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Stoichiometry of the Pyrimidine Deoxyribonucleoside 2′-Hydroxylase Reaction and of the Conversions of 5-Hydroxymethyluracil to 5-Formyluracil and of the Latter to Uracil-5-carboxylic Acid†

Chen Kao Liu, Patricia M. Shaffer, Robert S. Slaughter, R. P. McCroskey, and M. T. Abbott*

ABSTRACT: The oxidative demethylation of thymidine by *Neurospora* is thought to involve the following α-ketoglutarate- and O_2-dependent conversions: thymidine to thymine ribonucleoside (2′-hydroxylase reaction), thymine to 5-hydroxymethyluracil (7-hydroxylase reaction), 5-hydroxymethyluracil to 5-formyluracil, and 5-formyluracil to uracil-5-carboxylic acid. Subjection of extracts of *Neurospora* to a purification scheme, which includes calcium phosphate gel, ammonium sulfate, Sephadex G-150, and DEAE-cellulose fractionation procedures, yielded several enzyme fractions. One contained the 2′-hydroxylase and none of the other enzymes involved in the demethylation of thymidine and another fraction contained the 7-hydroxylase as well as the capacity to oxidize 5-hydroxymethyluracil and 5-formyluracil but no detectable 2′-hydroxylase activity. Using these enzyme fractions the oxidation of thymidine, 5-hydroxymethyluracil, and 5-formyluracil was shown to be coupled to the decarboxylation of α-ketoglutarate so that the oxidized product, succinate, and CO_2 are produced in a 1:1:1 molar ratio. In addition, the 2′-hydroxylase reaction was shown to be stimulated by inclusion of catalase in the incubation mixture.

The conversion of thymidine to the pyrimidines of RNA (Fink and Fink, 1962) is thought to be effected by the following enzymatic reactions: thymidine to thymine ribonucleoside, pyrimidine deoxyribonucleoside 2′-hydroxylase reaction (Shaffer *et al.*, 1968); thymine ribonucleoside to thymine plus ribose (Shaffer *et al.*, 1972); thymine to 5-hydroxymethyluracil, thymine 7-hydroxylase reaction (Abbott *et al.*, 1967); 5-hydroxymethyluracil to 5-formyluracil (Abbott *et al.*, 1968); 5-formyluracil to uracil-5-carboxylic acid (Watanabe *et al.*, 1970); and uracil-5-carboxylic acid to uracil and CO_2, uracil-5-carboxylic acid decarboxylase reaction (Palmatier *et al.*, 1970). The 2′-hydroxylase, which catalyzes the first step in this pathway, also converts deoxyuridine to uridine and requires α-ketoglutarate, Fe^{2+}, ascorbate (Shaffer *et al.*, 1968), and O_2 for activity (Shaffer *et al.*, 1972). Although the role of α-ketoglutarate had not been studied in this reaction, it has been shown that α-ketoglutarate is decarboxylated in the thymine 7-hydroxylase reaction (Holme *et al.*, 1970; Mc-

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Croskey *et al.*, 1971) and that, in this process, molecular oxygen is incorporated into the 5-hydroxymethyluracil and succinate which are produced (Holme *et al.*, 1971). In the conversions of 5-hydroxymethyluracil to 5-formyluracil and of 5-formyluracil to uracil-5-carboxylic acid, the stoichiometry of the formation of succinate and CO_2 from α-ketoglutarate had not been studied, but the oxidation of each of these pyrimidines had been shown to result in one atom of oxygen, derived from molecular oxygen, being incorporated into succinate. It also was shown that one atom of oxygen, from molecular oxygen, is incorporated into uracil-5-carboxylic acid (Holme *et al.*, 1971). A purification scheme is described in this paper that separates the 2′-hydroxylase from the other enzymes of the oxidative pathway. Since the resultant, partially purified enzyme preparation does not metabolize the thymine ribonucleoside produced from thymidine, the assay for the 2′-hydroxylase is much facilitated, and stoichiometry studies have been carried out with this preparation. The separation of this enzyme from the thymine 7-hydroxylase is interesting since it was a tenable hypothesis that thymidine and thymine interact with the same binding site in the hydroxylation reaction, even though the 2′-hydroxylase activity was less stable during purification (McCroskey *et al.*, 1971) and neither 5-hydroxymethyldeoxyuridine nor 5-hydroxymethyluridine was detected as a product (Shaffer *et al.*, 1972). Whether thymine 7-hydroxylase catalyzes each of the sequential reactions converting thymine to uracil-5-carboxylic acid is not known, although a pyrimidineless mutant of *Neurospora* has been developed which will grow in media supplemented with 5-formyluracil but not in media in which 5-hydroxymethyluracil is the only pyrimidine source (Williams and Mitchell, 1969). This paper also reports the results of studies of the stoichiometry of the reactions converting 5-hydroxymethyluracil to 5-formyluracil and the latter to uracil-5-carboxylic acid. Some of these data have been described in an abstract (Liu *et al.*, 1972).

Experimental Section

Materials. The specific activities (Ci/mole) to which radioactive compounds were adjusted are the following: [6-^{3}H]-thymidine, 16.5; [2-^{14}C]thymidine, 3.0; [2-^{14}C]deoxyuridine, 3.0; [2-^{14}C]thymine ribonucleoside, 3.0; [2-^{14}C]uridine, 3.0; [2-^{14}C]thymine, 3.0; 5-[7-^{14}C]hydroxymethyluracil, 3.0; 5-[6-^{3}H]formyluracil, 25.0; α-[1-^{14}C]ketoglutarate, 0.5; and α-ketoglutarate enriched with ^{14}C in both C-1 and C-5, 1.0 and 1.0. The methods of synthesis, the purification, and the commercial sources of the above compounds have been reported (Watanabe *et al.*, 1970; McCroskey *et al.*, 1971). Catalase (beef liver, C-30), DEAE-cellulose, and bovine albumin were obtained from the Sigma Chemical Co.

Enzyme Purification. The growth of *Neurospora crassa*, strain 1A, under nonaerated conditions, harvesting of the mycelia, preparation of a crude extract, and subsequent purification procedure yielding the Sephadex G-150 enzyme fraction, which catalyzes all of the reactions converting thymidine to uracil-5-carboxylic acid, have previously been described (McCroskey *et al.*, 1971). DEAE-cellulose was washed with alkali and acid, degassed, freed of "fines" by decantation, and packed into a column (30 × 1.5 cm) as described by the manufacturer (Whatman Technical Bulletin IL2). The Sephadex G-150 fraction was applied to this DEAE-cellulose column after it had been equilibrated with 0.02 M sodium phosphate buffer (pH 7.5), containing 0.1 mM ascorbate, 1.0 mM GSH, and 0.05 mM EDTA. Following application of the sample, 200 ml of the equilibration buffer was passed through the column. Then the enzymes were eluted (0.5 ml/min) with a linear gradient of 400 ml of the equilibration buffer containing sodium chloride, reaching a final concentration of 0.2 M. Fractions (3 ml) were collected and monitored with respect to the enzymatic activities and ultraviolet absorption (280 nm). In some experiments the fractions eluted from the DEAE-cellulose column were made 1 mg/ml with respect to albumin and then concentrated using a saturated solution of ammonium sulfate as previously described (McCroskey *et al.*, 1971).

Enzymatic Assays. To prepare the standard incubation mixture a 0.1-ml portion of the enzyme preparation was pipetted into a 10 × 75 mm test tube which contained the substrate and cofactors in 0.125 ml of 0.02 M sodium phosphate buffer (pH 7.5). The resultant incubation mixture was 0.2 mM in radioactive substrate, 0.9 mM in α-ketoglutarate, 0.9 mM in ferrous sulfate, and 0.4 mg/ml in catalase.[1] When [2-^{14}C]thymine ribonucleoside or [2-^{14}C]uridine was used as substrate, the α-ketoglutarate, ascorbate, ferrous sulfate, and catalase were omitted from the standard incubation mixture.

After addition of the enzyme preparation, the test tube was placed in a Dubnoff incubator and shaken at 37°. The enzymatic reaction was stopped by heating it to 100° for 3 min or by adding 0.8 ml of absolute ethanol. Following removal of the coagulated protein, appropriate nonradioactive compounds were added as chromatographic markers to the supernatant fluid. Paper chromatography was carried out on an aliquot of this supernatant which contained from 0.2 to 0.8 μmole of each of the marker compounds. The solvent systems used for development have previously been described (Fink and Adams, 1966). A thin-window Geiger tube was used for measuring the radioactivity of the chromatographically separated compounds. Duplicate assays of a given enzyme preparation usually agreed within ±10% of the mean.

For measuring the specific activity of an enzyme preparation, the protein concentration of the incubation mixture and the time of incubation were varied to determine conditions under which the rate of reaction was linear and proportional to the enzyme concentration. This rate was determined at a time in the incubation period when the amount of substrate utilized did not exceed 10%. Except for the assay of the 2′-hydroxylase, none of the assays for the oxygenases were complicated by the products of the enzymatic reaction being further metabolized. Preliminary studies indicate that thymine inhibits the conversion of 5-hydroxymethyluracil to 5-formyluracil. Similarly, 5-hydroxymethyluracil appears to inhibit the conversion of 5-formyluracil to uracil-5-carboxylic acid (Holme *et al.*, 1971). The uracil-5-carboxylic acid produced, when 5-formyluracil was used as substrate, was not converted to uracil since the decarboxylase had been removed during the purification procedure (Palmatier *et al.*, 1970). In order to measure the 2′-hydroxylase content of all enzyme preparations, except the DEAE-cellulose fraction, the radioactivity of thymine and 5-hydroxymethyluracil, as well as of thymine ribonucleoside, had to be measured. However, the rate calculated, as a result of these measurements, was

[1] With more purified enzyme preparations, standard incubation mixtures were prepared which contained nonlabeled substrate and α-[1-^{14}C]ketoglutarate so that a relatively rapid, preliminary assay for radioactive CO_2 could be carried out. When an incubation mixture contained α-[1-^{14}C]ketoglutarate, the 10 × 75 mm test tube was sealed in a 12-ml centrifuge tube which contained a 1 × 4 cm rectangle of Whatmann No. 3MM filter paper, saturated with Nuclear-Chicago solubilizer reagent.

TABLE I: Purification of Pyrimidine Deoxyribonucleoside 2′-Hydroxylase.

Step	Fraction	Vol (ml)	Protein (mg/ml)	Act. (Units)	Sp Act. (Units/mg)	Yield (%)	Purifcn (-fold)
1	Extract[a]	180	10	1480	0.8	100	1
2	Calcium phosphate I	180	2.5	1080	2.5	72	3
3	Calcium phosphate II	130	1.5	1700	8.7	115	11
4	Ammonium sulfate	18	11	1380	7.3	93	9
5	Sephadex G-150	100	0.95	1210	13	82	16
6	DEAE-cellulose	24	0.35	450	53	30	67

[a] The preparation of the extract from *Neurospora crassa* and the purification procedure yielding the Sephadex G-150 enzyme fraction has previously been described (McCroskey *et al.*, 1971).

usually proportional to the amount of the 2′-hydroxylase present in the incubation mixture. A unit of each of the α-ketoglutarate-dependent oxygenases is defined as the amount of the enzyme which catalyzes the oxidation of 1 nmole of substrate per min under the standard assay conditions.

Stoichiometry Studies. To determine the stoichiometric relationship between the production of CO_2 and of the oxidized pyrimidine or nucleoside, the period of incubation of the standard incubation mixture was varied. This incubation mixture contained α-[^{14}C]ketoglutarate and [6-^{3}H]thymidine, [2-^{14}C]deoxyuridine, 5-[7-^{14}C]hydroxymethyluracil, or 5-[6-^{3}H]formyluracil. One portion of the deproteinized incubation mixture was sujected to paper chromatography, as described above, to determine the extent to which the pyrimidine or nucleoside was oxidized. The α-[1-^{14}C]ketoglutarate did not have to be separated from the tritiated compounds in the reaction mixture since radioactivity measurements distinguished between the ^{3}H and ^{14}C labels as previously described (McCroskey *et al.*, 1971). Only those portions of the 2′-hydroxylase fraction in which no hydrolase activity could be detected were used in the studies of the 2′-hydroxylase. The standard incubation mixture, used to determine the stoichiometry of the relationship between the production of succinate and CO_2, contained nonradioactive substrate and α-ketoglutarate labeled in both C-1 and C-5. The paper chromatographic procedure used to separate and the method of measuring the radioactivity of the dicarboxylic acids has previously been described (McCroskey *et al.*, 1971). The procedures used for trapping CO_2 and the subsequent determination of its radioactivity with the use of a scintillation counter have also been described (Palmatier *et al.*, 1970). To further establish the identity of the enzymatic product of α-[5-^{14}C]-ketoglutarate in the 2′-hydroxylase reaction, a standard incubation mixture was prepared using nonlabeled thymidine and the 2′-hydroxylase fraction which had been concentrated with ammonium sulfate using albumin as a "carrier," as indicated above. The mixture was incubated for 30 min and then chromatographed on a column of silicic acid as previously described (McCroskey *et al.*, 1971). Control incubations, *i.e.*, incubation mixtures which were deproteinized at zero time or in which the enzyme preparation was replaced with buffer, were also subjected to silic acid chromatography. The amount of succinate produced in these control runs did not exceed 6% of that produced in the experimental runs.

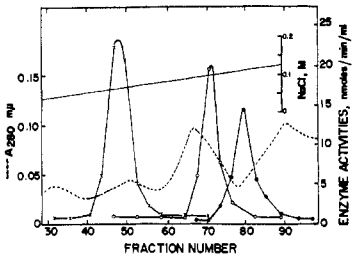

FIGURE 1: Effect of DEAE-cellulose chromatography on thymine 7-hydroxylase and pyrimidine 2′-hydroxylase activities. The Sephadex G-150 enzyme preparation which was applied to the DEAE-cellulose column had a volume of 100 ml and a protein concentration of 0.85 mg/ml. See the Experimental Section for details. 7-Hydroxylase activity (×); 2′-hydroxylase activity (○); hydrolase activity (●).

Results

Purification Procedure. The 2′-hydroxylase was typically purified as shown in Table I. While the calcium phosphate gel fractionation procedure (steps 2 and 3) freed the 2′-hydroxylase from uracil-5-carboxylic acid decarboxylase (Palmatier *et al.*, 1970) no detectable separation of any of the other enzymes, involved in the oxidative demethylation of thymidine, was achieved until the DEAE-cellulose chromatography step was carried out. Three peaks of enzymatic activity were eluted from the DEAE-cellulose column as depicted in Figure 1. The peak which was first eluted from the column contained the enzymes which catalyze the sequential reactions converting thymine to uracil-5-carboxylic acid. The specific activity of thymine 7-hydroxylase in this fraction was usually about 50 but values as high as 200 have been obtained. The typical specific activities of this fraction with respect to the substrates 5-hydroxymethyluracil and 5-formyluracil are 40 and 25, respectively. This fraction, however, did not catalyze the 2′-hydroxylase reaction nor the hydrolysis of either thymine ribonucleoside or uridine. The 2′-hydroxylation of thymidine and of deoxyuridine was catalyzed by the fraction constituting the second peak eluted from the column. Preliminary competition experiments suggest that both of the substrates are hydroxylated by the same enzyme. While the activities found in the first peak were not detected in the 2′-hydroxylase fraction, the second peak did to a small extent overlap the third peak eluted from the column. The fraction constituting the third peak catalyzed the hydrolysis of both thymine ribonucleoside and uridine. In experiments in which the fractions constituting the 2′-hy-

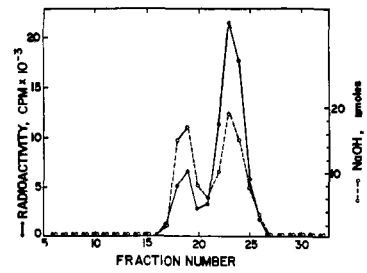

FIGURE 2: Identification of enzymatic product of α-ketoglutarate in the pyrimidine deoxyribonucleoside 2′-hydroxylase reaction using silicic acid chromatography. The standard incubation mixture contained nonradioactive thymidine, α-[5-^{14}C]ketoglutarate, and the ammonium sulfate concentrate of the 2′-hydroxylase fraction eluted from the DEAE-cellulose column (3 mg of protein/ml of incubation mixture). The incubations were carried out for 30 min. Before chromatography 5 mg of α-ketoglutarate and 3 mg of succinate were added to the radioactive enzymatic products. The 14.5 × 130 mm chromatography column of silicic acid was eluted with benzene–*tert*-butyl alcohol (9:1, v/v) in 2-ml fractions. The radioactivity of 0.5-ml aliquots of each fraction was measured with a scintillation counter, and the remainder of each fraction was titrated with 0.02 M NaOH. The firsk peak eluted from the column contained the succinic acid. The second peak contained α-ketoglutaric acid.

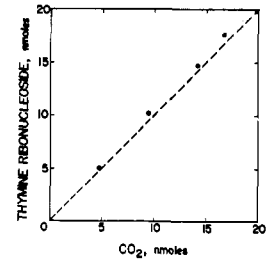

FIGURE 4: The stoichiometry of the production of thymine ribonucleoside and CO_2 in the pyrimidine deoxyribonucleoside 2′-hydroxylase reaction. The standard incubation mixture contained [6-^{3}H]thymidine, α-[1^{14}C]ketoglutarate and the 2′-hydroxylase fraction eluted from the DEAE-cellulose column (0.18 mg of protein/ml of incubation mixture). The period of incubation was varied.

droxylase and 7-hydroxylase activities were recombined, no inhibitory or stimulative effects were detected.

Pyrimidine Deoxyribonucleoside 2′-Hydroxylase Reaction. Succinic acid was identified as a product of the 2′-hydroxylase reaction with the use of silicic acid chromatography. Figure 2 shows that the enzymatic product and authentic succinic acid were eluted simultaneously from a silicic acid column. Typical data showing that succinate and CO_2 are produced in a 1:1 molar ratio in the 2′-hydroxylase reaction are depicted in Figure 3. CO_2 and thymine ribonucleoside are also produced in a 1:1 molar ratio (Figure 4). The same stoichiometry was observed when deoxyuridine was used as substrate. Since thymine 7-hydroxylase is stimulated by catalase (Holme *et al.*, 1970), the effect of catalase on the hydroxylation of thymidine was studied. As shown in Figure 5, the inclusion of catalase in the incubation mixture increased the amount of thymine ribonucleoside produced threefold. Catalase similarly stimulated the conversion of deoxyuridine to uridine. The specificity of this catalase effect has not been studied.

Conversions of 5-Hydroxymethyluracil to 5-Formlyuracil and of the Latter to Uracil-5-carboxylic Acid. When 5-hydroxymethyluracil was used as substrate and the time of incubation was varied, linear plots were obtained, as above, for the production of CO_2 and succinate and for the production of CO_2 and 5-formyluracil. These data showed that 5-formyluracil, succinate, and CO_2 are produced in a 1:1:1 molar ratio. Similarly in experiments in which 5-formyluracil was used as substrate, linear plots were obtained which indicated that uracil-5-carboxylic acid, succinate, and CO_2 are produced in a 1:1:1 molar ratio.

Discussion

In the 2′-hydroxylase reaction, as in the 7-hydroxylase reaction, molecular oxygen, α-ketoglutarate, Fe^{2+}, and ascorbate are required, catalase has a stimulative effect, and the decarboxylation of α-ketoglutarate is coupled to the hydroxylation of the substrate. These properties are consistent with a mechanism which is similar to that originally proposed for the γ-butyrobetaine hydroxylase (Lindstedt *et al.*, 1968) and collagen proline hydroxylase (Rhoads and Udenfriend, 1968) reactions. According to this mechanism thymine ribonucleoside, succinate, and CO_2 are produced as a result of a peroxide anion of thymidine making a nucleophilic attack on the carbonyl carbon of α-ketoglutarate. A similar mechanism was postulated for the enzymatic reactions in which 5-hydroxymethyluracil and 5-formyluracil are oxidized when it was realized that these reactions too are stimulated by molecular oxygen, α-ketoglutarate, Fe^{2+}, and ascorbate (Watanabe *et al.*, 1970). The results of the ^{18}O

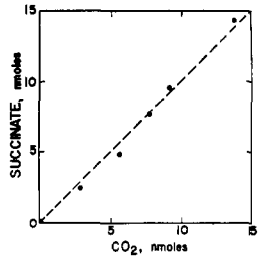

FIGURE 3: The stoichiometry of the production of succinate and CO_2 in the pyrimidine deoxyribonucleoside 2′-hydroxylase reaction with thymidine as substrate. The standard incubation mixture contained nonradioactive thymidine, α-ketoglutarate enriched with ^{14}C in both C-1 and C-5, and the 2′-hydroxylase fraction eluted from the DEAE-cellulose column (0.20 mg of protein/ml of incubation mixture). The period of incubation was varied.

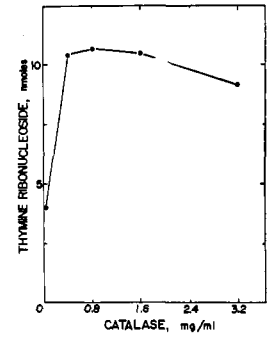

FIGURE 5: The effect of catalase on the pyrimidine deoxyribonucleoside 2′-hydroxylase reaction. Standard incubation mixtures were incubated for 10 min in the presence of various concentrations of catalase. The 2′-hydroxylase fraction eluted from the DEAE-cellulose column was used (0.18 mg of protein/ml of incubation mixture).

studies referred to above (Holme *et al.*, 1971) and the stoichiometry of these reactions are in accord with this mechanism.

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Denaturation of Horse Spleen Ferritin in Aqueous Guanidinium Chloride Solutions†

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ABSTRACT: The denaturation of ferritin and apoferritin in aqueous medium at room temperature has been described in terms of certain structural aspects of the protein. In aqueous solution, the circular dichroism spectra of native apoferritin and of ferritins with varying iron content were indistinguishable. The shape of the curves and magnitude of the far-ultraviolet ellipticity bands suggested that these proteins have substantial helical contents, and that almost 90% of their polypeptide chains may be in ordered structures. Sedimentation velocity studies showed that the iron-containing ferritin protein molecules were dissociated into subunits in 7.0 M aqueous guanidinium chloride solution of pH 7.5, but apoferritin remained in an aggregated state under these conditions. At pH 4.5, however, guanidinium chloride did induce the formation of apoferritin subunits. Circular dichroism studies indicated that extensive disruption of ordered structures accompanied subunit formation. The dissociation process was reversed significantly by removal of the guanidine. The reaggregated product exhibited electrophoretic and sedimentation properties similar to those of native apoferritin and its morphological appearance as viewed by the electron microscope, also resembled native apoferritin. Circular dichroism data obtained with the reassembled apoferritin suggested that most of its helical structure had been restored.

Ferritin is a well-characterized iron storage protein found in spleen, liver, and other mammalian tissues (Granick, 1942; Harrison, 1964; Crichton, 1971). The iron occurs as a hydrated ferric oxide–phosphate micelle, about 70 Å in diameter (Farrant, 1954; Fischbach and Anderegg; 1965; Spiro and Saltman, 1969) contained in the central cavity of a protein shell resembling a uniform hollow sphere with a diameter of approximately 120 Å (Harrison, 1963). Mechanisms of incorporation, retention and subsequent release of the iron are not fully understood. The molecular weight of the protein has been estimated to be 430,000–480,000 (Rothen, 1944: Richter and Walker, 1967), and for many years it was thought to consist of 20 subunits situated at the vertices of a pentagonal dodecahedron (Harrison, 1963; Hofmann and Harrison, 1963; Easterbook, 1970). Recent evidence, however, favors a structure containing 24 subunits (Bjork and Fish, 1971; Bryce and Crichton, 1971). Horse spleen ferritin preparations, considered to be highly purified, are heterogeneous with respect to iron content, and show three or more protein components on gel electrophoresis (Richter, 1963; Harrison and Gregory, 1965). The latter are considered to be stable aggregates of ferritin corresponding to dimers, trimers, and higher oligomers (Williams and Harrison, 1968).

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