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Inhibition of Orotidine-5′-phosphate Decarboxylase by 1-(5′-Phospho-β-D-ribofuranosyl)barbituric Acid, 6-Azauridine 5′-Phosphate, and Uridine 5′-Phosphate†

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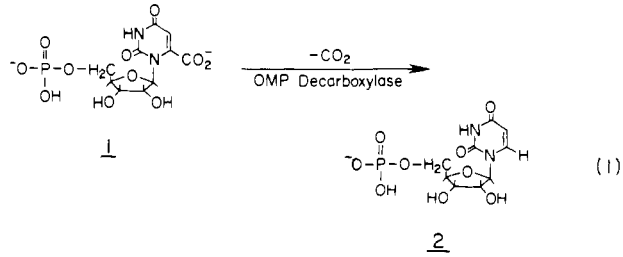
ABSTRACT: 1-(5′-Phospho-β-D-ribofuranosyl)barbituric acid, an analogue of orotidylic acid, binds to orotidine-5′-phosphate decarboxylase about 100 000 times as strongly as does the substrate. The K_i at pH 6 is 9×10^{-12} M and the half-time for dissociation at 4 °C is about 10 h. The binding of the barbiturate analogue to the enzyme is thus one of the strongest interactions between small molecules and proteins that have been measured. The possibility that the inhibitor is a transition-state analogue is discussed.

Orotidine-5′-phosphate decarboxylase (OMP decarboxylase,[1] EC 4.1.1.23) catalyzes the conversion of orotidine 5′-phosphate (**1**) to uridine 5′-phosphate (**2**) (eq 1), in the de



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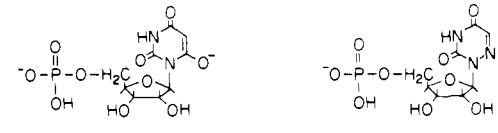
novo biosynthesis of pyrimidines (Lieberman et al., 1955). The enzyme from yeast has recently been purified to homogeneity by affinity chromatography (Brody & Westheimer, 1979; Reyes & Sandquist, 1978). In the course of the development of our affinity column (Brody & Westheimer, 1979), 1-(5′-phospho-β-D-ribofuranosyl)barbituric acid (**3**) was prepared.



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This compound has been shown to be a potent inhibitor of OMP decarboxylase from rat brain with a K_i of 4.1×10^{-9} M at pH 7.4 (Potvin et al., 1978). We now report that BMP is an extraordinarily powerful inhibitor of the yeast enzyme, binding so tightly that the stoichiometric enzyme–inhibitor complex can be purified by gel filtration with no hint of dissociation. The dissociation constant which we have measured for the binding of this inhibitor to yeast OMP decarboxylase is 9×10^{-12} M at pH 6 and 4 °C. Although the constant is much too small to be measured directly, it could be determined by a series of relays. The barbiturate inhibitor was equilibrated

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[1] Abbreviations used: OMP decarboxylase, orotidine-5′-phosphate decarboxylase; OMP, orotidine 5′-phosphate; UMP, uridine 5′-phosphate; BMP, 1-(5′-phospho-β-D-ribofuranosyl)barbituric acid; azaUMP, 6-azauridine 5′-phosphate; $NaDodSO_4$, sodium dodecyl sulfate.

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with enzyme against 6-azauridine 5′-phosphate (**4**), a previously known potent inhibitor of the enzyme (Handschumacher, 1960). This inhibitor was in turn equilibrated with the enzyme against uridine 5′-phosphate; the inhibition constant of the latter, 4.6×10^{-4} M, was determined by standard kinetic procedures. The rate of dissociation of BMP from the enzyme was measured by using radioactive BMP and "chasing" it with high concentrations of nonradioactive BMP.

The unusually tight binding of the barbiturate inhibitor is discussed in connection with the mechanism of action of the enzyme.

Experimental Procedures

Materials. 6-Azauridine 5′-phosphate and 1-(5′-phospho-β-D-ribofuranosyl)barbituric acid are commercially available from Sigma Chemical Co., but both were synthesized here as described below. [2-^{14}C]Barbituric acid (5 Ci/mol) was purchased from California Bionuclear Corp., 6-aza[5-^{3}H]-uridine (17 Ci/mmol) from Moravek Biochemicals, City of Industry, CA, and [*carboxyl*-^{14}C]OMP (34.9 Ci/mol) from New England Nuclear. The [2-^{14}C]barbituric acid and 6-aza[5-^{3}H]uridine were diluted with cold carrier before use. [*carboxyl*-^{14}C]OMP was used without isotopic dilution.

General Methods. UV spectra were recorded on either Cary 15 or Gilford 240 spectrophotometers, IR spectra on a Perkin-Elmer 137 spectrophotometer, and ^{1}H NMR spectra on either Varian HFT-80 or HA-100 spectrometers. Chemical shifts are reported in parts per million downfield from sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$. ^{31}P NMR spectra were recorded on a Varian XL-100 spectrometer with a phosphorus probe at 40.5 MHz. Chemical shifts are reported relative to 85% phosphoric acid as standard. Thin-layer chromatography was carried out on Eastman silica gel plates containing a fluorescent indicator and were visualized with short-wavelength UV light. Determinations of pH, at room temperature unless otherwise stated, were performed on a Radiometer pH meter.

2,4,6-Tris(trimethylsilyl)barbituric Acid (Winkley & Robins, 1969; Wittenburg, 1964). Barbituric acid (7.1 g, 0.066 mol) and trimethylchlorosilane (15 mL) were refluxed in 150 mL of hexamethyldisilazane until all of the solid had dissolved (~0.5 h). The excess hexamethyldisilazane was removed by rotary evaporation, and the sample was then heated in vacuo (0.1 mm) at 50 °C for 1 h. The 2,4,6-tris(trimethylsilyl)-barbituric acid crystallized on cooling and was used in the next step without further purification.

1-(2′,3′,5′-Tribenzoyl-β-D-ribofuranosyl)barbituric Acid. The unpurified sample from the previous step was dissolved in 1300 mL of dry 1,2-dichloroethane. 1-Acetyl-2,3,5-tribenzoyl-β-D-ribofuranoside (von Recondo & Rinderknecht, 1959) (25.2 g, 0.05 mol) and stannic chloride (4.2 mL) were added and the solution was stirred under nitrogen overnight (Niedballen & Vorbruggen, 1970). The solution was then extracted once with saturated aqueous sodium bicarbonate followed by three extractions with saturated aqueous sodium chloride. The organic layer was dried over magnesium sulfate and filtered through Celite. Rotary evaporation yielded 27.2 g (95%) of oily white crystals. Thin-layer chromatography on silica gel [1-butanol, saturated with water or 2-propanol/concentrated ammonium hydroxide/water (7:1:2)] showed only trace impurities (<5%).

1-β-D-Ribofuranosylbarbituric Acid (Winkley & Robins, 1969; Otter et al., 1968). A solution of 25.2 g (0.044 mol) of 1-(2′,3′,5′-tribenzoyl-β-D-ribofuranosyl)barbituric acid in 100 mL of methanol was saturated with ammonia at 0 °C in a Teflon-lined brass pressure bomb and allowed to stand at room temperature for 3 days. At this time, the methanol and ammonia were removed by rotary evaporation. The resulting oil was partitioned between ethyl acetate and water, and the aqueous layer was passed over a 140-mL column of cation-exchange resin (Bio-Rad AG 50-X8; H^+ form). The effluent was lyophilized to give 9.6 g (84%) of a yellow powder that was recrystallized from water by evaporation. The crystals were collected and washed once with cold 95% ethanol and twice with 100% ethanol to give 2.5 g (26%) of product as dull white crystals (mp 117–119 °C). A small sample was recrystallized a second time from water: mp 125–127 °C [lit. (Winkley & Robins, 1969) mp 124–126 °C]; UV λ_{max} (pH 7.0) 260 nm; ^{1}H NMR (D_2O) δ 6.15 (d, J = 3.4 Hz, $H_{1'}$), 3.8 (m, $H_{5'}$, $H_{5''}$).

1-(5′-Phospho-β-D-ribofuranosyl)barbituric Acid (**3**). Trimethyl phosphate (17 mL), phosphoryl chloride (1.82 mL, 0.02 mol), and water (0.12 mL, 0.007 mol) were combined and cooled to −5 °C. 1-β-D-Ribofuranosylbarbituric acid (1.73 g, 0.007 mol) was added and the solution stirred at 0 °C for 20 h (Yoshikawa et al., 1969). The solution was then poured into 200 mL of cold water containing 5.6 g of sodium bicarbonate (0.067 mol). After the solution had warmed to room temperature, the pH was raised to 8.0 with 1 M sodium hydroxide. The water was removed in vacuo and the remaining solid washed with ether and dissolved in 150 mL of water. The pH of the aqueous solution was raised to 8.5 with 1 M sodium hydroxide, and a 25-mL solution of barium acetate (5.1 g, 0.02 mol) in water was slowly added with stirring. The barium phosphate precipitate was removed by centrifugation, slurried with warm water, and centrifuged again. Ethanol (328 mL) was added to the combined supernatants (164 mL) and the mixture stored at 0 °C for several hours. The precipitate which had formed was collected by centrifugation at 0 °C, washed successively with 70% ethanol and 95% ethanol, and redissolved in 5 mL of water with the aid of approximately 1 mL of cation-exchange resin (Bio-Rad AG 50-X8; H^+ form). After being passed over a 20-mL column of this same resin, the solution was adjusted to pH 7.0 with 1 M sodium hydroxide and lyophilized to dryness. The resulting oily solid completely solidified on standing in ca. 20 mL of 100% ethanol. The solid was filtered and washed with 100% ethanol. After being dried in a desiccator under vacuum (0.1 mm), the product weighed 1.7 g (ca. 60%). Thin-layer chromatography on silica gel [1-propanol/concentrated ammonium hydroxide/water (6:3:1)] showed a single major spot (R_f 0.17) and a faint trace of an impurity (~1%). A molybdate phosphate analysis (Ames, 1966) revealed that the sample contained 5% inorganic phosphate.

The pH of a 10-mL solution containing 0.4 g of the nucleotide ($\sim 10^{-3}$ mol) was adjusted to 7.5 with 1 M sodium hydroxide and the solution applied to a 24 × 1.5 cm column of DEAE-Sephadex A-25 anion-exchange resin (HCO_3^- form). Elution was carried out with a linear gradient of 400 mL of 0.05 M and 400 mL of 0.5 M triethylammonium bicarbonate buffers (pH 7.5), and fractions were monitored spectrophotometrically at 263 nm. Fractions containing product were pooled and lyophilized. The resulting solid was redissolved in water and passed over 42 mL of cation-exchange resin (Bio-Rad AG 50-X8; H^+ form). After the product had been eluted from the column with water, the pH was adjusted to 8.0 with barium hydroxide. The solution was then reduced to 40 mL by rotary evaporation and filtered. The addition of two volumes of ethanol to the filtrate precipitated the product, which was then filtered and washed twice with cold 70% ethanol. After being dried in a desiccator under vacuum (0.1 mm), 0.39 g (70% from column) of **3** was recovered: UV

λ_{max} (pH 6.0) 260 nm ($\epsilon = 1.97 \times 10^4$ M^{-1} cm^{-1}); ^1H NMR (D_2O) δ 6.16 (d, J = 3 Hz); ^{31}P NMR (D_2O–H_2O, pH 10) δ −4.0 (pH 10), −3.2 (pH 7.0). Anal. Calcd for $C_9H_{10}N_2O_{10}PBa_{1.5}\cdot H_2O$: C, 19.26; H, 2.15; N, 4.99; P, 5.51; Ba, 36.71. Found: C, 19.41; H, 2.08; N, 4.92; P, 5.42; Ba, 36.80.

1-(5′-Phospho-β-D-ribofuranosyl)[2-^{14}C]barbituric acid (1.4 Ci/mol) was prepared from [2-^{14}C]barbituric acid as described above for the unlabeled material.

6-Azauridine 5′-Phosphate (**4**). 6-Azauridine 5′-phosphate was prepared from 6-azauridine as described previously (Brody & Westheimer, 1979). 6-Aza[5-^{3}H]uridine 5′-phosphate (1.1 Ci/mol) was prepared from 6-aza[5-^{3}H]uridine as described for the unlabeled material.

Purification of OMP Decarboxylase. OMP decarboxylase was purified from baker's yeast either as described by Brody & Westheimer (1979) or by the modified procedure described below. The enzyme obtained by either procedure was pure, as judged by $NaDodSO_4$ gel electrophoresis, and had a specific activity of 33–38 units/mg.

The modified purification scheme consisted of substituting Blue Sepharose chromatography similar to that described by Reyes & Sandquist (1978) for the affinity chromatography step in the original procedure. The 45–80% ammonium sulfate fraction obtained from 4 lb of pressed baker's yeast was dissolved in 64 mM Tris-HCl and 5 mM β-mercaptoethanol, pH 7.9 (0 °C), and applied to a 3.7 × 25 cm Blue Sepharose CL-6B (Pharmacia) column. The column was washed with this buffer until $A_{280} < 0.05$; OMP decarboxylase was then eluted with 50 μM azaUMP in the same buffer. The enzyme at this point had a specific activity of about 15 units/mg. After ion-exchange chromatography and gel filtration as described by Brody & Westheimer (1979), pure enzyme was obtained that was identical with that obtained by the original procedure.

Protein Determinations. Protein concentrations were determined by the method of Warburg & Christian (1942; Layne, 1957), using a molecular weight of 55 000 for OMP decarboxylase (Umezu et al., 1971; Brody & Westheimer, 1979).

Enzyme Assay. OMP decarboxylase activity was determined by the method of Lieberman et al. (1955), with the addition of β-mercaptoethanol to fully activate the enzyme (Umezu et al., 1971). One unit is defined as the amount of enzyme required to convert 1 μmol of OMP to product per minute, based on a value of $\Delta\epsilon_{285}$ of 2250 M^{-1} cm^{-1} (Brody & Westheimer, 1979).

Stoichiometry of Association of BMP with OMP Decarboxylase. OMP decarboxylase (0.8–100 nM) in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0, was incubated at 25 °C with various concentrations of BMP for 20 min. Residual activity was then determined by adding an aliquot of OMP (final concentration 50 μM) and recording the decrease in absorbance at 285 nm as in the standard assay.

Rate of Dissociation of BMP from the OMP Decarboxylase–BMP Complex. The enzyme–inhibitor complex was prepared by adding 2.2 mol of [2-^{14}C]BMP/mol of enzyme to a 7.6 μM solution of the enzyme in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0. After 30 min, the mixture was applied to a 1 × 20 cm column of Sephadex G-25 preequilibrated with 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0. The column was then washed with this buffer, and fractions of 1 mL were collected. The fractions were monitored by liquid scintillation counting, and those fractions containing the enzyme–inhibitor complex were combined and used immediately.

For kinetics, the enzyme–inhibitor complex (0.1–3 μM) was equilibrated at the desired temperature, and a large excess of unlabeled BMP was added (final concentration 30–400 μM). At given time intervals, 100 μL of the reaction solution was withdrawn and centrifuged in an Amicon CF 25 Centriflo ultrafiltration cone at 800g for 15 min. The filter was then washed with 200 μL of buffer and centrifuged again. The dry ultrafiltration cone was placed in a scintillation vial with 15 mL of Aquasol and counted. Control experiments showed that 70% of the applied enzyme–inhibitor complex was retained on the cone by this procedure, while no uncomplexed inhibitor was retained. Semilog plots of the loss of radioactivity vs. time yielded straight lines from which the rate constant for dissociation of BMP was calculated.

Reversibility of the Binding of BMP to OMP Decarboxylase. One milliliter of a 0.77 μM solution of the OMP decarboxylase–BMP complex prepared and isolated as described above was placed on one side of a Fisher Equilibrium-Type dialysis cell (2 mL total volume). In the other side was placed 1 mL of a 0.2 M solution of azaUMP in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0. The cell was then incubated at 4 °C with gentle shaking for 24 h. At this time, the contents of the cell compartment without enzyme were removed and 1 mL of buffer was added. The cell was again incubated at 4 °C for 24 h and the buffer solution removed. Another addition of 1 mL of 0.2 M azaUMP was made, followed by another incubation against buffer. This cycle of dialysis against azaUMP followed by dialysis against buffer was repeated nine times, after which the enzyme solution was removed and dialyzed against 1 L of 2 mM azaUMP in buffer followed by several changes of buffer without inhibitor. Finally, the enzyme solution was assayed as usual for decarboxylase activity.

Determination of Dissociation Constants. The apparent equilibrium constant for the competitive binding of BMP and azaUMP to OMP decarboxylase was determined with the aid of Fisher Equilibrium-Type dialysis cells (2-mL total volume). In one half of the cell was placed 1 mL of solution of the OMP decarboxylase–[2-^{14}C]BMP complex (0.6–3.0 μM), prepared and isolated as described above. In the other side was placed 1 mL of 50 mM sodium phosphate–5 mM β-mercaptoethanol, pH 6.0, containing various amounts of azaUMP (final concentation 8–95 μM). The cell was incubated with gentle shaking at 4 °C for 36 h. Aliquots from each compartment were then withdrawn and counted in 5 mL of Aquasol. The amount of BMP bound to the enzyme at equilibrium was thus determined, and K_{BMP}/K_{azaUMP} was calculated from eq 2. The

$$\frac{K_{BMP}}{K_{azaUMP}} = \frac{[E\cdot azaUMP][BMP]}{[E\cdot BMP][azaUMP]} \qquad (2)$$

range of azaUMP concentrations used was such that at equilibrium the ratio of free to bound BMP (i.e., [BMP]/[E·BMP]) in eq 2 ranged from 0.15 to 8.5. In order to ensure that equilibrium had been reached after 36 h, several determinations of K_{BMP}/K_{azaUMP} were also made after 48 and 72 h. The results of these longer incubation times were practically identical with those measured after 36 h.

The apparent equilibrium constant for the competitive binding of azaUMP and UMP to OMP decarboxylase was determined in an analogous manner to that described above for the BMP/azaUMP equilibrium. One side of the equilibrium dialysis cell initially contained 1 mL of a mixture of [5-^{3}H]azaUMP and OMP decarboxylase (1–9 μM) in the ratio 2:1 in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0, while the other side contained 1 mL of

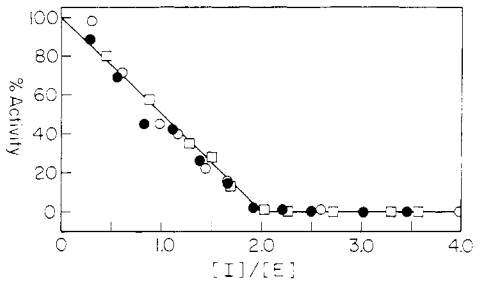


FIGURE 1: Stoichiometric inhibition of OMP decarboxylase by BMP. Inhibition was measured as described under Experimental Procedures. Concentrations of OMP decarboxylase dimer: (●) 1.1×10^{-7} M, (■) 1.5×10^{-8} M, and (○) 8.7×10^{-10} M.

various concentrations of UMP (final concentration 0.2–10 mM) in the same buffer. After gentle shaking of the solution at 4 °C for 24 h, aliquots from each compartment were withdrawn and counted as before. The ratio of bound to free azaUMP at equilibrium ranged from 0.15 to 8.5, and the apparent equilibrium constant, K_{azaUMP}/K_{UMP}, was calculated from eq 3.

$$\frac{K_{azaUMP}}{K_{UMP}} = \frac{[E{\cdot}UMP][azaUMP]}{[E{\cdot}azaUMP][UMP]} \quad (3)$$

The dissociation constant for the OMP decarboxylase–UMP complex was determined kinetically by measuring the inhibition by UMP of the rate of production of $^{14}CO_2$ from [*carboxyl*-^{14}C]OMP. A mixture of enzyme (0.1–1 nM) and various concentrations of UMP in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0, was equilibrated at 4 °C and the reaction was initiated by adding an aliquot (10–50 μL) of a 60 μM solution of [*carboxyl*-^{14}C]OMP (final concentration 0.5–3.0 μM) in deionized water (final volume 1.1 mL). The reaction was quenched at various times by the addition of 0.2 mL of 2 M perchloric acid, and the amount of $^{14}CO_2$ that had been produced was determined by liquid scintillation counting as described by Prabhakararao & Jones (1975). The initial rate of decarboxylation was taken as the slope of the initial portion of a plot of the amount of $^{14}CO_2$ released vs. time. The dissociation constant for UMP was then determined from either Dixon plots of v_i^{-1} vs. [UMP] or Lineweaver–Burk plots of v_i^{-1} vs. $[OMP]^{-1}$. The dissociation constant calculated by the two methods was the same.

Results

Stoichiometry of Association of BMP with OMP Decarboxylase. When the amount of BMP added to a solution of OMP decarboxylase is increased, decarboxylase activity is correspondingly decreased until finally, at a molar ratio of inhibitor to dimeric enzyme of 2:1, the enzyme is completely inhibited (Figure 1). Since Brody & Westheimer (1979) have shown that OMP decarboxylase is a dimer composed of two subunits of the same molecular weight, it seems most likely that each subunit contains one active site capable of interacting with BMP.

Reversibility of the Inhibition of OMP Decarboxylase by BMP. Gel filtration of the OMP decarboxylase–BMP complex on Sephadex G-25 does not restore decarboxylase activity, suggesting that the binding of BMP might be irreversible. However, the UV spectrum of the enzyme–inhibitor complex shown in Figure 2 is simply the sum of the spectra of OMP decarboxylase and BMP individually, indicating that the pyrimidine ring of BMP remains intact upon binding to the enzyme. Furthermore, denaturation of the enzyme–inhibitor complex with either 10% $NaDodSO_4$ or 8 M urea results in quantitative release of BMP from the complex. These facts

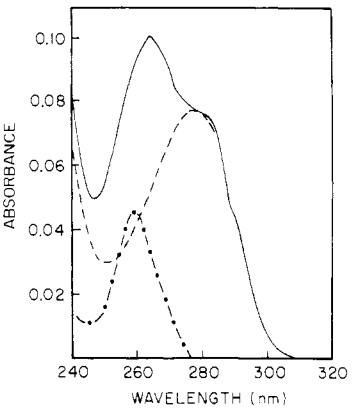


FIGURE 2: Ultraviolet spectrum of the OMP decarboxylase–BMP complex (1.6 μM, —) in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0. The enzyme–inhibitor complex was prepared and isolated as described under Experimental Procedures. The spectra of free BMP (3.2 μM, –·–) and uninhibited OMP decarboxylase (1.6 μM, – – –) in the same buffer are also shown. The sum of the spectra of BMP and the native enzyme (not shown) is identical within experimental error with that of the enzyme–inhibitor complex.

imply that BMP is not a covalent irreversible inhibitor but rather a very tightly bound reversible inhibitor.

The reversibility of BMP inhibition was demonstrated in two ways. A large excess of azaUMP was added to a solution of the OMP decarboxylase–BMP complex. After sufficient time to allow equilibration of the enzyme with both inhibitors, unbound inhibitor was removed by dialysis and another addition of azaUMP was made. This process of addition of azaUMP followed by removal of unbound inhibitor was repeated nine times, and then the enzyme solution was dialyzed against several changes of buffer without inhibitor. Using the equilibrium constant determined below for the competition between azaUMP and BMP for OMP decarboxylase, we can estimate that about 35% of bound BMP should be released in the first cycle of addition of azaUMP. In fact, a 0.81 μM solution of the OMP decarboxylase–BMP complex regained 34% activity in one cycle. Theoretically, then, the enzyme should be completely freed of BMP and should regain full activity after nine cycles; the enzyme actually isolated showed 95% of full activity.

The reversibility of BMP binding was also demonstrated by measuring the rate of release of [2-^{14}C]BMP from the enzyme–inhibitor complex prepared with [2-^{14}C]BMP when a large excess of unlabeled BMP was added. The rate of release of [2-^{14}C]BMP from the OMP decarboxylase–BMP complex was found to be first order in the complex and independent of the concentration of added inhibitor. The rate constants for the release of BMP from the complex at 4 and 25 °C in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0, are 2.2×10^{-5} s^{-1} (14 determinations, SD = $\pm 0.7 \times 10^{-5}$ s^{-1}) and 5.8×10^{-5} (16 determinations, SD = $\pm 1.1 \times 10^{-5}$ s^{-1}), respectively. The rate constant for dissociation of the inhibitor from the complex determined at 4 °C by using 2.6 μM complex and 0.194 mM BMP deviated from the mean value reported here by more than two standard deviations but was, nevertheless, included in the calculation of the rate constant. The cause of the deviation of this one of the 14 determinations is not known and was not investigated further. The concentration of enzyme–inhibitor complex used in these determinations ranged from 0.1 to 3.0 μM.

Determination of the Binding Constant of UMP to OMP Decarboxylase. The dissociation constant for the OMP decarboxylase–UMP complex, K_{UMP}, was determined kinetically. As shown in Figure 3, UMP is a competitive inhibitor of OMP

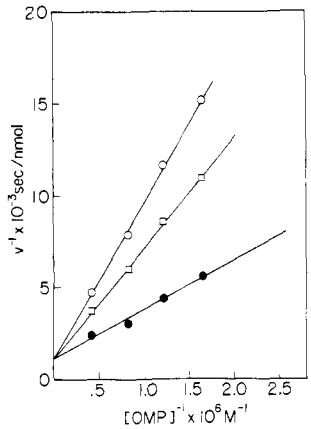


FIGURE 3: Kinetic study of the inhibition of OMP decarboxylase by UMP. Reactions were conducted at 4 °C as described in the text with 4.8×10^{-10} M OMP decarboxylase and UMP concentrations of (●) 0 M, (□) 7.26×10^{-4} M, and (○) 1.45×10^{-3} M. The Lineweaver–Burk plots presented here are for illustration purposes only; K_i was determined from Dixon plots of these and other data.

decarboxylase with a K_{UMP} of 4.6×10^{-4} M at 4 °C in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0.

Determination of the Dissociation Constants of Complexes Formed between OMP Decarboxylase and BMP and azaUMP. In order to measure the binding constants of BMP and azaUMP to OMP decarboxylase, we have used a competitive equilibrium method similar to that used by Vincent & Lazdunski (1972) to measure the binding constant for pancreatic trypsin inhibitor to trypsin. The OMP decarboxylase–BMP complex was prepared by using [2-^{14}C]-BMP, and various amounts of azaUMP were added. The ratio K_{BMP}/K_{azaUMP}, calculated as explained under Experimental Procedures, has a value of 1.7×10^{-5} (34 determinations, SD = $\pm 0.4 \times 10^{-5}$) at 4 °C in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0.

The dissociation constant of azaUMP was also measured by the competition equilibrium technique with UMP as the second inhibitor. This equilibrium constant K_{azaUMP}/K_{UMP} was found to be 1.1×10^{-3} (36 determinations, SD = $\pm 0.3 \times 10^{-3}$) at 4 °C in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0.

From the value of K_{BMP}/K_{azaUMP}, K_{azaUMP}/K_{UMP}, and K_{UMP}, the dissociation constants K_{BMP} and K_{azaUMP} for the complex formed between OMP decarboxylase and BMP and azaUMP, respectively, can be calculated. The values are listed in Table I.

Table I: Dissociation Constants for the Complexes of UMP, azaUMP, and BMP with OMP Decarboxylase[a]

inhibitor	K_i (M)	K_i*[b] (M)
UMP	4.6×10^{-4}	1.5×10^{-7}
azaUMP	5.1×10^{-7}	4.6×10^{-8}
BMP	8.8×10^{-12}	8.8×10^{-12}
OMP	1.5×10^{-6}[c]	

[a] At 4 °C in 50 mM sodium phosphate and 5 mM β-mercaptoethanol, pH 6.0. [b] Calculated intrinsic dissociation constants for the anionic form of each inhibitor, based on pK_a values of 9.5 for UMP, 7.0 for azaUMP, and 4.5 for BMP. [c] The actual dissociation constant for the enzyme–substrate complex is not known, but the kinetically determined K_M (Brody & Westheimer, 1979) represents an upper limit for this constant.

Discussion

Product inhibition in the decarboxylation of OMP by OMP decarboxylase was first reported by Creasy & Handschumcher (1961) and Blair & Potter (1961). These workers reported K_i values for UMP of 0.4 mM and 2.1 mM, respectively, at pH 8.0. Umezu et al. (1971), however, reported that OMP decarboxylase was not inhibited by 1 mM UMP at pH 6.0. We have reinvestigated UMP inhibition at pH 6.0 and find a value of 0.46 mM for K_i at this pH.

We have also reinvestigated the inhibition of OMP decarboxylase by azaUMP. Handschumacher (1960) originially reported that the inhibition of OMP decarboxylase by this inhibitor was pH dependent, with the inhibitor being most effective when its triazine ring (pK_a = 7.0) was ionized. He found a K_i value of 0.7–0.8 μM at pH 8.0. Brody & Westheimer (1979), using their highly purified enzyme, found a K_i of 0.03 μM for azaUMP at pH 8.0. Our value of 0.51 μM at pH 6.0 is consistent with this latter value when the difference in anion concentration at pH 6.0 vs. pH 8.0 is taken into consideration.

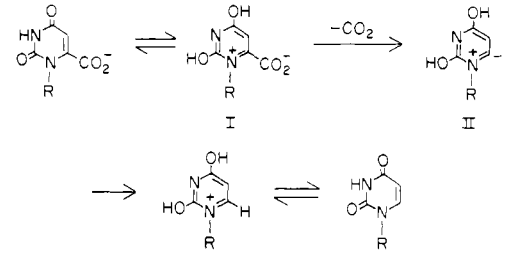
The potent inhibition of OMP decarboxylase by BMP ($K_i = 8.8 \times 10^{-12}$ M at pH 6.0) represents one of the strongest protein–ligand interactions that have been measured. The dissociation constants for the avidin–biotin and pancreatic trypsin inhibitor–trypsin complexes are given as 10^{-15} M (Green, 1973) and 6×10^{-14} M (Vincent & Lazdunski, 1972), respectively. The smallest dissociation constant that had been reported for a synthetic inhibitor is 3×10^{-11} M for the methotrexate–dihydrofolate reductase complex (Werkheiser, 1961). Other examples of tight binding between enzymes and inhibitors can be found in the recent reviews of Leinhard (1973) and Wolfenden (1976).

In an effort to understand the tight binding of BMP to the enzyme, we have measured the rate of dissociation of the inhibitor from the enzyme–inhibitor complex. Using this rate constant and the dissociation constant for BMP, we calculate that the rate of association of BMP with OMP decarboxylase is 2.7×10^6 M^{-1} s^{-1} at 4 °C. This rate is comparable to that measured for other protein–ligand associations (Eigen & Hammes, 1963). From the dissociation rate constants at 4 and 25 °C we can also estimate an Arrhenius activation energy of 6–7 kcal/mol for the dissociation of BMP from the enzyme–inhibitor complex. This relatively small activation energy implies a large negative entropy contribution to the free energy of binding of BMP to the enzyme. A similar large negative entropy of binding has also been observed in the binding of both NAD^+ and NADH (Schmid et al., 1978) to alcohol dehydrogenase, where it has been ascribed to a conformational change that occurs upon binding of the coenzyme to the enzyme (Subramanian & Ross, 1977). Such a large negative entropy contribution can also be understood on the basis of desolvation on forming the enzyme–substrate complex or of specific ionic and hydrogen-bonding interactions that fix the inhibitor (substrate) very precisely in the enzyme's active site.

Desolvation of the substrate upon binding to the enzyme might imply that the enzyme's active site is hydrophobic. On the basis of the proposed mechanism of action of OMP decarboxylase (Beak & Siegel, 1976; see below), a hydrophobic active site would be expected to facilitate decarboxylation. Alternatively, desolvation could result from electrostatic binding of the phosphate group of the inhibitor to a cationic site on the enzyme, since the electrically neutral zwitterionic complex would be less highly solvated than the separated ions.

Since OMP is completely ionized over the entire pH range in which OMP decarboxylase is active and azaUMP is most effective as an inhibitor above the pK_a of its triazine ring, it seems reasonable that a negatively charged substituent at either the 2 or 6 position of the pyrimidine ring is essential for

Scheme I

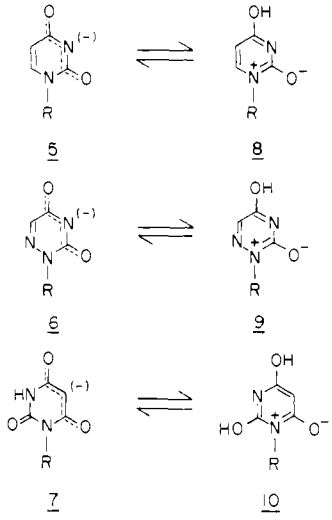


substrate (inhibitor) binding to OMP decarboxylase. This would explain the poor inhibition of the enzyme by UMP. At pH 6.0, less than 1% of the product exists in the ionized form.[2] If we assume that the contribution to the observed inhibition by the un-ionized pyrimidine is negligible, then we can calculate an intrinsic dissociation constant for the anionic form of UMP of 1.5×10^{-7} M. Similarly, we can calculate a dissociation constant of 4.6×10^{-8} for the anionic form of azaUMP.

These calculated values of the binding constants for the anionic forms of the various inhibitors are listed in the last column of Table I. It appears from these calculated values of K_i that the anionic forms of UMP, as well as of azaUMP and BMP, have a greater affinity for OMP decarboxylase than does OMP ($K_M = 1.5 \times 10^{-6}$ M). The absence of severe product inhibition in the enzymatic reaction under normal conditions is not then due to the inability per se of UMP to bind to the enzyme, but rather to the fact that only anions bind at the active site and the pK_a of UMP is several units higher than that of the substrate. Furthermore, the differences in inhibitory power observed for the various inhibitors should be pH dependent. At pH values low enough to result in substantially reduced quantities of ionized BMP, the apparent inhibition caused by it relative to azaUMP should also decrease. Unfortunately, OMP decarboxylase rapidly loses activity below pH 5; since the pK_a of BMP is ~4.5, this prediction could not be tested. But, at pH 8 where the anion concentration of BMP is essentially identical with that at pH 6, the dissociation constant of BMP appears to be unchanged (*results not shown*) while that of azaUMP decreases (Handschumacher, 1960; Brody & Westheimer, 1979).

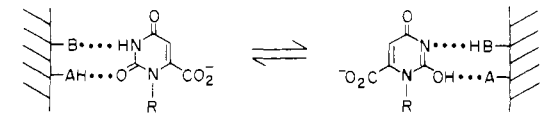
Beak & Siegel (1976) have proposed that the decarboxylation of OMP proceeds via the mechanism shown in Scheme I. According to this mechanism, OMP decarboxylase catalyzes this reaction by stabilizing the zwitterion I and the nitrogen ylide II which is generated by loss of CO_2 from I. Subsequent protonation of the ylide II affords the product, UMP. Such stabilization requires that the enzyme increase the basicity of the 4-oxygen of the substrate and possess an acidic residue capable of "pushing" protons onto the substrate. Our results, while they do not establish this mechanism, are consistent with it and will be discussed in terms of this mechanism.

The negative charge on each of the inhibitors resides, in part, on the 4-oxygen as illustrated in **5**–**7**. This increased electron density on the 4-oxygen would enhance the binding of the inhibitors via electrostatic interactions with the above postulated acidic residue. Furthermore, protonation of the inhibitors in the enzyme's active site leads to the zwitterions **8**–**10** which resemble the putative transition state for the decarboxylation of OMP. Therefore the anionic forms of the inhibitors studied



here may be regarded as transition-state analogues of OMP decarboxylase.

If the pair of hydrogen bonds illustrated in eq 4 are also



(4)

important in binding the substrate to the enzyme, then the greater affinity of BMP for the enzyme relative to azaUMP and UMP can be understood. The major structural difference between OMP and the inhibitors UMP and azaUMP lies in the uncharged portion of their pyrimidine rings. While OMP has an amide functionality, UMP and azaUMP have an alkene and imine functionality, respectively. UMP is thus unable to form any hydrogen bonds with the enzyme. In azaUMP, the lone pair on N-6 is an excellent potential hydrogen-bond acceptor, although this interaction might be minimized if the inhibitor is bound in the active site in such a manner that the residue that donates the hydrogen bond is too far away to form a strong bond. Unlike UMP and azaUMP, BMP possessess an amide moiety similar to that of the substrate, and therefore might be able to achieve maximum interaction with this portion of the enzyme's active site. The combination of favorable hydrogen bonding and electrostatic interactions could then result in the extremely tight binding of BMP to OMP decarboxylase that is observed.

While the relative binding strengths of the various inhibitors studied here can be rationalized as discussed above, it is possible that subtle conformational differences between the inhibitors and the substrate may be present which could affect their ability to bind the enzyme.[3] These differences may be at least partly responsible for the observed differences in the dissociation constants for UMP, azaUMP, and BMP.

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[2] The pK_a of UMP is approximately 9.5. See Jencks & Regenstein (1970).

[3] See Brody (1973) and Saerger (1973) for discussion of the conformational analysis of pyrimidine nucleotides and its relevance to the OMP decarboxylase reaction.

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Major Component of Acetylcholinesterase in *Torpedo* Electroplax Is Not Basal Lamina Associated†

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ABSTRACT: Electroplax tissue from *Torpedo californica* contains two major structural forms of the enzyme acetylcholinesterase. One form, composed of tetrameric protomers which are further aggregated by interactions among associated collagenous "tail fibers", has been well characterized previously. This form is associated in situ with the basal lamina. The other form is described and characterized herein. This latter form accounts for at least 50% of the acetylcholinesterase activity of the tissue. This enzyme associates with the tissue phospholipids. It aggregates in aqueous solution but readily dissociates to dimers in 1% sodium cholate solution, a solvent in which it is both soluble and catalytically fully active. The same dimer is obtained in sodium dodecyl sulfate solution where the enzyme is denatured. Denaturation in the presence of the reductant dithiothreitol results in the formation of a single 80 000-dalton subunit. The purified enzyme contains no collagenous component. It is not derivable from the collagenous "tailed-enzyme" form in the tissue homogenate. However, the two enzymes have similar molecular weight catalytic subunits and the same substrate-dependent turnover numbers (per active site) for a variety of choline esters which are generally utilized to distinguish specific esterase function. In the tissue homogenate each form of the enzyme is associated with a characteristic structural component (phospholipid or collagen). By implication, acetylcholinesterase function is localized in situ in the phospholipid membrane as well as at the basal lamina.

Both the structure and the catalytic activity of acetylcholinesterase, derived from a variety of sources, have received considerable attention. A major impetus for structural studies derives from physiological interest in the spatial relationships among three components of the synapse, the acetylcholine receptor, acetylcholine, and acetylcholinesterase. Most structural and catalytic studies have focused on two enzyme- and receptor-rich organs, the electroplax from the species *Electrophorus* and *Torpedo*. Since quantitative enzymological assays are usually carried out in homogeneous solution (an environment different from that of the structured synaptic cleft), most studies of catalysis and its modulation have been performed under environmental conditions designed for homogenization of the enzyme solution. Homogeneity can be achieved by a variety of different procedures. Soluble acetylcholinesterase protein can be isolated and purified by treatment of electroplax homogenates with trypsin, a treatment which is successful both for *Electrophorus electricus* (Rosenberry, 1975) and *Torpedo californica* (Taylor et al., 1974). This trypsin-solubilized enzyme has been utilized in most studies of enzyme activity and of the effects of ligand on the binding of substrates and substrate analogues to the active site (Rosenberry, 1975; Taylor & Lappi, 1975; Taylor et al., 1975). A solubilized form of the enzyme can also be produced by treatment of the electroplax homogenates with collagenase (Dudai & Silman, 1974; Lwebuga-Mukasa et al., 1976), a

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