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7-HYDROXYLATION OF THYMINE IN A NEUROSPORA STRAIN COUPLED TO OXIDATIVE DECARBOXYLATION OF 2-KETOGLUTARATE

E. HOLME, G. LINDSTEDT, S. LINDSTEDT AND M. TOFFT

Department of Physiological Chemistry, University of Lund, Lund, and Department of Clinical Chemistry, University of Gothenburg, Gothenburg (Sweden)

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SUMMARY

1. An enzyme preparation catalyzing the 2-ketoglutarate-dependent hydroxylation of thymine was prepared from a Neurospora strain.

2. The apparent K_m value for 2-ketoglutarate was found to be 0.2 mM. Optimal concentrations of Fe^{2+}, ascorbate and catalase were 1 mM, 1 mM, and 0.4 mg/ml, respectively. Little or no enzymic activity was found when the gas phase of the incubations involved argon instead of air. The hydroxylation of thymine was inhibited by 5-hydroxymethyluracil.

3. On hydroxylapatite chromatography of an extract of ground mycelia, two peaks were obtained with 2-ketoglutarate-decarboxylating activity, one of which coincided with thymine 7-hydroxylating activity. A stoichiometric relationship was found between hydroxylation of thymine and decarboxylation of 2-ketoglutarate. CO_2 and succinate were found as products of 2-ketoglutarate degradation.

4. It is proposed that thymine 7-hydroxylase belongs to the class of oxygenases which utilize a 2-keto acid for O_2 reduction.

INTRODUCTION

The formation of 5-hydroxymethyluracil from thymine in Neurospora was first observed by FINK AND FINK[1], ABBOTT AND FINK[2] and ABBOTT *et al.*[3], and extracts of a Neurospora strain were found to catalyze this reaction when NADPH and GSH had been added. Subsequently, ABBOTT *et al.*[4] demonstrated that 2-ketoglutarate and Fe^{2+} could replace NADPH and GSH and that the hydroxylase was also stimulated by ascorbate.

A requirement for 2-ketoglutarate, Fe^{2+} and stimulation by ascorbate has also been observed for collagen-proline hydroxylase[5–7] and for γ-butyrobetaine hydroxylase from liver and from a Pseudomonas strain[8–11]. In the case of γ-butyrobetaine hydroxylase, the requirement for 2-ketoglutarate and Fe^{2+} appears to be highly specific, whereas ascorbate may be replaced by several other reductants[11,12]. A reaction mechanism for 2-ketoglutarate-requiring reactions was proposed[13] on the basis of the

findings that 2-ketoglutarate is degraded to succinate concomitantly with the hydroxylation of γ-butyrobetaine[14] and that succinic semialdehyde is not a free intermediate in this reaction[13]. Evidence for this reaction mechanism could then be obtained in experiments with isotopic O_2[15].

These findings prompted a study of other 2-ketoglutarate-dependent hydroxylases. Because of the ready availability of the substrate for thymine hydroxylase as compared with collagen-proline hydroxylase, the former enzyme was chosen. As the enzymic activity was low in crude extracts of the Neurospora mycelia under the conditions used in earlier studies[4] and as these extracts catalyzed significant thymine-independent decarboxylation of 2-ketoglutarate, the enzyme was partially purified and its cofactor requirements were studied in more detail. As briefly reported previously[15], the results then obtained indicate that 2-ketoglutarate is degraded to CO_2 and succinate in amounts stoichiometric with the oxygenation of thymine to 5-hydroxymethyluracil. When this work was in progress, RHOADS AND UDENFRIEND[16] reported degradation of 2-ketoglutarate concomitantly with the hydroxylation of peptide-bound proline.

MATERIALS AND METHODS

Materials

Compounds were obtained from the following sources: thymine and 5-hydroxymethyluracil from Calbiochem AG, Luzerne, Switzerland; 2-ketoglutaric acid and catalase from C. F. Boehringer and Soehne GmbH, Mannheim, West Germany; sodium ascorbate from Dr. Th. Schuchardt GmbH, München, West Germany; silicic acid from Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.; hydroxylapatite from BioRad Laboratories, Inc., Richmond, Calif. U.S.A.; Hyamine from Packard Instrument Company, Downers Grove, Ill., U.S.A.; [2-^{14}C]thymine (59.9 mC/mmole) and sodium 2-keto[5-^{14}C]glutarate (17.1 mC/mmole) from The Radiochemical Centre, Amersham, Bucks, England; 2-keto[^{14}C]glutaric acid (11.0 mC/mmole) from Calbiochem AG, Luzerne, Switzerland. The labeled 2-ketoglutaric acid was purified by partition chromatography on silicic acid.

Chromatographic procedures

Descending paper chromatography was carried out on Munktell No. 312 filter paper with *sec.*-butanol saturated with water as the mobile phase[17]. Spots were made visible with ultraviolet light and radioactivity scanned with a paper chromatogram scanner (Labor. Prof. Dr. Berthold, Wildbad, West Germany). The R_F values were: thymine, 0.70 and 5-hydroxymethyluracil, 0.50. Silicic acid chromatography of pyrimidines with 0.25 M H_2SO_4 as stationary phase and chloroform with varying concentrations of *tert.*-butanol as mobile phase was performed according to BOVÉ AND RAVEUX[18]. In the eluate the concentration of pyrimidines was determined by reading the absorbance at 265 nm, and the radioactivity was determined with a liquid scintillation counter (Packard Instrument Co.). Thymine was eluted with 15% *tert.*-butanol in chloroform and 5-hydroxymethyluracil with 40% *tert.*-butanol in chloroform (see Fig. 1). Succinic acid was separated from 2-ketoglutaric acid by silicic acid chromatography according to PRIOR FERRAZ AND RELVÂS[19]. Desalting of proteins was performed on columns of Sephadex G-25 (coarse) in 5 mM potassium

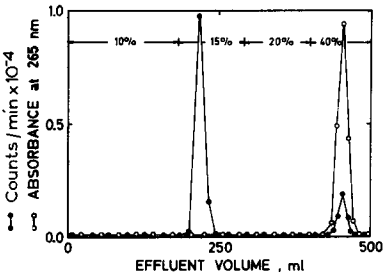


Fig. 1. Fractionation by silicic acid chromatography of the incubation mixture after incubating an extract of ground Neurospora mycelia with [2-^{14}C]thymine, 2-ketoglutarate, Fe^{2+}, ascorbate, and catalase in potassium phosphate buffer at pH 7.5 as described under *Assay*. The column was prepared from 8 g of silicic acid and 5 ml of 0.25 M H_2SO_4 and was eluted with increasing concentrations of *tert.*-butanol in chloroform (see figure). The fraction volume was 4 ml. 5-Hydroxymethyluracil (1 mg) had been added before chromatography.

phosphate buffer (pH 6.5) at 4°. Hydroxylapatite chromatography was carried out at 4° with a column of hydroxylapatite initially equilibrated with 5 mM potassium phosphate buffer at pH 6.5. Proteins were eluted with a convex gradient (5–50 mM) of potassium phosphate buffer at pH 6.5. Protein concentration was assayed by photometry at 280 nm.

Enzyme preparation

Neurospora crassa, Strain STA 4 (FGSC 262 A), was grown in minimal medium[20] for 4 days at 25°, each batch in 2 l of medium in a 5-l erlenmeyer flask. The mycelia were collected by suction filtration, washed twice with 5 mM potassium phosphate buffer at pH 6.5 and homogenized at 4° by grinding with sand[3]. The crude extract was centrifuged at 100 000 × *g* for 60 min at 4°. The supernatant fraction was desalted by filtration through a column of Sephadex G-25 (coarse) and then fractionated by means of hydroxylapatite chromatography (see above). Fractions containing thymine-hydroxylating activity (between about 20 and 40 mM phosphate, see Fig. 2) were pooled and pH adjusted to 7.5 with Tris. The protein solution was then concentrated with a Diaflo ultrafiltration apparatus. Storage of the concentrated protein solution in 0.1 M potassium phosphate buffer at pH 7.5 at −15° for several weeks resulted in little loss of activity. Storage for 2 weeks in phosphate buffer at pH 6.5 resulted in an almost complete loss of activity.

Assay

The enzyme was incubated in 5-ml test tubes with cofactors and with labeled thymine and/or labeled 2-ketoglutarate. Labeled 5-hydroxymethyluracil was separated from thymine by paper chromatography. Labeled succinic acid was separated from 2-ketoglutaric acid by silicic acid chromatography. $^{14}CO_2$ was trapped on 1-cm^2 pieces of filter paper attached to a wire in a rubber stopper of the test tubes. 20 μl of a 1 M solution of Hyamine in methanol had been pipetted onto the filter papers. After the incubations, 0.2 ml of a 10% water solution of trichloroacetic acid was added and after 1 h the filter paper was 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-

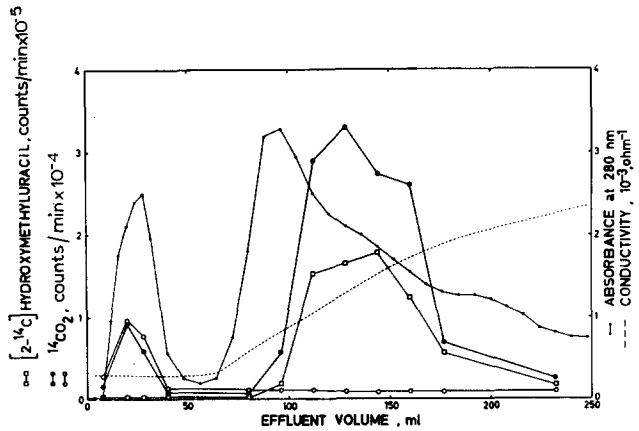


Fig. 2. Fractionation of a desalted extract of ground Neurospora mycelia on a column of hydroxylapatite by elution with increasing concentrations of potassium phosphate buffer at pH 6.5 (see MATERIALS AND METHODS). The thymine-hydroxylating activity (□—□) was determined in incubations with 100-μl aliquots of the fractions, [2-^{14}C]thymine (10 nmoles, 225 000 counts/min), 2-ketoglutarate, Fe^{2+} and ascorbate for 4 h at 37°. The 2-ketoglutarate-decarboxylating activity was determined in incubations with 2-keto[1-^{14}C]glutarate (4.1 nmoles, 50 000 counts/min), Fe^{2+}, and ascorbate with (●—●) and without (○—○) thymine. The protein concentration (·—·) was estimated by photometry at 280 nm, and conductivity (– – – –) by resistance measurements.

methyl-5-phenyloxazolyl)]benzene (0.3 g), toluene (1000 ml) and ethylene glycol monomethyl ether (600 ml).

The composition of the incubation medium was: enzyme preparation (0.02–0.5 mg of protein/ml), [2-^{14}C]thymine (1 μC/ml, 0.5 mM), 2-keto[1-^{14}C]glutarate or 2-keto[5-^{14}C]glutarate (1 μC/ml, 1 mM), $FeSO_4$ (1 mM), ascorbate (1 mM), catalase (0.4 mg/ml), and potassium phosphate buffer at pH 7.5 (20 mM). The total volume was 0.2 ml. The incubations were carried out at 37° for 30 min and were terminated by the addition of either 0.8 ml of ethanol (assay of thymine-hydroxylating activity) or 0.2 ml of 10% trichloroacetic acid (assay of 2-ketoglutarate-decarboxylating activity).

RESULTS

Reaction product

With paper and silicic acid chromatography, labeled 5-hydroxymethyluracil could be identified as a metabolite of [2-^{14}C]thymine in incubations with crude extracts of the mycelia, to which had been added 2-ketoglutarate, Fe^{2+}, ascorbate and catalase (Fig. 1). This confirms previous findings[1–3]. When more than about 20% of thymine had been consumed, two other metabolites were found with the same chromatographic properties[17] as 5-formyluracil and 5-carboxyuracil (to be published). Irregularly, small amounts of a labeled compound were eluted immediately before 5-hydroxymethyluracil from the silicic acid columns, which on paper chromatography had an R_F value about 10% lower than 5-hydroxymethyluracil. This material has not been identified.

Enzyme

A soluble enzyme preparation was obtained by hydroxylapatite chromatography of the desalted extract of the ground mycelia (Fig. 2). 2-Ketoglutarate-degrading activity (see below) was obtained in two distinct peaks in the chromatogram. The ratio between the areas of these peaks varied between different preparations. The activity in the first peak was much lower when the crude extract had been subjected to centrifugation at 100 000 $\times g$ for 1 h. This activity was not affected by the presence or absence of thymine in contrast to the activity in the second peak, which required thymine. The thymine-hydroxylating activity was eluted together with the second peak of 2-ketoglutarate-degrading activity. The material in these fractions was used for the subsequent studies.

With optimal cofactor concentrations the thymine-hydroxylating activity was 1–2 munits/mg of protein which is at least 20 times higher than that reported for the Neurospora strain used previously[4].

As shown in Fig. 3, there was significant inhibition of thymine hydroxylation by the product, 5-hydroxymethyluracil. The hydroxylase was therefore assayed under such conditions that less than about 20% of thymine was hydroxylated. The thymine-hydroxylating activity was proportional to the concentrations of protein in the incubations (Fig. 4), and a constant rate of hydroxylation was observed during the first 30 min of incubation (Fig. 5).

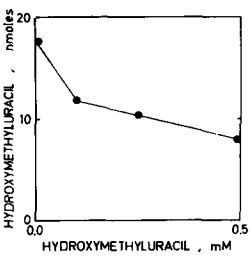


Fig. 3. Effect on hydroxylation of [2-^{14}C]thymine to labeled 5-hydroxymethyluracil of adding different concentrations of nonlabeled 5-hydroxymethyluracil. For incubating conditions, see *Assay*.

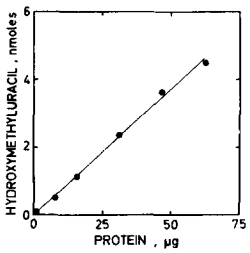


Fig. 4. Formation of labeled 5-hydroxymethyluracil from [2-^{14}C]thymine in incubations with different amounts of the enzyme preparation. For incubating conditions, see *Assay*.

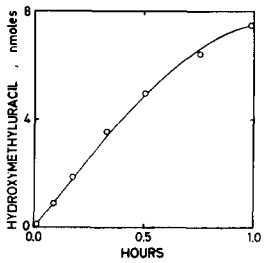


Fig. 5. Formation of labeled 5-hydroxymethyluracil from [2-^{14}C]thymine after different times of incubation. For incubating conditions, see *Assay*.

O_2 requirement

Table I shows that a very low formation of 5-hydroxymethyluracil was obtained in the absence of O_2.

Ascorbate, Fe^{2+} and catalase

As shown in Fig. 6, the optimal concentration of both ascorbate and Fe^{2+} was about 1 mM.

A stimulation by catalase has previously been observed with γ-butyrobetaine hydroxylases from rat liver and from a Pseudomonas strain[8–10,21] and with collagen-

TABLE I

7-HYDROXYLATION OF [2-^{14}C]THYMINE IN INCUBATIONS WITH AIR OR ARGON IN THE GAS PHASE

The enzyme was incubated with [2-^{14}C]thymine, 2-ketoglutarate, Fe^{2+}, ascorbate and catalase at 37° as described under *Assay*. The incubation mixtures had been kept in ice for 2 h before incubation and the tubes flushed with moistened argon. In Expt. 1, the argon atmosphere was replaced by air before incubation, whereas in Expt. 2, the incubation at 37° was carried out with continued flushing with moistened argon.

Expt.	*Gas phase*	*5-Hydroxymethyluracil (nmoles)*
1	Air	1.6
2	Argon	0.1

proline hydroxylase[22]; the effect of catalase on thymine hydroxylation was therefore studied. As shown in Fig. 7, the yield of 5-hydroxymethyluracil increased 3-fold when 0.4 mg/ml of catalase had been added to the incubations.

2-Ketoglutarate

Fig. 8 shows the effect on the formation of 5-hydroxymethyluracil of adding different concentrations of 2-ketoglutarate to incubations with the enzyme, ascorbate, Fe^{2+}, catalase and different concentrations of thymine. A Lineweaver–Burk plot gave an apparent K_m value for 2-ketoglutarate of about 0.2 mM. Both [2-^{14}C]thymine and 2-keto[1-^{14}C]glutarate were then incubated under the same conditions as those in Fig. 5, and the yield of $^{14}CO_2$ was plotted against the yield of 5-hydroxymethyluracil (Fig. 9). The results indicate a stoichiometry between degradation of 2-ketoglutarate and hydroxylation of thymine. However, in other experiments in which more than 20% of thymine had been hydroxylated, the amount of CO_2 formed exceeded the amount of thymine consumed. In preliminary experiments we have noted a hydroxymethyluracil-dependent decarboxylation of 2-ketoglutarate.

2-Keto[5-^{14}C]glutarate was incubated under the standard conditions (see above) and the incubation mixtures were then fractionated by means of silicic acid chromato-

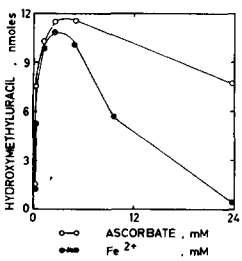


Fig. 6. Effect of various concentrations of ascorbate (○—○) and of Fe^{2+} (●—●) on the 7-hydroxylation of [2-^{14}C]thymine. For incubating conditions, see *Assay*.

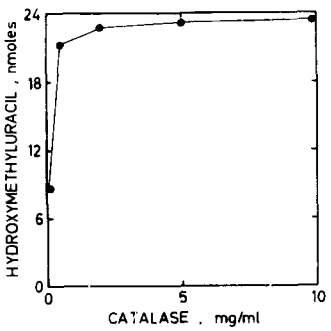
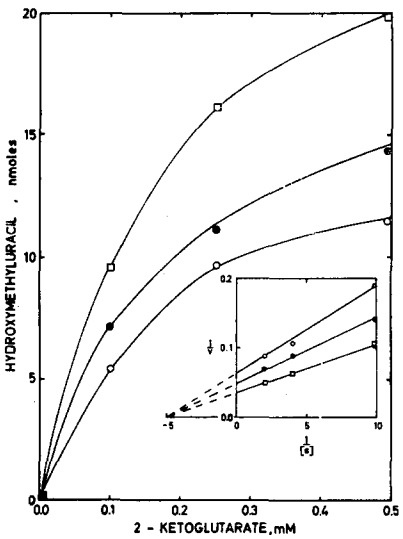


Fig. 7. Effect of various concentrations of catalase on the 7-hydroxylation of [2-^{14}C]thymine. For incubating conditions, see *Assay*.



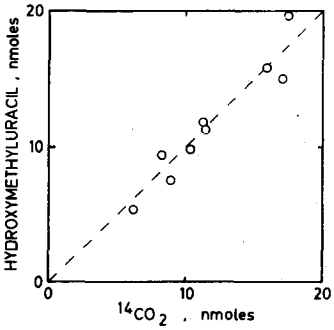


Fig. 8. Effect of various concentrations of 2-ketoglutarate on 7-hydroxylation of [2-^{14}C]thymine. The incubations were carried out as described under *Assay* with the following initial concentrations of [2-^{14}C]thymine: ○—○, 0.125 mM; ●—●, 0.275 mM; □—□, 0.525 mM. In the Lineweaver–Burk plots, v is the amount of 5-hydroxymethyluracil formed during the incubation and [S] the initial concentration of 2-ketoglutarate.

Fig. 9. Relationship between 7-hydroxylation of [2-^{14}C]thymine and decarboxylation of 2-keto-[1-^{14}C]glutarate. The incubations (see *Assay*) were carried out with labeled thymine and labeled 2-ketoglutarate of the same initial concentrations as those in Fig. 8.

graphy as described previously[14]. Only one metabolite was found and this was eluted together with added unlabeled succinic acid. The radioactive material had the same R_F value as succinate on paper chromatography.

DISCUSSION

In studies with isotopic O_2, evidence was obtained that 2-keto acids may serve as specific reductants for O_2 in some hydroxylation reactions, *viz.* the hydroxylation of γ-butyrobetaine to carnitine[15] and the formation of homogentisate from p-hydroxyphenylpyruvate[23]. 2-Ketoglutarate is used for γ-butyrobetaine hydroxylase and the pyruvate side chain of the substrate for p-hydroxyphenylpyruvate hydroxylase. The fact that 2-ketoglutarate is degraded also during the hydroxylation of peptide-bound proline[16] and during the 7-hydroxylation of thymine in amounts stoichiometric with the formation of hydroxylated product indicates that the previously proposed mechanism for γ-butyrobetaine hydroxylase[13] may be a general one for 2-ketoglutarate-dependent hydroxylations.

In the course of the present study it was observed that 5-hydroxymethyluracil may be further metabolized in a 2-ketoglutarate-dependent reaction. Studies of the nature of the reaction products as well as of the cofactor requirements are in progress.

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