 500 g of Dry Ice with a MSE Atommix (Measuring and Scientific Equipment, Ltd., London, England). The homogenate was centrifuged at 15,000 x g for 1 hour at 4°C. The sediment (270 g) was ground with 50 g of sand in an ice-cooled mortar for 10 min with intermittent additions of 50-ml portions of cold 5 mM potassium phosphate at pH 6.5 (in all 150 ml). After centrifugation as above the combined supernatant fractions were recentrifuged for 60 min at 100,000 x g. The supernatant (14g of protein, about 400 ml) was dialyzed against 5 mM potassium phosphate, pH 6.5, and fractionated by chromatography on a column (5 x 25 cm) of hydroxylapatite (about 250 g). The column was eluted with a linear gradient between 10 and 80 mM potassium phosphate at pH 6.5 (total volume, 4.0 liters). The fractions with thymidine hydroxylase activity (between about 40 and 60 mM phosphate, 500 ml) were pooled and the pH value was adjusted to 7.5 with Tris. The protein solution was concentrated with a Diaflo (Amicon, N.Y., The Hague, Holland) ultrafiltration apparatus to 10 to 15 ml and then frozen in aliquots. This preparation ("the hydroxylapatite fraction") contains thymidine 2'-hydroxylase, thymine 7-hydroxylase, and thymidine nucleosidase (phosphorylase) activities.

After desalting on a column (1.5 x 30.0 cm) of Sephadex G-25 (coarse) in 25 mM Tris-HCl at pH 6.5 with 110 mM potassium chloride, 90 mg of protein were fractionated on a column (2.5 x 12.5 cm) of DEAE-Sephadex A-50 (about 3 g). The column was eluted with a linear gradient of potassium chloride (110 to 175 mM) in 25 mM Tris-HCl at pH 6.5. The fractions with thymidine hydroxylase activity (between about 150 and 160 mM potassium chloride) were pooled, the pH value adjusted to 7.5
with Tris, and the solution then concentrated by ultrafiltration to a protein concentration of about 2 mg per liter. The resulting preparation had an activity of about 16 units per g for thymidine 2′-hydroxylation. No thymine 7-hydroxylase thymidine nucleosidase, or thymine riboside nucleosidase activities were detected.

**Incubations**—The incubation mixture ("complete system") usually contained about 30 μg of protein, a pyrimidine nucleoside (usually 0.5 mM), 2-ketoglutarate (usually 0.25 mM), ferrous sulfate (5 mM), ascorbate (5 mM), catalase (2 mg per ml), and 50 mM potassium phosphate at pH 7.6 in a total volume of 0.2 ml. The pH value in the incubations was usually around 7.2. In the studies on the variation in enzymic activity with variation of pH, the pH values given result from measurements, after incubations, with a micro glass electrode (pH-meter AMT 1, Radiometer A/S, Copenhagen, Denmark). During the incubations the change in pH value was less than 0.05 units. The low value for the 2-ketoglutarate concentration when the 2-ketoglutarate decarboxylation assay was used (0.25 mM, the value found for apparent Kₚ, see "Results") was a compromise between economy, sensitivity, and desire to obtain satisfactory kinetics.

Incubations were carried out at 37°C for 30 min. Assays for thymidine hydroxylation contained 0.5 μCi of [14C]thymidine or [2-14C]thymidine and assays for 2-ketoglutarate decarboxylation, 0.05 μCi of 2-ketoglutarate (2-[14C]glutarate).

In the 2-ketoglutarate decarboxylation assay, the 14CO₂ formed from labeled 2-ketoglutarate was trapped on a piece of filter paper attached to a wire in the rubber stopper of the test tube. Twenty microliters of a 1 M solution of Hyamine in methanol had been pipetted onto the filter paper. The incubations were stopped by the addition of 0.2 ml of 10% aqueous trichloroacetic acid. Diffusion of carbon dioxide was allowed to proceed for 60 min at 37°C, and the filter papers were then transferred to a scintillation vial, containing 10 ml of a solution of the following composition: 2,5-diphenyloxazole (10 g), 1,4-bis[2-(4-methyl-5-phenylloxazolyl)]benzene (0.3 g), toluene (1000 ml), and methyl Cellosolve (600 ml). Radioactivity measurements were done in a Packard Tri-Carb liquid scintillation counter. In the thymidine hydroxylase assay the incubation was stopped by adding 0.8 ml of 96% ethanol. [14C]Thymidine was separated from [14C]thymine riboside by means of thin layer chromatography as discussed above.

**RESULTS**

**Reaction Product**—Thymidine was converted to a more polar compound after incubation with enzyme, 2-ketoglutarate, ferrous sulfate, ascorbate, and catalase. The product had the same mobility as thymine riboside on thin layer chromatography on silica gel (cf. Reference 1). Fig. 1 shows an anion exchange chromatogram from an incubation of a trace amount of [14C]thymidine. Nonradioactive thymidine and thymine riboside had been added before chromatography. The radioactive material was eluted together with thymine riboside. This material was hydrolyzed after catalytic hydrogenation as described under "Experimental Procedures." After paper chromatography, the radioactive material was then found at the positions of ribose, 5,6-dihydrothymine, and 5,6-dihydrothymine riboside (Fig. 2). No radioactive arabinose or 5-hydroxymethyl-5,6-dihydrouracil could be detected. These findings establish thymine riboside as the product of thymidine hydroxylation. No labeled riboside could be detected after incubation of heat-inactivated enzyme with [2-14C]thymidine and cofactors.

**Enzyme Concentration, Incubation Time, and pH Optimum**—The hydroxylating activity was proportional to the concentration of enzyme protein up to 0.25 g per liter. A lag period of about 2 min was observed, the rate was then unchanged during 45 min. No lag period was observed when the enzyme had previously been incubated for 5 min at 37°C with the incubation mixture.
minus 2-ketoglutarate. As shown in Fig. 3, the optimal pH value was around 6.5. The activity at pH 7.2 was about 80% of that at pH 6.5. In preliminary experiments with crude extracts from Neurospora mycelia, thymine, and thymidine hydroxylases had been shown to be more stable at pH values up to 1 pH unit higher than 6.5, and 7.2 was therefore chosen as pH value during the incubations.

2-Ketoglutarate as Cofactor—Labeled carbon dioxide was obtained in incubations with 2-keto[1-14C]glutarate, thymidine, and cofactors. Fig. 4 shows the effect of varying the concentrations of 2-keto[1-14C]glutarate. The apparent $K_m$ value was found to be 0.2 to 0.3 mM. No labeled thymine riboside was obtained from [2-14C]thymidine after replacement of 2-ketoglutarate with other organic ions, namely pyruvate, 2-ketobutyrate, 2-ketovalerate, oxaloacetate, 2-ketoisocitrate, 2-ketoacidipate, 2-ketoisocitrate, glutamate, glutamine, iminodiacetate, diglycolate, or thio-diglycolate. Of these ions, those given in Table I were moderately inhibitory in 2.5 mM concentration, i.e. 10 times that of 2-ketoglutarate. The other ions mentioned above inhibited less than 5% under these conditions.

Chromatography on columns of silicic acid was used for the fractionation of the reaction mixture after incubating 2-keto[5-14C]glutarate with enzyme, thymidine, and cofactors. The results were identical with those obtained in previous studies with γ-butyrobetaine hydroxylases from Pseudomonas (7) and rat liver (11) and with thymine 7-hydroxylase from Neurospora (12); i.e., labeled succinic acid was found as the sole reaction product.

Deoxyribonucleoside Substrate—An apparent $K_m$ value of 0.09 mM was found in experiments with thymidine as the nucleoside substrate (Fig. 5). In these experiments the initial concentration of labeled 2-ketoglutarate was 0.25 mM, i.e. the same as the apparent $K_m$ for 2-ketoglutarate. This low value was a compromise between the requirement for sufficient sensitivity in the assay and for kinetically satisfactory concentrations. Identical values for apparent $K_m$ for thymidine were found when the initial concentration of 2-keto[1-14C]glutarate was 0.5 mM, 0.25 mM, or 0.125 mM. No formation of labeled carbon dioxide was found when thymidine was replaced by deoxythymidine, deoxyguanosine, deoxycytidine, deoxyadenosine, deoxyinosine, 1-methyluracil, 1-ethyluracil, or deoxyribose. The deoxyribose derivatives of uracil, 5-hydroxymethyluracil, 5-bromouracil, and 6-azathymine were active as substrates for the present enzyme preparation; thymidylate was also a substrate for the enzyme, albeit a poor one. These findings support the preliminary data on the substrate specificity in Reference 3. Table II shows the values obtained for apparent $K_m$ and $V_{max}$ in incubations with 0.25 mM 2-keto[1-14C]glutarate, enzyme, and cofactors.

A moderate degree of substrate inhibition was observed. This inhibition was more pronounced in incubations with higher concentrations of 2-ketoglutarate (see also Fig. 4). No inhibition was found when uracil, uridine, cytidine, or deoxycytidine

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been added in 5 mM concentration to incubations with 0.5 mM thymidine. Initial experiments with thymine riboside showed appreciable inhibition; however, no inhibition was found when the nucleoside had first been passed through a Chelex 100 resin, indicating that the inhibition was caused by an impurity. A series of incubations with various nucleotides was performed to elucidate whether any specific nucleotide exerted a biological control of thymidine 2'-hydroxylation. Deoxyuridine monophosphate, deoxyuridine triphosphate, and cytidine triphosphate were used as well as the mono-, di-, and triphosphates of the following nucleosides: thymidine, uridine, adenosine, and deoxyadenosine. Inhibition varying between 15 and 30% was noted when equal concentrations (0.5 mM) were used of thymidine and inhibitor. The nature of this moderate inhibition was not further studied.

**Metal Ion Requirement**—For most preparations of thymidine 2'-hydroxylase, there was an absolute requirement for added ferrous ion as shown in Fig. 6. The apparent $K_m$ in 50 mM phosphate buffer at pH 7.2 was 0.45 mM. $Fe^{2+}$ could not be replaced by Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, or Zn$^{2+}$. For some preparations, there was appreciable activity without added ferrous sulfate, and these preparations were slightly stimulated by several metal salts. However, the addition of 20 $\mu$M 1,10-phenanthroline resulted in complete inhibition, and this inhibition could be relieved only by ferrous salts. Higher concentrations of ferrous ion were moderately inhibitory. The other transitional metal ions were potent inhibitors; in a series of incubations with the "complete system" including 2.5 mM Fe$^{2+}$, more than 95% inhibition was caused by 0.25 mM Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$ and 60% inhibition by 0.25 mM Mn$^{2+}$. The inhibition by metal ions has not been studied further.

**Stimulation by Ascorbate and Catalase**—The stimulation by ascorbate is shown in Fig. 7. The specificity for ascorbate has not been tested for the present enzyme; however, ascorbate may be replaced by several other reductants in the case of $\gamma$-butyrobetaine hydroxylase from rat liver and Pseudomonas (11, 13) and collagen proline hydroxylase (14). The stimulation by catalase (1 to 2-fold after the addition of 2 mg per ml) is less than that found with $\gamma$-butyrobetaine hydroxylase (13, 15) and thymine 7-hydroxylase (12).

**Stoichiometry**—As indicated by the results in Table III, 1 mole of thymine riboside was formed from thymidine concomitantly with the formation of 1 mole of carbon dioxide from 2-ketoglutarate.

**Isoelectric Point and Molecular Weight of Enzyme**—The enzyme
preparation after the initial step of purification (the hydroxylapatite chromatography) was subjected to isoelectric focusing in a sucrose-stabilized pH gradient between 3 and 6 (Fig. 8). Thymidine 2'-hydroxylase was then eluted in a solution with pH 4.6. The isoelectric point of thymine 7 hydroxylase was found to be 4.9. An estimate of the molecular weight by gel filtration on Sephadex G-100 gave a value of 47,000 for thymidine 2'-hydroxylase (Fig. 9).

**DISCUSSION**

Previously identified biosynthetic reactions for ribose involve various monosaccharide phosphates (16, 17). The present reaction with a deoxyribonucleoside precursor has so far been demonstrated only in *Neurospora*. Its biological significance is unknown. *Neurospora* lacks a thymidine kinase, and thymidine may therefore be metabolized only by hydroxylation to thymine riboside or by cleavage to thymine and deoxyribose 1 phosphate. Thymidine phosphorylase may be separated from a riboside phosphorylase (unpublished). So far its appearance in the mycelial extracts has been erratic; different batches of *Neurospora* mycelia have given highly variable activities of thymidine phosphorylase. Further studies in this field are warranted. The fate of thymine riboside besides cleavage by a nucleoside phosphorylase is unknown.

The reverse reaction to the presently studied one, i.e. the formation of deoxyribose derivatives from ribose derivatives appears to be a general reaction in biological systems (for references, see Reference 18). A ribonucleoside diphosphate (in the case of *Escherichia coli* B and the Novikoff rat hepatoma) or a ribonucleoside triphosphate (*Lactobacillus leichmannii*) is reduced by a protein complex under allosteric control by nucleoside triphosphates. A hydride ion is furnished by NADPH via a dithiol, which, in the bacterial systems, is the low molecular weight protein, thioredoxin. The hydroxyl group on the 2'-carbon atom is eliminated followed by insertion of the hydride ion with retention of configuration.

The present results indicate that thymidine 2'-hydroxylase catalyzes the 2-hydroxylation of deoxyribose which is attached to a 2,4-diketo heterocyclic compound such as uracil and 6-aza-uracil (Scheme I). Substitution in the C-5 position of the ring with bromine, a methyl, or a hydroxymethyl group leads to compounds which are slightly less active as substrates. The sole nucleotide tested, thymidylate, is attacked only slowly. Compounds with an amino group instead of oxygen in the C-4 position...
are apparently inactive as substrates as are purine deoxyribo-
nucleosides. A requirement for oxygen at C-2 and C-4 in the
pyrimidine substrate has also been found with thymine 7-hy-
droxylase. This enzyme is inhibited by several pyrimidines
with polar substituents at carbon atoms 2 and 4 in contrast to
the present enzyme.

Although experiments with oxygen isotopes have not been car-
ried out with thymidine 2'-hydrolase, it seems safe to assume that
2-ketoglutarate is oxygenated to succinate concomitantly with
the hydroxylation reaction, which was shown first with the hy-
drosylase which acts on y-butyrobetaine (19), and later with
those acting on thymine, 5-hydroxymethyluracil, and 5-formyl-
uracil (4) as well as on collagen proline (20). In experiments
with 18O2 it could be established that p-hydroxyphenylpyruvate
hydroxylase catalyzes a similar type of reaction (21). All of
these enzymes have similar cofactor requirements, i.e. a specific
requirement for ferrous ion and a 2-ket'o acid (2-ketoglutarate
and the pyruvate side chain of p-hydroxyphenylpyruvate, re-
spectively). There is also a nonspecific requirement for a re-
ductant and for a hydrogen peroxide destroying system.

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