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STUDY OF THYMIDYLATE SYNTHETASE-FUNCTION BY LASER RAMAN SPECTROSCOPY

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Summary

The Laser-Raman spectra of thymidylate synthetase have been obtained with 488 nm excitation from an argon ion laser. Raman bands observed in the range 600—800 cm^{-1} have been assigned to functional groups of constituent amino acids. The band positions and intensities in the Amide I (1600—1700 cm^{-1}) and Amide III (1200—1300 cm^{-1}) regions, suggest that the enzyme is a mixture of α-helical and unordered conformations. Low levels of β-structure cannot be excluded.

The spectra of the ternary complex formed by reacting thymidylate synthetase with (+)-L-methylenetetrahydrofolate and fluorodeoxyuridylate reveals a new band at 1618 cm^{-1} assigned to the C=N stretching vibration. This band may be due to formation of dihydrofolate or an iminium ion.

The overall secondary structure of thymidylate synthetase does not change on formation of the ternary complex. However, the spectrum of the complex indicates local changes in groups such as ionized carboxyl (1400 cm^{-1}), tryptophan (1003 cm^{-1}) and CH_3, CH_2 deformation modes (1440—1470 cm^{-1}).

Thymidylate synthetase catalyzes the reaction of 5,10-methylenetetrahydrofolate with deoxyuridylate to form thymidylate and dihydrofolate [1]. H_4-folate serves as the reductant to convert the methylene group to a methyl group which displaced the H on the 5 carbon of dUMP.

The pyrimidine nucleotide analog 5-fluoro-2′-deoxyuridylate, a potent inhibitor of thymidylate synthetase, forms a ternary complex with the enzyme in the presence of methylenetetrahydrofolate [2—8].

Abbreviations: (±)-L-H_4-folate = mixture of diastereoisomers of 5,6,7,8 tetrahydrofolate at carbon 6; (+)-CH_2-H_4-folate = natural diastereoisomer of N_5,N_{10}-methylenetetrahydrofolate; H_2-folate = 7,8 dihydrofolate; FdUMP = 5-fluoro-2′-deoxyuridylate.

Spectrophotometric studies [2,6—8] indicate that H_4-folate is chemically altered upon formation of the ternary complex. Comparison of the difference spectrum between H_4-folate and H_2-folate [2] with the spectral change occurring upon formation of the ternary complex suggests that the new folate species might be H_2-folate. Another possible interpretation is that an iminium ion ($N^+=CH_2$) is formed [9,10].

We report here the Raman spectra of thymidylate synthetase and ternary complex which yield information on the structure of the protein and the nature of the tetrahydrofolate present in the complex.

Experimental

Materials

Folic acid (Nutritional Biochemicals) was used to prepare (±)-L-H_4-folate by catalytic hydrogenation [11] over platinum catalyst. (+)-L-H_4-folate was prepared enzymatically from dihydrofolate by the method of Mathews and Huennekens [12], as modified [13]. FdUMP was obtained from the Terra-Marine Bioresearch. Hydroxylapatite was purchased from Bio Rad Laboratories.

Methods

Enzyme preparation

Crude bacterial extracts containing thymidylate synthetase were prepared at the New England Enzyme Center [14] from a methotrexate-resistant strain of *Lactobacillus casei* developed in this laboratory [15]. Thymidylate synthetase was purified according to the method of Leary and Kisliuk [16] through the hydroxylapatite chromatography step. The pooled fractions were concentrated [16] and dialyzed overnight against 80 mM potassium phosphate, 100 mM KCl, 1 mM disodium EDTA, pH 6.8. Further purification was obtained by gel filtration on Sephadex G-100. Approx. 80—100 mg protein in 4—6 ml was applied to the column (2.6 × 86) and eluted with the same solution. This method was developed by Dr Richard Leary in this laboratory. Fractions with specific activities of 126—145 were pooled and concentrated by ammonium sulfate precipitation, dialyzed as above and the gel filtration procedure was repeated. This protein (yield 26 mg) showed a single band when 180 μg were subjected to acrylamide gel electrophoresis at pH 8.5 [17]. The extinction coefficient of enzyme at 280 nm was taken as 108 000 based on amino acid analyses, performed by Dr B.H. Davis and Dr J. Ozols of the University of Connecticut. Tryptophan analysis of the enzyme by magnetic circular dichroism [18] was carried out by Dr Barton Holmquist (Biophysics Research Laboratory, Harvard Medical School, Boston, Mass.) showed 12 tryptophan residues per M_r 68 000.

Enzyme assay

Thymidylate synthetase activity was measured spectrophotometrically [19]. Specific activity is defined as the number of micromoles of thymidylate formed per hour per mg of protein at 30° C.

Raman spectroscopy

Aqueous solutions of thymidylate synthetase $1.8 \cdot 10^{-4}$ M, (+)-CH_2-H_4-

folate $4.5 \cdot 10^{-4}$ M, FdUMP $4.5 \cdot 10^{-4}$ M and H_2-folate $4.5 \cdot 10^{-4}$ M alone or in various combinations, were transferred to 0.9—1 mm ID Kimex capillaries. After sealing, the sample capillaries were placed in a Miller-Harney cell [20] for temperature control. The temperature was regulated at 20° ± 2° by a flow of nitrogen regulated by a telethermometer. Temperature control was checked by melting point measurements on standard lipids in the laser beam.

Raman spectra were recorded with a Ramalog 4 Raman spectrometer (Spex Industries, Metuchen, N.J.) interfaced to an Interdata Computer (Model 70). An argon-ion laser (Spectra Physics model 164), tuned at 488 nm, was used as an excitation source, generally at 100 mW power. The Raman scattering was detected by a thermoelectrically cooled photomultiplier (RCA) and was recorded in terms of photons/s. The "dark" counts of the photo cell were <100 counts/s and "light" counts were of the order of 10^4 counts/s. The scanning was done through the computer at frequency intervals of 1 cm^{-1}/s. The dwell time at each frequency step was maximally 1 s. The photon counts were stored in the computer memory during scanning (2—3 scans). The averaged, stored, spectra was then plotted as on the Ramalog recorder after adjustment of plotting parameters (see figure legends). The time required to obtain spectra from 500—1800 cm^{-1} was approx. 22 min. We kept the enzyme in the laser light for about 5—10 min before scanning. This time is sufficient for a constant baseline which otherwise varies due to fluorescence. Carbon tetrachloride was used to check frequency calibrations. The enzyme retained its original activity after Raman spectroscopy.

Infrared spectroscopy

Aqueous solutions of the enzyme or the constituents of the ternary complex were deposited on AgCl plates, frozen and lyophilized. Infrared spectra were recorded at 30°C and 55% relative humidity, using a Perkin-Elmer 621 spectrophotometer.

Results and Discussion

The Raman and infrared spectra of thymidylate synthetase

A computer-averaged plot of the thymidylate synthetase Raman Spectrum is represented in Fig. 1. Spectral assignments based on reported Raman frequencies of different proteins and polypeptides are given in Table I.

Amide I and Amide III regions

The Amide I vibrations, due to the peptide linkages of proteins and polypeptides (predominantly C=O stretching) yield bands between 1600 cm^{-1} and 1700 cm^{-1}. Thymidylate synthetase exhibits three bands in this region. These center at 1630 cm^{-1}, 1655 cm^{-1} and 1680 cm^{-1}, but are somewhat diffused by the broad water peak centered at 1640 cm^{-1}.

The positions of Amide bands are known to depend on the secondary structures of the peptide linkages [21]. Thus, α-helical poly-L-lysine in aqueous solution is characterized by a very strong Amide I band at 1647 cm^{-1}, whereas the anti-parallel β-structure of this polypeptide exhibits a major Amide I peak at 1670 cm^{-1} [21]. With aqueous solutions of "unordered" poly-L-lysine one observes three Amide I bands at 1653 cm^{-1}, 1665 cm^{-1}, and 1683 cm^{-1} [22].

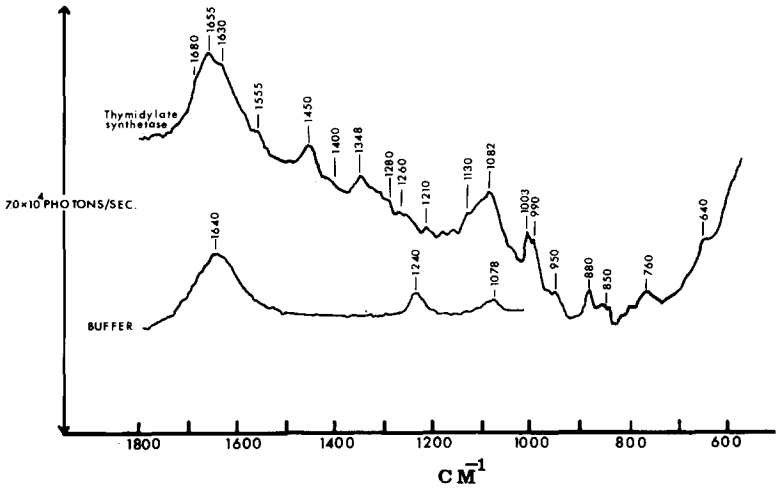

Fig. 1. Raman spectrum of thymidylate synthetase ($1.8 \cdot 10^{-4}$ M) in 0.08 M potassium phosphate, 0.1 M KCl and 0.0001 M EDTA (disodium salt) containing 0.1 M 2 mercaptoethanol, pH 6.8. The lower spectrum is of the same solvent alone. Ordinate full scale $7.0 \cdot 10^4$ photons/s. Abscissa represents frequency shift (Δ cm^{-1}) from laser excitation frequency 488.0 nm (20492 cm^{-1}); power 100 mW; plotting step = 50 cm^{-1}/s; resolution 8 cm^{-1}; temperature 20° C.

This conformational sensitivity of the Amide I region should, in principle, allow a conformational analysis of thymidylate synthetase but, at the protein concentrations feasible here the large scattering contribution of water relative to the Amide I scattering intensity makes assignment of band positions difficult and precludes extensive conclusions. However, the lack of a sharp 1670 cm^{-1} peak suggests that the β-structure is not dominant in thymidylate synthetase. This is consistent with the infrared spectra. Our spectra do not allow an evaluation of the relative proportions of helical and "unordered" structure.

To eliminate water interference we lyophilized thymidylate synthetase and recorded the Raman spectra of the enzyme redissolved in ^2H_2O. (Fig. 2). The Amide I peaks (Amide I') now lie at 1630 cm^{-1} and 1665 cm^{-1}. No band is observed at 1658 cm^{-1}, the frequency characteristic of β-structured poly-L-lysine in ^2H_2O solution [21]. The 1630 cm^{-1} and 1665 cm^{-1} bands of thymidylate synthetase are, however, consistent with a mixture of α-helical and "unordered" peptide, since the principal Amide I bands of α-helical and "unordered" poly-L-lysine in ^2H_2O lie at 1632 cm^{-1} and 1660 cm^{-1} [21].

Infrared spectra (Amide I region) of lyophilized thymidylate synthetase films (not shown) show a single, strong band maximal at 1650 cm^{-1}, lacking irregularities at 1630—1640 cm^{-1}, the absorption frequency of the β-structure [23]. Since pure helical polypeptides absorb maximally at 1650—1655 cm^{-1} and the "unordered" conformation yields an Amide I peak at 1656 cm^{-1}, we cannot distinguish between these conformations. The data are consistent with the conclusions drawn from the Raman spectra, that thymidylate synthetase is primarily a mixture of helical and "unordered" peptide arrays.

The Amide I region of thymidylate synthetase also exhibits bands not of peptide origin. Thus, the 1608 band (^2H_2O; Fig. 2) probably arises from Trp and the peaks at 1712 cm^{-1} and 1735 cm^{-1} derive from unionized carboxyl groups (C=O stretch).

TABLE I

FREQUENCY ASSIGNMENTS OF THYMIDYLATE SYNTHETASE, TERNARY COMPLEX (THYMIDYLATE SYNTHETASE + (+)-CH_2-H_4-FOLATE + fdFUMP) AND ITS CONTROLS

Numbers in parenthesis refer to thymidylate synthetase in ^2H_2O

Frequency (cm^{-1})						
Thymidylate synthetase		(+)-CH_2-H_4-folate	Thymidylate synthetase + (+)-CH_2-folate	Ternary complex	H_2-folate	Tentative assignment
	(1735)					ester
	(1712)					
1680				1678		
	(1665)	1660		1660	1665	amide I
1655			1655			and H_2O
1630	(1630)		1630			
		1620		1618	1620	ν(C=N)
	(1608)					
1555	(1553)		1555	1558		Trp
	(1540)					
				1530		
	(1512)					
		1480		1470	1485	
1450	(1455)	1440	1455			CH_2 deformation
				1430	1430	
1400	(1403)		1410	1400		COO^-
	(1385)	1380				
				1360	1358	
1348			1340			Trp and C-H deformation
	(1315)	1310			1303	
				1293		
1280	(1280)	1280				
1260						Amide III
		1245	1250	1240	1240	
					1185	
1210		1200	1210			Tyr and Phe
1130			1120			
1082		1090	1080	1070	1075	ν(C-N)
		1015		1020		
1003						Trp
990						
950						ν(C-C)
880						
850						Tyr and Trp
760						Trp
640						ν(C-S)

The Amide III vibrations (mainly C-N stretching plus N-H deformation) yield scattered peaks between 1200 cm^{-1} and 1300 cm^{-1}. Thymidylate synthetase exhibits two weak rather broad bands in this region at 1260 cm^{-1} and 1280 cm^{-1}. (Fig. 1). These again do not permit detailed conformational analyses [24—26].

Ring vibrations

The strong bands at 1608 cm^{-1} (^2H_2O) (Fig. 2) and 1003 cm^{-1} (Fig. 1) are attributable to the indole rings of the twelve thymidylate synthetase trypto-

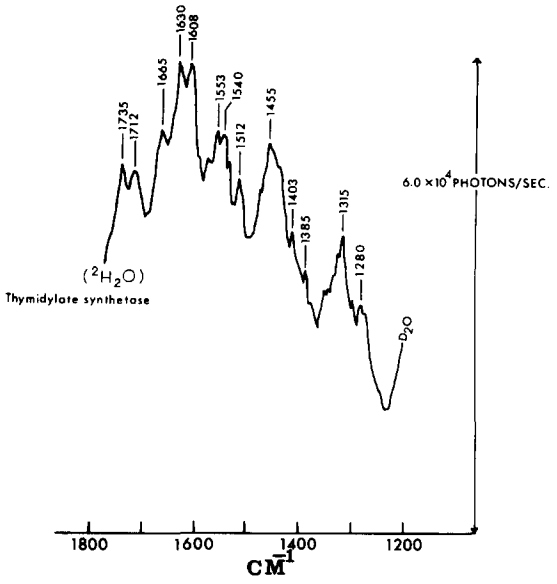

Fig. 2. Raman spectrum of thymidylate synthetase ($1.8 \cdot 10^{-4}$ M) in ^2H_2O. Ordinate full scale $6.0 \cdot 10^4$ photons/s. Other conditions were the same as in Fig. 1.

phans (see Methods). These residues also account for the bands at 1555 cm^{-1}, 1348 cm^{-1}, 880 cm^{-1}, and 760 cm^{-1} (Table I). The 880 cm^{-1} band cannot be securely assigned since SH deformation (cysteine) produces a peak at 875 cm^{-1}. The *p*-hydroxyphenyl ring of tyrosine also yields a characteristic band at 880 cm^{-1}.

C-H deformation modes (1300—1500 cm^{-1})

The Raman spectrum of thymidylate synthetase between 1300 cm^{-1} and 1500 cm^{-1} resembles that of bovine serum albumin [25]. The strongest CH deformation band lies at 1348 cm^{-1}. The broad band between 1440 cm^{-1} and 1470 cm^{-1} is attributed to the CH_2 and CH_3 deformation modes. The broad shoulder near 1400 cm^{-1} may be due to ionized carboxyl groups.

C-N and C-C stretching (900—1100 cm^{-1})

Thymidylate synthetase exhibits a strong C-N stretching band at 1082 cm^{-1}; as discussed below this varies with enzyme "state". The bands at 990 cm^{-1} and 950 cm^{-1} are assigned to C-C stretching vibrations.

C-S stretching and SH deformation (600—900 cm^{-1})

Thymidylate synthetase contains four residues of cysteine and 12 residues of methionine which may contribute to Raman scattering in this region. We observe four distinct peaks between 600 cm^{-1} and 900 cm^{-1}, at 880 cm^{-1}, 850 cm^{-1}, 760 cm^{-1} and 640 cm^{-1}. The peaks at 850 cm^{-1} and 760 cm^{-1} are attributable to the ring vibrations of Trp. As noted, the peak at 880 cm^{-1} cannot be unambiguously assigned since both the SH-deformation vibration of cysteine and a ring vibration of aromatic residues produce Raman scattering at

this frequency. Since there are 38 residues of Trp + Tyr and only 4 residues of Cys SH, we are inclined to assign the 880 cm^{-1} band to the aromatic residues.

The C-S stretching bands of cysteine lie between 690 cm^{-1} and 680 cm^{-1}, and those of Met at 724 cm^{-1}, 701 cm^{-1} and 655 cm^{-1} [26]. The shoulder in the thymidylate synthetase spectrum at approx. 690 cm^{-1} might thus be assigned to C-S stretching. We suspect that the 640 cm^{-1} band is also due to Met since C-S stretching bands can vary widely in frequency depending on the micro-environment of the C-S linkage [27].

Differences in Raman scattering between thymidylate synthetase and the ternary complex

FdUMP does not yield a resolved Raman Spectrum, at the concentrations used. Mixtures of thymidylate synthetase and (+)-CH_2-H_4-folate, or of thymidylate synthetase and FdUMP yield spectra identical to that of thymidylate synthetase without additions. However, the spectrum of the ternary complex (enzyme + CH_2-H_4-folate + FdUMP) is distinctly different from that of thymidylate synthetase in the following respects (Fig. 3):

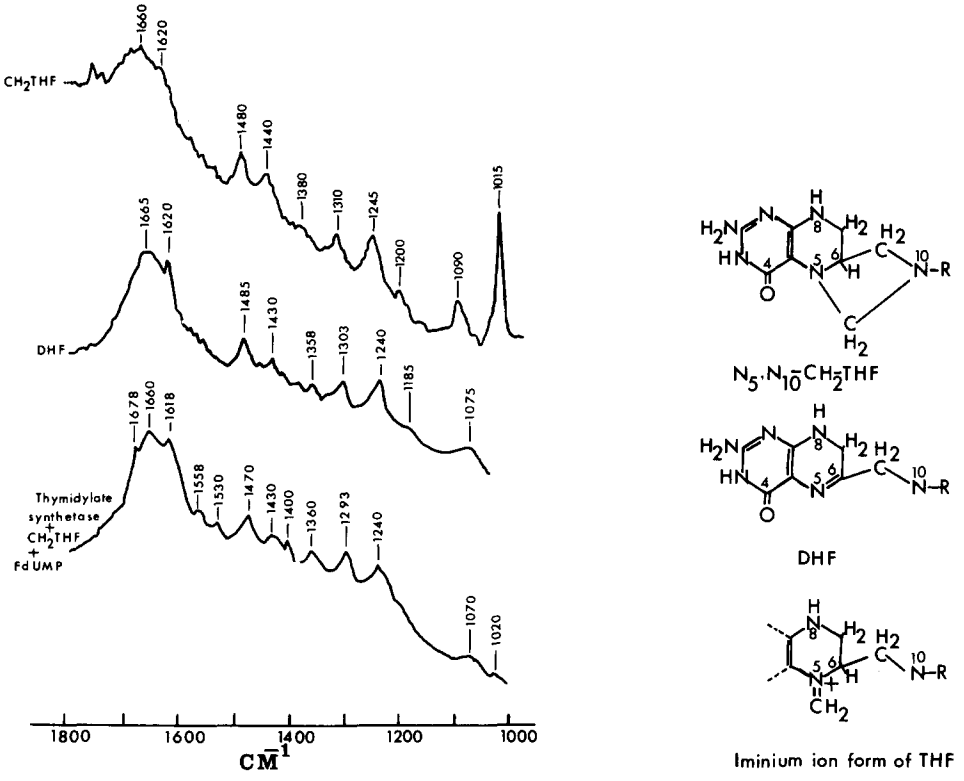

Fig. 3. Raman spectrum of ternary complex (thymidylate synthetase $1.8 \cdot 10^{-4}$ M + (+)-CH_2-H_4-folate (CH_2THF), $4.5 \cdot 10^{-4}$ M + FdUMP $4.5 \cdot 10^{-4}$ M), H_2-folate (DHF), ($4.5 \cdot 10^{-4}$ M) and (+)-CH_2-H_4-folate ($4.5 \cdot 10^{-4}$ M) in the same buffer as used in Fig. 1. Other conditions were same as in Fig. 1, except in (+)-CH_2-H_4-folate ordinate full scale = $3 \cdot 10^3$ photons/s.

Fig. 4. Structural formula of CH_2-H_4-folate (N_5N_{10}-CH_2-THF), H_2-folate (DHF) and iminium ion. The iminium ion could also occur on the N^{10} position.

1. A new band appears at 1618 cm^{-1}.

2. The 1082 cm^{-1} band of thymidylate synthetase disappears and the intensity of the 1003 cm^{-1} Trp peak decreases markedly.

3. The peak at 1450 cm^{-1} and shoulder at 1400 cm^{-1} are replaced by three bands at 1470 cm^{-1}, 1430 cm^{-1} and 1400 cm^{-1}.

The position of the 1618 cm^{-1} band is characteristic of the C=N stretching vibration [28]. In support of this assignment is the fact that CH_2H_4-folate, which has a C=N bond (Fig. 4) exhibits a weak Raman band at 1618 cm^{-1}—1620 cm^{-1} (Fig. 3). In H_2-folate which has an additional -C=N- bond per molecule (Fig. 4), the 1618 cm^{-1} band is sharp and intense (Fig. 3). One could thus assign the 1618 cm^{-1} band of the ternary complex to the formation of H_2-folate. However, the 1618 cm^{-1} band in the complex appears rather broad compared with that of H_2-folate. One should therefore consider possible formation of an iminium ion [9,10]. The $C=N^+$ vibrations of iminium ions yield Raman scattering bands at approximately the same frequency as C=N vibrations [29], but protonation of the N atom generally causes a reduction of scattering intensity as well as considerable band broadening [30—32]. Our data are thus compatible with the suggestion that an iminium ion derivative rather than H_2-folate is the folate intermediate in the ternary complex. However, we cannot clearly discriminate between the two possibilities at this stage.

The remaining alterations at 1003 cm^{-1}, 1082 cm^{-1} and between 1400 cm^{-1} and 1500 cm^{-1} are most reasonably attributed to structural alterations within thymidylate synthetase. This is particularly true for the reduction in intensity of the 1082 cm^{-1} and 1003 cm^{-1} bands and for the appearance of the 1400 cm^{-1} peak. The latter is most reasonably attributed to an increase in the proportion of ionized carboxyls. Whatever the structural modifications in the enzyme, however, we see no spectral alterations indicative of a change in secondary structure.

Acknowledgement

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