Resonance Raman Characterization of the Binary and Ternary Complexes of Thymidylate Synthase with 5-Nitrodeoxyuridylate†

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ABSTRACT: Resonance Raman (RR) spectra are reported for the binary complex of *Escherichia coli* thymidylate synthase (TS) with the substrate analog inhibitor 5-nitrodeoxyuridylate (NDU). The TS/NDU binary complex RR spectrum shows many similarities to the RR spectra of thiol adducts of NDU or of 5-nitro-1-methyluracil formed in solution, providing strong evidence in support of the formation of a covalent link between Cys146 of TS and C_6 of NDU. Spectral differences between the model compounds and the binary complex reflect the consequences of fixing the conformations of the uracil and ribose rings at the enzyme active site. The RR spectra of the ternary complexes of TS/NDU with either tetrahydrofolate (H_4-folate) or the cofactor 5,10-methylenetetrahydrofolate (CH_2H_4-folate) show that a covalent link is not formed between C_{11} of CH_2H_4-folate and C_5 of NDU. Neither does the methylene bridge of CH_2H_4-folate remain intact in the ternary complex; either CH_2H_4-folate is present as the N_5 iminium cation species or the methylene group is lost as formaldehyde. A shift in the NO_2 symmetric stretching frequency in the ternary complex indicates expulsion of water molecules from the region of the NO_2 group by the cofactor.

Thymidylate synthase (TS) catalyzes the methylation of deoxyuridylate (dUMP) to deoxythymidylate (dTMP), through the conversion of the cofactor 5,10-methylenetetrahydrofolate (CH_2H_4-folate) to 7,8-dihydrofolate. The early steps of TS catalysis are the binding of dUMP to TS and the formation of a covalent bond between a cysteine residue and C_6 of the uracil ring (Cisneros et al., 1988; Santi & Danenberg, 1984). In TS catalysis, the subsequent steps involve binding of the cofactor CH_2H_4-folate to form a covalent ternary complex (Moore et al., 1986). Many studies of TS have made use of the high stability of ternary complexes of TS with 5-substituted dUMP analogs, such as FdUMP (Donato et al., 1976; James et al., 1976; Santi & Danenberg, 1984; Matthews et al., 1990a). The spectroscopic and crystallographic analyses of these complexes have shown the presence of covalent links between cysteine and C_6 of FdUMP and between C_5 of FdUMP and C_{11} of the cofactor (see Figure 1). Unfortunately, the binary complex formed between TS and dUMP (or FdUMP) is relatively unstable and is not amenable to crystallographic investigations. Some spectroscopic investigations of the TS/FdUMP complex have been undertaken and have shown evidence for a mixture of covalent and noncovalent binary complexes (Lewis et al., 1980, 1981). 5-Nitro-dUMP (NDU) is an unusual inhibitor of TS, because it forms a stable binary complex with TS. The NDU/TS complex is very amenable to spectroscopic investigations since it has a unique absorption at 338 nm, due to the enzyme-bound NDU. The TS/NDU binary complex is also unusual in that it is not significantly further stabilized by the addition of the cofactor, CH_2H_4-folate (Wataya et al., 1980).

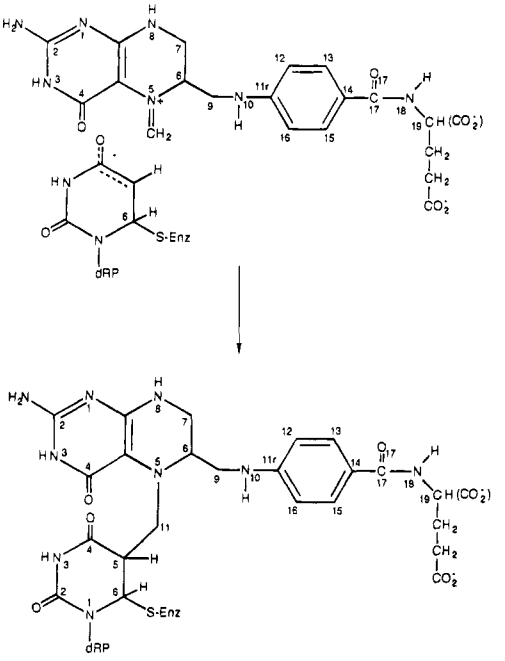

FIGURE 1: Structures of the ternary complex of TS with dUMP and CH_2H_4-folate (bottom) and of the N_5 iminium cation species (top) that has been proposed as the precursor to the covalent ternary complex (Santi & Danenberg, 1984).

RR spectroscopy has been used in the present study to investigate the structure and environment of NDU in the TS binary complex and to establish the nature of the ternary complex formed with CH_2H_4-folate. The vibrational spectrum of the NDU group is selectively obtained by tuning the laser excitation to the absorption of NDU in the binary

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or ternary complex. The RR spectrum of the TS/NDU complex shows clear evidence for formation of the covalent link between NDU and the enzymes' Cys146. The RR spectrum also reflects the specific environment that TS provides for NDU in the binary complex and the changes in the NDU environment that occur on binding H_4-folate or CH_2H_4-folate.

EXPERIMENTAL PROCEDURES

Synthesis of NDU and 5-Nitro-1-methyluracil. NDU was synthesized from dUMP (US Biochemical Co.) following the procedure of Evans and Haley (1987). Typically, 100 mg of dUMP (sodium salt) was desalted on a Dowex 50 column and evaporated to dryness under reduced pressure. The dUMP was dissolved in 5−10 mL of dry dimethylformamide and reacted with *ca.* 1 g of nitrosonium tetrafluoroborate (Aldrich) in an N_2 atmosphere. The reaction was quenched by the addition of 2 mL of water. The subsequent separation and purification of NDU was performed as described by Evans and Haley (1987). The reaction time was less than 5 min; longer reaction times resulted in the formation of other products which exhibited absorption spectra similar to that of NDU but did not bind to TS. NDU was identified by its absorption maxima at 306 nm in acidic solutions (pH 1−6) and at 322 nm in alkaline solutions (pH > 8) and from its ability to bind to TS.

5-Nitro-1-methyluracil was synthesized from 1-methyluracil (Aldrich) using the procedure of Evans and Haley (1987). The initial desalting step was unnecessary, and the final separation on a BD-cellulose column was omitted.

Enzyme Preparation and Formation of Binary and Ternary Complexes. E. coli TS was prepared and stored as described elsewhere (Austin et al., 1995; Matthews et al., 1990a). Prior to formation of binary and ternary complexes, TS was dialyzed overnight (4 °C) in a buffer (pH 7) containing 0.05 M potassium phosphate, 1 mM EDTA, 25 mM KCl, and 75 mM β-mercaptoethanol. The TS/NDU binary complex was formed by the addition of a 5−10-fold excess of NDU to TS. Concentrations were estimated using the extinction coefficients $\epsilon_{282} = 9.5 \times 10^4$ M^{-1} cm^{-1} for TS and $\epsilon_{306} =$ 9000 M^{-1} cm^{-1} for NDU (Huang & Torrence, 1977). The *E. coli* TS extinction coefficient was estimated from the known extinction coefficient for the *L. casei* enzyme (Donato et al., 1976) by taking into account the different numbers of aromatic amino acid residues. The binary complex was separated from unbound NDU by passage down a sephadex G25 column or by repeated washing and reconcentration steps in centricon-10 filters (Amicon). The binary complex had absorbance maxima at 282 and 338 nm; the ratio of absorbances varied from 2.8:1 to 3.8:1 in different preparations, but was normally close to 3:1.

The ternary complexes of TS and NDU with either H_4-folate or CH_2H_4-folate were formed from the binary complex. The ternary complex with H_4-folate was formed by the addition of a 30−50-fold excess of H_4-folate (Calbiochem) to the binary complex. The ternary complex was allowed to form over several hours at 4 °C; then, either the sample was passed down a G25 column or several concentration and washing steps using Centricon 10 filters were employed to remove free H_4-folate. The ternary complex with CH_2H_4-folate was formed in an identical manner. CH_2H_4-folate was formed by dilution of H_4-folate into a buffer containing 0.05 M sodium bicarbonate, 0.07 M formaldehyde, and 10 mM dithiothreitol (DTT).

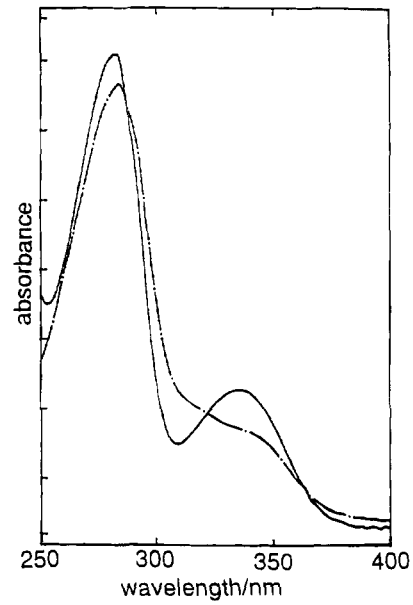

FIGURE 2: Absorption spectra of (—) TS/NDU binary complex (λ_{max} at 282 and 338 nm) and (— • —) TS/NDU/CH_2H_4-folate ternary complex (λ_{max} at 282 nm) in phosphate buffer, pH 7.

Raman Spectroscopy. Samples for Raman measurements were contained in a quartz cuvette and were stirred continuously during irradiation. Enzyme samples were maintained between 4 and 10 °C by blowing cold N_2 on the sample cuvette. Samples were irradiated using 135° backscattering geometry with 5−10 mW of 337.5-nm excitation from a Kr^+ laser (Coherent 100) or with 1 mW of 333.6-nm excitation (5-nitro-1-methyluracil samples only) from an Ar^+ laser (Spectra Physics 2025). The other laser lines and strong plasma lines were separated from the desired laser line by the use of a pellin broca prism and a prism monochromator (Anaspec). Raman scattering was dispersed using a Spex Triplemate (1877) spectrometer equipped with a 2400 grooves/mm spectrograph stage grating. Spectral resolution was *ca.* 8 cm^{-1}. Raman scattering was detected using a cooled (−30 °C) diode array detector (EG&G PAR). Spectra were calibrated using standard wavenumber values for dimethylformamide and ethanol, giving a wavenumber accuracy of ±1.5 cm^{-1}.

RESULTS AND DISCUSSION

Absorption Spectra. The absorption spectra of the binary complex of TS with NDU and of the ternary complex with CH_2H_4-folate are shown in Figure 2. The binary complex has an absorption maximum at 338 nm due to the complexed NDU group. This absorption maximum is similar to the absorption maximum of NDU in thiol solutions (Wataya et al., 1980; Maggiora et al., 1981; Matsuda et al., 1978) and can be explained by the formation of a thiol adduct species, B, depicted in Figure 3. It has been suggested that the covalent binary complex species formed between TS and 5-substituted dUMPs is not the enolate adduct species (as depicted in Figure 3B), but is in fact a 5,6-dihydro adduct (Lewis et al., 1980). This suggestion is not consistent with the observation of a red-shifted absorption for NDU bound to TS. The 5,6-dihydroadduct of NDU would have a blue-shifted absorption (i.e., $\lambda_{max} < 306$ nm), as observed in 5,6-

dihydrouridine (Dawson et al., 1984). Resonance stabilization of the enolate by the nitro substituent is no doubt responsible for the exceptional stability of the binary complex with NDU.

The absorption spectrum of the TS/NDU binary complex indicates that both sites of the TS dimer contain NDU that is covalently linked via an enzyme cysteine residue. The ratio of the 282- and 338-nm absorptions (A_{280}:A_{338}) varied from *ca.* 3.8:1 to 2.8:1 in different preparations and was normally close to 3:1. If an extinction coefficient of $\epsilon_{280} = 9.5 \times 10^4$ M^{-1} cm^{-1} is assumed for *Escherichia coli* TS, then two-site binding would produce an A_{280}:A_{338} ratio of 2.8:1 if $\epsilon_{338} = 17\,000$ M^{-1} cm^{-1}. This value is much larger than the reported extinction coefficient for NDU in acidic solution ($\epsilon_{306} = 9000$ M^{-1} cm^{-1}) or in alkaline solution ($\epsilon_{320} = 12\,000$ M^{-1} cm^{-1}). Maggiora et al. (1981) have reported a 64% increase in the extinction coefficient of neutral solutions of NDU as the thiol adduct of NDU with β-mercaptoethanol is formed, yielding an extinction coefficient in the range observed for NDU bound to TS. Thus, the absorption spectrum is most consistent with two-site binding of NDU to TS. Non-covalently bound NDU would absorb in the region of the free NDU species (306 nm) and would raise the A_{280}:A_{338} ratio and fill in the trough in the absorption spectrum. A small proportion (e.g., 10%) of non-covalent binding could remain undetected in the RR and absorption spectra, but it appears that covalently bound NDU occupies the majority of the sites that contain NDU.

On formation of the ternary complex with either CH_2H_4-folate or H_4-folate, the absorption at 338 nm is reduced in intensity and is overlapped by the absorption of the bound cofactor or H_4-folate (Figure 2). The bound cofactor has an absorption maximum at *ca.* 320 nm (not resolved), which is similar to the absorption maximum observed in the TS ternary complex with FdUMP (Donato et al., 1976). The ratio A_{282}:A_{338} increases to 3.5:1. If the extinction coefficient of bound NDU remains approximately the same, then the increased A_{280}:A_{338} ratio reflects a loss of NDU binding (the absorption of the cofactor is not expected to contribute significantly to the absorption at 280 nm). However, the ratio is not sufficiently elevated to indicate that formation of the ternary complex can only occur at one site of the TS dimer. It is also possible that the bound NDU extinction coefficient is diminished because of decreased polarity in the ternary complex, RR evidence for which is discussed below. These results are at variance with those reported by Wataya et al. (1980), who were not able to observe significant binding of CH_2H_4-folate to the TS/NDU complex.

RR Spectra. (1) NDU and NMU. The introduction of the nitro group onto the uracil ring of dUMP shifts the lowest energy dUMP absorption from 262 to 306 nm. In dUMP, the lowest energy electronic transition is localized on the O=C_4–C_5=C_6 enone portion of the uracil ring (see Figure 1 for numbering). In NO_2dUMP, we expect that the 306-nm transition will also be localized on the enone portion of the molecule, but that the nitro group will be involved in the transition. Consequently, we expect to observe high RR intensity for nitro group vibrations and for modes involving vibrations of the enone. In the thiol adduct species (and the hydroxide adduct; see below), the enone structure is lost (see Figure 3). In this case, the chromophore must be formed from the O–C_4–C_5–NO_2 portion of NDU.

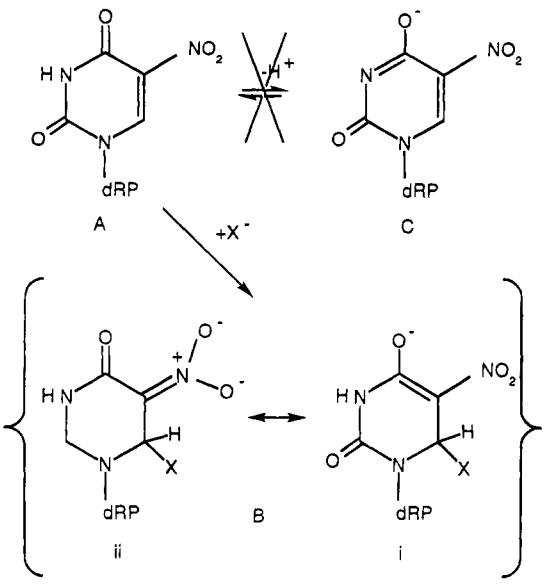

FIGURE 3: Structures of NDU and NDU adducts: X^- = OH^- or RS^- (β-mercaptoethanol or dithiothreitol.) Alternative resonance forms, Bi and Bii, are shown for the adducts. Hydroxide adduct formation overrides deprotonation at N_3 (structure C).

Table 1: RR Band Frequencies (cm^{-1}) of NDU and NMU, and Suggested Assignments

species	NDU H_2O	NDU D_2O	NMU, H_2O	assignment
NDU or NMU (pH 5)	1616	1618	1624	C_5=C_6 stretch
		1466	1443	ring
		1389		ring
	1354	1355	1367	NO_2 symmetric stretch
			1319	
		957		N_3D bend
	902			
	870	867	881	C–NO_2 stretch
	788	787	771	NO_2 + ring breathing + N_1–R[a]
OH adduct	1402	1409		NO_2 symmetric stretch + ring stretching
	1346	1347		NO_2 symmetric stretch
	1260	1280		C_2–N_3 stretch + N_3–H bend + C_2=O bend
		1043		N_3–D bend
	860	830		C–NO_2 + ring breathing + N_1–R
thiol adduct	1418[b]	1428[b]	1420	NO_2 symmetric stretch + ring stretching
	1393	1397	1387	NO_2 symmetric stretch
	1261	1269	1263	C_2=N_3 stretch + N_3–H bend + C_2=O bend
			1203	
			1108	
		1034		N_3–D bend
	847		872	C–NO_2 + N_3–H bend
	802	798	778	NO_2 + ring breathing + N_1–R

[a] R = deoxyribose 5′-monophosphate or methyl. [b] Determined from curve fitting.

The NDU RR spectrum is profoundly influenced by the nitro group and shows few similarities to the spectra of dUMP and dTMP, indicating that most of the observed vibrational modes involve the nitro group. Some tentative assignments are discussed below and are also listed in Table 1. The RR spectra of NDU under various conditions are compared in Figure 4. The corresponding spectra obtained in D_2O solutions are shown in Figure 5. The RR spectrum

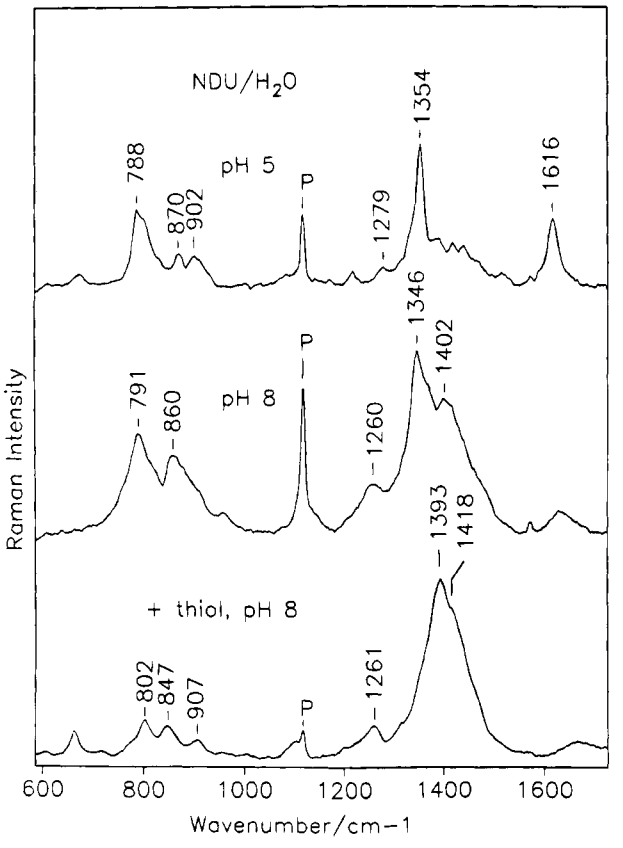

FIGURE 4: 337.5-nm-excited RR spectra of NDU at pH 5 and 8 and in 1 M β-mercaptoethanol at pH 8. A laser plasma line is labeled P.

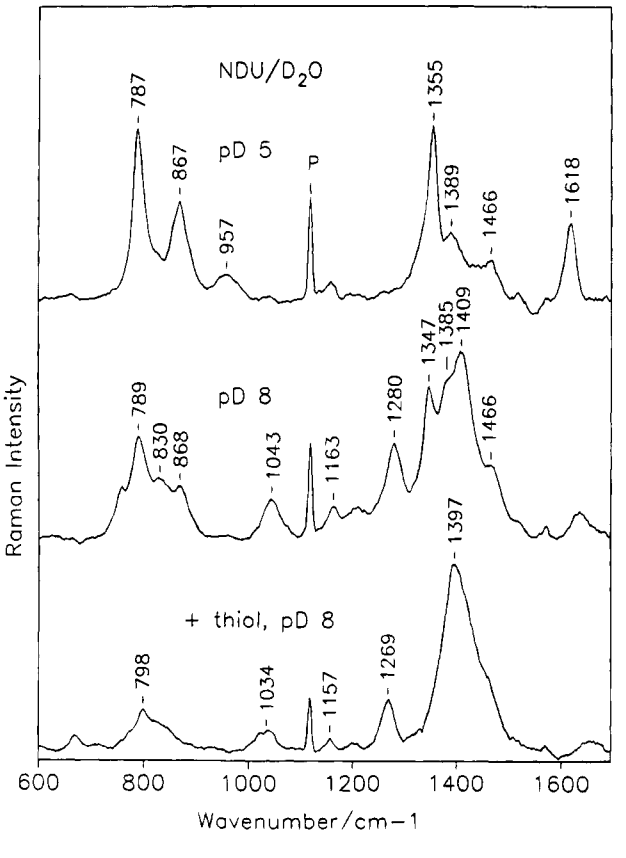

FIGURE 5: Spectra recorded as in Figure 4, but in D_2O solution.

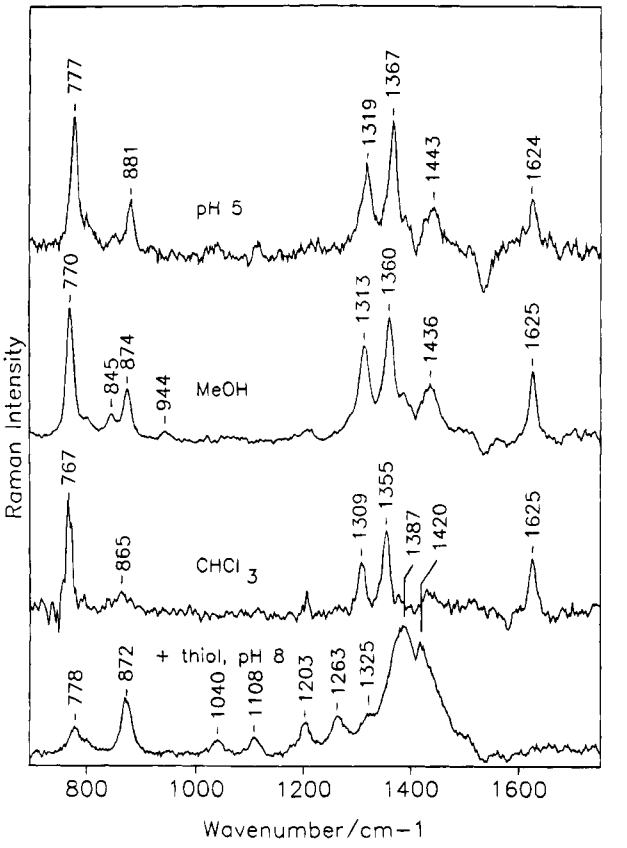

FIGURE 6: 333.6-nm-excited RR spectra of 5-nitro-1-methyluracil in water, MeOH, $CHCl_3$, and 100 mM dithiothreitol, pH 8.

of the acidic species is consistent with the neutral structure A shown in Figure 3, since the Raman band at 1616 cm^{-1} arises from the C_5=C_6 stretching vibration (Toyama et al., 1991; Grygon & Spiro, 1990). The C_4=O stretch is also expected to lie close to this frequency, but may not be strongly enhanced. Other prominent features of the NDU spectrum at pH 5 are the 1354-cm^{-1} band and the 788- and 870-cm^{-1} bands. The 1354-cm^{-1} band can be assigned to a mode involving mostly the nitro symmetric stretch, which is found in the 1300−1400-cm^{-1} region in nitro compounds and is always strong in Raman spectra (Dollish et al., 1974). The 788-cm^{-1} band is close in frequency to bands observed in the RR spectra of dUMP, dTMP, and FdUMP that have been assigned to ring breathing vibrations, also containing N_1-ribose stretching character (Perno et al., 1989; Tsuboi et al., 1973). The 870-cm^{-1} band is a candidate for the ring−NO_2 stretching vibration. It also reflects some involvement of N_3H bending, since it shifts 3 cm^{-1} and intensifies substantially in D_2O. The N_3H/D replacement in D_2O has other effects on the spectrum, indicating a pervasive influence of N_3H bending: bands at 1279 and 902 cm^{-1} disappear, as does a shoulder on the 788-cm^{-1} band, while new bands appear at 957, 1389, and 1466 cm^{-1}.

The nitrouracil RR spectrum is surprisingly sensitive to the substituent at N_1, as can be seen from the 5-nitro-1-methyluracil (NMU; Figure 6). Substitution of the deoxyribose 5′-monophosphate by a methyl group produces 8−13-cm^{-1} upshifts in the bands assigned to C_5=C_6, NO_2, and ring−NO_2 stretching (1624, 1367, and 881 cm^{-1}) and an 11-cm^{-1} downshift in the ring breathing mode (777 cm^{-1}). These shifts must reflect altered electron distributions and/or normal mode compositions. For NMU an additional prominent band

is seen at 1319 cm^{-1}, which is not present in NDU. It is possible that this band results from a near-resonant vibrational interaction between the NO_2 symmetric stretch and the CH_3 umbrella mode (not present in NDU), which is expected at about the same frequency, resulting in a pair of mixed modes at 1319 and 1367 cm^{-1}. Both bands shift down, and by the same amount, between water and methanol, and again between methanol and chloroform. This solvent sensitivity indicates involvement of polar substituents in the modes. These substituents might be the carbonyl or NH groups, but the likeliest candidate is the NO_2 group. Solvent sensitivity of the symmetric NO_2 vibration has been observed in other studies (Epstein et al., 1982).

(2) Hydroxide and Thiolate Adducts. At alkaline pH, NDU forms a hydroxide adduct, analogous to the thiolate adduct (Figure 3B). The formation of the hydroxide adduct occurs at a sufficiently low pH to prevent observation of the N_3 deprotonated species (shown as structure C in Figure 3). Hydroxide adduct formation has been reported elsewhere (Pitman et al., 1974), but has been overlooked in some recent papers (Wataya et al., 1980; Maggiora et al., 1981). Facile hydroxide adduct formation explains several observations. It explains the large pH-induced shift of the absorption spectrum (306−322 nm), which is not observed for dUMP. A similar red shift is observed for 1,3-dimethyl-5-nitrouracil, which contains no titratable protons (Pitman et al., 1974). In addition, adduct formation must be responsible for the sensitivity of the NDU RR spectrum (Figures 4 and 5) to D_2O at alkaline pDs. If N_3 deprotonation were occurring, there would be no exchangeable protons to explain the D_2O sensitivity. Finally, the formation of the adduct explains the loss of the $C_5{=}C_6$ vinyl stretching band at 1616 cm^{-1}.

The strongest band in the RR spectrum of the hydroxide adduct is the NO_2 symmetric stretch, at 1346 cm^{-1}, shifted down by 8 cm^{-1} from its frequency in NDU. The downshift is consistent with a contribution of the resonance form shown as Bii in Figure 3, in which the N−O bonds become single bonds. This resonance form may also explain the absence of any RR band above 1500 cm^{-1} that could be assigned to the $C_4{=}C_5$ stretch, as would be expected for resonance form Bi. There is, however, a strong broad band at 1404 cm^{-1}, which must arise from one or more ring modes, mixed with the NO_2 stretch. In D_2O (Figure 5), this broad band resolves into three components at 1385, 1409, and 1466 cm^{-1}. A band at 1260 cm^{-1} shifts up to 1280 cm^{-1} in D_2O, reminiscent of the behavior of the 1236-cm^{-1} mode of uracil (Bowman & Spiro, 1980). The uracil mode is a combination of $C_2{-}N_3$ stretching, $C_2{=}O$ bending, and $N_3{-}H$ bending , and its upshift in D_2O is attributed to the removal of the $N_3{-}H$ bending interaction (Bowman & Spiro, 1980). The 791-cm^{-1} band is assigned to the ring breathing mode. As in NDU, it narrows and intensifies in D_2O. A broad band at 860 cm^{-1}, which may contain the ring−NO_2 stretch, 10 cm^{-1} lower than in NDU, is replaced by two bands, at 869 and 830 cm^{-1} in D_2O. Additional bands are seen only in the D_2O spectrum, at 1043 and 1163 cm^{-1}. The 1043-cm^{-1} band is suggested to be the N_3D bend.

Figures 4c and 5c show spectra of the β-mercaptoethanol adduct of NDU. The same spectrum was obtained when dithiothreitol was used to form the adduct. As in the case of the hydroxide adduct, the RR spectrum of the thiolate adducts is dominated by a broad complex band in the NO_2 stretching region. The peak frequency is substantially higher for the thiol adducts, however, 1392 vs 1346 cm^{-1}, and the high-frequency shoulder, at 1418 cm^{-1}, is less well resolved. In D_2O, the peak frequency is even higher, 1397 cm^{-1}, and the high-frequency component is not resolved at all. Thus, the electronic structure, and the normal mode composition, differs somewhat for the thiol and hydroxide adducts. The higher frequency for the NO_2 stretch in the thiol adduct suggests diminished importance for resonance form Bii relative to Bi (Figure 3), resulting in stronger N−O bonds. Consistent with this interpretation, the band at 847 cm^{-1}, which may contain ring−NO_2 stretching, is lower than the corresponding band, 860 cm^{-1}, in the hydroxide adduct. The ring breathing mode is slightly higher for the thiol adduct, 802 vs 791 cm^{-1}. As in the hydroxide adduct, the 1261-cm^{-1} band of the thiol adduct shifts up in D_2O, reflecting relief of the N_3H bending interaction, but the shift is smaller, 8 vs 19 cm^{-1}. Again new bands are seen in D_2O, at 1034 and 1157 cm^{-1}, the former being tentatively assigned to N_3D bending.

As in the NDU RR spectrum, the ribose phosphate substituent is a significant determinant of the band frequencies and shapes, as can be seen by comparing the spectrum of the β-mercaptoethanol adduct of 5-nitro-1-methyluracil, shown in Figure 6. The broad NO_2 stretch is found at nearly the same frequency, 1387 cm^{-1}, and again has a prominent high-frequency shoulder, at 1420 cm^{-1}. Likewise, the 1263-cm^{-1} band is at the NDU-thiol position. But the breathing mode is at a much lower frequency, 778 vs 802 cm^{-1}, while the ring−NO_2 candidate band is much higher, 872 vs 847 cm^{-1}. Moreover, there are three additional bands which are not seen in the NDU-thiol spectrum, at 1203, 1108, and 1040 cm^{-1}. Again, the marked differences between NDU and the 1-methyl analog implicate the substituents as being significantly involved in the electronic or vibrational structure of the chromophores.

(3) TS/NDU Binary Complex. The RR spectra of the TS/NDU binary complex formed in H_2O and D_2O solutions are compared in Figure 7. These spectra show both similarities and differences with respect to the NDU-thiol spectra (Figures 4c and 5c). The peaks of the latter find close frequency correspondences in the former. Thus, the NO_2 stretch, the ring−NO_2 stretch, and the ring breathing modes are all at essentially the same frequencies: 1392, 847, and 802 cm^{-1} vs 1390, 849, and 797 cm^{-1}. Likewise, the D_2O shifts of these bands are essentially the same. The close frequency matches establish that the nitrouracil electronic structure is the same in the binary complex as in the model thiol adduct in solution. There are, however, many more bands in the RR spectra of the binary complex, and all the bands are much sharper than those seen in the NDU-thiol solution spectrum. Some of the extra bands find correspondences in the spectrum of NMU-thiol at shifted frequencies (Table 1), but others do not, e.g., 900, 940, 969, and 1378 cm^{-1}. Likewise the spectra in D_2O are richer and reveal more changes in frequency and intensity than are evident in the NDU-thiol spectra.

In view of the evidence from the NDU/NMU spectral comparison for sensitivity of the RR bands to the ribose phosphate substituent, one factor in explaining the spectral differences between the solution adduct and the binary complex is the ordering of the substituent conformation upon binding to the protein. The ribose and phosphate groups have clear hydrogen bonding interactions with the enzyme

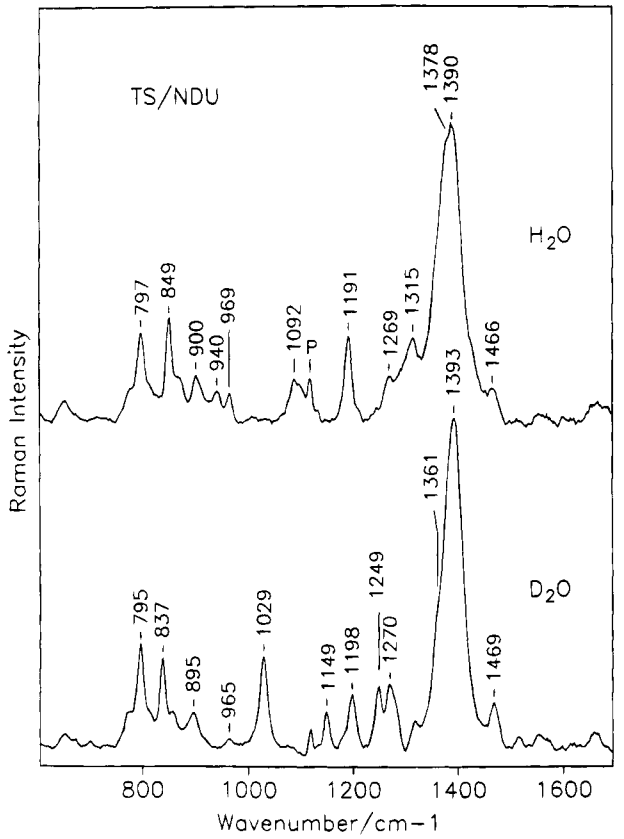

FIGURE 7: 337.5-nm-excited RR spectra of the TS/NDU binary complex in H_2O (top) and D_2O (bottom). The TS concentrations were *ca.* 0.3 mM in 50 mM phosphate buffer, pH (pD) 7, with 1 mM EDTA and 75 mM β-mercaptoethanol.

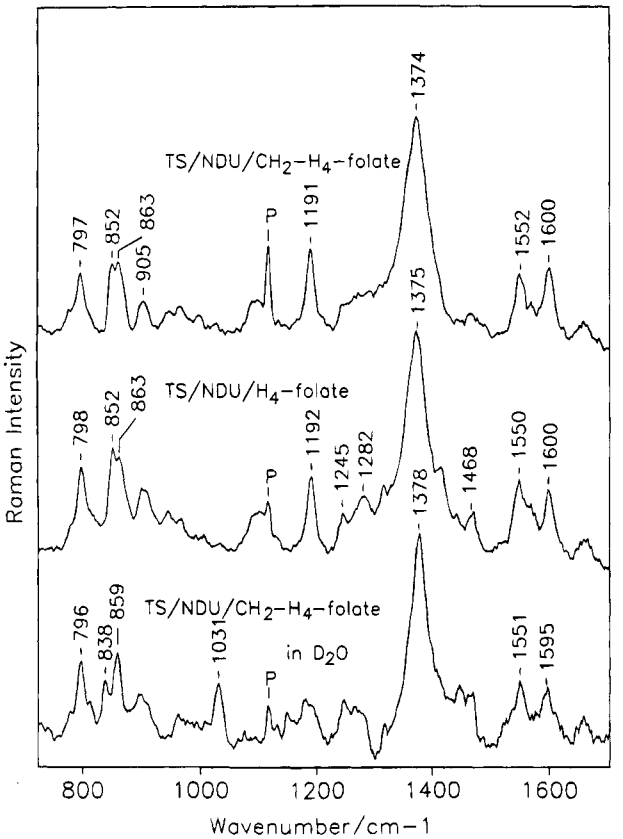

FIGURE 8: 337.5-nm-excited RR spectra of the TS/NDU/CH_2H_4-folate ternary complex in H_2O (top) and D_2O (bottom) and of the TS/NDU/H_4-folate ternary complex in H_2O (middle).

(Finer-Moore et al., 1993; Matthews et al., 1990a,b) that are known to be crucial for binding of the nucleotide to TS (Santi & Danenberg, 1984). These interactions fix the sugar conformation and its orientation with respect to the uracil ring. In solution, however, many sugar conformations and orientations are available to the nucleotide. To the extent that the ribose ring participates in the vibrational modes active in the RR spectra, this distribution of conformations is expected to produce a distribution of frequencies, broadening the spectral bands. Thus, some of the band narrowing and increased resolution in the binary complex spectra can be attributed to the freezing out of a single conformation by the interactions with the protein.

The ribose conformation cannot be the only factor, however, since the binary complex RR bands are also narrower than those of the NMU-thiol adduct (Figure 6), which has only a methyl group at N_1. Indeed the NO_2 stretching band of the latter is as broad as that of the NDU-thiol adduct, whereas the binary complex band is narrow. A likely reason for this difference is that the uracil ring becomes nonplanar in the thiol adducts and can adopt a variety of conformations, which might have different NO_2 stretching frequencies. Again, one of these conformations is frozen out in the binary complex due to interactions with the protein residues, producing narrowed RR bands. Matthews et al. (1990a) observe a half-chair conformation for the uracil ring of FdUMP bound to TS in the presence of the cofactor analog CB3717. The ordering of the uracil as well as the ribose conformation probably accounts for the band narrowing and the appearance of extra bands observed for the binary complex. In the NDU-thiol spectra these extra bands may be broadened beyond recognition by the conformational fluctuations in solution.

Despite these differences, it is striking that the NO_2 symmetric stretching frequency is essentially the same, 1390 vs 1392 cm^{-1} in the binary complex and NDU-thiolate spectra. It can be concluded that not only is a thiolate adduct formed in the binary complex, but the environment of the NO_2 group is essentially the same in the binary complex and in solution. The NMU-thiolate spectra (Figure 6) show this frequency to be sensitive to the polarity of the solvent, shifting down significantly as water is replaced by methanol and by chloroform. The close frequency match therefore implies that the NO_2 group is exposed to water molecules in the binary complex.

We note that the 1261-cm^{-1} NDU-thiolate band is at a higher frequency, 1269 cm^{-1}, in the binary complex, possibly due to H-bonding of the N_3H and C_2=O groups. Crystal structure of the FdUMP/TS ternary complex (Matthews et al., 1990; Finer-Moore et al., 1993) reveals specific H-bonds between these groups and the amide side chain of Asn177 and a main-chain NH, respectively. Similar H-bonding has been implicated in flavoproteins via similar upshifts in the analogous uracil-like RR band (Bowman & Spiro, 1980).

(4) TS/NDU Ternary Complexes with CH_2H_4-folate and H_4-folate. Figure 8 shows the effect of adding CH_2H_4-folate or H_4-folate to form a ternary complex with TS/NDU. The two spectra are essentially the same. Three new bands appear, at 1600, 1552, and 863 cm^{-1}, which arise from the *p*-aminobenzoylglutamate (PABA-glu) group of the folate. The frequencies are the same as those observed for the TS/FdUMP/CH_2H_4-folate ternary complex (Fitzhugh et al., 1986;

Austin et al., 1995). The remainder of the bands in the RR spectra of the TS/NDU ternary complexes arise from the NDU. They are at the same frequencies observed for the binary complexes (some of the weaker bands are not resolved due to lower signal/noise), with the singular exception of the dominant NO_2 stretching band. In the binary complex, the frequency is 1390 cm^{-1}, whereas in the ternary complexes it is 1375 cm^{-1}. The upshift in D_2O is 3 cm^{-1} in both cases, showing that the normal mode composition is unaltered. The frequency lowering in the ternary complex must therefore be an electronic effect. The 15-cm^{-1} shift is greater than that observed between water and chloroform for 5-nitro-1-methyluracil (Figure 6), suggesting a large reduction in the polarity of the NO_2 environment when cofactor is added to the binary complex. This reduction implies that cofactor binding displaces all the water molecules in the vicinity of the NO_2 group.

The close correspondence in the NDU bands between the ternary and binary complexes establishes that the cofactor does not form a covalent bond to the uracil C_5 atom, as it does in the ternary complex with FdUMP (Figure 1). If such a bond did form, the electronic structure of the chromophore would be profoundly affected by the sp^3 hybridization at C_5.

At the same time, the close correspondence of the PABA-glu bands with those of the TS/FdUMP/CH_2H_4-folate ternay complex implies that the cofactor is activated upon binding to the TS/NDU complex. These bands are quite different in character from those displayed by CH_2H_4-folate in solution, and they have been interpreted in terms of enzyme stabilization of the PABA-glu quinoid resonance structure via steric interactions that enforce planarity (Austin et al., 1995). These same interactions are evidently maintained in the TS/NDU ternary complex. The fact that the PABA-glu frequencies are the same when H_4-folate is substituted for CH_2H_4-folate implies that the methylene$-N_{10}$ bond of CH_2H_4-folate is broken in the ternary complex with NDU, even though no covalent bond to the uracil C_5 atom is formed. The bound CH_2H_4-folate might exist as the N_5-iminium ion, believed to be an intermediate on the enzyme pathway (Figure 1), but it is possible that the methylene group is released as formaldehyde by a reversal of the process whereby H_4-folate is converted to CH_2H_4-folate. The RR spectra are uninformative about these alternatives, since only PABA-glu modes are enhanced. The K_d for dissociation of CH_2H_4-folate into H_4-folate and HCHO is 4.76×10^{-5} M in solution (Dawson et al., 1986), but the equilibrium is likely to shift toward dissociation when the methylene bridge is opened by the activating forces in the protein.

We note that the D_2O shifts of the 1552- and 1600-cm^{-1} bands, 1 and 5 cm^{-1}, are smaller than those observed for the TS/FdUMP ternary complexes, 4 and 10 cm^{-1}. In view of the evidence for low polarity of the active site environment near the NDU nitro group, it is possible that D_2O exchange at the PABA-glu amine group is incomplete. Although broadened bands are expected when exchange is incomplete, the data are of insufficient quality in this spectral region to distinguish shifts from broadening.

CONCLUSIONS

Because of its unique electronic properties, NDU inhibits TSase and reveals the active site interactions in a particularly dramatic way. The NO_2 substituent is strongly electron withdrawing and promotes attack by nucleophiles, with formation of adducts at the C_6 position. The electronic effect is strong enough to produce a hydroxide adduct at a pH as low as 8, and thiolate adducts are readily formed in aqueous solution. Thus, the active site cysteine, Cys146, forms a covalent bond with NDU without any additional activation, producing an exceptionally stable binary complex.

Adduct formation alters the NDU absorption and RR spectra substantially, permitting detailed spectroscopic characterization of the binding to TSase. The absorption spectrum is similar for the binary complex and for NDU-thiolate adducts in solution. Moreover, the RR band frequencies are nearly the same, establishing that the thiolate bond at the active site is unexceptional. Indeed, the close match of the NO_2 symmetric stretching frequency and its sensitivity to the local polarity, as seen in the solvent sensitivity of this band in the model NMU, establishes that the NO_2 group must be in contact with water molecules in the TS/NDU binary complex. On the other hand, the RR spectrum is much richer for the binary complex than for NDU-thiolate adducts in solution, an effect that is attributed to the multiple conformations accessible to both the uracil and the ribose rings in solution. These multiple conformations broaden the RR bands, sometimes beyond recognition. At the enzyme active site, however, a single conformation is stabilized by the numerous steric and H-bonding contacts with protein residues, leading to sharp, well-resolved bands.

The NDU RR bands are not significantly altered when CH_2H_4-folate is added to the binary complex, except that the NO_2 symmetric stretching frequency is shifted down by 15 cm^{-1}. This shift is slightly greater than that seen for NMU when the solvent is chloroform instead of water. It implies that binding of the cofactor displaces water from the active site, leaving the NO_2 group in a hydrophobic environment. The altered environment may be responsible for the apparent reduction in optical absorptivity in the 338-nm absorption band of the ternary complex, relative to the binary complex (Figure 2). A hydrophobic environment is consistent with the NO_2 group being juxtaposed with the pterin ring of the folate cofactor, in an orientation favorable for covalent bond formation between the uracil C_5 atom and the methylene group bound to the pterin N_5, as required in the enzymatic process (Figure 1).

This covalent bond is not formed between the cofactor and NDU, however. If it were, the chromophore would be drastically altered by the sp^3 rehybridization at C_5, and the RR spectrum would be greatly affected. Rehybridization is resisted by the NO_2 group, via the resonance form shown as Bii in Figure 3, which has a C=N double bond. This resistance and the electron-withdrawing effect of the NO_2 group are the factors that inhibit covalent bond formation from the incipient iminium ion of the CH_2H_4-folate cofactor. Consistent with this inhibition, the stability of the TS/NDU complex is not increased significantly by cofactor binding (Wataya et al., 1980). This binding pattern is in marked contrast to that of FdUMP, for which the ternary complex is much more stable than the binary complex. There is a covalent bond in the TS/FdUMP/CH_2H_4-folate ternary complex.

Despite the absence of covalent bond formation, the CH_2H_4-folate cofactor is activated toward iminium ion formation upon binding to the TS/NDU binary complex. PABA-glu RR bands are seen at the same positions as those

of the TS/FdUMP/CH_2H_4-folate ternary complex, which have been attributed to the quinoid resonance form. This form is stabilized by protein-enforced coplanarity of the benzene ring and the amide and amine substituents. Quinoid formation, as well as the steric effects of the protein binding site (Matthews et al., 1990b), promotes opening of the imidazoline ring and iminium ion formation. It is possible that the ternary complex contains the iminium ion (Figure 1), blocked in its attack on the uracil C_5 by the NO_2 group. Alternatively, the iminium ion may decompose by loss of formaldehyde, leaving a H_4-folate in place of CH_2H_4-folate in the ternary complex. Consistent with this possibility, the same RR spectrum is obtained whether the ternary complex is made with CH_2H_4-folate or with H_4-folate. The RR spectrum is not sensitive to the substituent at the pterin N_5, however, because it is dominated by resonance enhanced bands of the PABA-glu chromophore.

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