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^{31}P NMR STUDIES ON THE INTERACTION OF DEOXYURIDYLATE WITH THYMIDYLATE SYNTHASE

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Summary

The ^{31}P nuclear magnetic resonance signal of deoxyuridylate was studied in the presence and absence of thymidylate synthase. In the absence of enzyme the chemical shift of deoxyuridylate is pH dependent with a pK_a of 6.25. In the presence of enzyme, a peak corresponding to the dianionic form of deoxyuridylate is observed which is independent of pH between pH 5.7 and pH 7.4. The pK_a of the phosphate in the deoxyuridylate-thymidylate synthase complex is therefore less than 5. The release of inorganic phosphate from deoxyuridylate catalyzed by contaminating phosphatase was also observed.

Thymidylate synthase (5,10-methylenetetrahydrofolate:deoxyuridylate *C*-methyltransferase, EC 2.1.1.45) catalyzes the following reaction

$$N^5, N^{10}\text{-methylenetetrahydrofolate} + \text{deoxyuridylate} \rightarrow \text{thymidylate} + \text{dihydrofolate}$$

This enzyme catalyzes the rate limiting step of the de novo synthesis of thymidylate [1,2]. Circular dichroic [3] and equilibrium dialysis studies [4] show that a binary complex is formed between thymidylate synthase and deoxyuridylate, and that one mol of deoxyuridylate binds per mol of enzyme. We have undertaken a phosphorus magnetic resonance study to determine the pK_a of the phosphate group of deoxyuridylate bound to thymidylate synthase. This provides information on the mode of binding of the phosphate in the binary complex.

Thymidylate synthase was isolated from extracts of methotrexate resistant *Lactobacillus casei* [5] which were provided by the New England

Enzyme Center [6]. Crude extracts were purified on phosphocellulose [7] by the procedure of Beaudette et al. [8]. The final purification was obtained by chromatography on Sephadex G-100 [8]. The material purified in this manner had a specific activity of 232 units of activity per mg of enzyme. A unit of activity is defined as that amount of enzyme necessary to catalyze the synthesis of 1 μmol of thymidylate per hour at 30°C as measured by the spectrophotometric assay of Wahba and Friedkin [9]. The enzyme concentration is determined using the extinction coefficient of $1.08 \cdot 10^{-5}\ M^{-1} \cdot cm^{-1}$ at 280 nm [10]. The enzyme was concentrated to $6.15 \cdot 10^{-4}$ M for nuclear magnetic resonance studies by use of a Schleicher and Schuell collodion bag with a molecular weight cutoff of 25 000. The enzyme preparation showed a single band when 50 μg protein was subjected to nondenaturing [11] gel electrophoresis.

Phosphatase activity was assayed spectrophotometrically by following the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol plus phosphate [12]. Activity was determined at 37°C in 0.05 M tris(hydroxymethyl)aminomethane, 0.001 M EDTA buffer pH 7.4. A unit of phosphatase is defined as that amount necessary to produce 1 μmol of nitrophenol per hour.

All ^{31}P nuclear magnetic resonance spectra were obtained at ambient temperature, at 24.3 MHz using a Bruker WP 60 Spectrometer. Proton decoupling, quadrature detection and Fourier transform techniques were utilized. The sample tube diameter was 10 mm and spectra were obtained in buffer containing 0.05 M tris(hydroxymethyl)aminomethane, 0.1 M KCl, and 0.001 M EDTA. EDTA is added to preclude problems associated with extensive linebroadening caused by paramagnetic ion contamination [13]. The sample buffer contained 10% ^2H_2O to provide an instrument lock signal. Chemical shifts are reported as ppm from 85% H_3PO_4 as the external standard. No correction for the deuterium isotope effect upon pH was made.

The resonance due to the ^{31}P nucleus in both deoxyuridylate and inorganic phosphate moves downfield as pH is increased from pH 4 to pH 8. The changes in chemical shifts correspond to changes in the protonation states in the phosphate moiety of the compounds. The pK_a values are 6.25 for deoxyuridylate and 7.4 for inorganic phosphate (Fig. 1). The pK_a of 6.25 for deoxyuridylate is in agreement with those determined in other ^{31}P NMR studies [14] and by calorimetry [15].

The ^{31}P NMR spectrum of thymidylate synthase in the absence of added deoxyuridylate shows no phosphorus peaks. This indicates that the ^{31}P NMR peaks observed after the incubation of deoxyuridylate with thymidylate synthase are due to added deoxyuridylate.

The ^{31}P NMR spectrum of a mixture of 2 parts deoxyuridylate to one part thymidylate synthase at pH 6.2 is characterized by three peaks (Fig. 2A). Peaks b (—1.87 ppm) and c (—0.48 ppm) have chemical shifts corresponding to deoxyuridylate and inorganic phosphate respectively. Peak a (—3.74 ppm) is broadened and shifted downfield from the resonance of deoxyuridylate and is assigned to the deoxyuridylate-thymidylate synthase complex.

A titration of the phosphate moiety of deoxyuridylate in the presence of thymidylate synthase is reported in Table I. The average chemical shift

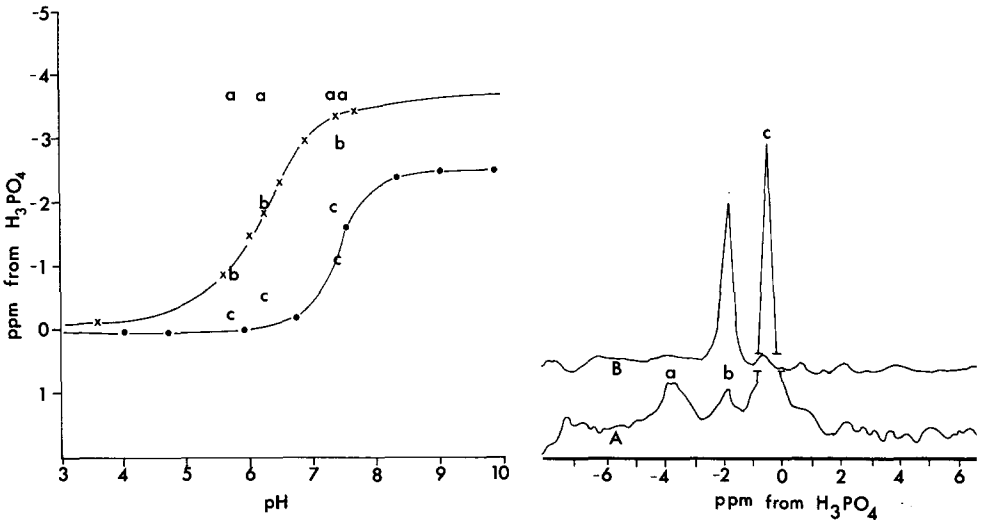

Fig. 1. The pH dependence of the chemical shift of the ^{31}P resonances of deoxyuridylate and inorganic phosphate in the absence and presence of thymidylate synthase. X and • denote deoxyuridylate and inorganic phosphate, respectively, in the absence of thymidylate synthase, a denotes the binary complex of deoxyuridylate and thymidylate synthase. b and c denote excess deoxyuridylate and inorganic phosphate, respectively in the presence of thymidylate synthase. Spectra were obtained at 24.3 MHz.

Fig. 2. A. The ^{31}P NMR spectra of deoxyuridylate in the presence of thymidylate synthase at pH 6.2. Peaks labeled a, b, c, correspond to binary complex, excess deoxyuridylate, and inorganic phosphate respectively. The spectrum was acquired over a period of 8 hours. B. The ^{31}P NMR spectrum of deoxyuridylate in the absence of enzyme at pH 6.2.

TABLE I

^{31}P CHEMICAL SHIFT OF PEAKS OVSERVED WHEN DEOXYURIDYLATE IS MIXED WITH THYMIDYLATE SYNTHASE

Peaks a, b, c correspond to binary complex, excess deoxyuridylate and inorganic phosphate respectively (Fig. 1).

pH	Ratio enzyme: dUMP	Chemical shift of peaks		
		a	b	c
5.7	1:0.7	—	—	—0.14
	1:2	—	—0.89	—0.24
	1:3	—3.69	—*	—0.31
6.2	1:0.7	—3.69	—	—0.56
	1:2	—3.74	—1.87	—0.48
	1:2.8	—3.66	—1.95	—0.59
	1:2.8	—3.66	—2.03	—0.69
7.3	1:0.7	—3.74	—	—1.95
7.4	1:10	—3.74	—2.92	—1.05

*Excess deoxyuridylate is not observed due to the presence of contaminating phosphatase.

for each pH studied is plotted in Fig. 1. The chemical shifts of peaks b and c are pH dependent and correspond to the titration curves of deoxyuridylate and inorganic phosphate respectively. The small differences in the chemical shift of inorganic phosphate in the presence and absence of enzyme shown in Fig. 1 may be due to a weak interaction between inorganic phosphate and the enzyme. The chemical shift of peak a, assigned to the deoxyuridylate-thymidylate synthase complex, is independent of pH in the range pH 5.7—7.4 and corresponds to the dianionic form of deoxyuridylate.

The pK_a of deoxyuridylate in the complex is therefore less than 5 in contrast to the pK_a of 6.25 for the free nucleotide. This shows that the phosphate of deoxyuridylate is fully ionized in the deoxyuridylate-thymidylate synthase complex over the pH range 5.7—7.4.

The appearance of ^{31}P NMR peaks (Fig. 2) corresponding to both bound and free deoxyuridylate indicates that the exchange between free and bound ligand is slow on the NMR time scale and from the absence of exchange broadening we can put an upper limit of 20 s^{-1} on this exchange rate.

During the 8-h period necessary to obtain high resolution ^{31}P NMR spectra, the intensity of peak c increases as that of peak b decreases, which shows that phosphate is being cleaved from deoxyuridylate. Phosphatase assays indicate an activity of 0.06 units per ml in the preparation used to obtain the ^{31}P NMR spectra. This activity is only 0.006% of the activity of thymidylate synthase, however it would suffice to cause the observed inorganic phosphate peak. The phosphatase activity could be separated from thymidylate synthase activity by affinity chromatography [16].

The results obtained in this study provide direct evidence that deoxyuridylate is bound to thymidylate synthase as the dianion.

Additional phosphorylated ligands shown by ^{31}P NMR studies to bind to enzymes as dianions are: (1) Pyridoxal phosphate binding to aspartate transaminase [17], (2) dihydroxyacetone phosphate binding to trioseisomerase [18] and (3) the binding of NADPH to dihydrofolate reductase [19]. The binding of deoxyuridylate as the dianion was postulated in two other studies involving indirect determination: (1) analog studies [20] showed that a structural requirement for the interaction of nucleotides and thymidylate synthase from *Escherichia coli* is the presence of a phosphate group capable of forming a dianion, (2) calorimetric studies of deoxyuridylate binding to *L. casei* thymidylate synthase [21] showed a release of protons when the complex was formed at pH 5.8.

The interaction of deoxyuridylate and thymidylate synthase may be analogous to the binding of 3′-CMP to ribonuclease A. Calorimetric studies [22] indicate that the 3′-CMP is bound as the dianion and that the complex is stabilized by the interaction of the dianion with at least two histidine residues as the active site.

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