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THYMINE 7-HYDROXYLASE FROM *NEUROSPORA CRASSA*

SUBSTRATE SPECIFICITY STUDIES

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Summary

A partially purified preparation of thymine 7-hydroxylase (thymine, 2-oxoglutarate : oxygen oxidoreductase (7-hydroxylating), EC 1.14.11.6) from *Neurospora crassa* was incubated with a number of pyrimidines chemically related to thymine.

1. Pyrimidines with oxygen or sulfur substituents on atoms Nos. 2 and 4 as well as an alkyl group on atom Nos. 1 or 5 were substrates.

2. K_m values were determined for 1-methyluracil, 1-ethyluracil, thymine, 6-azathymine, 1-methylthymine, 1-ethylthymine, 5-formyluracil and 5-hydroxymethyluracil.

3. Uracil was identified as one of the metabolites after incubation with 1-methyluracil. The one-carbon metabolite has not been characterized.

4. Several pyrimidines with polar groups on atoms Nos. 2 and 4 were inhibitory.

5. Addition of 1-methyluracil, 1-methylthymine, 1-ethylthymine or 5-hydroxymethyluracil to incubations with thymine and 2-oxo[1-$^{14}C_1$]glutarate did not result in additional formation of $^{14}CO_2$, indicating that the same enzyme acts on the different compounds. It has previously been found (Bankel, L., Holme, E., Lindstedt, G. and Lindstedt, S. (1972) FEBS Lett. 21, 135—138) that a mutant strain of *N. crassa* which is devoid of thymine 7-hydroxylase activity also lacks ability to perform the coupled oxygenation of 2-oxoglutarate and 1-methyluracil, 5-hydroxymethyluracil and 5-formyluracil, respectively. It is concluded that one and the same oxygenase is responsible for the activities studied.

Introduction

Neurospora crassa cells are able to convert thymine to 5-carboxyuracil in three consequtive oxygenase reactions, which are coupled to the oxidative

decarboxylation of 2-oxoglutarate to succinate [1—8]. Results from protein fractionation studies [9] as well as from studies of a mutant strain of *N. crassa*, which lacks ability to utilize thymine [10,11], indicate that the three reactions are catalyzed by one enzyme. The specificity of this enzyme is low: an alkane as well as an alcohol and an aldehyde are substrates. We also found that 1-methyluracil could act as 'cofactor' for the oxidative decarboxylation of 2-oxoglutarate in incubations with a wild strain of *N. crassa*. With the mutant strain, there was no degradation of 2-oxoglutarate when 1-methyluracil, had been added indicating that the same oxygenase acts on 1- and 5-alkyluracils. It therefore appeared to us to be of interest to analyze the substrate specificity of this unique enzyme, utilizing an assay based on the degradation of the co-substrate, 2-oxoglutarate [12].

Methods

Materials. Compounds were obtained from the following sources: thymine and 5-hydroxymethyluracil from Calbiochem AG, Luzerne, Switzerland; 5-nitrouracil and 5-aminouracil from Nutritional Biochemicals Corporation, Cleveland, Ohio; 1-methyluracil, 1-ethyluracil, 1-benzyluracil, 1-cyclohexyluracil, 1,3-dimethyluracil, 3-methyluracil, 5-carbethoxyuracil, 5-hydroxyuracil, 5-mercaptouracil, 5-methylaminouracil, 5-bromo-1-methyluracil, 6-methyluracil, 6-hydroxyuracil, 1-methylthymine, 1-ethylthymine, 1-cyclohexylthymine, 6-azathymine, 2,4-dimethoxy-5-methylpyrimidine, 2,4-dimercapto-5-methylpyrimidine, cytosine,1-methylcytosine, 3-methylcytosine, 5-methylcytosine, 6-methylisocytosine, 4-hydroxy-2-methylmercaptopyrimidine, 4-hydroxypyrrolo-(2,3d)-pyrimidine, and 6-methyl-2-thio-4-hydroxypyrimidine from Cyclo Chemical Division, Travenol Laboratories Inc., Los Angeles, Calif.; uracil, 4,6-dihydroxypyrimidine, and 5,6-dimethyluracil from Fluka AG, Buchs, Switzerland; 5-bromouracil from Serva Feinbiochemica, GmbH, Heidelberg, West Germany; 5-carboxyuracil, 5,6-dihydrothymine, 4-methylpyrimidine, 5-methylpyrimidine, 6-methylpurine, 2-hydroxy-4-methylpyrimidine, 4,6-dihydroxy-2-methylpyrimidine, 4-methyl-2-methylthiopyrimidine, and sodium ascorbate from Dr. Theodor Schuchardt, GmbH, Munich, West Germany; 2-oxoglutaric acid and catalase from Boehringer und Soehne GmbH, Mannheim, West Germany; kieselgel GF 254 from E. Merck AG, Darmstadt, West Germany; hydroxyapatite and the anion exchanger AG 2-X8 (200—400 mesh) from Bio-Rad Laboratories, Inc., Richmond, Calif.; bis(trimethylsilyl)trifluoroacetamide from Pierce Chemical Company, Rockford, Ill.; [2-$^{14}C_1$]thymine (60 Ci/mol) from the Radiochemical Centre, Amersham, Bucks., England; 2-oxo[1-$^{14}C_1$]-glutaric acid (14.2 Ci/mol) from New England Nuclear, Boston, Mass. The purity of the pyrimidines active as substrates was checked by thin-layer chromatography and gas-liquid chromatography.

Chromatographic procedures. Thin-layer chromatography of pyrimidines was carried out on glass plates coated with kieselgel GF 254 which were heated to 105°C for 1 h. The mobile phase used was benzene/acetic acid/99.5% ethanol (4 : 1 : 2). The plates were scanned for ultraviolet absorption with a Zeiss Chromatogram Spectralphotometer M4QIII at 260 nm and for radioactivity

with a thin-layer chromatogram scanner, LB 2722 (Labor Professor Dr. Berthold, Wildbad, West Germany).

Gas-liquid chromatography was carried out at 120 or 130° C on a coiled glass column (6 feet long, internal diameter 3 mm) packed with Chromosorb W (100—200 mesh) coated with 2% of the silicon polymer SE-30. Silylation of pyrimidines in pyridine solution was carried out with bis(trimethylsilyl)trifluoroacetamide. Methylation was performed with diazomethane. Retention times of the derivatives were 2—10 min.

Enzyme. N. crassa strain STA 4, FGSC 262 A (Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif.) was cultured and harvested as described previously [5]. Mycelial extracts were partially purified by hydroxylapatite chromatography [11]. The specific activity of these preparations was 0.03—0.2 μkat per g of protein for thymine hydroxylation.

Incubations. The incubation mixture contained 40—50 μg of protein from the enzyme preparation, a pyrimidine (1—5 mM), 2-oxo[1-$^{14}C_1$]glutarate (0.05 μCi, 0.25 mM), ferrous sulfate (5 mM), ascorbate (5 mM), catalase (2 g/l), and 50 mM potassium phosphate at pH 7.6 in a total volume of 0.2 ml. Incubations were carried out at 37° C for 30 min. Apparent K_m values were calculated from results from incubations with six different pyrimidine concentrations (0.025—2.5 mM) in three series of incubations with 0.125 mM, 0.25 mM and 0.50 mM 2-oxoglutarate, respectively. V values were calculated from Lineweaver-Burk plots of data from the incubations with 0.25 mM 2-oxoglutarate. Collection of $^{14}CO_2$ and determination of radioactivity was performed as described previously [5]. Inhibition studies of those pyrimidines which were substrates for the enzyme were performed with [2-$^{14}C_1$]thymine (0.6 μCi, 0.25 mM) and nonradioactive 2-oxoglutarate. The inhibitor concentration was 5 mM. After 30 min the incubations were stopped by adding 0.4 ml of 96% ethanol in the cold. The labeled 5-hydroxymethyluracil was separated from thymine by thin-layer chromatography and the plates were scanned for radioactivity. Preliminary purification after incubation with 1-methyluracil was performed on columns (1.2 × 2.6 cm) of the anion exchanger AG 2-X8 (200—400 mesh, acetate form). After sample application the columns were eluted with 20 ml of water and then with 30 ml of 0.5 M aqueous acetic acid. The eluate was collected in fractions of 3 ml and analyzed for absorbance at 266 nm. Fractions containing a pyrimidine were taken to dryness and dried in a desiccator at 25° C over potassium hydroxide and phosphorus pentoxide. The residue was dissolved in 100 μl of pyridine to which was added 100 μl of bis(trimethylsilyl)trifluoroacetamide and analyzed by gas-liquid chromatography-mass spectrometry.

Results and Discussion

A summary of the results is given in Table I. Pyrimidines, which are substrates for thymine 7-hydroxylase have certain features in common. They have polar substituents such as hydroxyl (oxo), methoxy or mercapto groups in positions 2 and 4. Those with hydroxyls in these positions are better substrates than those with methoxy or mercapto groups. Pyrimidines with an amino group at position 4 are not substrates for the enzyme. 1-alkylated and 5-alkylated uracils

TABLE I

Activity of pyrimidines as substrate and inhibitor of thymine 7-hydroxylase from *N. crassa*. The left column gives amounts of $^{14}CO_2$ from 2-oxo-[1-$^{14}C_1$]glutarate in incubations with the respective compound in 1 mM concentration in % of that formed in incubations with 0.25 mM thymine. The right column gives inhibition of thymine 7-hydroxylase in % using 5 mM concentration of the inhibitor and 0.25 mM concentration of thymine.

	Relative amount of CO_2	Inhibition (%)
Uracil	1	50
1-Benzyluracil	0	7
1-Cyclohexyluracil	0	0
1-Ethyluracil	76	60
1-Methyluracil	68	80
1,3-Dimethyluracil	9	19
3-Methyluracil	0	12
5-Aminouracil	6	100
5-Bromouracil	0	95
5-Carboxyuracil	0	1 *
5-Carbethoxyuracil	0	9
5-Formyluracil	13	79 *
5-Hydroxyuracil	1	92
5-Hydroxymethyluracil	51	73
5-Mercaptouracil	3	60
5-Methylaminouracil	2	100
5-Nitrouracil	0	70
6-Hydroxyuracil	0	7
6-Methyluracil	0	18
5-Bromo-1-methyluracil	31	87
Thymine	100	—
1-Ethylthymine	89	70
1-Methylthymine	70	73
1-Cyclohexylthymine	0	5
5,6-Dihydrothymine	4	73
6-Methylthymine	0	41
6-Azathymine	19	94
Cytosine	0	6
1-Methylcytosine	0	12
3-Methylcytosine	0	12
5-Methylcytosine	0	16
6-Methylisocytosine	0	11
4-Methylpyrimidine	0	0
5-Methylpyrimidine	0	0
4,6-Dihydroxypyrimidine	0	5
2-Hydroxy-4-methylpyrimidine	0	0
2,4-Dimethoxy-5-methylpyrimidine	8	68
2,4-Dimercapto-5-methylpyrimidine	7	47
6-Methylpurine	0	11
6-Methyl-2-thio-4-hydroxypyrimidine	1	19
2-Methyl-4,6-dihydroxypyrimidine	0	5
4-Hydroxy-2-methylmercaptopyrimidine	0	8
4-Hydroxy-pyrrolo-(2,3d)-pyrimidine	0	8
4-Methyl-2-methylthiopyrimidine	0	6

* The pyrimidine concentration was 2.5 mM.

are almost as good as substrate (Table I). It may be speculated that the enzyme does not recognize a difference between substituents at C-1 and C-5 and between polar groups at C-2 and C-4 since the molecule is nearly symmetrical around an axis through atoms 3 and 6.

3-Alkyl and 6-alkyl uracils are inactive as substrate. Alkylation of thymine and 1-methyluracil at these positions, particularly at C-6, decreases activity as substrate. It appears that the configuration at atom 6 is critical, since both 5,6-dihydrothymine and 6-azathymine are less active as substrate than thymine.

Pyrimidines with the above-mentioned substitutions at atoms 2 and 4 are also active as inhibitors. Several 5-substituted uracil derivatives were potent inhibitors but substitution at position 3 and 6 reduced the inhibition compared to uracil. Preliminary findings indicate that resonance-stabilized intermediates with free electron pairs at atoms 6 and 7 are involved in the reaction. We therefore find it interesting that 5,6-dihydrothymine and 6-azathymine are both potent inhibitors but poor substrates for the enzyme. The K_m values for 6-azathymine and thymine are about the same, but the V for the reaction with 6-azathymine as substrate is much lower than that for thymine (Table II).

Thymine hydroxylation is an ordered sequential reaction [8]. 5-Carboxyuracil is a noncompetitive inhibitor against thymine and 5-hydroxymethyluracil a competitive inhibitor in low concentrations. In the present study 1-methyluracil and 1-methylthymine were found to be noncompetitive inhibitors against thymine. It is thus not possible from present studies of the type of inhibition to prove that one and the same enzyme acts on the different substrates. Addition of 0.5 or 2.5 mM 1-methyluracil to incubations with 0.5 mM thymine and 2-oxo[1-$^{14}C_1$]glutarate did not result in increased formation of labeled carbon dioxide (Fig. 1) indicating that the same enzyme attacks 1- and 5-methyluracil. The yields of $^{14}CO_2$ were somewhat lower in the presence of 1-methyluracil, which inhibited thymine hydroxylation with about 25% in 0.5 mM concentration. Similar results were obtained in experiments with 1-ethyluracil, 1-methylthymine, 1-ethylthymine and 5-hydroxymethyluracil. The results are in accord with the previous finding that extracts from cells of the uc-3 mutant *Neurospora* strain, which do not metabolize thymine [10,11,13] do not degrade 5-hydroxymethyluracil, 5-formyluracil or 1-methyluracil under the present assay conditions [11].

The results shown in Table I were obtained with an assay based on the decarboxylation of 2-oxoglutarate. We have not identified the pyrimidine products except when 1-methyluracil was the substrate. In this case uracil was identified as the product by gas chromatography-mass spectrometry (Fig. 2) and the following reactions may therefore be envisaged (Scheme 1): 1-methyl-

TABLE II

Values of apparent K_m and V of substrates for thymine 7-hydroxylase from *N. crassa*.

	K_m app (mM)	V (nkat/g protein)
1-Methyluracil	0.50	107
1-Ethyluracil	1.00	96
5-Formyluracil	0.22	15
5-Hydroxymethyluracil	1.00	65
Thymine	0.20	110
1-Methylthymine	0.45	107
1-Ethylthymine	0.60	85
6-Azathymine	0.25	19

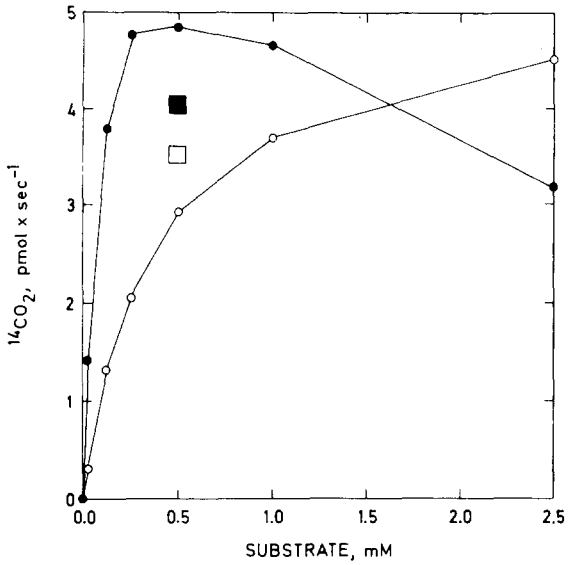


Fig. 1. Formation of $^{14}CO_2$ from 2-oxo[1-$^{14}C_1$]glutarate in incubations with different initial concentrations of thymine (●——●) and 1-methyluracil (○——○). To incubations with 0.5 mM concentration of thymine were also added 0.5 mM (■) and 2.5 mM (□) 1-methyluracil.

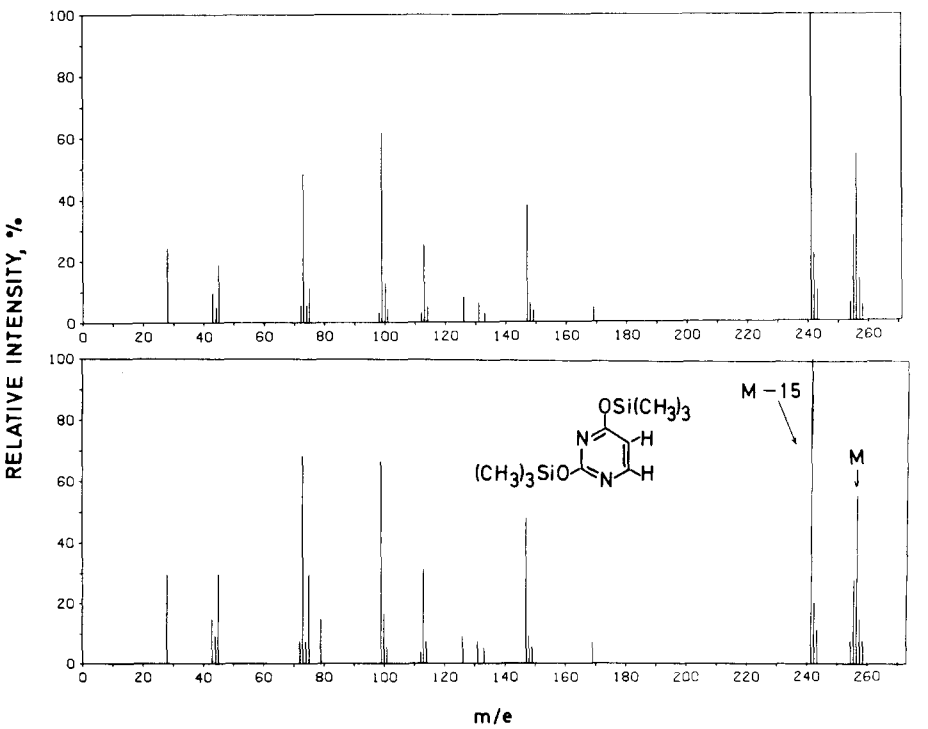
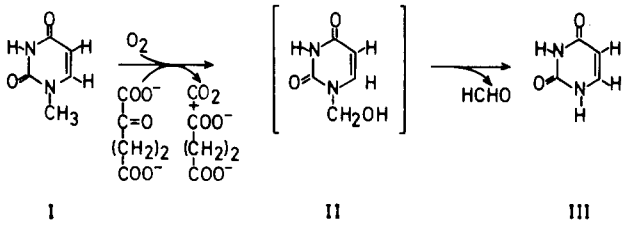


Fig. 2. The mass spectrum of the trimethylsilyl derivative of the pyrmidine formed during incubation of 1-methyluracil (upper part) and that of uracil (lower part). 1-Methyluracil (0.5 mM) was incubated for 30 min with 2-oxoglutarate, ferrous ion, ascorbate, catalase and a partially purified preparation of thymine 7-hydroxylase from *N. crassa* in 0.05 M potassium phosphate buffer at pH 7.6. After purification on an anion exchanger the pyrimidine was silylated with bis(trimethylsilyl)trifluoroacetamide and chromatographed on a column of 2% SE 30 at 120°C. The mass spectrum was recorded on a gas chromatograph-mass spectrometer type 9000 (LKB-produkter AB, Stockholm, Sweden).



I II III

Scheme 1

uracil (I) is hydroxylated to 1-hydroxymethyluracil (II) concomitantly with the oxidative decarboxylation of 2-oxoglutarate. 1-Hydroxymethyluracil is then nonenzymically cleaved to uracil and formaldehyde. In this scheme it has been assumed that 1-hydroxymethyluracil has low stability, similar to that of aliphatic hydroxymethylamines. Alternatively, one may envisage further oxygenation of 1-hydroxymethyluracil to 1-formyluracil and 1-carboxyuracil before cleavage of the one-carbon fragment from uracil. Characterization of the one-carbon fragment has not been attempted, as the incubations are performed in the presence of Fe^{2+} and high concentrations of ascorbate.

The presently studied enzyme activities have been classified [14] as thymine, 2-oxoglutarate : oxygen oxidoreductase (7-hydroxylating) (EC 1.14.11.6) and 5-hydroxymethyluracil, 2-oxoglutarate : oxygen oxidoreductase (EC 1.14.11.5). We feel that there is now sufficient evidence to ascribe these activities to a single enzyme as discussed above. A systematic name might be 1(5)-alkyl(5-alkanol, 5-alkanal)uracil, 2-oxoglutarate : oxygen oxidoreductase [1(7)-hydroxylating]. A recommended name might be thymine, 2-oxoglutarate dioxygenase.

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